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Abstract
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Keywords
dynamics, cellular, specific, scattering, neutron, vivo, water, nmr, observed, CMMB

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Specific cellular water dynamics observed *in vivo* by neutron scattering and NMR

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Neutron scattering, by using deuterium labelling, revealed how intracellular water dynamics, measured *in vivo* in *E. coli*, human red blood cells and the extreme halophile, *Haloarcula marismortui*, depends on the cell type and nature of the cytoplasm. The method uniquely permits the determination of motions on the molecular length (~ångstrøm) and time (pico- to nanosecond) scales. In the bacterial and human cells, intracellular water beyond the hydration shells of cytoplasmic macromolecules and membrane faces flows as freely as liquid water. It is not "tamed" by confinement. In contrast, in the extreme halophile archaeon, in addition to free and hydration water an intracellular water component was observed with significantly slowed down translational diffusion. The results are discussed and compared to observations in *E. coli* and *Haloarcula marismortui* by deuteron spin relaxation in NMR—a method that is sensitive to water rotational dynamics on a wide range of time scales.

Introduction

Water, which is essential for all known living organisms, has extraordinary properties compared to other liquids that make it a fascinating and important theme to study. Unravelling the structural and dynamic properties of water on the molecular scale constitutes a major scientific challenge. In particular within the cytoplasmic matrix, where the molecular interactions fundamental to biology take place, the properties of intracellular water continue to provoke controversy after more than five decades of study.[1] It is essential, therefore, to underpin statements about these properties with strong experimental data.

Free and confined water in biology

It is general to assume that water in the cell can be either in its free, bulk state or/and confined by macromolecular and membrane surfaces of highly diverse physical chemical nature. The surface of any protein, for example, is not homogeneous with respect to charge density, hydrophobic character, amino acid side-chain packing *etc*. Models that predict water dynamics in confinement are necessarily over-simplifications as well as being specific to a given type of surface (reviewed by ref. 1). In the case of protein–water interactions, the models implicitly assume common behaviour for all soluble proteins, when the main biological property of a protein is its adaptation through selection to fulfil a certain function and activity, *i.e*. to be different from other proteins, including in its surface and other physical and chemical properties.

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Neutrons

Neutrons scatter strongly off the nuclei in H atoms, like billiard balls bouncing off each other to provide important information on water molecular dynamics. Thermal energy neutrons have wavelengths of the order of atomic fluctuation amplitudes and are perfectly suited to probe dynamics in condensed matter. There is a useful trick used in neutron scattering that enabled the study complex molecular systems as well as their interactions with water. Deuterium, an isotope of hydrogen, scatters neutrons with a very different cross-section. By replacing hydrogen by deuterium in components of a complex system, it becomes possible to separate their contributions to the scattering signal. Inelastic neutron experiments to study water dynamics were performed in the late 1950s, close to the very beginning of neutron beam applications to the study of liquids[2,3] and neutrons continue to contribute significantly in the field, the study by ref. 4 being a recent example.

Coherent and incoherent neutron scattering

The effective neutron scattering amplitude of a nucleus depends amongst other parameters also on the spin states of the neutron and nucleus. The effect is particularly strong for the proton. In most experiments, neither the neutron beam nor the sample atoms are polarized, so that their spins take up random orientations. Even for a single type of nucleus, therefore, scattering will take place with different effective amplitudes. This is because part of the scattered beam is coherent and able to interfere with waves scattered by other atoms, and part of it is incoherent and not able to interfere with waves scattered by other atoms. Interestingly, however, in incoherent scattering, the wave is coherent with waves scattered by the *same atom* as

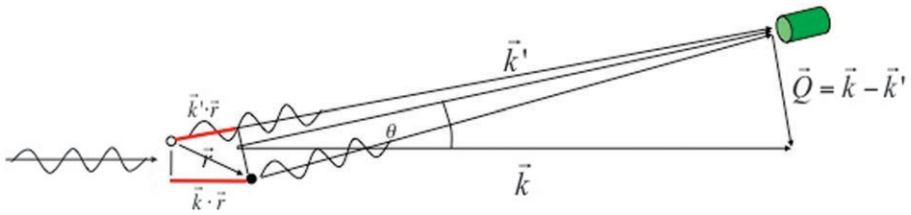

Fig. 1 Single particle scattering. (a) The particle scatters neutron waves, which interfere with each other before detection, as it moves along vector $\boldsymbol{r}$ in a given time period t. $\boldsymbol{k}$ and $\boldsymbol{k}'$ are the incident and scattered wave-vectors, respectively. $\boldsymbol{Q}$ is the scattering vector. The experiment yields information on how far the particle has moved in time t.

