Time scales of water dynamics at biological interfaces: peptides, proteins and cells

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Water ^{2}H and ^{17}O spin relaxation is used to study water dynamics in the hydration layers of two small peptides, two globular proteins and in living cells of two microorganisms. The dynamical heterogeneity of hydration water is characterized by performing relaxation measurements over a wide temperature range, extending deeply into the supercooled regime, or by covering a wide frequency range. Protein hydration layers can be described by a power-law distribution of rotational correlation times with an exponent close to 2. This distribution comprises a small fraction of protein-specific hydration sites, where water rotation is strongly retarded, and a dominant fraction of generic hydration sites, where water rotation is as fast as in the hydration shells of small peptides. The generic dynamic perturbation factor is less than 2 at room temperature and exhibits a maximum near 260 K. The dynamic perturbation is induced by H-bond constraints that interfere with the cooperative mechanism that facilitates rotation in bulk water. Because these constraints are temperature-independent, hydration water does not follow the super-Arrhenius temperature dependence of bulk water. Water in living cells behaves as expected from studies of simpler model systems, the only difference being a larger fraction of secluded (strongly perturbed) hydration sites associated with the supramolecular organization in the cell. Intracellular water that is not in direct contact with biopolymers has essentially the same dynamics as bulk water. There is no significant difference in cell water dynamics between mesophilic and halophilic organisms, despite the high K^+ and Na^+ concentrations in the latter.

Introduction

At the molecular level, most of biology happens at interfaces where water makes contact with macromolecules or molecular aggregates. We therefore need to understand in detail how water behaves at such interfaces. Taking bulk water as the reference state, we want to know to what extent and in what way the physical properties of water are modified at the interface. Liquid water has an exceptionally high cohesive energy density which tends to minimize the effect of solutes on its H-bonded network structure. The perturbation induced by a biological interface is therefore of short range, essentially limited to the first coordination shell.[1,2] Even within this hydration layer, only subtle structural changes are usually seen.[3]

The dense H-bond network makes water structurally robust without compromising fluidity. Cold water has nearly the same short-range structure as ice I_h, but molecular rotation is six orders of magnitude faster in the liquid. Molecular mobility remains high in liquid water because the H-bond network is restructured by a cooperative

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mechanism where H-bond partners are interchanged in a concerted way, thereby circumventing the high energy barriers that would have to be surmounted if several H-bonds were broken simultaneously.[4–6] An interface interferes with this cooperative mechanism, leading to a dynamic perturbation that is usually much larger and more amenable to experimental characterization than the structural perturbation.

Rotational diffusion is a more localized motion than translational diffusion and it therefore reports more accurately on the local mobility of the hydration layer. By ^{17}O NMR, water molecules can be monitored selectively in systems of arbitrary complexity and the single-molecule rotational correlation time τ can be accurately determined from spin relaxation experiments.[7] Provided that solute–water hydrogen exchange is not an issue, ^{2}H NMR can be used in the same way. Water exchange between the hydration layer and bulk water is invariably fast on the NMR time scale so the measured spin relaxation rate is a population-weighted average over these environments. The consequent loss of "spatial resolution" is not a serious handicap, because the dynamic perturbation is essentially confined to the (first) hydration layer. This has been established by experiments on simple model interfaces[1,2] and by molecular dynamics simulations.[8–10] Given an estimate of the number (ν_H) of water molecules in the hydration layer, *e.g.* from a simulation, we can obtain, in an essentially model-free way, the rotational correlation time $\langle\tau_H\rangle$ averaged over all sites in the hydration layer. The rotational correlation time τ_0 in bulk water is determined from a pure-water reference sample that is routinely measured in parallel with the sample. As a convenient measure of water dynamics in the hydration layer, we use the dynamic perturbation factor (DPF), defined as

$$\xi_H \equiv \frac{\langle\tau_H\rangle}{\tau_0} \quad (1)$$

The most important biological interface is that between water and proteins. Protein surfaces have a complex topography, leading to a strong dynamical heterogeneity in the hydration layer[10–12] with rotational correlation times ranging from picoseconds to nanoseconds (at room temperature). Very little experimental information is available about this dynamical heterogeneity. NMR cannot frequency-resolve sub-nanosecond correlation times (as can be done for less mobile internal water molecules[7,13]), so a different strategy is needed to dissect the hydration-layer average $\langle\tau_H\rangle$. The strategy adopted here for the proteins ubiquitin and β-lactoglobulin is to measure the ^{17}O spin relaxation rate at a fixed high frequency over a wide temperature range extending into the deeply supercooled regime.

Protein surfaces differ in topographical details but their average properties, such as polarity and curvature, are similar. We therefore expect both protein-specific and generic contributions to the hydration-layer-averaged DPF. One aim of this study is to isolate the generic component and to compare it with small solutes. For this purpose, we studied, over the same wide temperature range, the hydration dynamics of two small peptides: *N*-acetylglycine-*N*′-methylamide (NAGMA) and *N*-acetylleucine-*N*′-methylamide (NALMA). The hydration shells of these peptides contain only 5–10% of the number of water molecules in the hydration layers of the two proteins (Fig. 1). Furthermore, the peptides have a relatively simple surface topography, free from concave depressions (grooves and pockets). For these reasons, we can neglect dynamical heterogeneity in the peptide hydration shell.

Our results show that, excluding a small fraction of protein-specific hydration sites, the protein hydration layer differs little from the hydration shell of small peptides. In other words: the vast majority of the water molecules at a protein surface "don't know" if they are hydrating a protein or a small peptide. New principles need not be invoked, except to account for a small fraction of protein-specific hydration sites. Of course, these few hydration sites may sometimes be important for protein function.

Both peptides and proteins were studied here in dilute aqueous solution. We have thus characterized water dynamics in "free" hydration layers sandwiched between

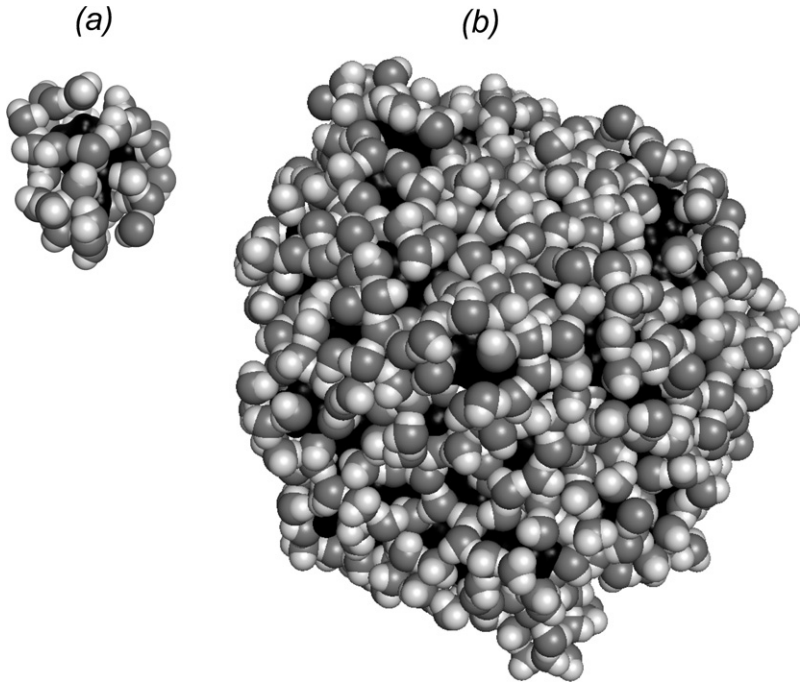

Fig. 1 Hydration shells of (a) NALMA and (b) BLG taken from MD simulations at 300 K. The solute, represented by its molecular surface, is covered by 43 (NALMA) or 740 (BLG) water molecules, the O atoms of which are within 3.3 (O), 3.5 (N) or 5.0 Å (C) of a non-H solute atom. The Figure was rendered with PyMOL (www.pymol.org).

the solute's surface and a bulk-water phase. These model systems are well-defined, but one could question their relevance for what goes on in the real, *in vivo* world. In a typical biological cell, ~30% of the volume is occupied by proteins and other biomolecules that self-assemble into supramolecular structures of stupendous complexity. The remaining 70% is water, some or all of which might differ substantially from bulk water. The view that most cell water differs radically from bulk water has a long history[14–16] and has recently received support from a quasielastic neutron scattering (QENS) study of the extreme halophilic archaeon *Haloarcula marismortui*, which indicated that the mobility of most of the cell water is two orders of magnitude lower than in bulk water.[17] If correct, this finding would force us to rethink much of what we have learnt from studies of model systems such as dilute protein solutions.

To clarify the contentious cell water issue, we have used ^{2}H NMR to selectively study water dynamics in living cells of *Escherichia coli* and *H. marismortui* cultivated in D_2O. To resolve water dynamics on different time scales, we recorded the ^{2}H spin relaxation rate over five orders of magnitude in frequency. Such magnetic relaxation dispersion (MRD) data have not been reported previously for any microorganism. The essentially model-independent picture of cell water dynamics that emerges from these NMR data differs drastically from the view supported by QENS measurements.[17]

Materials and methods

Sample preparation

The peptides NAGMA and NALMA (>99% purity, Bachem) were dissolved in D_2O (99.9% ^{2}H, CIL) at pH* 4.5 (pH* is the pH meter reading, uncorrected for H/D isotope effects). At this pH, the amide deuterons exchange too slowly to contribute to the measured water ^{2}H relaxation rate. The peptide concentrations, determined

gravimetrically and by ^{1}H NMR spectroscopy, were kept below 220 mM to avoid self-association and hydration shell overlap. In this dilute regime, the relaxation rate R_1 increases linearly with solute concentration.

Highly purified ubiquitin (expressed in *E. coli*)[13] and bovine β-lactoglobulin (BLG) isoform A (Sigma) were dissolved in H_2O (19% ^{17}O, Isotec) at pH 5.0 and 2.7 respectively. Protein concentrations were determined by amino acid analysis. We verified that neither protein undergoes cold denaturation in the investigated temperature range.

Relaxation measurements at temperatures below the equilibrium freezing point of water were made on emulsified aqueous solutions with heptane as the carrier phase and sorbitan tristearate as the emulsifier.[18] Control experiments gave identical results for solution and emulsion samples at the same temperature.

E. coli (strain K-12 RV308) and *H. marismortui* from DSMZ were cultured aerobically at 310 K and pH* 7.6 in rich media prepared with D_2O (99.9% ^{2}H, Spectra Stable Isotopes). After incubation, the cell suspensions were centrifuged and the cell pellets washed twice with D_2O–saline buffers. Water-content determination, elemental analysis and amino acid analysis were performed on a portion of the cell mass. To minimize cell death, all NMR measurements were completed within 6 h. Control experiments showed that 70 ± 7% of the *E. coli* cells were viable after this period. Furthermore, repeated measurements of R_1 at a fixed high frequency showed no significant variation during the 6 h period.

NMR experiments

For the peptide and protein solutions, the relaxation rate R_1 of the water ^{2}H or ^{17}O longitudinal magnetization was measured at 55.5 or 81.3 MHz respectively. R_1 was determined with 0.5–1.0% accuracy from three-parameter fits to single-exponential inversion–recovery curves with 30 delay times in nonmonotonic order. At each temperature, measurements of R_1^0 for a pure-water reference sample were alternated with solution R_1 measurements. The sample temperature was regulated to ±0.1 K by a precooled stream of dry air and was determined before and after R_1 measurements with a thermocouple in an NMR tube containing a water–ethanol mixture.

For the cell samples, the water ^{2}H relaxation rate R_1 was measured from 1.5 kHz to 76.8 MHz using a field-cycling spectrometer and five fixed-field spectrometers. As a control, the water ^{17}O relaxation rate R_1 was measured at 67.8 MHz on an *E. coli* sample, showing that labile hydrogens make a negligible contribution to the water ^{2}H rate at the highest frequency. All measurements were performed at 300.0 ± 0.1 K with an estimated uncertainty in R_1 of less than 1%.

Molecular dynamics (MD) simulations

The rectangular simulation box contained 1631 (NAGMA) or 1858 (NALMA) SPCE water molecules and one peptide molecule with geometrical and Lennard-Jones parameters from the general Amber force field and partial charges determined with the AM1-BCC method. The MD simulations were performed at 300 K and 1 atm with periodic boundary conditions, particle-mesh Ewald summation for long-range electrostatics, 12 Å cutoff for nonbonded interactions, constrained X–H bonds and 2 fs time step. Atomic coordinates were saved every 1 ps of the stable 1 ns production trajectory.

