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ORIGINAL PAPER

Solvent isotope effect on macromolecular dynamics in *E. coli*

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Abstract Elastic incoherent neutron scattering was used to explore solvent isotope effects on average macromolecular dynamics in vivo. Measurements were performed on living *E. coli* bacteria containing H_2O and D_2O, respectively, close to physiological conditions of temperature. Global macromolecular flexibility, expressed as mean square fluctuation (MSF) values, and structural resilience in a free energy potential, expressed as a mean effective force constant, $\langle k' \rangle$, were extracted in the two solvent conditions. They referred to the average contribution of all macromolecules inside the cell, mostly dominated by the internal motions of the protein fraction. Flexibility and resilience were both found to be smaller in D_2O than in H_2O. A difference was expected because the driving forces behind macromolecular stabilization and dynamics are different in H_2O and D_2O. In D_2O, the hydrophobic effect is known to be stronger than in H_2O: it favours the burial of non-polar surfaces as well as their van der Waals' packing in the macromolecule cores. This may lead to the observed smaller MSF values. In contrast, in H_2O, macromolecules would present more water-exposed surfaces, which would give rise to larger MSF values, in particular at the macromolecular surface. The smaller $\langle k' \rangle$ value suggested a larger entropy content in the D_2O case due to increased sampling of macromolecular conformational substates.

Keywords Neutron scattering · In vivo macromolecular dynamics · H_2O/D_2O isotope effect · Global macromolecular flexibility · Structural resilience · Hydrophobic effect

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Introduction

Water is the solvent of biological systems, without which macromolecules cannot fold and function correctly. Heavy water (D_2O) is often used in biophysical studies to replace H_2O to prevent signal contamination by water H nuclei (NMR and neutron scattering) or to study exchange or other isotope effects (mass spectrometry, FTIR etc.). The solvent environment plays a fundamental role in macromolecular stability, through hydration, hydrogen bonds, van der Waals interactions, ion binding and the hydrophobic effect. These weak interactions govern structure formation and atomic fluctuations, which occur in the pico- to nanosecond (ps–ns) time-scale and act as the lubricant of slower, millisecond, conformational changes necessary for biological activity (Brooks et al. 1988). The achievement of a good understanding of macromolecular stability is therefore intimately correlated to the understanding of dynamics. Elastic incoherent neutron scattering (EINS) spectroscopy is a technique uniquely suited for the measurement of atomic fluctuations in the ps–ns time-scale, on

samples that need not be crystalline or even monodisperse. The atomic fluctuations in proteins have been explored successfully in hydrated powders and in solutions using EINS (Doster et al. 1989; Ferrand et al. 1993; Gabel et al. 2004; Paciaroni et al. 2002; Tehei et al. 2001, 2005, 2006; Tehei and Zaccai 2007). Tehei et al. 2004 have performed EINS measurements on bacterial cells adapted to extreme temperatures, and explored global macromolecular dynamics. Their results led to the hypothesis that evolution had selected the forces required for macromolecular stability and activity at physiological temperatures. The present study addressed solvent isotope effects on macromolecular dynamics in vivo, by direct incoherent neutron scattering measurements on living *E. coli*.