it moves in time (Fig. 1). The process provides a powerful tool to observe single atoms as they move in a length-time window, defined by the instrument scattering vector range and energy resolution, respectively. In practice, neutron spectrometers have length-time windows on the ångstrøm to nanometre—pico- to nanosecond scale. The proton, which has the largest incoherent cross-section, dominates the scattered signal. It is highly informative on the internal molecular dynamics of proteins, because H atoms are quite homogeneously distributed in the structure, and in the observed length-time windows, they move with the larger groups to which they are bound, such as amino acid side chains, for example (review by ref. 5). Thermal linear and rotational diffusion motions of water molecules are also within the neutron scattering length-time scales.

Quasi-elastic neutron scattering (QENS)

In QENS, the intensity is plotted as a function of scattering vector modulus, Q, and energy transfer, E. The information is obtained from B, the broadening on the E scale, compared to the instrument-resolution-defined width of the incident beam. In the case of diffusive motion described by an exponential function in time, B has a Lorentzian shape as a function of energy. In wave scattering, energy and time are related by Fourier transformation. The Lorentzian mathematical function is the Fourier transform of the exponential function. B may be fitted by a set of Lorentzians of different widths, Γ, describing diffusive motions on different time scales. The behaviour of each Γ value with Q is subsequently interpreted in a model approach.[6]

Scattering in the elastic window

The elastically scattered intensity measured as a function of Q contains information on the geometry of the motions occurring within the time defined by the energy resolution of the spectrometer. This is expressed as a mean square displacement (MSD) within this time window for all single atoms contributing to the scattering. An effective force constant is calculated from the MSD plotted as a function of temperature.[7]

NMR and neutron scattering

NMR and neutron scattering are complementary for the study of dynamics at the atomic level.[8] The spin relaxation rate of ^{2}H nuclei or ^{17}O in isotopically labelled water, when measured for a wide magnetic field range reveals water dynamics on a wide range of time scales, from an overlap with neutron scattering in the pico- to nanosecond range to the much slower microsecond range. The method is sensitive to the rotation of the water molecule in the bulk and when it is interacting with solutes and surfaces.[9,10] The neutron scattering contribution of our laboratory towards understanding in the complex world of biological water has been to focus on experimental, quantitative measurements of water dynamics in different biological environments, and to correlate them to specific biological function. To do this we determine fluctuation amplitudes and diffusion coefficients in various time-scales by neutron spectroscopy, which as outlined above for QENS, lead to the evaluation of diffusion coefficients. By using specific deuterium labelling, we separate contributions from different sample components—for example, the incoherent neutron scattering from a sample of bacteria grown in fully deuterated culture medium and then soaked in natural abundance H_2O will be strongly dominated by water molecule motions and informative on the intracellular water dynamics, in spite of the huge complexity of the system. Neutron scattering and NMR are arguably the only methods that can provide atomic length and time scale information on water dynamics *in situ* in biological environments.

In this perspective article

We present neutron scattering results from our laboratories on water dynamics in *E. coli*,[11] human red blood cells (RBC) and haemoglobin (Hb)[12,13] and the extreme halophile archaeon first isolated from the Dead Sea, *Haloarcula marismortui* (*Hmm*)[14] and discuss them[15] with reference to work by NMR on cell water by Persson and Halle.[9] When we consider the importance of water for biological interactions at the molecular level,[1] the requirement and importance of such experimental data on its intracellular structure and dynamics become self-evident.

Neutron scattering experimental results

Spectrometers

Instruments used for QENS experiments were IN16 at ILL, energy resolution 0.9 μeV, corresponding to a time scale of 1 ns, IRIS at the ISIS spallation neutron source at the Rutherford Laboratory (energy resolution of 17 μeV, corresponding to a time scale of ~40 ps (http://www.isis.stfc.ac.uk/instruments/iris/) (IN6 at ILL (energy resolution of 90 μeV, corresponding to a time scale of ~8 ps, (http://www.ill.eu/instruments-support/instruments-groups/instruments/in6/)), TOFTOF at the Munich FRM2 reactor, energy resolution of 100 μeV, similar to IN6 at 5.1 Å (http://www.frm2.tum.de/en/science/spectrometry/toftof/index.html), and FOCUS at PSI

(energy resolution of 50 μeV, time scale ~13 ps (http://spectroscopy.web.psi.ch/focus/)). Elastic scattering was analysed on IN13 at ILL, energy resolution of 8 μeV corresponding to a time scale of ~100 ps (http://www.ill.eu/instruments-support/instruments-groups/instruments/in13/). The spectrometer used in each study is given in the caption of the corresponding results figure.