The number (ν_H) of water molecules in the hydration shell of the peptides was determined to be the mean number of water molecules satisfying at least one of the following geometric criteria: $R(O_W–O) < 3.3$ Å, $R(O_W–N) < 3.5$ Å and $R(O_W–C) < 5.0$ Å. These cutoff distances are close to the first minimum in the O_W–X radial distribution functions.

For the proteins, the number of water molecules in the hydration layer was calculated as $\nu_H = A_S/a_W$, where A_S is the solvent-accessible surface area (SASA) of the

protein and a_W is the amount of SASA occupied by one water molecule on average. Applying this procedure to the peptides and comparing with the MD-derived ν_H values, we find $a_W = 10.75$ Å^2. We thus obtained $\nu_H = 443$ for ubiquitin and $\nu_H = 735$ for BLG. For BLG, we obtained $\nu_H = 745$ from a 4 ns MD trajectory[19] (using the same cutoffs as for the peptides). The close agreement with the SASA-derived ν_H value indicates that the a_W value established for peptides also applies to proteins.

Results and discussion

Peptide hydration dynamics

The hydration-layer DPF ξ_H for NAGMA and NALMA was determined from the linear variation of the water ^{2}H relaxation rate R_1 with solute concentration according to:

$$\frac{R_1 - R_1^0}{R_1^0} = \frac{\nu_H}{N_W} (\xi_H - 1) \quad (2)$$

where N_W is the water/peptide mole ratio in the sample and ν_H is the number of water molecules in the hydration shell. The hydration number was determined from MD simulations of ~30 mM peptide solutions (see Materials and methods), yielding $\nu_H = 33.3$ (NAGMA) and 42.6 (NALMA). This corresponds to a single layer of water molecules covering the solute (Fig. 1a).

The temperature dependence of the DPFs for NAGMA and NALMA is shown in Fig. 2. The physical significance of the DPF maximum at ~256 K is clear from eqn (1), which yields

$$\frac{d\xi_H(T)}{dT} = \frac{\xi_H(T)}{k_B T^2} [E_0(T) - E_H(T)] \quad (3)$$

where E_H is the apparent Arrhenius activation energy for the hydration shell, defined as

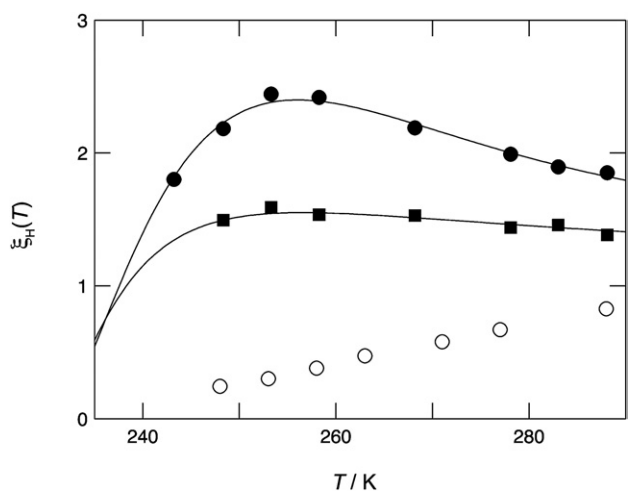

Fig. 2 Temperature dependence of the hydration-shell DPF for NAGMA (■) and NALMA (●), derived from water ^{2}H spin relaxation measurements at 55.5 MHz on dilute (<0.22 M) aqueous solutions. The curves were obtained from separate fits to the monotonic temperature dependence of $\langle \tau_H \rangle$ and τ_0. The open circles are results derived from QENS data for 1.0 M NALMA[28] as described in the text.

$$E_H(T) = -k_B T^2 \frac{d \ln\langle \tau_H \rangle}{d T} \quad (4)$$

and similarly for the bulk-water activation energy E_0. The DPF maximum thus occurs at the crossover temperature T_X where these two activation energies are equal.

At room temperature, water rotation in the peptide hydration shell is slowed down by less than a factor of 2 as compared to bulk water (Table 1), as previously found for a wide range of organic solutes.[20–23] As is generally the case, we see that the most hydrophobic solute (NALMA) has the largest effect on water dynamics. But even at the maximum, the largest DPF is only 2.4. Furthermore, our data suggest that water rotation is *faster* in the hydration shell than in bulk water (that is, $\xi_H < 1$) at temperatures below 237–238 K (Fig. 2). This can be inferred by extrapolating the monotonic curves obtained from separate fits to the temperature dependence of $\langle \tau_H \rangle$ and τ_0. The curves shown in Fig. 2 were calculated from these fitted curves by means of eqn (1).

When bulk water is cooled, the apparent activation energy E_0 increases because the liquid structure is gradually transformed toward more open configurations with higher tetrahedral order. This subtle and gradual structural change interferes with the cooperative rotation mechanism, causing τ_0 to increase dramatically.[24] In the hydration shell, the slowing down of water rotation can also be attributed to interference with the cooperative rotation mechanism, partly because of the reduced number of nearby water molecules with which to swap H-bonds and partly because H-bond partners in the solute are either absent (at apolar sites) or else are geometrically constrained (at polar sites). Because these constraints are essentially temperature-independent, the activation energy E_H does not depend as strongly on temperature as E_0.

The hydration dynamics of NAGMA and NALMA have previously been studied by QENS and MD simulations[25–29] at or above the concentrations (1.0 M for NAGMA and 0.5 M for NALMA) where we find that R_1 no longer depends linearly on solute concentration. Whereas our results pertain to the hydration shell of the "isolated" solute, the QENS and MD results may thus be influenced by peptide self-association and hydration shell overlap. For ~1.0 M NAGMA, the simulation gave $\langle \tau \rangle = 2.27$ ps for the average over all $N_W = 55$ water molecules.[29] Attributing the deviation from bulk-water dynamics, with $\tau_0 = 1.90$ ps, to the $\nu_H = 33.3$ (Table 1) water molecules in the hydration shell, we find that the MD result corresponds to $\xi_H = 1.32$, not far from our (dilute-solution) result $\xi_H = 1.37$ (Table 1). Similarly, for ~0.5 M NALMA ($N_W = 92.4$), the simulation[29] yields $\xi_H = 1.46$ while we find $\xi_H = 1.70$ (Table 1).

For a 1.0 M NALMA solution (where $N_W = 45.9 \approx \nu_H$ so we can set $\langle \tau \rangle = \langle \tau_H \rangle$), QENS data[28] acquired in the temperature range 248–288 K gave rotational correlation times substantially *shorter* than the rotational correlation time τ_0 for bulk H_2O.[24] The resulting DPF has a qualitatively different temperature dependence and differs by an order of magnitude at low temperatures from our NMR results

Table 1 Generic hydration dynamics of proteins and peptides

			Ubiquitin		BLG	
Property	NAGMA	NALMA	$p = 0.5$	$p = 0.9$	$p = 0.5$	$p = 0.9$
ν_H	33.3	42.6	222	399	368	662
ξ_H(298 K)	1.37	1.70	1.21	1.86	1.26	2.09
$\xi_H(T_X)$	1.55	2.40	1.58	2.43	1.62	2.70
T_X/K	256.6	256.0	262.5	262.5	263.1	263.1

(Fig. 2). This discrepancy may result from the high peptide concentration used in the QENS study, but more likely is a consequence of the strong model-dependence in the interpretation of the QENS data.[29,30]

Protein hydration dynamics

The water ^{17}O spin relaxation rate R_1 in a protein solution exceeds the bulk-water value R_1^0, not only because water molecules in the hydration layer rotate more slowly than in bulk water, but also because a small number of water molecules are buried inside the protein. Because they interact strongly with the protein, the orientational time correlation function of these internal water molecules has a long-time tail that decays with the protein's rotational correlation time τ_R.[7] Because τ_R is more than three orders of magnitude longer than τ_0, even a single internal water molecule can have a large effect on the observed magnetic relaxation dispersion (MRD) profile $R_1(\omega_0)$ (Fig. 3). Here, we are interested in water dynamics in the external hydration layer and we therefore measure R_1 at a fixed high frequency (81.3 MHz) where the frequency-dependent internal-water contribution to R_1 is negligibly small (Fig. 3).

Even though the internal-water contribution is eliminated by measuring at a high frequency, R_1 still depends on the resonance frequency ω_0 because a small fraction of the several hundred water molecules in the hydration layer have correlation times of order $1/\omega_0 = 2$ ns. We are thus led to introduce an *apparent* DPF:

$$\begin{aligned} \xi_H(\omega_0, T) &\equiv 1 + \frac{N_W}{\nu_H}\left[\frac{R_1(\omega_0, T) - R_1^0(T)}{R_1^0(T)}\right] \\ &= \frac{1}{5\,\tau_0(T)}\left\langle \frac{\tau_H(T)}{1 + [\omega_0\,\tau_H(T)]^2} + \frac{4\,\tau_H(T)}{1 + 4\,[\omega_0\,\tau_H(T)]^2}\right\rangle \end{aligned} \tag{5}$$

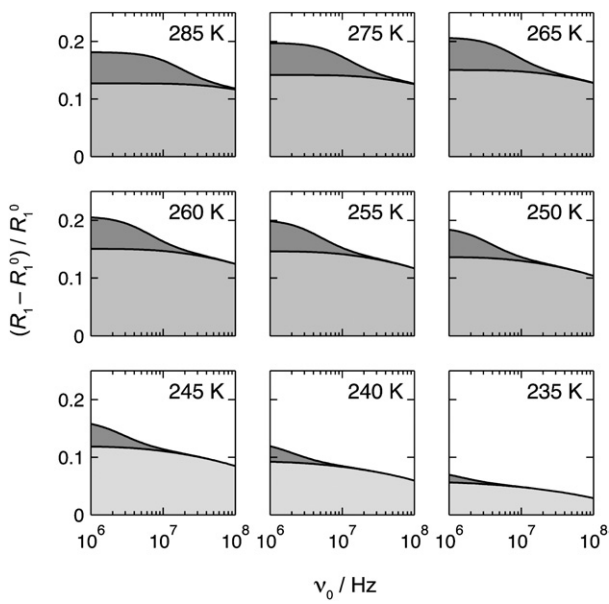

Fig. 3 Normalized water ^{17}O MRD profiles at different temperatures for a dilute (5 mM) aqueous solution of ubiquitin, showing the contributions from the external hydration layer (light gray) and from the single internal water molecule (dark gray). The curves were calculated with parameter values determined here and elsewhere.[13]

The number ν_H of water molecules in the protein's hydration layer was calculated from the solvent-accessible surface area (SASA) of the protein and, for BLG, also from MD simulations. The two methods agreed to within 1% (see Materials and methods).

As seen from eqn (5), the apparent DPF reduces to the true DPF in eqn (1) in the limit $\omega_0 = 0$, or when $(\omega_0\tau_H)^2 \ll 1$ for all sites in the hydration layer. However, at such low frequencies, the relaxation enhancement $R_1 - R_1^0$ is usually dominated by internal water molecules (Fig. 3). For proteins, the apparent DPF in eqn (5) is therefore the only way to quantitatively characterize water dynamics in the external hydration layer. Although the apparent DPF is perfectly well defined, its dependence on the resonance frequency makes it less useful for comparing results obtained on different NMR spectrometers or with other techniques. Moreover, it is not straightforward to interpret the temperature dependence of the apparent DPF. For these reasons, we adopt a model that allows us to convert the apparent DPF to a true DPF (and to determine the parameters in the model). Two model assumptions are needed.

First, we need to specify the mathematical form of the distribution $f(\tau_H,T)$ of correlation times in the hydration layer. Guided by MD simulations that indicate a power-law distribution in the picosecond–nanosecond range,[11,12] we assume that $f(\tau_H,T) \propto \tau_H^{-\nu}$ for $\tau_- < \tau_H < \tau_+$. Second, we must specify how the limits $\tau_\pm$ vary with temperature. For simplicity, we assume that they obey the Arrhenius law: $\tau_\pm(T) \propto \exp[E_H^\pm/(k_B T)]$. Consistent with experimental observations, we also postulate that $\tau_-(T^*) = \tau_0(T^*)$ and $\tau_+(T^*) = \tau_R(T^*)$ at the reference temperature $T^* = 293.2$ K. The model then contains only two free parameters, which we choose as the power-law exponent ν and the activation energy E_- at the short-τ end of the distribution. Our calculations indicate that the results of the model-dependent analysis are robust. Specifically, they are not significantly altered by allowing for a linear temperature dependence in the activation energies $E_\pm$ or by reasonable variations in the values of the limits $\tau_\pm$ at the reference temperature T^*.