H_2O and D_2O molecules are close in properties, with respect to their shape, size, bond lengths and dipole moment. They differ, however, in their vibrational frequencies due to their differences of mass and moments of inertia (Conway 1981; Némethy and Scheraga 1964). As a result, the deuterium bond is 0.24 kcal mol^{-1} of higher energy than the hydrogen bond, which leads to stronger D_2O–D_2O deuterium bond interactions, as well as stronger hydration-bond interactions in D_2O. The hydrophobic effect is enhanced in D_2O, which results from the stronger association between D_2O molecules (Parker and Clarke 1997) and leads to a lower solubility of apolar groups. The hydrophobic effect has long been regarded as one of the major driving forces behind macromolecular folding and stabilisation (Baldwin 2007). While an entropic contribution brings together apolar groups in the macromolecule core to minimize water-exposed surfaces, van der Waals' packing of apolar groups in the core leads also to an enhanced enthalpic contribution. The differences in properties between H_2O and D_2O are, therefore, expected to influence macromolecular stabilisation and dynamics. In vitro studies have reported various results on the stabilizing influence of D_2O on proteins (Bonneté et al. 1994; Chakrabarti et al. 1999; Efimova et al. 2007; Kuhlman et al. 1998; Maybury and Katz 1956; Parker and Clarke 1997; Tehei et al. 2001); in few cases, stability can be unchanged or lower (Kern et al. 1980; Makhatadze et al. 1995). Sasisanker et al. have shown that proteins adopt a more compact form in D_2O (Sasisanker et al. 2004). In contrast to the wealth of data on protein stability, the role of solvent isotope substitution on protein dynamics has not been thoroughly examined. The few studies carried out to date in solution or in hydrated powder samples, have reported that dynamics-stability relationships were complex. Heavy water can increase protein rigidity (Cioni and Strambini 2002; Tehei et al. 2001) as well as reduce it (Tehei et al. 2001). Tehei et al. have revealed that higher stability was not necessarily associated to higher resilience (Tehei et al. 2001). Furthermore, although a few in vitro studies have been reported (Cioni and Strambini 2002; Gabel et al. 2004; Tehei et al. 2001), the isotope effect on dynamics remains unexplored in the cellular environment. In the present work, we measured macromolecular flexibility and resilience in living *E. coli* containing H_2O or D_2O, to explore the isotope effect on the average macromolecular behaviour in the cell interior.

Experimental

Sample preparation

E. coli (BLE21 (DE3) strain) were cultivated at 37°C to an optical density of two, in Enfors minimum growth medium with glycerol as the carbon source. Cells were pelleted by centrifugation at 5,000 rpm in a Beckman centrifuge (JLA10500 rotor) for 20 min at 4°C. The supernatant was discarded and the cells were washed twice with 100 ml of H_2O or D_2O buffer solution (150 mM NaCl, 5 mM KCl, 10 mM Tris–H(D)Cl pH 6.6). The cells were pelleted via 20 min centrifugation and transferred to aluminium sample holders ($4 \times 3 \times 0.03$ cm^3) for the neutron measurements. After the experiments, a small amount of the pellet was resuspended in the buffer and layered on Petri dishes after several dilutions steps. The number of colonies was compared to that obtained for cells from the fresh culture, and found to be similar, which indicated that most of the cells remained intact and viable after the total beamtime exposure.

Neutron measurements and data analysis

Elastic incoherent neutron scattering

Hydrogen nuclei have an incoherent cross section about 40 times larger than that of any other nucleus or isotope, and dominate the neutron scattering signal. The dynamics of hydrogenated components can, therefore, be explored in complex macromolecular systems such as living cells (Tehei et al. 2004). Hydrogen atoms represent about 30–50% of the atoms present in macromolecules, and are uniformly distributed in the structure. On the appropriate length and time domains, hydrogen fluctuations reflect the motions of the side chain and backbone atoms to which they are bound (Smith 1991). The length and time windows of the accessible motions are determined by the wave vector transfer modulus Q range and the energy resolution of the spectrometer, respectively. At a given temperature, the analysis of the elastic incoherent scattering signal yields a value of the mean square fluctuation amplitude (MSF), $\langle u^2 \rangle$. The motions that are too fast or too slow as

compared to the accessible time domain do not contribute to the scattering signal. As pure solvent diffuses in a time-scale two orders of magnitude shorter than macromolecular thermal motions, the measurements can be performed in H_2O in an appropriate length-time window, without contamination by water scattering (Tehei et al. 2006). Macromolecules make up ca. 96% of the total dry weight of *E. coli*, of which about 55% are proteins. Considering that the hydrogen percentage is higher in proteins compared to every other type of macromolecules, it is reasonable to assume, therefore, that protein thermal motions dominate macromolecular scattering signal at 75–80%.