Water in *E. coli*

E. coli of a strain that grows well under deuterated conditions was cultivated with deuterated glycerol as the carbon source. Recall that the neutron scattering signal of H is much stronger than that of D. It was important, therefore, to replace H with D in all the cellular components except for water so that water motions would dominate the scattering. *E. coli* water dynamics was studied in deuterated cell pellets resuspended in H_2O and D_2O buffers, respectively. Cells were washed in H_2O or D_2O buffer, and pelleted to reduce strongly the quantity of extracellular water in the sample that was found to represent less than 7% of the total water (see ref. 11 for details). The pellets were transferred to aluminium sample holders ($4 \times 3 \times 0.02$ cm^3), and sealed with an indium 'O' ring for the neutron measurements. The water scattering signal was obtained to a good approximation by subtracting the spectra of D_2O samples from those of the corresponding H_2O sample, scaled by the sample mass, and other appropriate corrections. The QENS measurements were carried out on the IRIS and IN6 neutron spectrometers, to cover diffusive motions from those of interfacial water to pure water.[11] Similar water translational diffusion parameters were extracted on the two spectrometers, by using a jump diffusion model that describes diffusion between sites for the water protons with a mean residence time at each site.[6] Data are in Fig. 2.[11] Translational diffusion coefficients were found to be very close to those of pure water at corresponding temperatures, with residence times about twice longer.[16] The higher residence times may reflect the longer times spent by the protons in the first hydration shells of internal cellular structures. The rotational parameters were extracted on IN6 at two temperatures. The rotational correlation times were close to the values extracted for the buffer under the same conditions and of the same order as the values measured for pure water. We concluded, therefore, that proton exchange between water layers take place with the diffusion rates of pure-like water.