Fig. 4a shows the apparent DPF obtained *via* eqn (5) from water ^{17}O relaxation measurements on dilute solutions of β-lactoglobulin (BLG) and ubiquitin in a 50 K temperature range extending down to 238 K. As for the peptides (Fig. 2), we observe a maximum. The model fits shown in Fig. 4a yield $\nu = 2.15$ for BLG and 2.32 for ubiquitin and $E_- = 27$ kJ mol^{-1} for both proteins. These exponents are close to the value ($\nu = 2.3$) obtained from an MD simulation of cytochrome *c*.[11] The slightly smaller exponent for BLG, corresponding to a wider distribution, is consistent with the known[19] presence in BLG of an unusually large fraction of hydration sites with correlation times of order 1 ns.

From the model parameters, we can calculate the temperature variation of the true (rather than apparent) DPF $\xi_H(T)$. As seen from Fig. 4b, the DPF is substantially larger for BLG than for ubiquitin and both are larger than for the peptides (Fig. 2). These differences are caused by a minor fraction of protein-specific hydration sites with correlation times of order $1/\omega_0$ or longer, as seen directly in low-temperature MRD profiles.[31] Since the power-law exponent is close to 2, equal intervals of $\log(\tau_H)$ make equal contributions to $\langle\tau_H\rangle$ so the long-τ_H tail of the distribution contributes substantially to the DPF. In MD simulations,[12,32,33] the water molecules with the longest correlation times are generally found in concave depressions or pockets on the protein surface. Such secluded hydration sites do not occur in the peptides so the DPF is smaller.

The majority of the several hundred water molecules in the protein's hydration layer are associated with convex protrusions on the surface, where the local environment differs little from the peptide's hydration shell (Fig. 1). To compare this more exposed part of the protein hydration layer with the peptides, we calculate the *partial* DPF $\xi_H(T,p)$ for the most mobile fraction p of the ν_H water molecules in the hydration layer. This quantity is shown in Fig. 4b for $p = 0.5$ and in Fig. 5 as a function of p at two temperatures. We see that the DPFs for the two proteins are nearly the same

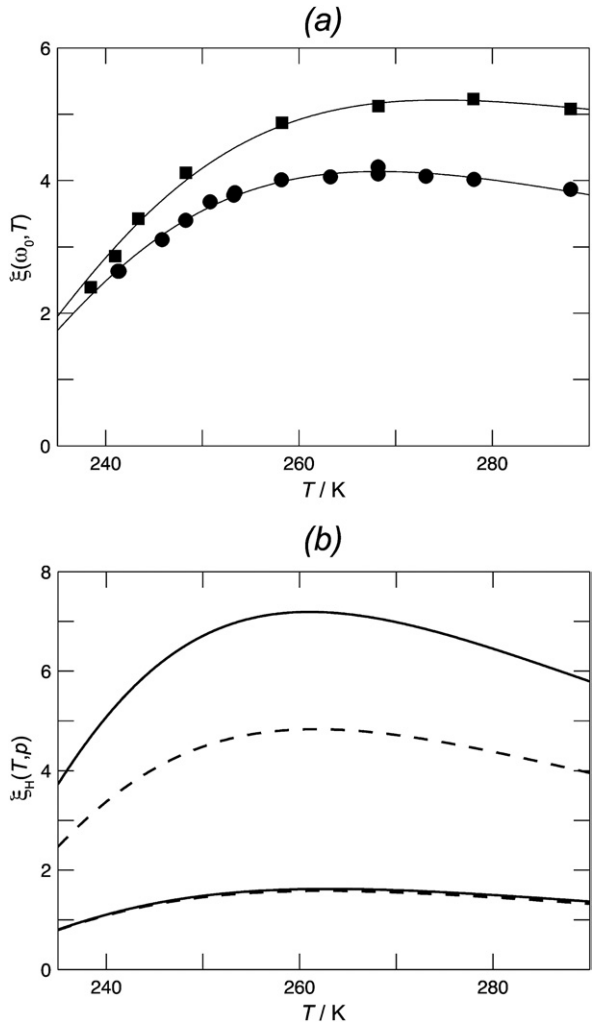

Fig. 4 (a) Temperature dependence of the apparent DPF for BLG (■) and ubiquitin (●), derived from water ^{17}O spin relaxation measurements at 81.3 MHz on dilute (0.98 and 5.1 mM respectively) aqueous solutions. The curves were obtained by fitting the two model parameters. (b) Temperature dependence of the full ($p = 1$, upper pair of curves) and partial ($p = 0.5$, lower overlapping curves) hydration-layer DPF for BLG (solid curves) and ubiquitin (dashed curves), calculated from the parameters determined in (a).

if we disregard the ~10% most strongly perturbed water molecules. In fact, the DPFs for the most mobile half of the hydration layer in the two proteins are virtually identical. Furthermore, with the exception of a small fraction of secluded hydration sites, the protein hydration layer differs little from the hydration shell of peptides and other small organic solutes. In both cases, the dynamic perturbation factor is less than 2 at room temperature and exhibits a maximum near 260 K (Table 1).

Hydration dynamics *in vivo*

In the protein solutions studied here, only 1–4% of the water molecules are in contact with the protein surface. It has been estimated that ~15% of the water in an *E. coli* cell belongs to the first hydration layer of proteins and other macromolecular

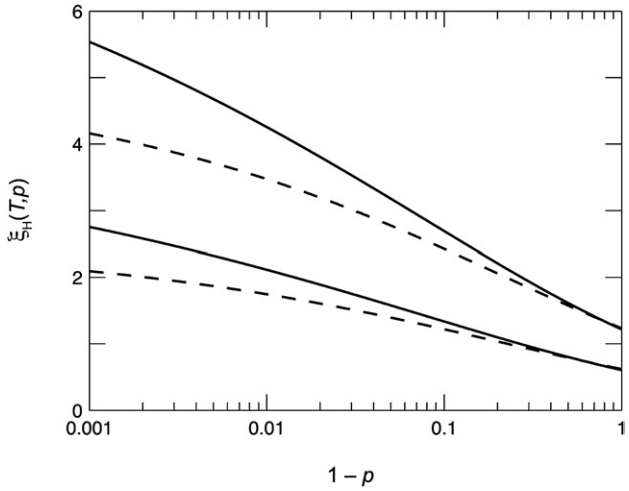

Fig. 5 Partial hydration-layer DPF at 260 K (upper pair of curves) and at 235 K (lower pair of curves) for BLG (solid curves) and ubiquitin (dashed curves). The partial DPF pertains to the most mobile fraction p of the hydration layer.

structures.[34] Most solvent-exposed surfaces in the cell are thus likely to be surrounded by multiple water layers. The long-standing question that we now address is whether the water in a living cell is similar to the water in a protein solution, with a modest dynamic perturbation in the hydration layer and bulk-water properties outside this layer. We have chosen to study the bacterium *Escherichia coli*, because of the wealth of information available about this organism, and the extreme halophilic archaeon *Haloarcula marismortui*, because of reports of unusual hydration behavior of halophilic proteins[35] and of extremely slow water diffusion in *H. marismortui* cells.[17]

Even a prokaryotic cell exhibits a high degree of spatial organization. Whether or not this structural complexity impacts on the water dynamics, it fundamentally alters the spin relaxation behavior for the biopolymers and the water molecules that are buried inside them.[13,36] The reason is that a large fraction of the biopolymers in a cell are rotationally immobilized on the NMR time scale. In fact, we have exploited the sensitivity of ^{1}H–^{14}N cross-relaxation to rotational immobilization to demonstrate that $50 \pm 10\%$ of the peptide groups in the two cell preparations studied here are immobilized.[34] Because of immobilization, the interpretation of water ^{1}H relaxation data from biological materials is a complicated task.[37] We therefore cultured the cells in D_2O and measured the water ^{2}H relaxation rate. To frequency-resolve contributions from different water populations, we varied the resonance frequency over five orders of magnitude.

Fig. 6 shows the water ^{2}H MRD profiles from living *E. coli* and *H. marismortui* cells. The MRD profile $R_1(\omega_0)$ is essentially a mapping in the frequency domain of the distribution of rotational correlation times for all water molecules in the sample. At a given frequency ω_0, R_1 reports on water motions with correlation times shorter than $1/\omega_0$. The dramatic increase in R_1 in the kHz–MHz range corresponds to water dynamics on the 0.1–10 μs time scale. Similar low-frequency ^{2}H relaxation dispersions are observed in biopolymer gels (but not in solutions of freely tumbling proteins) and have been quantitatively linked to the exchange of internal water molecules in rotationally immobilized proteins.[13] The R_1 dispersion below ~1 MHz is a frequency mapping of the residence time distribution of these internal water molecules. An essentially model-independent analysis[34] shows that the low-frequency dispersions in Fig. 6 are quantitatively consistent with the internal-water content

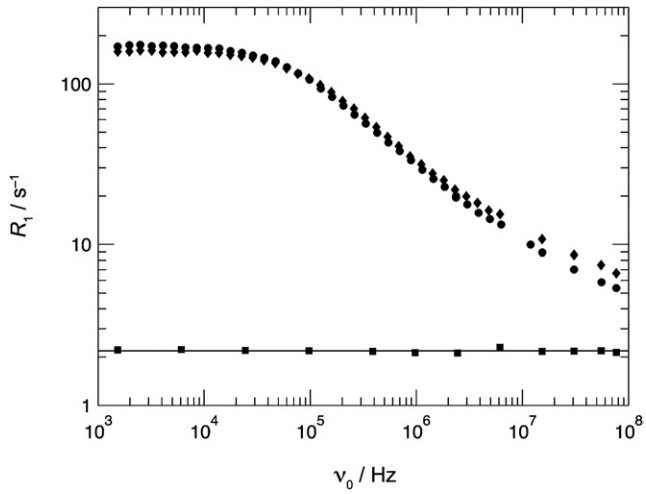

Fig. 6 Water ^{2}H MRD profiles of cells in stationary phase at 300 K: *E. coli* (●) and *H. marismortui* (◆). The results of parallel measurements on a pure-D_2O reference sample are also shown (■). Note the logarithmic R_1 scale.

of the cell samples, predicted from their molecular composition and the crystallographically-determined mean abundance of one internal water molecule per 29 amino acid residues.[38]

Our focus here is not on internal water molecules, but on water dynamics in the external hydration layers. As in the case of protein solutions (Fig. 3), this information is contained in the relaxation rate measured at high frequencies. To obtain the apparent hydration-layer DPF in eqn (5), we redefine N_W as the total number of water molecules per gram dry cell mass (DCM) in the sample and ν_H as the total number of water molecules in hydration layers per gram DCM. N_W was obtained by drying the sample at 130 °C and ν_H was calculated as A_S/a_W, as for the protein solutions. The total SASA per gram DCM, A_S, was estimated from a detailed molecular inventory of the *E. coli* cell and from elemental and amino acid analyses of our samples.[34]

The apparent hydration-layer DPFs for the two cell samples are shown in Fig. 7. At lower frequencies, $\xi_H(\omega_0,T)$ contains contributions from internal water molecules and labile hydrogens in freely tumbling biopolymers, as in a protein solution.[7] (At frequencies below 1 MHz, water molecules buried in immobilized biopolymers dominate R_1.) However, at the highest accessed ^{2}H frequency of 76.8 MHz, corresponding to a correlation time of 2 ns, the apparent DPF is expected to differ by at most 10% from the true DPF $\xi_H(T)$.[34] The convergence at high frequencies of the apparent DPFs for *E. coli* and *H. marismortui* (Fig. 7) demonstrates that the hydration water in these cells has essentially the same mobility on average. The hydration-layer DPFs are 16 ± 3 for *E. coli* and 15 ± 3 for *H. marismortui*. These results are obtained from the measured spin relaxation rates in a straightforward way, without invoking motional models. The only other input is the SASA-based estimate of the amount of hydration water (ν_H), which accounts for the ± 20% uncertainty in the DPF.

