The effect of water on protein dynamics

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Neutron diffraction and spectroscopy were applied to describe the hydration and dynamics of a soluble protein and a natural membrane from extreme halophilic Archaea. The quantitative dependence of protein motions on water activity was clearly illustrated, and it was established that a minimum hydration shell is required for the systems to access their functional resilience, i.e. a dynamics state that allows biological activity.

Keywords: neutron; dynamical transition; hydration shell; halophilic malate dehydrogenase; bacteriorhodopsin; purple membrane

1. INTRODUCTION (THOUGHTS ABOUT LIFE... SALT WATER AND NEUTRONS)

The central dogma of molecular biology is presented in figure 1. The cell is the fundamental component of all life forms. Genetic information, encoded in cellular DNA, is passed from one generation to the next by *replication* of the mother molecule into two daughters identical to herself. For the information in the DNA to be acted upon, it is first *transcribed* into RNA. The dogma is quite open to change. In the first scheme, the flow of information was in the DNA to RNA direction only, then *reverse transcription* was discovered in retroviruses, and the white arrow in figure 1 was added. The RNA, in turn, is *translated* into proteins, the molecular machines, which assume the vital specific functions required to maintain the cell alive: enzyme or receptor activities, small molecule or ion transport and pumping, etc.

Each process of *replication*, *transcription* and *translation* is, in fact, a highly catalysed and finely controlled process, involving a large set of proteins. Replication is catalysed by DNA polymerases. Various repressor and activator proteins, transcription factors and DNA-dependent RNA polymerases control and catalyse transcription. Ribosomes, large protein–RNA molecular machines, are the main seats of translation, which also brings into play aminoacyl-tRNA synthetases, tRNA, and several initiation, elongation and other factors.

The control and regulation of gene expression represents an essential function of proteins, which they ensure, in large part, through specific protein–nucleic acid interactions. The protein products of the DNA to RNA to protein scheme in figure 1, therefore, are unavoidable requirements for all steps of the scheme: replication, transcription and translation. A logical consequence is that the scheme represents a current *end product* of evolution. Because it is found in all known organisms, from the prokaryotic Archaea and Bacteria to human beings, it follows

that we are all already highly evolved, and require the full set of macromolecules, ions, small molecules and *water*, to be alive. There are no known examples of really primitive life forms, in the sense that they predate the rules of the central dogma, and we can only speculate about them.

The question put by the Discussion Meeting organizers refers to whether or not *water* is necessary for life. As was discussed in the previous paragraph, we are certain of the existence of only one type of life, which has evolved by adapting to the environment to a point where it has many apparent fundamental requirements that have to be supplied by this environment, including carbon compounds, sodium and potassium ions and, of course, water. So, what is the question? Is it: are there alternative life forms, perhaps on other planets, that could do without water? Or, is it: can life on Earth, as we know it, do without water? The first is a fascinating question, but certainly beyond the competence of at least the author of this paper. The second question can be addressed by examining how living organisms fare in dry environments and the effect of water on molecular events involved in life processes.

From an anthropomorphic viewpoint, living organisms have adapted to some very strange environments: cold polar and glacier waters, very hot volcanic springs, high pressures in deep ocean trenches, saturated salt ponds. Such environments are called extreme, and the organisms living in them are called extremophiles. They are subdivided into psychrophiles (cool-loving), barophiles (pressure...), halophiles (salt...), thermophiles (heat...) and hyperthermophiles (even hotter...).

In *Voyage of H.M.S. Beagle*, Charles Darwin wrote: ‘Parts of the lake seen from a short distance appeared of a reddish colour, and this perhaps was owing to some infusorial animalcula... How surprising it is that any creatures should be able to exist in brine, and that they should be crawling among crystals of sulphate of soda and lime! ... Thus we have a little living world within itself, adapted to these inland lakes of brine...’.