Neutron scattering experiments and analysis

The neutron experiments were carried out on the back-scattering spectrometer IN13, Institut Laue Langevin (ILL, Grenoble; see http://www.ill.fr for further information). The full-width at half-maximum (FWHM) energy resolution defines the upper limit of the associated time-scale of motions. IN13 has a FWHM = 8 μeV and is sensitive to motions that occur in a time-scale up to about 0.1 ns. The elastic incoherent neutron scattering signal was measured and analysed over $1.27\ \text{Å}^{-1} < Q < 1.87\ \text{Å}^{-1}$ ($Q = 4\pi \sin\theta/\lambda$ for elastic scattering, where 2θ is the scattering angle and λ is the incident neutron wavelength). The elastic intensities were corrected for sample holder and buffer scattering, normalised to a vanadium sample (a purely elastic scatterer) and corrected for sample absorption by using the IN13 data reduction program ELASCAN (information on the program is available on the ILL web site at http://www.ill.fr). For each sample, the elastic intensity $I(Q, \theta \pm \Delta\omega)$ was obtained as a function of temperature, T, rising from 279 to 315 K, and ln $I(Q, \theta \pm \Delta\omega)$ was plotted against Q^2. The mean square fluctuation amplitude (MSF), $\langle u^2 \rangle$, was calculated from the slope of the straight-line fit to the experimental data according to the Gaussian approximation (Zaccai 2000):

$$I(Q, 0 \pm \Delta\omega) = \text{constant} \times \exp(-1/6\langle u^2 \rangle Q^2) \quad (1)$$

and linearised as:

$$\ln I(Q, 0 \pm \Delta\omega) = \text{constant} - 1/6\langle u^2 \rangle Q^2 \quad (2)$$

The approximation is valid for localized motions of any shape, provided $\langle u^2 \rangle\ Q^2$ is smaller or equal to 2. The validity range can be extended, however, to significantly higher $\langle u^2 \rangle\ Q^2$ values in the case of certain asymmetric motions such as ellipsoidal motions with axial ratios 1:1:a, with $0.6 < a < 1.7$, which could be reasonable approximations for H displacements in protein internal motions within the IN13 length-time window. The value of the root MSF quantifies the global flexibility. It contains contributions from both diffusive and vibrational motions, whose amplitudes remain within the length-time window defined by the instrumental Q range and energy resolution (Gabel 2005). Because of the time-scale resolution of the IN13 spectrometer, the macromolecular global diffusion within the living cells contribution to the MSF values is expected to be small compared to the internal motions contribution (Gabel 2005; Tehei et al. 2001). Furthermore, the H_2O solvent contamination to the scattering signal is restricted to Q values lower than 1 Å^{-1} (Tehei et al. 2006). The mean resilience, $\langle k' \rangle$, which corresponds to a mean effective force constant and defines the average resilience in a free energy potential (Bicout and Zaccai 2001; Zaccai 2000), was extracted from the slope of $\langle u^2 \rangle$ as a function of T, using the following relation:

$$\langle k' \rangle = \frac{0.00276}{\mathrm{d}\langle u^2 \rangle / \mathrm{d}T} \quad (3)$$

Results and discussion

The fits from which the MSF values were extracted are shown in Fig. 1. The MSF are plotted as a function of temperature in Fig. 2, for cells in H_2O (triangles) and in D_2O (squares). MSF from cells in H_2O are above those from cells in D_2O, with mean values of about 2 and 1.5 Å^2, respectively. Note that macromolecules have larger amplitude motions in H_2O than in D_2O.

The mean resilience values were extracted from linear fits performed on the MSF data, using Eq. 3. A smaller slope corresponds to a larger resilience and vice versa. The mean resilience defines the average macromolecular resilience for all macromolecules inside the cell, mostly dominated by the protein fraction. Resilience is 0.38 N·m^{-1} for cells in H_2O, compared to 0.19 N·m^{-1} for cells in D_2O. The value in H_2O is in good agreement with the previous value found by Tehei and collaborators for *E. coli* (MRE600 strain) in H_2O, 0.42 N·m^{-1} (Tehei et al. 2004). The result established that *E. coli* macromolecules have more resilient structures in H_2O than in D_2O; they are "softer" in D_2O.