As seen from Table 2, the hydration-layer DPF for the cells is a factor of 3 larger than for small (6.5–18.4 kDa) monomeric proteins,[39] but only ~70% larger than for a set of more representative proteins (intestinal fatty-acid-binding protein, carbonic anhydrase II, β-trypsin and serum albumin),[39] which have a larger fraction of secluded hydration sites than the small proteins. (These sites are reflected directly in the MRD profiles of these proteins.) We therefore conclude that hydration layers in a living cell do not differ in any essential way from the hydration layers in a dilute

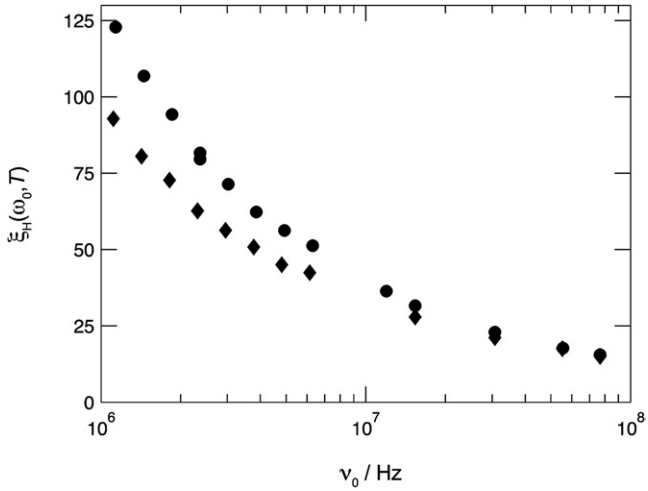

Fig. 7 Convergence at high frequencies of the apparent hydration-layer DPF for *E. coli* (●) and *H. marismortui* (◆), obtained from the data in Fig. 6, and estimates of the fraction hydration water in the cell samples.

Table 2 Hydration-layer DPF for proteins (at 298 K) and cells (at 300 K)

System	$\xi_H(T)$
Ubiquitin	3.6
BLG	5.2
11 small proteins	4.9 ± 0.6
4 larger proteins	9 ± 2
E. coli	16 ± 3
H. marismortui	15 ± 3

protein solution. The modestly larger DPF in the cells can be attributed to a larger fraction of secluded hydration sites at subunit interfaces in enzyme complexes, ribosomes, cytoskeleton and other supramolecular assemblies.

In deriving the hydration-layer DPF from the relaxation data, we tacitly assumed that the (intracellular and extracellular) water that does not belong to hydration layers has the rotational correlation time τ_0 of bulk water. This assumption is supported by the finding that it yields a hydration-layer DPF of the magnitude expected from studies of protein solutions. A more general and definite result that does not rely on our ν_H estimate can be obtained by defining a *cell-averaged* DPF by replacing ν_H in eqn (5) by the number (N_W^{cell}) of intracellular water molecules per gram DCM in the sample. The fraction of intracellular water, N_W^{cell}/N_W, is 0.74 for both samples, yielding $\xi_{cell} = 3.02 \pm 0.04$ for *E. coli* and 3.77 ± 0.04 for *H. marismortui*. Averaged over the whole cell, water rotation is thus slowed by a factor of 3 in *E. coli*. As we have seen, this average perturbation can be reasonably attributed to the 15% of the intracellular water (or 10% of the water in the sample) that belongs to hydration layers, leaving little or no room for a significant perturbation of the dominant water fraction.

The somewhat larger ξ_{cell} value for *H. marismortui* does not indicate slower water dynamics in this organism, but is a trivial dilution effect. Because of the very high salt concentration in the *H. marismortui* sample, the water/protein mass ratio is lower than in the *E. coli* sample. According to the elemental analysis, the *H. marismortui* sample contains only 12 water molecules per Na^+ or K^+ ion. Despite their

high concentration, these ions have a negligible effect on water dynamics, partly because of cancellation (Na^+ ions slow down, but K^+ ions speed up water rotation in the hydration shell).[40] In striking contrast to our results, a recent QENS study of a *H. marismortui* sample similar to ours concluded that 76% of the cell water is slowed down in its short-range translational diffusion by a factor of 260 at 300 K.[17] It was speculated that this extreme slowing down reflected a specific structure of cell water that is responsible for the selective accumulation of K^+ ions in the halophile cell.[17] This viewpoint, which can be traced back to the time before ion pumps had been discovered,[41] is not supported by our results. In fact, we find no significant difference in water dynamics between *E. coli* and the extreme halophile *H. marismortui*.

Conclusions

We have used ^2H and ^{17}O spin relaxation to selectively probe water dynamics in the hydration layers of peptides, proteins and cells. We report our findings in terms of the hydration-layer-averaged dynamic perturbation factor $\xi_H = \langle\tau_H\rangle/\tau_0$, a model-independent quantitative measure of the slowing of single-molecule water rotation in the hydration layer relative to bulk water. Our principal findings are as follows:

(1) Below 256 K, the activation energy for water rotation is lower in the hydration shell of a hydrophobic peptide than in bulk water. This finding challenges the classical "iceberg" view of hydrophobic hydration. The lower activation energy implies that hydration water rotates faster than bulk water at sufficiently low temperatures. Our data suggest that this is the case below 237 K for NALMA.

(2) The strong dynamical heterogeneity of the protein hydration layer can be described by a power-law distribution of rotational correlation times with an exponent close to 2.

(3) The long-τ tail of the power-law distribution is contributed by a protein-specific small population of slowly rotating water molecules, consistent with the finding from MD simulations that the most strongly perturbed water molecules reside in secluded sites.

(4) Most water molecules in the protein hydration layer exhibit a weak and generic dynamic perturbation. At room temperature, the average dynamic perturbation is a factor of $\sim$2 for 90% of the hydration layer and only $\sim$1.3 for the most mobile half of the layer.

(5) The hydration layer can be regarded as a defect in the H-bond network of bulk water, induced by a protein surface that provides fewer and less flexible H-bonding opportunities for the adjacent water molecules. These constraints slow down water rotation because they interfere with the cooperative mechanism that facilitates rotation in bulk water.

(6) Because the constraints are essentially temperature-independent, hydration water does not follow the strongly super-Arrhenius temperature dependence of bulk water. In this sense, hydration water is less anomalous than bulk water.

(7) With the exception of a small fraction of secluded hydration sites, the protein hydration layer differs little from the hydration shell of peptides and other small organic solutes. In both cases, the dynamic perturbation factor is less than 2 at room temperature and exhibits a maximum near 260 K.

(8) Water in the hydration layers of living cells behaves as expected from studies of simple model systems, the only difference being a somewhat larger fraction of secluded (strongly perturbed) hydration sites, associated with the supramolecular organization in the cell.

(9) Intracellular water that is not in direct contact with biopolymers has essentially the same dynamics as bulk water. This applies to >80% of the cell water.

(10) There is no significant difference in cell water dynamics between *E. coli* and *H. marismortui*, despite the high K^+ and Na^+ concentrations in the latter organism.

Acknowledgements

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General discussion

Professor Luzar opened the discussion of the paper by Professor Halle: We should not question the validity of QENS in comparison with NMR. The results are good in both cases and one must find what is the best description that is compatible with *both* techniques. In your present careful analysis, what is measured is the relative change of rotational relaxation times of water molecules, which is *not* measured directly by QENS. Also the time window in your case (Fig. 6 and 7 in your paper) is centered on long times: microseconds and nanoseconds, while in QENS the time scales are picoseconds. QENS measures exactly, and directly. Nothing more. The value of this correlation function is normally dominated by (i) diffusion (not seen in your case), and (ii) rotations of hydrogen around the center of mass. That is why this method is also more local and less dependent on concentration.

Professor Halle replied: No-one is questioning the "validity of QENS"; it is a powerful technique for probing single-particle dynamics on relatively short time and length scales. What is at issue here is rather the *interpretation* of QENS data from a very complex system and, in particular, the inference that 76% of the cell water in *H. marismortui* (55% of the water in the sample) has a translational diffusion coefficient that is 250 times smaller than the bulk-water value.[1] As you know, translation and rotation in liquid water take place on the same time scale (because they are strongly coupled by the H-bond network). Therefore, if the QENS interpretation is correct, also the water rotation that we measure should be 250 times slower. At the highest frequency (76.8 MHz), we should then have $R_1 = 0.45 \times 2.2 + 0.55 \times 2.2 \times 250 \approx 300\ s^{-1}$, whereas the measurement[2] gives $R_1 = 6.66 \pm 0.03\ s^{-1}$. On the other hand, the interpretation of QENS data from *E. coli* suggested that the visible cell water does not differ significantly from bulk water.[3] Yet, the primary NMR data (Fig. 6 of our paper) from *E. coli* and *H. marismortui* are virtually superimposable (and the small difference is removed when the data are concentration-normalized). Complacency in the face of such order-of-magnitude discrepancies is simply bad science.

1 M. Tehei, B. Franzetti, K. Wood, F. Gabel, E. Fabiani, M. Jasnin, M. Zamponi, D. Oesterhelt, G. Zaccai, M. Ginzburg and B.-Z. Ginzburg, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 766.
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Dr Zaccai commented: You stated, with a certain sense of humour that since it is the parameter that you measure with your technique, water rotational diffusion being a more localised motion reports more accurately on the hydration layer. Certainly, when measured by NMR, translational motions are on a long length scale (~μm), which is not informative on local mobility. With neutron scattering, however, translational and rotational diffusion are measured on an atomic length scale (~Å–nm) on different time scales (~ps–ns). The primary data from QENS are line widths, which provide information on time scale, measured as a function of scattering vector Q, which informs us on the length scale. At small Q values, the Q^2 dependence of the line-width yields the atomic scale translational diffusion. This is a direct visualisation of a trajectory according to a space–time correlation function. Note that such trajectories are what is calculated in MD simulations, so that a direct comparison with experiment is possible. See for example the paper of Professor Tobias *et al.* in this volume. Because of this, contrary to another of your assertions, the primary interpretation of QENS data is not model-dependent.

Dr Zaccai then said: You have several times pointed out the contradiction between neutron scattering and NMR results. The two methods measure different parameters or effects and I cannot see how their results can be in contradiction. On the contrary, assuming that the data has been collected and treated correctly, an interpretation that is compatible with both sets can only enrich our knowledge of a complex system. In your paper you compare rotational correlation times from NMR and neutrons. Incoherent neutron scattering measures the single particle space-time correlation of protons. What does NMR measure?

Professor Halle replied: I think we agree that translation diffusion coefficients derived from water displacements on the micrometre length scale, whether determined by pulsed-gradient spin echo NMR or any other technique, tells us little about cell water dynamics. Indeed, the clinical value of the apparent diffusion coefficients measured in MRI examinations lies in their dependence on tissue morphology and cell ultrastructure.

Like QENS, NMR relaxation probes single-particle dynamics on a molecular length scale. But there are important differences in how the dynamical information is extracted from the primary data. In both NMR and QENS this is a two-step process, which can be schematically illustrated as follows:

$$\text{NMR: } M(t) \rightarrow R(\omega) \rightarrow \upsilon_{\mathrm{R}}$$

$$\text{QENS: } S(Q,\omega) \rightarrow \Gamma(Q) \rightarrow (D, \upsilon_0, \upsilon_{\mathrm{R}}, \ldots)$$

In NMR, the first step is a single-exponential fit of the magnetization curve $M(t)$ as a function of evolution time t. The exponential form is found in all water-rich systems, even if they are dynamically heterogeneous, and the resulting frequency-dependent spin relaxation rate $R(\omega)$ is a model-independent quantity. In QENS, the first step is to fit the spectrum $S(Q,\omega)$ to an empirical function that may include one or more Lorentzians in addition to an elastic peak (due to unresolved and/or confined motions) and a uniform background (which includes motions faster than the dynamic range of the instrument). Whereas the exponential form of $M(t)$ follows from the general theory of nuclear spin relaxation, the precise shape of $S(Q,\omega)$ cannot be predicted without specifying a model for the proton motions in the particular sample that is being investigated. Therefore, already the choice of fitting function introduces a model-dependence in the quasielastic linewidths $\Gamma(Q)$.

The second step in NMR depends strongly on the nuclear isotope used and the frequency range covered. For isotopes with a nuclear electric quadrupole moment (like ^{2}H and ^{17}O), the spin relaxation behavior is particularly simple. The relaxation dispersion $R(\omega)$ is then a direct mapping of the spectral density function, which is the Fourier transform of an orientational time correlation function (TCF). In the case of ^{2}H relaxation, the TCF describes the rotational motion of the water O–H bond. With current magnet technology, the highest accessible ^{2}H or ^{17}O frequency is ~100 MHz. This means that rotational motions faster than ~1 ns do not produce a frequency dependence in $R(\omega)$. The relaxation rate then yields the time integral of the TCF, which defines a model-independent rotational correlation time υ_{R} (equal to $1/(6D_{\mathrm{R}})$ for a rotational diffusion model).

In QENS, the interpretation of the empirically fitted linewidths $\Gamma(Q)$ is an open problem even for bulk water. QENS probes displacements of protons, not water molecules. This distinction can be ignored on large length scales (small momentum transfer, Q), but not in the Q range 0.2–2 Å^{-1} usually probed in QENS studies. Water translation and rotation are certainly not statistically independent processes (since both are governed by H-bond rearrangements), as invariably assumed to simplify the interpretation of QENS data. As a result of these complications, the physical significance of the correlation times (υ_0 and υ_{R}) deduced with the aid of the standard QENS model remains obscure even for homogeneous systems.