Salt lakes and saline works ponds are often tinted red by the halophilic organisms that thrive in their hypersaline waters (Oren 1998). The main pigment is β -carotene, which enters the food chain and is responsible, for

One contribution of 16 to a Discussion Meeting Issue ‘The molecular basis of life: is life possible without water?’.

Table 1. Neutron energy, wave number, and frequency equivalents and wavelength corresponding to ambient temperature. ($1 \text{ \AA} = 1 \times 10^{-10} \text{ m.}$)

T (K)	energy ($\text{kJ mol}^{-1}/\text{kcal mol}^{-1}$)	energy (meV)	wavenumber (cm^{-1})	frequency (s^{-1})	wavelength ($\text{\AA}$)
300	2.49/0.59	25.8	208	6.2×10^{12}	1.8

Figure 1. The central dogma of molecular biology.

example, for the pink colour of flamingos and salmon. The extreme halophilic Archaea counterbalance the osmotic pressure due to the external multimolar NaCl concentration, by accumulating multimolar KCl in their cytoplasm. Considering the inside of a cell is already very crowded with macromolecules, which occupy close to 30% of its volume, it is probable that the state of water inside halophilic Archaea is very different from that of the bulk liquid. There are *ca.* 55 mol in a kilogram of water. In a six molal solution of a fully ionized 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 greatly reduced water activity.

Does the existence of extreme halophiles prove that if life has adapted to reduced water activity it may be able to do away with it all together, or, on the contrary, that they represent the limit for a minimum requirement of water, below which no adaptation is possible? The second proposal seems to be the most likely, because not only is there no evidence of a life form adapted to the total absence of water, but also organisms that are exposed to very dry conditions have evolved mechanisms to go into suspended animation during drought periods and to recover their usual metabolic state when water becomes available again (Cordone *et al.* 1999; Tehei *et al.* 2002, and references therein).

Life forms seem to have colonized almost all ecological niches possible. However, these environments all have in common that they contain water in its liquid state; environments at temperatures above 100°C , for example, are at high pressure. We should be careful, however, before jumping to firm conclusions. The fact that a life form that grows in the absence of water has not been discovered (yet!) does not prove that it does not exist.

Thermal energy neutrons have wavelengths close to $1 \text{ \AA}$, matching inter-atomic distances in condensed matter (table 1). Experiments using neutron radiation, therefore, provide simultaneous information on the amplitudes and energies of atomic motions. Neutrons have been privileged probes of the structure and dynamics of water in all its states, because they are sensitive to the positions and fluctuations of the hydrogen atoms in the molecule, as well as

of the oxygen atom (Bellissent-Funel *et al.* 1996; Bon *et al.* 1999, 2002). The neutron scattering cross-sections of hydrogen (H) and its heavy isotope deuterium (D), in contrast to the X-ray case, are different from each other and significantly comparable with those of the heavier elements. Neutron-scattering experiments, using $\text{H}_2\text{O} : \text{D}_2\text{O}$ exchange and/or specific H : D isotope labelling, have allowed the description of hydration structures associated to membranes and proteins (Zaccai & Gilmore 1979; Svergun *et al.* 1998; Bon *et al.* 1999), and of internal macromolecular dynamics associated with thermal fluctuations (Doster *et al.* 1989; Ferrand *et al.* 1993; Andreani *et al.* 1995; Daniel *et al.* 1999; Fitter 1999; Paciaroni *et al.* 1999; Réat *et al.* 2000; reviewed in Smith 1991; Gabel *et al.* 2002; Tehei *et al.* 2004). Neutron experiments led to the characterization in many proteins of the dynamical transition between harmonic and non-harmonic states, which had been discovered by Mössbauer spectroscopy in myoglobin (Parak *et al.* 1982).

Neutron diffraction and spectroscopy were applied to describe the hydration and dynamics of a soluble protein and a natural membrane from extreme halophiles. The results, by correlating dynamics and structural information on hydration, allowed the exploration of the effect of water on protein dynamics.