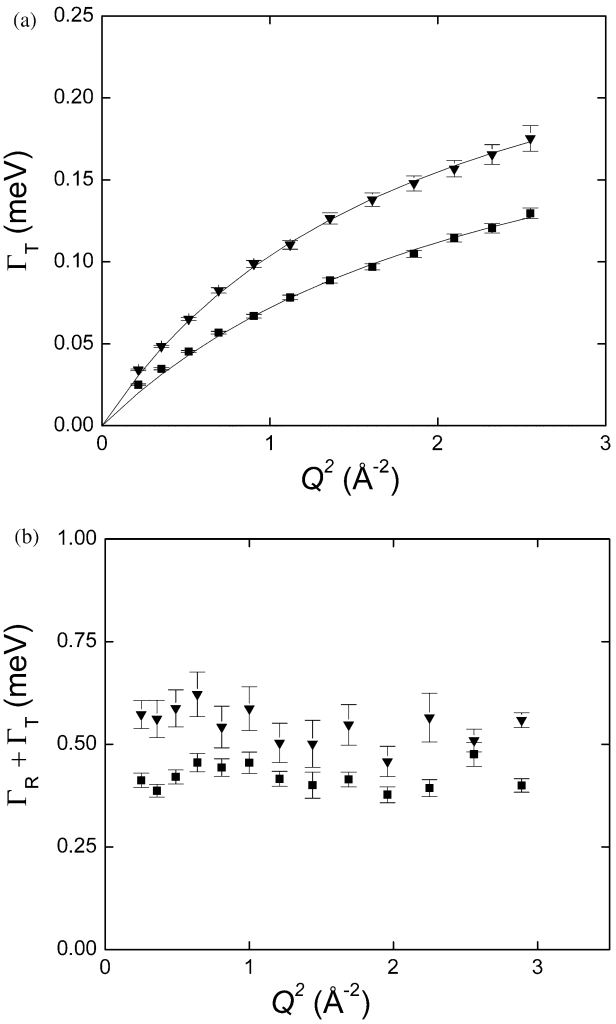

Fig. 2 (a) IRIS data. Half-width at half-maximum of the translational Lorentzian, Γ_T, as a function of Q^2, at 281 K (filled black squares) and 301 K (filled black triangles). Γ_T was best fitted (solid lines) using a jump diffusion model.[6] We found $D_T = (1.53 \pm 0.05) \times 10^{-5}$ cm^2 s^{-1}, $\tau_0 = 2.63 \pm 0.11$ ps at 281 K and $D_T = (2.39 \pm 0.05) \times 10^{-5}$ cm^2 s^{-1}, $\tau_0 = 2.16 \pm 0.05$ ps at 301 K. (b) IN6 data. Half-width at half-maximum of the Lorentzian, $\Gamma_R + \Gamma_T$, arising from both rotational and translational motions, as a function of Q^2, at 281 K (filled black squares) and 301 K (filled black triangles). The rotational correlation times, $\tau_{cor,R} = 1/\Gamma_R$, were extracted from the Lorentzian after correction for the broadening arising from the translational contribution, and found to be 1.96 ± 0.01 ps at 281 K, and 1.54 ± 0.01 ps at 301 K.

Water in Hmm, an extreme halophilic archaeon

Extreme halophilic archaea live in salt lakes and saline ponds in essentially saturated salt water; they counterbalance the osmotic pressure due to the external multimolar NaCl concentration by accumulating multimolar KCl in their cytoplasm. *Hmm* were originally isolated from the Dead Sea. The very existence of these organisms is proof of the adaptation of Life to highly reduced water activity. Previous work by certain of the authors of the[14] article, led to the conclusion that *Hmm* cells contain water in two distinct dynamic states: a ~24% state characteristic of a 3.5 M NaCl solution, and one ~76% fraction involving water with a significantly lower rate of mobility. A direct determination of intracellular water dynamics in *Hmm* was therefore attempted by QENS by using fully deuterated cultures of *Hmm* hydrated in H_2O. Deuterated *Hmm* cells were grown at 37 °C to an optical density of 0.8–1 (late logarithmic phase) in a standard medium in which yeast extract was replaced by deuterated algal extract produced at the Max Planck Institut fur Biochemie (Martinsried, Germany). For the neutron scattering experiments, the cells were pelleted in either D_2O or H_2O buffer (see ref. 14 for details). Measurements were performed on IN6 and IN16, respectively to cover time-scales from 10 ps to 1 ns respectively (Fig. 3). From IN6 (Fig. 3a), a translational diffusion constant of 1.3×10^{-5} cm^2 s^{-1} was determined at 285 K, close to the