In complex systems (like cells), water motions take place on multiple time scales. Such dynamic heterogeneity is manifested in quite different ways in NMR and QENS: while $R(\omega)$ is a fast-exchange population-weighted average, $S(Q,\omega)$ is a slow-exchange superposition of Lorentzians. In QENS, a small population of slow water produces a narrow peak of small amplitude that may easily escape detection. But in the NMR relaxation rate $R(\omega)$, the small population is multiplied by a long correlation time. It is therefore possible, for example, to detect a single water molecule buried inside a protein against a background of 10^5 bulk water molecules. In dynamically heterogeneous systems, a model is needed to describe exchange averaging of $R(\omega)$, but because the exchange is invariably fast on the spin relaxation time scale, there is no need to model the exchange dynamics. In most cases, a two-state model is sufficient, where the single parameter is the water fraction f in hydration shells, the remaining fraction $(1 - f)$ being considered as bulk water. The models that you used to interpret QENS data from cells ignore this dynamic heterogeneity.

Professor Finney addressed Professor Halle and Dr Zaccai: It is often claimed that interpreting neutron scattering data on the dynamics of water—particularly in biological systems—requires the use of models which may themselves force an interpretation that depends on the model used. In contrast, Halle claims his NMR results are essentially model-independent. Can Dr Zaccai and Professor Halle explore a little the way in which the models used to interpret their respective data could be influencing the physical conclusions being drawn from their data, both qualitatively and quantitatively?

In this context of model dependency we might recall the conventional wisdom in the early 1970s that water close to protein was "slowed down" by a factor of about 10^6 compared to the bulk. I believe that this conclusion was the result of the model(s) used to interpret NMR data, and these were shown (I believe by Professor Halle and others) to be internally inconsistent. As interpretation of data improved (removing model dependence?) the conventional wisdom changed dramatically from "slowing downs" of 10^6 to "a few".

Professor Halle responded: Regarding the first, general part of your question, I addressed it in my response to the previous question by Dr Zaccai. The historical literature on NMR relaxation studies of protein (and DNA) hydration makes fascinating and sobering reading. Much of the confusion resulted from incomplete understanding of the spin relaxation mechanism, rather than from the use of inappropriate models. Thus, the earliest NMR studies (1950s) measured proton linewidths, which may be influenced by a variety of relaxation mechanisms, in addition to dipolar relaxation. Many NMR spectroscopists were also lead astray by the confounding effects of labile protons, that "looked" like long-lived water molecules. These problems were sorted out in the mid 1990s by measuring the longitudinal relaxation rate of the ^{2}H and ^{17}O isotopes over a wide frequency range (magnetic relaxation dispersion, MRD). It was thus demonstrated that the only long-lived (more than a few ns) water molecules that exist in protein solutions are buried in cavities inside the protein and that water molecules in the external hydration layer are highly mobile.[1] This general picture has withstood the test of time and subsequent MRD studies have refined the dynamical characterization of internal water molecules[2] and the external hydration layer.[3]

The theory of NMR relaxation involves dynamics in the nuclear spin system, which must be described quantum-mechanically, and dynamics in the molecular system, and the coupling of the two *via* nuclear interactions rendered time-dependent by molecular motions. There are many different spin couplings and a universe of NMR experiments used for different purposes. All of this may deter the 'outsider' who just wants to know if a particular NMR study of, say, cell water dynamics, is trustworthy. But if we don't make an effort to understand other methods than our own, the scientific enterprise will degenerate into a collection of subcultures

which can neither communicate with nor benefit from each other. We will increasingly attend conferences catering exclusively to NMR spectroscopists, neutron scatterers, computational chemists and so on. Perhaps we are already there...

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3 C. Mattea, J. Qvist and B. Halle, *Biophys. J.*, 2008, **95**, 2951.

Dr Zaccai answered: I agree that we should look carefully at the NMR and neutron scattering data (which essentially measure different phenomena) in a model-independent way, in order to reach a better understanding of water behaviour in these complex systems.

Professor Halle opened the discussion of the paper by Dr Zaccai: In your QENS study of *E. coli*,[1] you find that the observed cell water is indistinguishable (within experimental accuracy) from bulk water as regards translational and rotational dynamics. However, on the basis of another parameter (υ_0) in the model that you used to establish the bulk-like dynamics, you infer that "water molecules spend longer times in the first hydration shell of macromolecular structures than in the bulk phase". Can you explain how you can extract information about hydration water from a model that does not take into account the dynamical heterogeneity of cell water?

In our NMR study of *E. coli*,[2] we found that hydration water (estimated to 15% of total cell water) is rotationally slowed down by a factor 15 on average. This substantial retardation is directly manifested as an increase (relative to bulk water) of the measured spin relaxation rate R_1 by a factor that is >250 times larger than our measurement error. If the NMR results are accepted, one must conclude that the QENS data[1] indicate bulk-like behavior for the trivial reason that they cannot resolve the hydration water. Do you share this view?

From your QENS study of *H. marismortui*,[3] you conclude that 45% of the water molecules in your cell pellet have bulk-like dynamics, while the remaining 55% (corresponding to 76% of the cell water) are slowed down 250-fold. According to your interpretation, this extremely slow motion occurs in confined spaces of 3.3 Å radius. This seems to imply that the ultrastructure of this halophile differs radically from that of other microorganisms. Is this view consistent with current cell biology?

Our NMR data[2] show that cell water dynamics are very similar in *E. coli* and *H. marismortui*. This model-independent result cannot be reconciled with your QENS interpretation.[3] We therefore question your assignment of the quasielastic broadening observed on the high-resolution instrument (IN16) to 55% of the water molecules in the sample. If this were so, would you not expect the elastic peak on the low-resolution instrument (IN6) to account for >55% of the integrated spectral intensity on IN6? But in your fit (Fig. 1a in ref. 3), the elastic intensity appears to be less than 10%.

1 M. Jasnin, M. Moulin, M. Härtlein, G. Zaccai and M. Tehei, *EMBO Rep.*, 2008, **9**, 543.
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Dr Zaccai answered: Regarding the QENS study of *E. coli*, as discussed earlier in this session, neutron scattering and NMR measure different rotational relaxation processes and there is reason to expect qualitative but not quantitative agreement between the two parameter sets. There have been various neutron studies of hydration water. In 1996 Bellissent-Funel *et al.*[1] found a proton residence time longer by a factor of 6 compared to bulk water in a 0.4 g per g hydrated powder of phycocyanin. Your factor of 15 for NMR rotational diffusion was found in solution in

which, because of rapid exchange, one might expect a smaller rather than larger slowing-down factor, but then again I believe these factors cannot be compared quantitatively. In the *E. coli* neutron scattering study, because of rapid exchange one expects to measure a mean residence time between the protons in hydration sites and the protons in the bulk. The published value of longer residence time by a factor of 2 compared to bulk, is in quantitative agreement with 20% of the cell water behaving as in the paper by Bellissent-Funel *et al.*[1]—like hydration water in rapid exchange with 80% of the cell water, which behaves like bulk -water.

Regarding the QENS study of *H. marismortui*, indeed it is consistent with current cell biology, with respect to the extreme halophilic archaeon *Haloarcula marismortui* and to the particular salt–ionic and hydration interactions of its proteins. Please see ref. 2–11.

With respect to your question about Fig. 1a in our *PNAS* paper, please note that the (integrated elastic)/(integrated quasi-elastic) ratio measured with a given energy resolution is dependent on the scattering vector, Q. The data shown in Fig 1a of our paper are for a large Q value (1.65 $Å^{-1}$). The ratio at a given Q value is not equal to the ratio of respective proton populations. Formally, the population ratio could be obtained from that ratio as Q tends to zero. We estimated the ratio for a Q value of 0.5 $Å^{-1}$ to be between 0.7 and 0.8, above the 55%, as expected.

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5 H. Morgan, M. Ginzburg and B.-Z. Ginzburg, *Biochim. Biophys. Acta*, 1987, **924**, 54.
6 S. Bone, B.-Z. Ginzburg, H. Morgan, G. Wilson and B. Zaba, *Phys. Med. Biol.*, 1996, **41**, 45.
7 B.-Z. Ginzburg and M. Ginzburg, in *Biomembranes*, ed. H. Eisenberg, E. Katchalski-Katzir and L. A. Manson, Plenum, New York, 1975, vol. 7, pp. 219–251.
8 C. Ebel, L. Costenaro, M. Pascu, P. Faou, B. Kernel, F. Proust De Martin and G. Zaccai, *Biochemistry*, 2002, **41**, 13234.
9 D. Madern, C. Ebel and G. Zaccai, *Extremophiles*, 2000, **4**, 91.
10 C. Ebel, P. Faou, B. Kernel and G. Zaccai, *Biochemistry*, 1999, **38**, 9039.
11 S. B. Richard, D. Madern, E. Garcin and G. Zaccai, *Biochemistry*, 2000, **39**, 992.

Dr Patel continued the discussion of the paper by Professor Halle: For halophiles, neutron scattering studies seem to indicate a 2 orders of magnitude slowing down of the hydration water dynamics. In your work, the dynamic perturbation factor is inferred from the high-frequency data in order to avoid contribution from internal water molecules that relax on the time-scale that the protein relaxes. My question is: if the 2 orders of magnitude slowing down in the hydration water dynamics seen by neutron scattering were indeed present, wouldn't its effect be manifest at lower frequencies and be indistinguishable from the signal due to the relaxation of the internal water molecules?

Professor Halle responded: This is a valid concern and it is the reason why it was important to measure the spin relaxation rate down to very low frequencies, even though it turned out that the strong frequency dependence comes from internal water molecules that make up merely ~0.1% of the cell water.[1] If the interpretation of the QENS data[2] were correct, the rotational correlation time for 76% of the cell water (55% of the water in the sample) would be $\upsilon = 250 \times 2.8 = 700$ ps. Even at our highest frequency of 76.8 MHz, $(\omega_0\upsilon)^2 = 0.11 << 1$, which means that these putative water molecules would not give rise to a frequency dependence but would increase R_1 to the same extent over the entire frequency range. At the highest frequency, where internal water molecules do not contribute, we measured $R_1/R_1^\circ = 3.06 \pm 0.02$ (where R_1° is the spin relaxation rate in pure D_2O). According to the QENS interpretation, we should have measured $R_1/R_1^\circ = 0.45 + 0.55 \times 250 = 138$ at this frequency. We therefore conclude that the QENS interpretation is incorrect.

It should be said that the QENS data that led to the proclamation of "extremely slow cell water" in *H. marismortui* were acquired on an instrument that cannot resolve water rotation.[2] The dynamical information was thus extracted in the form of a translational diffusion coefficient in a particular model of spherically confined diffusion. It might be argued that water translation is slowed 250-fold, while water rotation is bulk-like. However, because of the extensive H-bonding in liquid water, translation and rotation are strongly coupled and invariably occur on the same time scale.

1 E. Persson and B. Halle, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 6266.
2 M. Tehei, B. Franzetti, K. Wood, F. Gabel, E. Fabiani, M. Jasnin, M. Zamponi, D. Oesterhelt, G. Zaccai, M. Ginzburg and B.-Z. Ginzburg, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 766.

Dr Zaccai replied: The frequency ranges used in the *H. marismortui* experiments corresponded to time scales from the picosecond to the nanosecond on different spectrometers. The "slow" water was "seen" in the longer time scale. With respect to translational diffusion, internal water molecules would probably appear as immobile on this time scale. Bulk-like water was observed on the spectrometer with the shorter time scale. In *E. coli* there was no "slow" water component observed on the longer time scale spectrometers.[1,2]

1 M. Tehei, B. Franzetti, K. Wood, F. Gabel, E. Fabiani, M. Jasnin, M. Zamponi, D. Oesterhelt, G. Zaccai, M. Ginzburg and B.-Z. Ginzburg, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 766.
2 M. Jasnin, M. Moulin, M. Härtlein, G. Zaccai and M. Tehei, *EMBO Rep.*, 2008, **9**, 590.

Dr Nutt remarked: How do you unify the two apparently opposite effects described in your conclusions (1) and (5), whereby rotation of hydration water is faster at low T around NALMA, but disruption of the tetrahedrality of hydration water interferes with the cooperative rotation of water molecules, slowing rotation?