The lower mean resilience value found for *E. coli* macromolecules in D_2O is consistent with a previous in vitro study, in which the flexibility of mesophilic and halophilic proteins have been compared (Tehei et al. 2001). The mesophilic bovine serum albumin protein, BSA, was found to be six times less resilient in D_2O than in H_2O; the lower resilience in D_2O was coupled to higher stability through entropic effects arising from increased sampling of conformational substates in D_2O. In contrast, the halophilic malate dehydrogenase from *Haloarcula marismortui*, was more resilient and more stable in D_2O. Tehei et al. have

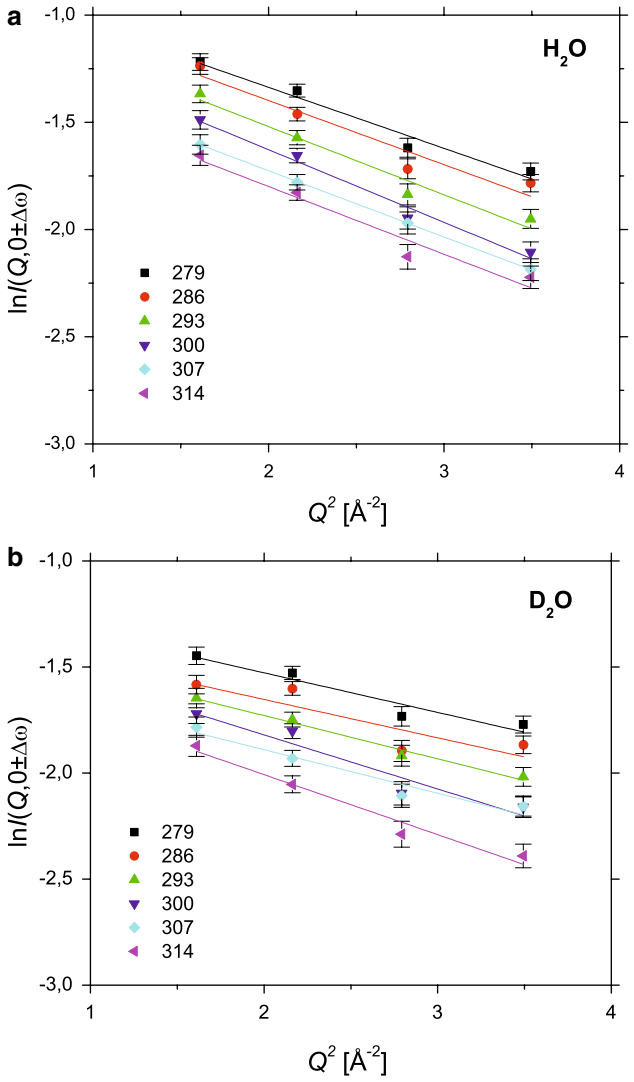

Fig. 1 Scattered normalised intensities plotted as ln $I(Q, \theta \pm \Delta\omega)$ as a function of Q^2 from cells in H_2O (**a**) and D_2O (**b**). Linear fits were performed over the ranges 1.6 $Å^{-2} < Q^2 < 3.5$ $Å^{-2}$ using Eq. 2

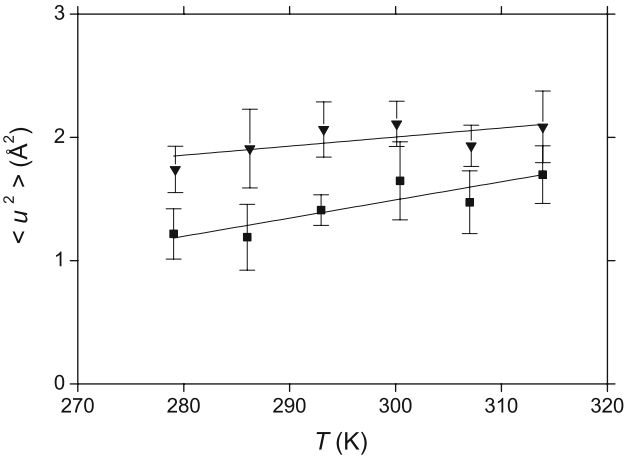

Fig. 2 MSF as a function of temperature, from cells in H_2O (*triangles*) and cells in D_2O (*squares*). Linear fits (*bold lines*) were performed between the temperature range 279–314 K using Eq. 3