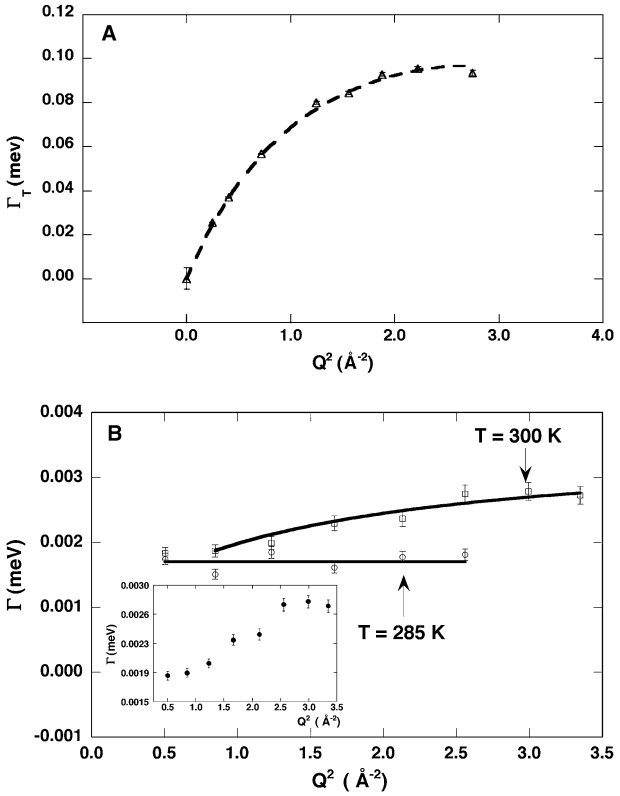

Fig. 3 (A) IN6 data. Half-widths at half-maximum Γ of the translational motions of water as a function of Q^2 at $T = 285$ K. The dashed line results from the fit using Singwi and Sjolander model.[6] A translational diffusion constant of 1.3×10^{-5} cm^2 s^{-1} was extracted from the fit. (B) IN16 data. Half widths at half maximum Γ as a function of Q^2 at $T = 285$ K and $T = 300$ K. At 300 K, the bold solid line results from the fit using the jump diffusion model[6] from $Q_0^2 = 0.9$ Å^{-2}. (*Inset*) A zoom-in on the data obtained at 300 K. At $T = 285$ K, the water protons displayed a residence time of 411 ps. At 300 K, the residence time dropped to 243 ps and was associated with a translational diffusion of 9.3×10^{-8} cm^2 s^{-1}.

value measured for water in the 3.5 M NaCl solution bathing the cells. A very slow water component was discovered from the IN16 data and attributed to translational motion of water (Fig. 3b). The water protons of this component displayed a residence time of 411 ps (compared with a few ps in bulk water). At 300 K, the residence time dropped to 243 ps and was associated with a translational diffusion of 9.3×10^{-8} cm^2 s^{-1}, or 250 times lower than that of bulk water. No such slow component was found in *E. coli*, measured on the BSS back-scattering spectrometer at Jülich (energy resolution of 0.9 μeV, similar to IN16). This instrument is no longer available since its host reactor has been shut down.

Water in red blood cells

Cell water dynamics in RBC was measured on IRIS, FOCUS and TOFTOF at FRM-II.[12] As fully deuterated RBC are not available, natural abundance RBC in H_2O buffer and in D_2O buffer were measured separately, and the water dominated contribution to the scattering was obtained by subtraction. RBC are highly specialised cells. They do not contain organelles, a nucleus or nucleic acid, and their main constituent is Hb, which constitutes 92% of their dry weight. The concentration of Hb in RBC is 330 mg ml^{-1}, which corresponds to a volume fraction of 0.25. After centrifugation, the extracellular water content of the human RBC preparation was found by weighing and comparing to fully dry material to be less than 10% of the total water content assuming a cellular Hb concentration of 330 mg ml^{-1}.[12] H_2O/D_2O exchange was obtained by repeated washing in the appropriate saline buffer. The QENS analysis revealed two populations of water in RBC: a major fraction of ~90% with dynamical properties similar to those of bulk water (time scale ~ps) and a minor fraction of ~10%, interpreted as bound hydration water with significantly slower dynamics (time scale ~40 ps) (Fig. 4). The translational diffusion coefficient of cellular water is nearly identical to H_2O buffer on all spectrometers (Fig. 4a). The dashed line indicates normal temperature behaviour of H_2O buffer following an Arrhenius law and serves as reference. As with the *E. coli* samples, residence times were found to be larger in intracellular than in bulk water. A fraction of about $10 \pm 2\%$ immobile water that is absent in H_2O buffer was identified in RBC from the QENS analysis (Fig. 4b). On the ~10 Å length scale of the measurement, confining effects of protein surface cavities or boundaries on water become observable, and the immobile fraction was attributed to water