Professor Halle responded: The picture of water rotation, inspired by computer simulations of bulk water,[1,2] that we use to rationalize our experimental results is as follows. Rotation in bulk water at ambient temperature is fast because high-energy configurations with one or more completely broken H-bonds can be avoided by concerted H-bond interchanges made possible by cooperative rearrangements of several neighboring water molecules. At the interface of an inert solute, a water molecule has fewer water neighbors with which to swap H-bonds and so the cooperative mechanism is impeded. This is the case even if the solute can H-bond with the water molecule, because the bulky solute is less mobile than a water molecule and thus cannot participate in the cooperative rearrangement. Therefore, water rotates more slowly at an interface. This is what we try to summarize in conclusion (5) in our paper. We do not mean to imply that "disruption of tetrahedrality" leads to slower water rotation. On the contrary: when bulk water is (super)cooled, water rotation slows down drastically (in a super-Arrhenius fashion) because the tetrahedral order increases. This ordering interferes with the cooperative rotation mechanism, which requires that a fifth water molecule can approach closely enough to replace one of the four nearest neighbors. For an interfacial water molecule, rotation is also slowed down on cooling. But below a certain temperature, which is 256 K for NALMA and ~260 K for the proteins that we studied, it slows down less than it does in bulk water. The reason for this difference, we believe is that the structure of the solute, which constitutes part of the environment of the interfacial water molecule, hardly changes with temperature. If water rotation in the hydration layer slows down less on cooling than it does in bulk water, there will come a temperature where hydration water actually rotates faster than bulk water. For NALMA, our data indicate that this temperature is 237 K. This is the substance of our conclusion (1).

1 F. Sciortino, A. Geiger and H. E. Stanley, *Nature*, 1991, **354**, 218.
2 D. Laage and J. T. Hynes, *Science*, 2006, **311**, 832.

Dr Ricci remarked: There is evidence from both simulations and experiments that structural and dynamical properties of water are influenced by the presence of solutes[1,2] or the vicinity of a substrate[3,4] well beyond the first hydration shell. Most of these studies are performed on solutions of small electrolites or in confinement within a matrix formed of molecules as simple as SiO_2. How is it possible that, as you say: "the perturbation induced by a biological interface is of short range, essentially limited to the first shell"? In particular this contrasts with the results reported in Professor Havenith's paper in this same volume.[5] Could you please tell us on which evidence your statement is based?

1 R. Mancinelli, A. Botti, F. Bruni, M. A. Ricci and A. K. Soper, *Phys. Chem. Chem. Phys.*, 2007, **9**, 2959.
2 J. Holzmann, R. Ludwig, A. Geiger and D. Paschek, *Angew. Chem., Int. Ed.*, 2007, **46**, 8907.
3 H. Thompson, A. K. Soper, M. A. Ricci, F. Bruni and N. T. Skipper, *J. Phys. Chem. B*, 2007, **111**, 5610.
4 P. Gallo, M. Rovere and E. Spohr, *J. Chem. Phys.*, 2000, **113**, 11324.
5 B. Born, S. J. Kim, S. Ebbinghaus, M. Gruebele and M. Havenith, *Faraday Discuss.*, 2009, DOI: 10.1039/b804734k.

Professor Halle answered: The solute-induced perturbations of water structure and dynamics surely decay gradually and do not vanish identically at any finite distance. A simple Born model estimate indicates that some 200 kJ mol^{-1} of the hydration free energy of a monovalent ion is contributed by water molecules beyond the first hydration shell. Yet, NMR relaxation measurements[1] show that the average rotational correlation time of the water molecules in a 4 molal KCl solution (with 14 water molecules per K^+Cl^- pair) differ by merely a few percent from that in pure water. When discussing the range of solute-induced perturbations, it is thus necessary to specify which solvent property is being considered and at what quantitative level the perturbation can be deemed negligibly small. A 0.2 Å shift of the second peak in the O–O RDF for water molecules outside the first shell of a Na^+ ion, as you found,[2] does not necessarily imply a significant dynamic perturbation. And, unlike the extended silica surfaces examined in two of the other studies that you cite, biological interfaces tend to induce less pronounced solvent layering. Around nonpolar groups, in particular, the boundary between the first and second hydration shells is quite fuzzy (that is, the first minimum in the RDF is shallow and very broad).

We do not imagine that the dynamical perturbation outside the first hydration shell is zero, but we believe that it is much smaller than in the first shell. Thus, if the slowing down is 50% (a typical value) in the first shell, it might be 5% in the second and 0.5% in the third shell. It is then a reasonable approximation to attribute the entire dynamical perturbation to the first shell. This viewpoint is strongly supported by experiments and simulations, see for example ref. 1, 2 and 8–10 in our paper.

1 J. R. C. van der Maarel, D. Lankhorst, J. de Bleijser and J. C. Leyte, *J. Phys. Chem.*, 1986, **90**, 1470.
2 R. Mancinelli, A. Botti, F. Bruni, M. A. Ricci and A. K. Soper, *Phys. Chem. Chem. Phys.*, 2007, **9**, 2959.

Dr Russo commented: In the Fig. 2 in your paper, you show NAGMA and NALMA DPF values as arising from spin relaxation measurement together to QENS data. You find a discrepancy which you attribute to the strong model dependence in the interpretation of the QENS data generalizing this idea to the all QENS technique.

It is true that the QENS NALMA data that you report in Fig. 2 have a strange behavior. The DPF is smaller than 1, which would suggest that pure bulk water

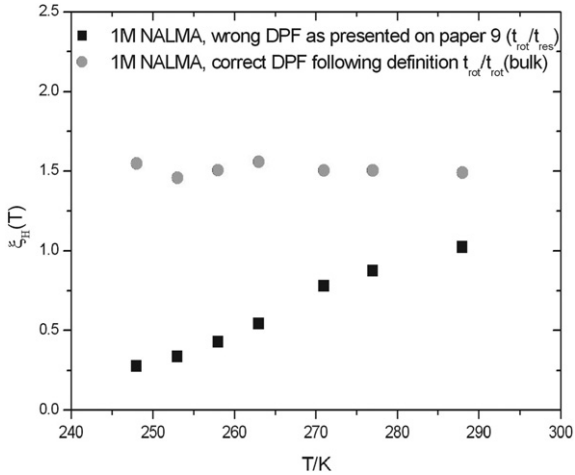

Fig. 1

has a rotational dynamics slower than hydration water. Most likely the problem on the Figure arises from a bad interpretation of the experimental data, from your side, instead of a model-dependence interpretation. Indeed you calculated the DPF factor using the wrong "t_0". Instead to normalize the t_{rot} data reported in the Table 1 of ref. 28 in your paper, for the t_{rot} of bulk water in the same range of temperature ($t_{bulk} = 0.0485 \exp(E_a/kT)$, $E_a = 1.85$ kcal mol^{-1} [1]) it seems that you normalized for the residential time t_0 reported on the same Table. Residential times arise from translational dynamics. In Fig. 1, I plot the QENS DPF as you reported on the paper and the DPF as should have been calculated following the information given in Table 1 of ref. 28 and the paper from Teixeira *et al.*[1] Correlation times are calculated from the following relation: $tE = 0.6582$ (where t is in ps and E in meV), and if we speak in term of the lifetime of the hydrogen bond we divide this result by a factor of 3.[2] Therefore it is not very clear in ref. 28 what the authors calculate and what they call t_{rot}. There is a discrepancy between their Fig. 4 and Table 1, in addition to the fact that the sample has an "undefined" concentration as a consequence of some experimental problems.

In Fig. 2, I report the DPF calculated in a smaller range of temperature for three different concentrations: 1 M, 2 M and 0.5 M.[3,4] The DPF dependence on the NALMA concentration and temperature suggests that only the "really first" hydration water layer has a rotational correlation which is affected from the presence of the biomolecule and that the outer layers (~2–3 shells) are bulk-like. The value reported in Fig. 2 for 2 M NALMA are similar to those that you account for the same peptide on the same range of temperature. No discrepancy seems to appear and most importantly the data presented do not arise from a model.

Can you clearly define what you mean by hydration shell and what you measure for it in your paper? Are your NALMA DPF data comparable with the 2 M NALMA (where there is only one hydration layer is in place), or with the 1–0.5 M NALMA (where ~2–3 hydration layers are in place, and the pair correlation function $g(r)$ is available)? Is your rotational time (NMR) directly comparable with our rotational correlation time (QENS)?

1 J. Teixeira, M.-C. Bellisent-Funel, S. H. Chen and A. J. Dianoux, *Phys. Rev. A*, 1985, **31**, 1913.
2 V. F. Sears, *Can. J. Phys.*, 1966, **44**, 1299.
3 D. Russo, R. K. Murarka, G. Hura, E. Verschnell, J. R. D. Copley and T. Head-Gordon, *J. Phys. Chem. B*, 2004, **108**, 19885.
4 D. Russo, data not yet published.

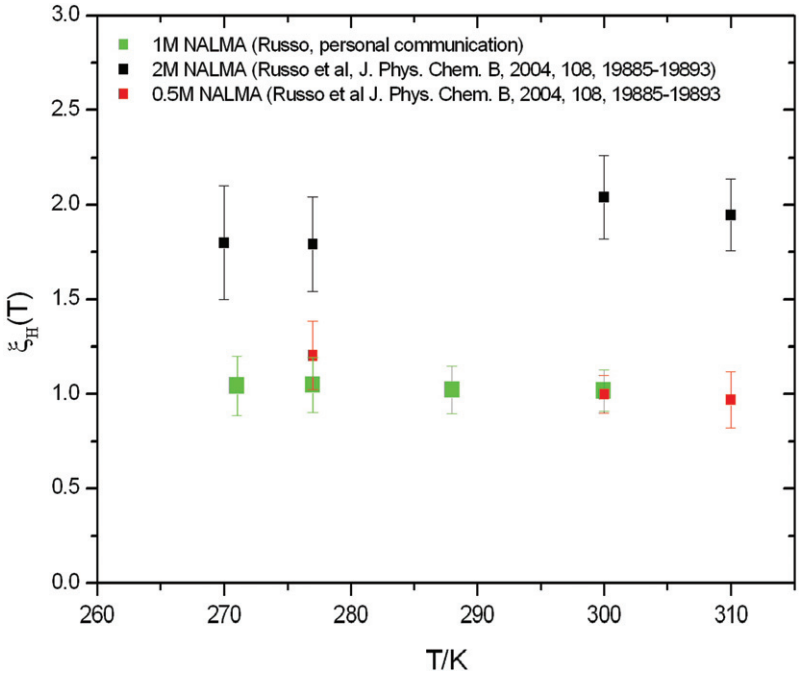

Fig. 2

Professor Halle responded: Your questions relate to Fig. 2 in our paper, which shows the temperature dependence of the dynamic perturbation factor (DPF) ξ_H for the hydration shell of the peptide NALMA. The DPF is obtained from the spin relaxation rate R_1 measured in dilute (<0.2 M) NALMA solutions and that (R_1^0) measured in pure water. In this concentration range R_1 increases linearly with the NALMA concentration, which means that the relaxation enhancement $R_1 - R_1^0$ can be attributed to the solute-induced perturbation of water rotation in the hydration shell of the solute at effectively infinite dilution of solute. These measurements do not allow us to separately determine the degree of rotational slowing down and the spatial range of this perturbation. However, the range of the linear dependence of R_1 on solute concentration in this and many other systems as well as many MD simulations demonstrate that the dynamic perturbation is essentially confined to the first water layer as I explained earlier to Dr Ricci. As a reasonable approximation, we therefore attribute the entire perturbation to the first layer of water molecules. The number of water molecules in the first layer, $\nu_S = 43$ for NALMA, is obtained from MD simulations. The DPF is then obtained directly from the measured data according to eqn (2) in our paper.

The interpretation of the DPF does not rely on a dynamical model (such as rotational diffusion or large-angle jumps), but follows from the rigorous link[1] between R_1 and the integral of the time correlation function $\langle P_2(\cos\theta(t))\rangle$, where $\theta(t)$ specifies the orientation of the O–H bond at time t. MD simulations show that this function drops from 1 to $S_{lib}^2 \approx 0.8$ as a result of subpicosecond O–H bond librations and thereafter decays exponentially with a rotational correlation time υ_R. The integral correlation time, which we denote by υ, is thus given by $S_{lib}^2\upsilon_R$ to an excellent approximation. The DPF is the ratio of the integral correlation time in the hydration shell (υ_H), averaged over the 43 molecules in the shell, and that in pure bulk water (υ_0). Since S_{lib} should be nearly the same in hydration shell and bulk, the DPF can also be regarded as the ratio of υ_R values in the two regions. Further details

about our NMR study of hydration dynamics for NALMA and three other solutes can be found in ref. 2.