proposed that, in the case of the halophilic malate dehydrogenase, stabilization in D_2O is dominated by the strong hydration bonds (Tehei et al. 2001). Their prediction was in good agreement with the large proportion of acidic residues present at the protein surface, and their known interactions with solvent ions and water. In *E. coli*, considering that both motion amplitudes and resilience are smaller in D_2O (Fig. 2), we suggested that the effect in D_2O could arise from the dominance of the hydrophobic effect, resulting in the burial of non-polar surface groups as well as more compact van der Waals' packing in the protein core. In contrast, in H_2O, stronger resilience associated to larger fluctuation amplitudes, suggested a bigger role of hydration bond interaction in dynamics. Because hydrophobic effect is lower than in D_2O, macromolecules in H_2O have the freedom to expose more surfaces to the solvent. Considering that surface amino acids do not pack very tightly (Gerstein and Chothia 1996) and that fluctuations are larger at the macromolecule surface than in the core (Pérez et al. 1999), an increase in water exposed surface in H_2O is in good agreement with the higher amplitudes found in H_2O. It should be mentioned, however, that the increased flexibility measured in the H_2O case can arise partly from the contribution of exchangeable hydrogens exposed to the solvent, which present fluctuation amplitudes accessible on the IN13 time and length domains. In D_2O, such hydrogens have been exchanged with deuterium atoms and do not contribute to the MSF values. We should discuss, furthermore, an eventual viscosity effect when the solvent is changed from H_2O to D_2O. In dilute solution, the viscosity is a factor about 1.25 higher in D_2O, which remains constant in the temperature range of the measurements. Such a viscosity difference would affect both internal and self-diffusion macromolecular dynamics contributions to the MSF values in dilute solution (Gabel 2005). Applying the ratio to the data in Fig. 2, would not influence the slopes and resilience values; in contrast, it would bring the MSF values for the two solvent conditions closer together. The *E. coli* cytoplasm, however, is a complex environment very far from a dilute aqueous solution; it is expected to have an internal viscosity that is strongly influenced by a high concentration of macromolecules as well as smaller organic solutes (Ellis 2001). We cannot exclude that there still exists a small viscosity change between H_2O and D_2O, which may influence MSF values slightly, as was discussed for concentrated solutions of malate dehydrogenase in high salt (Tehei et al. 2001). However, as explained above, the important point is that it would not change the difference in resilience observed between the H_2O and D_2O conditions.

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References

Baldwin RL (2007) Energetics of protein folding. J Mol Biol 371:283–301

Bicout DJ, Zaccai G (2001) Protein flexibility from the dynamical transition: a force constant analysis. Biophys J 80:1115–1123

Bonneté F, Madern D, Zaccai G (1994) Stability against denaturation mechanisms in halophilic malate dehydrogenase "adapt" to solvent conditions. J Mol Biol 244:436–447

Brooks CL, Karplus M, Pettitt BM (1988) Proteins: a theoretical perspective of dynamics, structure, and thermodynamics. Adv Chem Phys 71:1–249

Chakrabarti G, Kim S, Gupta ML Jr., Barton JS, Himes RH (1999) Stabilization of tubulin by deuterium oxide. Biochemistry 38:3067–3072

Cioni P, Strambini GB (2002) Effect of heavy water on protein flexibility. Biophys J 82:3246–3253

Conway BE (1981) Ionic hydration in chemistry and biophysics. In: Studies in physical and theoretical chemistry, vol 12. Amsterdam

Doster W, Cusack S, Petry W (1989) Dynamical transition of myoglobin revealed by inelastic neutron scattering. Nature 337:754–756