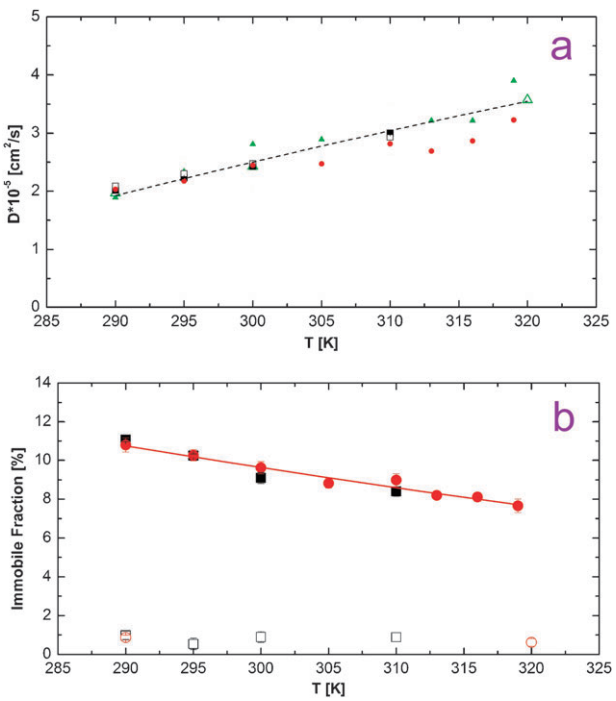

Fig. 4 (a) Translational diffusion coefficient, D, of H_2O in RBC measured on IRIS (filled black squares), FOCUS (filled red circles) and TOFTOF (filled green triangles); H_2O buffer was measured on the instruments IRIS (empty black squares) and TOFTOF (empty green triangles). The dashed line indicates normal temperature behaviour of H_2O buffer. (b) Percentage of immobile fraction of H_2O in RBC measured on IRIS at $q = 0.61$ Å^{-1} (filled black squares) and FOCUS at $q = 0.55$ Å^{-1} (filled red circles) are compared to values of H_2O buffer measured on IRIS (empty black squares) and FOCUS (empty red circles). The straight red line represents Arrhenius behaviour. From ref. 12.

that is dynamically bound to Hb surfaces. From the known Hb concentration inside RBC, we calculated that 10% of the total cytoplasmic water corresponds to ~50% of the hydration layers, in agreement with hydrodynamic experiments on the percentage of strongly bound hydration water.[17]

Hydration dependent Hb dynamics

QENS data on Hb dynamics in hydrated powders, solutions and RBC were measured on TOFTOF and FOCUS.[13,18] Elastic scattering data were measured on IN13. In the QENS experiments, global macromolecular diffusion is suppressed in the hydrated powder sample and not observable with the energy resolutions of the time-of-flight spectrometers in the concentrated Hb solutions. The QENS from the protein in these samples was interpreted in terms of its internal dynamics. In the RBC sample, global macromolecular diffusion contributed to the measured intensities and the scattering function used in the interpretation included a term for global Hb diffusion. The experiments revealed that increasing hydration of Hb has a strong influence on the rates of diffusive internal motions. The obtained activation energy of the residence times rises from 1.45 ± 0.18 kcal mol^{-1} for Hb solution to 1.70 ± 0.12 kcal mol^{-1} for hydrated Hb powder. The MSD of Hb dynamics in hydrated powders, solutions and RBC (all in D_2O buffer) were measured by elastic scattering on IN13 (Fig. 5a). Clearly, there is an effect on protein dynamics due to the increasing dilution, even beyond full hydration of the Hb surface, including a contribution from global diffusion. The diffusion coefficient of Hb has been measured as a function of concentration by ref. 19. At 350 g l^{-1} and 20 ± 0.2 °C (293 K) the value given is about 0.7×10^{-7} cm^2 s^{-1}. Its contribution to the MSD observed on IN13 (in a time window of 100 ps) is estimated at

$$0.7 \times 10^{-7} \times 10^{16} \times 10^{-10} = 0.07 \text{ Å}^2$$

The value is significantly smaller than the MSD difference between each concentration value in Fig. 4a, suggesting that increased dilution also increases the MSD due to internal protein motions on the 100 ps time-scale. In this range, an approximately linear influence of temperature on both MSD and global diffusion is expected, as seen in Fig. 5a. The effective force constants or resilience corresponding to the MSD temperature dependence are shown for the different Hb sample conditions in Fig. 5b. They indicate that associated with the increasing MSD seen in Fig. 5a, there is a softer response to temperature with increasing dilution.