Our Fig. 2 also includes a DPF derived from QENS data for a 1 M NALMA solution.[3] As in previous QENS studies[4,5] of peptide solutions, ref. 3 reported a quantity called υ_{rot}, defined as $1/(6D_{rot})$, and deduced from a model of unrestricted, torque-free, isotropic rotational diffusion (eqn (6) in ref. 3). If this model is valid, and if the analysis of the QENS data is correct also in other respects, then the QENS-derived quantity υ_{rot} should be equal, apart from the librational factor S_{lib}^2, to the integral rotational correlation time υ derived from NMR. To calculate a DPF, we need to divide υ_{rot} by the bulk rotational correlation time, υ_{rot}(bulk). Since the latter quantity was not reported in ref. 3 and since both methods should give the same rotational correlation time for bulk water (if the data are properly analyzed), we used the NMR-derived rotational correlation for bulk water, which we denote by υ_0. Unfortunately, the same symbol υ_0 is used for a parameter in the Singwi–Sjölander model[6] that was used to describe the translational part of the scattering function.[3–5] Thus, we did not, as you assume, use υ_0 values from Table 1 in ref. 3 to compute the DPF.

As seen from Fig. 2 in our paper, the DPFs derived from NMR and QENS depend on temperature in opposite ways and differ by an order of magnitude at low temperatures. This discrepancy might be caused by the >5-fold higher solute concentration used in the QENS study, but MD simulations at this concentration agree rather well with the NMR results. Therefore, we conclude that the quantity υ_{rot} derived from QENS data is, in fact, not the rotational correlation time of water. Even for bulk water,[8] the parameter υ_{rot} extracted from QENS data with the same model assumptions as in the peptide work, differs substantially from the rotational correlation time determined by NMR and other methods. In particular, υ_{rot} is found to obey the Arrhenius law with a small activation energy of ~8 kJ mol^{-1},[8] whereas the NMR-derived rotational correlation time has a much stronger temperature dependence with the (apparent) activation energy increasing from 19 to 33 kJ mol^{-1} between +20 and –20 °C. This discrepancy has been recognized for some time,[9,10] but the QENS model has persisted because "it provides an excellent fit to the data".[8] It has been suggested that υ_{rot} reflects "large-amplitude librations" or "hindered rotational diffusion",[9,10] but this is not what the model describes. Furthermore, librations in water occur on time scales <100 fs and are presumably taken into account *via* the Debye–Waller factor. It seems clear, therefore, that the interpretation of QENS data on bulk water remains an open problem. And the models that don't work for bulk water cannot be expected to work for hydration water.

As you point out, it would have been more consistent to calculate the QENS-DPF with υ_{rot}(bulk) values measured by QENS.[8] When this is done, the DPF is 1.5 with no significant temperature dependence. So again there is a qualitative discrepancy with the NMR-DPF. But the important point is that NMR measures the rotational correlation time of water molecules, whereas the standard analysis of QENS data yields a quantity (υ_{rot}) that is model-dependent and so far has not been convincingly linked to anything that can be measured by other experiments or obtained from MD simulations. Other QENS models have been proposed,[11,12] but they are of an empirical nature and therefore cannot resolve the issue.

The strong model-dependence in the interpretation of QENS data from systems as simple as peptide solutions is further illustrated by the conclusion that a large fraction (of order 50%) of the water molecules in 0.5–3 M solutions of NAGMA or NALMA are immobile on time scales up to 40 ps (the time scale corresponding to an energy resolution of 35 μeV).[5] If this were the case, the DPF would be at least an order of magnitude larger than what we find by NMR (where no water is outside the "time window").[2] Furthermore, the immobilized fraction was found to decrease with increasing solute concentration.[5] This unphysical behavior was deduced from small deviations of the (apparent) elastic incoherent structure factor from the Q-dependence of the rotational structure factor. These deviations may be caused by inadequacies of the QENS model at high Q values.

1 A. Abragam, *The Principles of Nuclear Magnetism*, Clarendon Press, Oxford, 1961.
2 J. Qvist and B. Halle, *J. Am. Chem. Soc.*, 2008, **130**, 10345.
3 C. Malardier-Jugroot and T. Head-Gordon, *Phys. Chem. Chem. Phys.*, 2007, **9**, 1962.
4 D. Russo, G. Hura and T. Head-Gordon, *Biophys. J.*, 2004, **86**, 1852.
5 D. Russo, R. K. Murarka, J. R. D. Copley and T. Head-Gordon, *J. Phys. Chem. B*, 2005, **109**, 12966.
6 K. S. Singwi and A. Sjölander, *Phys. Rev.*, 1960, **119**, 863.
7 R. K. Murarka and T. Head-Gordon, *J. Chem. Phys.*, 2007, **126**, 215101.
8 J. Teixeira, M.-C. Bellisent-Funel, S. H. Chen and A. J. Dianoux, *Phys. Rev. A*, 1985, **31**, 1913.
9 M.-C. Bellisent-Funel and J. Teixeira, *J. Mol. Struct.*, 1991, **250**, 213.
10 J. Teixeira, M.-C. Bellisent-Funel and S.-H. Chen, *J. Mol. Liq.*, 1991, **48**, 123.
11 S. H. Chen, C. Liao, F. Sciortino, P. Gallo and P. Tartaglia, *Phys. Rev. E*, 1999, **59**, 6708.
12 L. Liu, A. Faraone and S. H. Chen, *Phys. Rev. E*, 2002, **65**, 041506.

Professor Klein opened the discussion of the paper by Professor Tobias, addressing Professor Halle, Dr Zaccai and Professor Tobias: I have two questions for the three speakers.

Firstly, what are the implications of the rapid exchange dynamics of the water-of-hydration molecules together with their large dehydration energies? These can result in large hydration repulsion effects, as well as in interesting hydration lubrication phenomena (see *e.g.* Raviv and Klein[1]).

Secondly, were large variations in the relaxation times of hydration water molecules observed in the vicinity of multivalent ions?

1 U. Raviv and J. Klein, *Science*, 2002, **297**, 1540.

Professor Halle replied: In response to your first question, just as you contrast the fast hydration-to-bulk water exchange with the large dehydration energy, one could contrast the fast dynamics in bulk water with the large heat of vaporization. In both cases, the implication is that dynamics do not require dehydration. When a water molecule in the bulk liquid or in a hydration layer moves, it does not leave behind a cavity. Rather, the movement is a cooperative process where another water molecule enters the vacated space in a concerted manner. In this way, high (dehydration) barriers are avoided and the motion is therefore fast. However, dehydration energies are relevant to dynamics in two cases. The first is hydration/dehydration of a protein surface from the vapor phase (of limited biological relevance), where the water residence times are very long. The other case concerns secluded hydration sites, such a deep pocket or pore on the surface of a protein.[1] The geometry then prevents the new water molecule from entering the site until the old one has left, giving rise to a high barrier and long residence time. For this reason, the residence time distribution in protein hydration layer is strongly influenced by the surface topography.

Secondly, the water residence times in the hydration shell of monatomic ions (in bulk electrolyte solutions) span some 20 orders of magnitude.[2] For ions at surfaces, less is known. The residence time of water molecules coordinating the protein-bound Ca^{2+} ions in calbindin D_{9k} is in the ns–μs range, much longer than for Ca^{2+} in bulk solution.[3]

1 B. Halle, *Philos. Trans. R. Soc. London, Ser. B*, 2004, **359**, 1207.
2 L. Helm and A. E. Merbach, *Chem. Rev.*, 2005, **105**, 1923.
3 V. P. Denisov and B. Halle, *J. Am. Chem. Soc.*, 1995, **117**, 8456.

Dr Zaccai responded: Regarding your first question, indeed the rapid exchange dynamics of hydration water associated with their large dehydration energies has direct implications for neutron scattering observations. The dehydration energy leads to a time-averaged structural modification of the hydration layer with respect to bulk water that was observed by small angle neutron and X-ray scattering.[1] Rapid

exchange leads to an averaging of the times of residence observed in quasi-elastic neutron scattering.[2]

As to your second, a recent review on water around various ions, measured by neutron scattering, is by Collins *et al.*[3] A QENS paper on water diffusion on the presence of multivalent ions was published in 1987.[4] I note that the biological aspects in this discussion are dominated by proteins. Hydrated multivalent ions play an important role in the stabilisation of nucleic acid structures, and it would certainly be interesting to study the interactions involved in detail.

1 D. I. Svergun, M. H. J. Koch, S. Kuprin, S. Richard and G. Zaccai, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 2267.
2 M. Jasnin, M. Moulin, M. Härtlein, G. Zaccai and M. Tehei, *EMBO Rep.*, 2008, **9**, 543.
3 K. D. Collins, G. W. Neilson and J. E. Enderby, *Biophys. Chem.*, 2007, **128**(2–3), 95.
4 P. S. Salmon, W. S. Howells and R. Mills, *J. Phys. C.: Solid State Phys.*, 1987, **20**, 5727.

Professor Tobias answered: By large dehydration energies I assume you mean that a water molecule near a biomolecule has a much lower free energy than a water molecule in the bulk. Rapid exchange dynamics implies a low free energy barrier to move from next to the molecule to the bulk. I don't know what are the implications for hydration repulsion forces and lubrication phenomena, but I imagine it could be quite different for biomolecules from the case for the flat and rigid mica surfaces employed in the study you referred to, because biomolecular surfaces are rough on the length scale of a water molecule, and mobile on the time scale of water dynamics. We have not included multivalent ions in our simulations, but I would expect large variations based on experimental data (*e.g.* water mobility near calcium dications is orders of magnitude greater than near magnesium dications).

Professor Halle addressed Professor Tobias and Dr Zaccai: In the papers presented by Professor Tobias and Dr Zaccai, a large number of so-called "dynamical transitions" are identified from the temperature dependence of atomic mean-square displacements in hydrated biological systems. These transitions, variously referred to as "inflections" (Professor Tobias' paper) or "breaks in slope" (Dr Zaccai's paper), are not always apparent to the untrained eye. Could you therefore describe the objective quantitative procedure that you used to determine the transition temperature and its associated error, taking the data for nonexchangeable protons in maltose binding protein as an example (shown in Fig. 1A of both papers)?

Professor Tobias replied: So-called dynamical transitions in many proteins have been identified by various experimental techniques (most commonly neutron scattering) and discussed for more than two decades by numerous authors. As far as I am aware, there is no objective quantitative procedure for determining the temperature at which the transition(s) occur. In most cases it is clearly manifested (at least to a trained eye) by a change of slope in a plot of the mean-squared fluctuations *vs.* temperature. The maltose binding protein data plotted in our papers is, of all cases that I have seen, the one where the transition in the protein dynamics is least evident (although a corresponding transition in the water dynamics is more clear). In any case, the dynamical transition is not expected to be sharp, in the sense of a thermodynamic phase transition.

Dr Zaccai responded: We used these terms because of the discussion within the community concerning the nature of the transition. In the simplest approximation, the quantitative definition of the "transition" is where there is an observable deviation from harmonic behaviour. This happens at various temperatures depending on the protein, the energy resolution of and scattering vector range of the spectrometer.

Professor Jungwirth continued the discussion of the paper by Dr Zaccai: Extreme halophiles fill the intracellular region with potassium and the surface of intracellular

proteins has many carboxylate anionic groups. A single COO^- group prefers sodium over potassium. So, is the situation for halophiles a result of massive active pumping of sodium out of the cell or of a special arrangement of carboxylates to prefer potassium (like on the potassium channel) or both of these effects?

Dr Zaccai responded: The work of the Ginzburgs,[1–6] which was at the origin of our neutron study,[7] presented evidence that the high K^+ intracellular concentration was maintained somehow, even when the membrane barrier was diminished in *H. marismortui*. This would be in favour of a special structural arrangements of water and potassium ion "binding" rather than massive pumping.

1 M. Ginzburg, L. Sachs and B.-Z. Ginzburg, *J. Gen. Physiol.*, 1970, **55**, 187.
2 B.-Z. Ginzburg and M. Ginzburg, in *Biophysics of Water*, ed. F. Franks and S. F. Mathias, Interscience, London, 1982, pp. 340–343.
3 B.-Z. Ginzburg, *Thermochim. Acta*, 1981, **46**, 249.
4 H. Morgan, M. Ginzburg and B.-Z. Ginzburg, *Biochim. Biophys. Acta*, 1987, **924**, 54.
5 S. Bone, B.-Z. Ginzburg, H. Morgan, G. Wilson and B. Zaba, *Phys. Med. Biol.*, 1996, **41**, 45.
6 B.-Z. Ginzburg and M. Ginzburg, in *Biomembranes*, ed. H. Eisenberg, E. Katchalski-Katzir and L. A. Manson, Plenum, New York, 1975, vol. 7, pp. 219–251.
7 M. Tehei, B. Franzetti, K. Wood, F. Gabel, E. Fabiani, M. Jasnin, M. Zamponi, D. Oesterhelt, G. Zaccai, M. Ginzburg and B.-Z. Ginzburg, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 766.