2. MATERIAL AND METHODS (A HALOPHILIC PROTEIN, PURPLE MEMBRANES AND NEUTRON SCATTERING)

Hm MalDH, the malate dehydrogenase from the halophilic archaeon *Haloarcula marismortui* is, currently, the most-studied and best-characterized halophilic protein (Madern *et al.* 2000). It is obtained pure in relatively large quantities after expression in *E. coli* (in a non-active state) and renaturation by dialysis against high salt. It was the first protein found to belong to the tetrameric lactate dehydrogenase-like malate dehydrogenase (LDH-like MalDH) family, a newly discovered enzyme family.

The PM of *Halobacterium salinarum* is formed of a highly ordered two-dimensional lattice of archaeal specific lipids and the retinal binding protein, BR (Grigorieff *et al.* 1995; Oesterhelt 1998). BR functions as a light-driven proton pump. Its activity can be followed quite sensitively by spectroscopic methods because it is associated with a millisecond photocycle of protein conformational changes and retinal colour changes. PM samples are easily purified in large quantities from *H. salinarum* cultures, because of their relative rigidity compared with the rest of the membrane fractions.

EINS experiments provide information on mean-square fluctuations in a sample as a function of temperature (Zaccai 2000). The time-scale examined (0.1 ns on IN13, and 1 ns on IN16 at the Institut Laue Langevin) depends on the energy resolution of the spectrometer, whereas the scattering vector range in which the scattered intensity is measured defines the length scale

Figure 2. Quantifying internal forces in myoglobin. Mean square fluctuations ($\langle u^2 \rangle$) and effective force constants (k and k'), from neutron-scattering experiments in the ($\leq 1 \text{ \AA}^2$, 0.1 ns) length-time window. The data on hydrated myoglobin (triangles) and on myoglobin in a trehalose glass (circles) are from Doster *et al.* (1989) and Cordone *et al.* (1999), respectively. The force constant calculations are from Zaccai (2000).

(down to less than $1 \text{ \AA}$ for IN13 and *ca.* $1 \text{ \AA}$ for IN16). Plots of mean-square fluctuations as a function of absolute temperature for hydrated myoglobin and the protein in a trehalose glass, measured on IN13, are shown in figure 2. Mean-square fluctuations are in absolute $\text{\AA}^2$ units ($1 \text{ \AA} = 1 \times 10^{-10} \text{ m}$). The break denoting the dynamical transition in the hydrated sample is clearly seen between 180 and 200 K. The low-temperature regime is harmonic as shown by the linear dependence of $\langle u^2 \rangle$ on T . An effective dynamic resilience expressed as an elastic force constant (in newtons per metre) can be calculated from the inverse of the slope of $\langle u^2 \rangle$ versus T (Zaccai 2000; Bicout & Zaccai 2001). The dynamical transition is associated with a significant decrease in resilience.

3. RESULTS (HYDRATION SHELLS, STABILITY, ACTIVITY AND DYNAMICS)

The structure and dynamics of water at the vicinity of a macromolecular surface are strongly perturbed with respect to the bulk liquid state (Bellissent-Funel *et al.* 1996). It has been shown from small angle neutron and X-ray experiments, that hen egg white lysozyme and other soluble proteins are each surrounded by a $3 \text{ \AA}$ hydration shell (corresponding, on average, to a monomolecular layer), which is *ca.* 10% denser than bulk water (Svergun *et al.* 1998). This result has been simulated successfully (Merzel & Smith 2002) and is believed to be valid for all soluble proteins, at least those from mesophiles.