Efimova YM, Haemers S, Wierczinski B, Norde W, van Well AA (2007) Stability of globular proteins in H_2O and D_2O. Biopolymers 85:264–273

Ellis RJ (2001) Macromolecular crowding: obvious but underappreciated. Trends Biochem Sci 26:597–604

Ferrand M, Dianoux AJ, Petry W, Zaccai G (1993) Thermal motions and function of bacteriorhodopsin in purple membranes: effects of temperature and hydration studied by neutron scattering. Proc Natl Acad Sci USA 90:9668–9672

Gabel F (2005) Protein dynamics in solution and powder measured by incoherent elastic neutron scattering: the influence of *Q*-range and energy resolution. Eur Biophys J 34:1–12

Gabel F, Weik M, Doctor BP, Saxena A, Fournier D, Brochier L, Renault F, Masson P, Silman I, Zaccai G (2004) The influence of solvent composition on global dynamics of human butyrylcholinesterase powders: a neutron-scattering study. Biophys J 86:3152–3165

Gerstein M, Chothia C (1996) Packing at the protein-water interface. Proc Natl Acad Sci USA 93:10167–10172

Kern D, Zaccai G, Giege R (1980) Effect of heavy water substitution for water on the tRNAVal-valyl-tRNA synthetase system from yeast. Biochemistry 19:3158–3164

Kuhlman B, Luisi DL, Evans PA, Raleigh DP (1998) Global analysis of the effects of temperature and denaturant on the folding and unfolding kinetics of the *N*-terminal domain of the protein L9. J Mol Biol 284:1661–1670

Makhatadze GI, Clore GM, Gronenborn AM (1995) Solvent isotope effect and protein stability. Nat Struct Biol 2:852–855

Maybury RH, Katz JJ (1956) Protein denaturation in heavy water. Nature 177:629–630

Némethy G, Scheraga HA (1964) Structure of water and hydrophobic bonding in proteins. IV. The thermodynamic properties of liquid deuterium oxide. J Chem Phys 41:680–689

Paciaroni A, Cinelli S, Onori G (2002) Effect of the environment on the protein dynamical transition: a neutron scattering study. Biophys J 83:1157–1164

Parker MJ, Clarke AR (1997) Amide backbone and water-related H/D isotope effects on the dynamics of a protein folding reaction. Biochemistry 36:5786–5794

Pérez J, Zanotti JM, Durand D (1999) Evolution of the internal dynamics of two globular proteins from dry powder to solution. Biophys J 77:454–469

Sasisanker P, Oleinikova A, Weingärtner H, Ravindra R, Winter R (2004) Solvation properties and stability of ribonuclease A in normal and deuterated water studied by dielectric relaxation and differential scanning/pressure perturbation calorimetry. Phys Chem Chem Phys 6:1899–1905

Smith JC (1991) Protein dynamics: comparison of simulations with inelastic neutron scattering experiments. Q Rev Biophys 24:227–291

Tehei M, Madern D, Pfister C, Zaccai G (2001) Fast dynamics of halophilic malate dehydrogenase and BSA measured by neutron scattering under various solvent conditions influencing protein stability. Proc Natl Acad Sci USA 98:14356–14361

Tehei M, Franzetti B, Madern D, Ginzburg M, Ginzburg BZ, Giudici-Orticoni MT, Bruschi M, Zaccai G (2004) Adaptation to extreme environments: macromolecular dynamics in bacteria compared in vivo by neutron scattering. EMBO Rep 5:66–70

Tehei M, Madern D, Franzetti B, Zaccai G (2005) Neutron scattering reveals the dynamic basis of protein adaptation to extreme temperature. J Biol Chem 280:40974–40979

Tehei M, Daniel R, Zaccai G (2006) Fundamental and biotechnological applications of neutron scattering measurements for macromolecular dynamics. Eur Biophys J 35:551–558

Tehei M, Zaccai G (2007) Adaptation to high temperatures through macromolecular dynamics by neutron scattering. FEBS J 274:4034–4043

Zaccai G (2000) How soft is a protein? A protein dynamics force constant measured by neutron scattering. Science 288:1604–1607