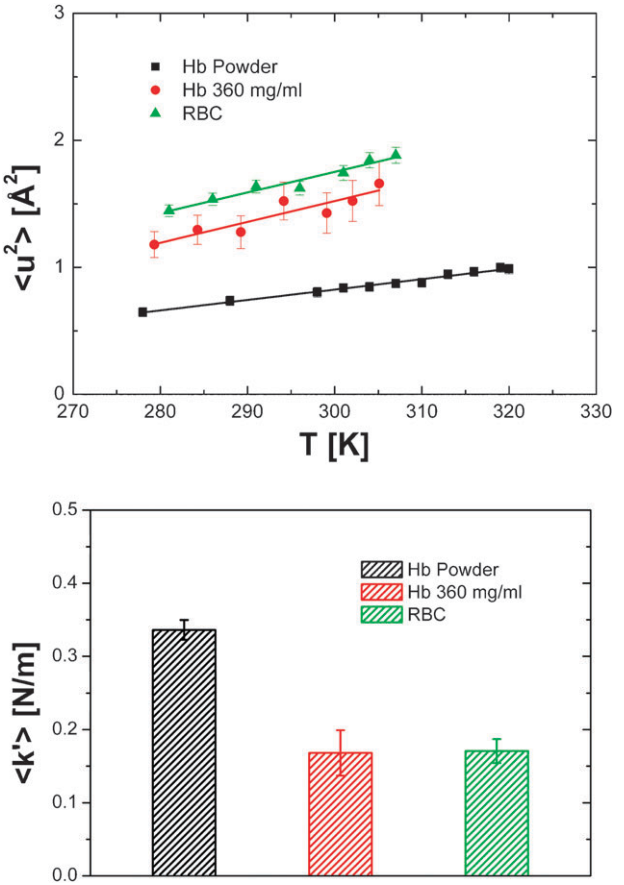

Fig. 5 (a) MSD of Hb in various solutions and hydrated powder (all in D_2O buffer) measured as a function of temperature on the IN13 spectrometer. (b) Effective force constants calculated from the temperature dependence of the MSD in (a).

NMR experimental results

Cell water in *E. coli* and *H. marismortui*

NMR experiments on cell water dynamics are described by Persson and Halle in ref. 9. These authors measured the deuteron (^2H) spin relaxation rate in living *E. coli* and *Hmm* cultured in heavy water (D_2O), in the magnetic field range 0.2 mT to 12 T. A model-independent analysis allowed them to characterise water dynamics *in situ* in the organisms on a wide range of time scales from below the nanosecond to above the microsecond. The intracellular environment is highly heterogeneous and water molecules were expected to rotate with a wide range of rates depending on how they interact with macromolecules, membrane surfaces and small solutes. The Larmor frequency of nuclear spin is proportional to applied magnetic field. In the ground breaking NMR study, the applied magnetic field was varied over five orders of magnitude in order to examine the magnetic relaxation dispersion (MRD) of the water nuclides, ^2H and ^{17}O, corresponding to the full range of water rotation rates. Careful calculations to take into account the composition of the samples and control experiments, comparing results from ^2H and ^{17}O allowed to justify that the signals were dominated by intracellular water.

Persson and Halle found similar water dynamics results for *E. coli* and *Hmm*: approximately 85% of cell water in both organisms displayed bulk-like rotational dynamics. The remaining approximately 15%, which was assumed to interact directly with biomolecular surfaces was found to be retarded in its rotation rate by an average factor of 15 ± 3, corresponding to a rotational correlation time of 27 ps. The observed dynamic perturbation was three times larger than for small monomeric proteins in solution, a difference that was attributed to secluded surface hydration sites in supramolecular assemblies. The relaxation data on the microsecond time scale, show that

a small fraction (~0.1%) of cell water exchanges from buried hydration sites, consistent with other observations on protein hydration in solutions and crystals.

Discussion

At a recent workshop on molecular dynamics in biology, we were asked if we were not bothered to have published contradictory results on water diffusion in cells. At first we did not understand to what the person asking the question was referring. He explained that in 2007 in ref. 14 we claimed that intracellular water in the extreme halophiles showed a major, strongly slowed down fraction; then, a year later, in ref. 11, we published that water dynamics in *E. coli* was mainly bulk-like. We realised the difficulties of interdisciplinary research. The person showing interest in our work was a physical chemist. Like others before him, notably those who study general aspects of protein dynamics in a few favourite 'model' proteins such as myoglobin or lysozyme, he assumed that intra-cellular water should behave in the same way in different cells. In biology it is the differences between systems and their specificity that is interesting, like the different structure and dynamics of proteins that evolved and have been selected for different biological function and activity in a given environment. The environmental aspect is essential because of the sensitivity of biomolecules to conditions, such as salt composition and concentration of the aqueous solvent, pH, temperature and pressure. The intracellular environments of *Hmm* and *E. coli* are so different that there is no reason to expect similar water dynamic behaviour in the two cell types. Considering the inside of a cell is already very crowded with macromolecules, which occupy close to 30% of its volume, what is the expected state of the water in the intracellular environment of the extreme halophiles compared to that in the bulk liquid? There are about 55 moles in a kilogramme of water. In a six molal solution of a fully ionised monovalent salt, therefore, there are 4.5 water molecules per ion. Ions may be coordinated by six water molecules, so that each and every water molecule has at least one ion as one of its nearest neighbours, leading to significantly reduced water activity. In order to estimate the influence of the solvent ions on the intracellular water dynamics in the extreme halophiles, the diffusion coefficients of water was measured by QENS in molal solutions of KCl and NaCl.[15] The results showed clearly that molar concentrations of either NaCl or KCl hardly affected the diffusion coefficient of water, and it was concluded that the high salt concentration in the cytoplasm of the extreme halophilic archaea, was not responsible by itself for the observed very slow water component. Proteins from the extreme halophilic archaea had been shown to display exceptional salt and water binding, necessary for their stabilisation and activity.[20] Hydrate salt-ion binding stabilisation is also reflected in halophilic protein dynamics, with significant differences between behaviour in high concentrations of KCl and NaCl.[21] A hypothesis to be verified is that the very slow water in *Hmm* is due to specific macromolecular structures that would also be responsible for the large amount of K^+ bound within the extreme halophile cells. The absolute requirement of *Hmm* for a high salt environment and its ability to bind potassium ions specifically then would appear to be closely related to the low mobility of water in these cells. Halophilic proteins have an excess of negative charge in carboxylic groups on their surface.[20] Interactions may be similar to those of structured water around potassium ions and protein carboxylic groups observed by ref. 22 in the potassium channel protein.