The halophilic *Hm* MalDH tetramer dissociates and unfolds in 'low' salt concentration, and is stable only in solutions containing higher than 2 M KCl or NaCl concentrations. Careful hydrodynamic and scattering measurements have established that the stable folded enzyme, in contrast to the mesophilic proteins, is surrounded by a solvation shell made up of water associated

Figure 3. Mean-square fluctuations as a function of temperature, measured by neutron scattering in the ($\leq 1 \text{ \AA}^2$, 0.1 ns) length-time window on IN13 at ILL (details at www.ill.fr/YellowBook/IN13) for *Hm* MalDH in various solutions (circles, 2 M NaCl- D_2O ; inverted triangles, 2 M KCl- D_2O ; diamonds, 2 M NaCl- H_2O). From Tehei *et al.* (2001).

with a high local concentration of salt ions, similar to the multimolar concentration in the solvent (Ebel *et al.* 2002). The solvation shell and specific ion-binding sites (seen in the crystallographic structure of the enzyme) participate in stabilizing the folded protein structure as well as maintaining its solubility in the high salt environment (Madern *et al.* 2000; Costenaro *et al.* 2002; Irimia *et al.* 2003). The internal thermal dynamics of *Hm* MalDH in different solvents was examined by EINS on the IN13 spectrometer (figure 3). The results indicate a strong correlation between the stability of the protein, the nature of its solvation shell, and the dynamical resilience of its structure in the temperature range of 280–320 K (7–50 °C; figure 4). The resilience is highest (0.5 N m^{-1}) in NaCl- D_2O , the solvent in which *Hm* MalDH is most stable; it is 0.2 N m^{-1} in KCl- D_2O , in which the protein is less stable than in NaCl- D_2O , and 0.1 N m^{-1} in NaCl- H_2O , in which, out of the three solvent conditions tested, it is the least stable (Tehei *et al.* 2001).

PM activity, hydration and dynamics have been measured in membrane stacks as functions of r.h. Below 60% r.h. functional conformational changes in BR do not occur and the photocycle cannot be completed (table 2). Hydration in the plane of the membrane is predominantly around the lipid head-groups and in the proton channel (Zaccai & Gilmore 1979; Papadopoulos *et al.* 1990; figure 5). Perpendicular to the membrane surface, above 60% r.h. there is, on average, one molecular layer of water between adjacent membranes in the stack; there are two layers at 86% r.h. and three layers at 93% r.h. The ($\leq 1 \text{ \AA}^2$, 0.1 ns) scale mean-square fluctuations in PM as a function of temperature are shown in figure 6 for different r.h. values. Note how a dynamical transition at *ca.* 260 K appears at 75% r.h. and becomes progressively more pronounced with increasing hydration, establishing a correlation between hydration level, dynamic flexibility and biological activity (Lehnert *et al.* 1998).

Figure 4. The relation between the composition of the solvation shell (from small-angle scattering and hydrodynamics experiments), stability (from circular dichroism and residual-activity measurements) and resilience (from neutron spectroscopy) for *Hm* MalDH.

Table 2. PM hydration and activity.

r.h. (%)	number of hydration layers between membranes	conformational changes and proton pumping?
86	2	yes
75	1	yes
57	0	no

4. DISCUSSION (THE EFFECT OF WATER ON HALOPHILIC PROTEIN AND MEMBRANE DYNAMICS)

The surprising observation, which followed the discovery of halophilic proteins, was not so much their stability in high salt, but that they remain soluble in very high salt concentrations and unfold when the solvent NaCl or KCl concentration falls below *ca.* 2 M (still a 'high' salt concentration). Initially, it was thought that a particularly robust hydration shell, excluding solvent salt ions, surrounded halophilic proteins. Careful experiments showed, however, that this is not the case. In fact, the solvation shell around halophilic *Hm* MalDH comprises, essentially, mobilized solvent salt ions and water molecules in interaction with the protein surface. The solvation shell includes a very high local ionic concentration (e.g. equivalent to 5 M NaCl in NaCl-containing solvents) and participates actively in protein stabilization and solubility (Ebel *et al.* 2002). In contrast to stabilization mechanisms in mesophilic soluble proteins, halophilic solvation–stabilization appears to be dominated by enthalpic (ion-binding and H-bonding) rather than entropic (hydrophobic force) effects (Bonneté *et al.* 1994). Thus, *Hm* MalDH is more stable in NaCl-D₂O because of the 'stronger' ionic hydration of the Na⁺ ion and 'stronger' H-bonding in D₂O. And this is reflected in the dynamics. The state with the more strongly associated solvation shell is more stable and more resilient (figures 3 and 4). If stabilization were entropic, the more stable state would display more fluctuations and appear to be less resilient, as was observed for bovine serum albumin in D₂O solvent (Tehei *et al.* 2001). Dynamics and stability are protein-concentration dependent. Neutron-scattering experiments to measure molecular dynamics require high protein concentrations (*ca.* 300 mg ml⁻¹). Although these are very high concentrations by biochemical standards, they are close to physiological intracellular concentrations.