Professor Lyashchenko commented: I am a specialist in high frequency dielectric properties of electrolyte and nonelectrolyte solutions so my comment will be from a solution point of view. We have a timescale break between the elementary dynamic processes in bulk water (less than 1 ps) and the water in the biological gel and glass structures (more than 1 ns). I think that we can use the dielectric data of simple aqueous systems for our understanding of this functional difference. There is a very interesting phenomenon in the simple water–electrolyte systems (maybe it is a common case). In the first concentration region with bulk tetrahedral water we have one Debye relaxation process with one relaxation time (about 2–8 ps). There is a concentration boundary around the first zone.[1] In the second concentration zone we have the hydration shells and clusters with a small number of water molecules. This cluster formation is connected with specific concentrations. In this case we have two relaxation processes. The time of relaxation therefore has two values: not only 2 ps (as in hydration shell) but also 20 or 40 ps (for the second process)![2] In addition, it is only in this concentration region with heterogeneous dynamics of water that we have gel and glass formation in water–electrolyte systems. It can be assumed that the concentration of bulk water is the main functional parameter in the case of biological aqueous systems. The absence of bulk water can determine the possibility of gel formation. What do you think about it?

1 A. Lyashchenko, *J. Mol. Liq.*, 2001, **91**, 21.
2 A. Lyashchenko and A. Zasetsky, *J. Mol. Liq.*, 1998, **77**, 61.

Dr Zaccai answered: Certainly, in our current understanding, the specific hydrogen bonding properties of bulk water that for example lead to the so-called hydrophobic effect, are essential for many fundamental biological processes such as protein folding and stabilisation, protein–protein and protein–nucleic acid interactions.

Professor Finney remarked: I'd like to make a further comment on the so-called "dynamical transition", perhaps suggesting it might have served its function as a concept and that it might be more productive to move on to something more sophisticated. From my own experience in obtaining plots of mean square deviation with temperature, locating a temperature at which a "transition" is clearly seen can involve almost an act of faith. In most systems there is a change in gradient as temperature is increased. However, in many cases, this looks like a gradual change in gradient, and to assign a specific temperature at which a gradient change occurs

may not only be unhelpful but could be misleading, putting the results in a conceptual straitjacket. As more measurements of MSD *vs. T* are made on a wider range of systems and over a wider range of conditions, we increasingly see that, if we do try to assign temperatures at which a "dynamical transition" may occur, these can be dependent on sample and timescale probed and more than one "transition" is sometimes found.[1] We should perhaps go beyond the two-well model used to explain the early hydrated myoglobin powder work.[2]

1 R. M. Daniel, R. V. Dunn, J. L. Finney and J. C. Smith, *Annu. Rev. Biophys. Biomol. Struct.*, 2003, **32**, 69.
2 W. Doster, S. Cusack and W. Petry, *Nature*, 1989, **37**, 754.

Professor Finney then continued the discussion of the paper by Professor Halle: The conclusion that water rotation might be faster than in the bulk is interesting but the apparent extrapolation to lower temperature of the DPFs for NAGMA and NALMA in your Fig. 2 are not convincing. Is this conclusion based on other arguments, and if so what?

On another topic, some of the results are dependent on the use of surface accessible area to obtain estimates of the number of water molecules "covering" a solute. In my experience, calculations of numbers of hydration waters based on surface accessible areas can vary over a wide range depending on the assumptions made. Can you say why you think the numbers you obtain for hydration shell water molecules are realistic, and also estimate the likely maximum error in these numbers? And how would this error propagate to affect your later conclusions?

Professor Halle replied: The dynamic perturbation factor (DPF) ξ_H shown in Fig. 2 is the ratio of the average correlation time in the hydration layer $\langle \upsilon_H \rangle$ and the bulk water correlation time υ_0, each of which was measured as a function of temperature. It is evident from these data (only their ratio is shown in Fig. 2) that, at low temperatures, the activation energy is higher in bulk water than in the hydration shell, which means that the DPF decreases on cooling in this range. Since this trend is not likely to be overturned a few degrees below the lowest measured temperature, we can confidently predict that $\xi_H < 1$ at temperatures below ~237 K. As briefly indicated in the Figure legend, we fitted the temperature dependence of $\langle \upsilon_H \rangle$ and υ_0 separately (and over a somewhat wider temperature range). These fits are displayed as Arrhenius plots in Fig. 3 of ref. 1. The curves shown in Fig. 2 of our paper were obtained by dividing these fits. Because $\langle \upsilon_H \rangle$ and υ_0 vary monotonically with temperature, we were brave enough to extrapolate the fitted curves 5–10 K below the 65 K data range.

Regarding the dependence on surface accessible area, the answer is yes: for small molecules (but not for proteins) the solvent accessible surface area (SASA) is rather sensitive to the choice of van der Waals radii. Partly for this reason, we determined the number of water molecules ν_H in the first hydration shell of each solute from MD simulations (1 ns trajectory with ~1700 SPCE water molecules per solute molecule). From simulations of four small solutes,[1] we found that the mean SASA occupied per water molecule, obtained by dividing the SASA (calculated with a standard set of vdW radii and a probe radius of 1.7 Å) by the simulation-based hydration number ν_H, were all within the narrow range 10.65–10.87 Å^2. Moreover, nearly the same value (10.61 Å^2) was obtained from simulations of the protein β-lactoglobulin.[2] It thus appears that accurate hydration numbers can be obtained for small organic solutes as well as for proteins with a "water area" of 10.75 Å^2 and with the SASA calculated as described. These hydration numbers include water oxygens that are inside the first minimum of the RDF for O–O_W (3.3 Å), N–O_W (3.5 Å) and C–O_W (5.0 Å) pairs. As seen from Fig. 1 in our paper, this prescription corresponds closely to the intuitive picture of a first water layer. The simulation-based hydration numbers depend mostly on the C–O_W cutoff, which is not very well-defined since the

RDF minimum is wide and shallow. An uncertainty of ±0.2 Å in this cutoff corresponds to ±10% in ν_H and to ±(3–5)% in ξ_H. This error is probably smaller than the one incurred by attributing the total dynamical perturbation to the first layer as I said earlier in response to Dr Ricci's question

1 J. Qvist and B. Halle, *J. Am. Chem. Soc.*, 2008, **130**, 10345.
2 C. Mattea, J. Qvist and B. Halle, *Biophys. J.*, 2008, **95**, 2951.

Professor Debenedetti continued the discussion of the paper by Professor Tobias: Fig. 6 of your paper and the accompanying discussion refers to "anomalous" diffusion, whereby the mean-squared displacement increases sub-linearly with time. In simulations in simple geometries (*e.g.* water between parallel slabs) one finds that the diffusion is normal in the direction parallel to the slabs (MSD ~ t) but not in the direction normal to the slabs. Have you tried a similar "decomposition" in your simulations?

Professor Tobias answered: The interlamellar space between membranes in purple membrane stacks seems at first glance to share some similarities with the "simple geometries" you refer to, namely the space between parallel, essentially planar slabs. One might therefore expect similarities in the decomposition of the water mean-squared displacement (and corresponding diffusion constant). However, the "slabs" in our case are very rough and chemically heterogeneous on the length scale of a water molecule, and the protein protrusions from the approximately planar membrane matrix presents barriers to lateral diffusion (see Fig. 3 of our paper). Thus, it is indeed very interesting to compare and contrast water dynamics between planar slabs and rough membrane surfaces. We have not done a systematic analysis as a function of temperature and hydration, but we have performed the decomposition of the water mean-square displacement into in-plane and out-of-plane (normal to the membrane surface) components for the purple membrane system with 1924 water molecules at 296 K. The plot shown here (Fig. 3) reveals that the water in the interlamellar space of purple membranes displays anomalous diffusion on the 100 ps time scale (sub-linear temporal evolution of mean-squared displacement) in both the in-plane and out-of-plane directions. Moreover, the plot shows that the diffusion is somewhat anisotropic, because the in-plane component is more than 2/3 of the total.

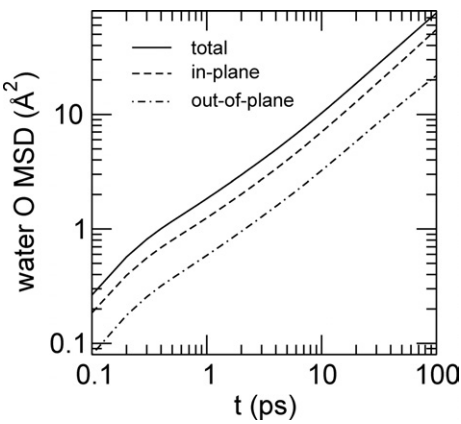

Fig. 3 Mean-squared displacements (MSD) computed from molecular dynamics trajectories of oxygen atoms of hydration water molecules between purple membrane stacks at 296 K. Solid: total MSD (three-dimensional). Dashed: components of the MSD in the plane of the membranes (two-dimensional). Dot-dashed: component of the MSD normal to the plane of the membranes.

Professor Debenedetti addressed Professor Tobias, Professor Halle and Dr Zaccai: In recent years the view has been advocated (by simulations from Stanley's group in Boston,[1] and in experiments by Chen at MIT[1]) that the so-called dynamical transition is associated with a change in water dynamics from super-Arrhenius at high temperature to Arrhenius at low temperature. Do you see evidence for this in your simulations/experiments?

1 P. Kumar, Z. Yan, L. Xu, M. G. Mazza, S. V. Buldyrev, S. H. Chen, S. Sastry and H. E. Stanley, *Phys. Rev. Lett.*, 2006, **97**, 177802.

Professor Halle responded: To make progress in this area, it is first of all necessary to distinguish between (1) bulk water, (2) water in "free" hydration layers of *e.g.* proteins in dilute aqueous solution, and (3) water molecules trapped/confined in (sub-)nanometer spaces in *e.g.* rehydrated solid protein powders or nanoporous silica matrices. The free hydration layer, being in contact with bulk water on one side, is expected to have properties intermediate between those of bulk and confined water. Some authors, including the ones you mention, argue that the physical mechanism underlying the anomalously strong temperature dependence of bulk-water dynamics (with an apparent divergence at 220–230 K) also operate in confined water. We would then reasonably expect this mechanism to prevail also in free hydration layers, where water dynamics differ much less from bulk water than in *e.g.* protein powders. We have recently studied water dynamics over a wide temperature range in the free hydration layers of several proteins (at millimolar concentration).[1] We find Arrhenius-like dynamics in the hydration layer down to at least 238 K (our limit of supercooling), while bulk water dynamics is highly super-Arrhenius in this temperature range. A similar behavior is found for the hydration shells of small organic solutes.[2] Going from bulk to confined water *via* "semiconfined" water (free hydration layer), the temperature dependence thus goes from super-Arrhenius to Arrhenius and back to super-Arrhenius. This nonmonotonic trend suggests that the underlying causes of the super-Arrhenius behavior in bulk and confined water are different. Deviations of the Arrhenius law can occur for many reasons, such as changes in the confining potential or structure. One should also bear in mind that low-temperature confined water is unfreezable rather than supercooled.

1 C. Mattea, J. Qvist and B. Halle, *Biophys. J.*, 2008, **95**, 2951.
2 J. Qvist and B. Halle, *J. Am. Chem. Soc.*, 2008, **130**, 10345.

Dr Zaccai replied: Our experimental work has shown the coincidence of protein and water "dynamical transitions" at about 200 K for the soluble maltose binding protein from *E. coli*.[1] We have to look more carefully at the data with respect to the Stanley and Chen results to which you refer.

1 K. Wood, A. Fröhlich, P. Paciaroni, M. Moulin, M. Härtlein, G. Zaccai, D. J. Tobias and M. Weik, *J. Am. Chem. Soc.*, 2008, **130**, 4586.

Professor Tobias replied: The behavior you refer to has been observed in a plot of the reciprocal of the diffusion constant of hydration water *vs.* the reciprocal of the temperature (see *e.g.* Fig. 3 in ref. 37 of our paper). The calculation of the diffusion constant from the MD trajectories was based on the Einstein relation. Given that the mean-square displacements of hydration water in our purple membrane systems displays a sublinear time-dependence (indicative of anomalous diffusion), at least up to the 100 ps over which we have calculated them, I am hesitant to extract a diffusion constant using the Einstein relation. Another dynamical quantity that could be expected to show the strong/fragile crossover is the relaxation time of the protein–water hydrogen-bonds (see Fig. 13 of our paper). The temperature dependence of this quantity (and the corresponding quantity for protein–lipid hydrogen bonds) reveals a change in behavior at ~240 K, which is close to the temperature of the