Water diffusion in *E. coli* and RBC was found to be similar, establishing that in these cells water, other than a confined fraction of about 10% corresponding to the macromolecular hydration shells, essentially flowed as freely as bulk water. A similar conclusion has been reached using NMR spectroscopy on *E. coli*.[9] Samples were essentially identical to the ones used in the neutron scattering experiments, except that only the water molecules and not cellular components were deuterated in the NMR study. Persson and Halle[9] also presented data on *Hmm* showing identical water rotational dynamics to that in *E. coli*, pointing out an inconsistency with the neutron study of Tehei *et al.*[14] As explained in the experimental section above, however, NMR is sensitive to re-orientational water motions, while QENS can measure translational and rotational diffusion separately. (See for example the study of water rotation and translational diffusion around hydrophobic and hydrophilic sites by Russo *et al.*[23]). Rotation rates for the fast water fraction in *Hmm* were found to be only slightly slowed down compared to bulk water.[14] We believe the results from NMR and QENS are complementary rather than inconsistent. In the hypothesis presented in the previous paragraph, the NMR and QENS results would suggest that water 'bound' to the halophilic carboxyl–salt ion structures would find their translational diffusion inhibited, with only slightly affected re-orientational motions.

Without hydration water, proteins would neither fold correctly nor acquire the conformational flexibility, which permits biological activity. Hydration dependent protein dynamics has been studied and discussed extensively in several systems mainly as hydrated powders, including myoglobin,[24] ribonuclease A,[25] deuterated maltose binding protein,[26] and deuterated phycocyanin.[27] There is a consensus concerning protein water dynamic coupling, and the requirement for a certain level of hydration for the protein to display the dynamical transition at about 200 K. The experimental neutron data on dynamics of Hb from hydrated powder to solution and inside RBC, however, led to the conclusion that protein dynamics, in this system, continues to evolve beyond one hydration layer. Dilution corresponding to up to three layers of water on the protein surface enhances the rates of diffusive internal motions, perhaps by lowering the activation barriers between conformational substates.[28] A similar conclusion on the dilution dependence of protein dynamics was reached by Jasnin *et al.*[29] from *in vivo* neutron measurements of internal and global macromolecular motions in *E. coli*. There is a growing body of theoretical chemistry and vibrational spectroscopy studies on hydration of amino acids and small peptides that are amenable to computational analysis (*e.g.* ref. 30), which provides hope for a future understanding of the role of water and hydration interactions within the complex intracellular chemical environment. The review of this work, however, is beyond the scope of the present

perspectives article, which is focused on the experimental characterisation of intracellular water dynamics in prokaryotes.

NMR and neutron experimental approaches to study water are based on hydrogen isotope labelling. Hydrogen and deuterium bonds have different energy profiles, which affects protein dynamics and stabilisation, as shown, for example, in a neutron scattering study of *E. coli* intracellular macromolecular dynamics.[31] For the same reason, water dynamics in H_2O and D_2O are expected to be different. At physiological temperatures, where the studies were performed, these differences are not expected to be significant with respect to the uncertainty in the experimental results. This is supported by the observation of similar rotational diffusion observed, on one hand, by NMR in *E. coli* samples containing D_2O and by neutron scattering, on the other, in *E. coli* samples containing H_2O.

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