Figure 5. Projection Fourier density maps of native PMs (a) from *Halobacterium salinarum* and (b) their hydration at 86% r.h. The data were from neutron diffraction experiments on the D16 instrument at Institut Laue Langevin (details at www.ill.fr/YellowBook/D16). The hydration map is from D₂O minus H₂O difference data. The lattice has P3 symmetry. The three BR helices perpendicular to the membrane plane appear as circles of high density in (a), whereas the four tilted ones are in the 'smile' shaped density. The areas where there is no density in the native map correspond to the lipid positions in between the protein molecules. The helix positions A–G are given in (b). Note how the hydration is predominantly around the lipid head-groups and in the proton channel through BR, in the pocket close to helix G. The maps are from Lehnert (2000).

Each protein has evolved to form a stable active particle in its particular environment. The solvation shell is an integral part of the particle, even though it is composed of solvent components that may be in rapid exchange with their bulk solvent partners. Solvation shell interactions participate actively in particle energetics, which define its

Figure 6. Relation between the number of hydration layers between adjacent membranes in the sample stack (from D16 data), and membrane dynamics (from IN13 data) for three values of r.h. The saturated salt solutions with which r.h. values were defined are also given. The mean-square fluctuations as a function of temperature are from Lehnert *et al.* (1998). Filled circles: PM dry; open diamonds: (a) PM 75% r.h.; (b) PM 86% r.h.; (c) PM 93% r.h.

stability and dynamics. The solvation shell may be composed of only water in some type of H-bonding network including the protein surface (as in mesophilic soluble proteins), or of water and other solvent components such as the ions observed for the halophilic case. However, water always appears to be present, even if its activity may be significantly reduced. It also appears probable that it is the solvation shell that provides the required fluid environment for the dynamical transition in a soluble protein to take place (Tournier *et al.* 2003).

What is the equivalent of a solvation shell for a membrane protein? The immediate environment of BR is interestingly diverse. The protein crosses the membrane in seven α -helices that display apolar surfaces towards

lipid chains (Grigorieff *et al.* 1995). Protruding loops on the cytoplasmic side are in an essentially saturated KCl solution, whereas protein residues on the extracellular side are exposed to very high NaCl concentration. Hydration in the membrane plane increases the surface area available to the lipid molecules (figure 5), which led to the suggestion that the increased lipid flexibility also liberated protein motions (Zaccai 1987). In the direction normal to the membrane plane, one layer of water (shared between two membrane faces) is sufficient to trigger a dynamical transition at *ca.* 260 K and the onset of BR activity. Both the 'amplitude' of the transition and activity kinetics, however, increase significantly with increasing hydration.

Figure 7. Mean-square fluctuations in PMs as a function of temperature measured by neutron scattering in the ($\geq 1 \text{ \AA}^2$, 1 ns) length–time scale on IN16 at ILL (details at www.ill.fr/YellowBook/IN16). The data are for 86% r.h. The 260 K transition is absent in the dry membranes (0% r.h.), but the transition at 130 K is still present. These data were collected by M. Weik and U. Lehnert (personal communication).

Experiments at various r.h. values are performed to study the effects of hydration on a biological system. It is usually assumed that physiological conditions are best approached in excess water or 100% r.h. Recalling the composition of the immediate environment of BR in the PM, it is legitimate to ask which r.h. condition best approaches physiological conditions in the halophilic case. The partial vapour pressure of water in a saturated NaCl solution at room temperature defines 75% r.h., whereas that of a saturated KCl solution defines 86% r.h. (figure 6). It is tempting to suggest, therefore, that the physiological dynamics of PM are better represented by the 75% and 86% r.h. data in figure 6, than by higher r.h. values.

It is accepted that a dynamical transition at *ca.* 200 K in soluble proteins is triggered only if a minimum hydration level is available. A similar conclusion was reached for PM dynamics in the ($\leq 1 \text{ \AA}^2$, 0.1 ns) length–time window (Ferrand *et al.* 1993; Lehnert *et al.* 1998). Experiments investigating larger amplitude fluctuations on a longer time-scale, however, revealed the existence of another dynamical transition at *ca.* 130–150 K (figure 7), which is present even in the dry membrane (Réat *et al.* 1997). The origin of this dynamical transition is currently not understood. However, its existence does establish the fact that a softening of a biological system from a harmonic to a non-harmonic (or quasi-harmonic) state can and does occur in the absence of water.

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- G. Zaccai. Yes, with about 12 water molecules (seen by low resolution neutron diffraction) in the proton channel. Only a few of these are more strongly ‘bound’ than others and can be seen in the high-resolution X-ray structures.
- A. Cupane (Department of Physical and Astronomical Sciences, University of Palermo, Palermo, Italy). You showed data that relate bacteriorhodopsin dynamics to the hydration level (i.e. to the number of water layers between membrane stacks). There is, however, a third component in your system, which is the lipid bilayer. What is known about the lipid dynamics and about its relationships with bacteriorhodopsin dynamics and hydration level?
- G. Zaccai. We have neutron scattering data (Ursula Lehnert’s thesis (Lehnert 2000); U. Lehnert, D. Oesterhelt and G. Zaccai, unpublished data) on specifically deuterated purple membranes (D-lipids and H-BR and vice versa) that showed the lipid component is significantly softer than the protein, with a stronger hydration dependence.
- R. Hooper (Nanoscale Function Group, SFI Physics Department, Trinity College, Dublin, Ireland). You mentioned how trehalose protects myoglobin at high temperatures. The previous speaker (Grant 2004) also mentioned how many organisms synthesize trehalose to survive dehydration. How does trehalose have this protective effect?
- G. Zaccai. There are solution data showing that trehalose appears to adapt perfectly to the hydration shell (C. Ebel and H. Eisenberg, and collaborators), probably by replacing part of the water and through the internal flexibility of its disaccharide structure. L. Cordone in Palermo has given a lot of thought to this stabilization through dynamics effect.
- F. Franks (BioUpdate Foundation, London, UK). Several references are being made during the course of this meeting to ‘flash cooling’. It is my understanding that this refers to the immersion of a specimen in liquid nitrogen, a notoriously indifferent cryogen. You have shown how this process leads to a shrinkage and a reduction in the membrane spacings due to freezing.
- I would suggest that much higher cooling rates can be achieved by the use of so-called ‘nitrogen slush’, a mixture of solid and liquid nitrogen. The preparation of such a cooling system is trivial. It might then become possible to achieve cooling rates of the order of those required for the vitrification of water (greater than 4000 K s⁻¹) which, in turn, would maintain the original membrane spacings and hydration water (not ice) content.
- G. Zaccai. The flash cooling was by plunging in liquid nitrogen. Thank you for the suggestion to use nitrogen slush for a faster effect.

Additional reference

- Grant, W. D. 2004 Life at low water activity. *Phil. Trans. R. Soc. Lond. B* **359**, 1249–1267. (DOI 10.1098/rstb.2004.1502.)

GLOSSARY

- BR: bacteriorhodopsin
EINS: elastic incoherent neutron scattering
PM: purple membrane
r.h.: relative humidity

Discussion

R. M. Daniel (Department of Biological Sciences, University of Waikato, Hamilton, New Zealand). Presumably the part of the bacteriorhodopsin in the membrane is ‘dry’?