Probing the role of water in protein conformation and function

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Life began in a bath of water and has never escaped it. Cellular function has forced the evolution of many mechanisms ensuring that cellular water concentration has never changed significantly. To free oneself of any conceptual distinction among all small molecules, solutes and solvents, means that experiments to probe water's specific role in molecular function can be designed like any classical chemical reaction. Such an 'osmotic stress' strategy will be described in general and for an enzyme, hexokinase. Water behaves like a reactant that competes with glucose in binding to hexokinase, and modulates its conformational change and activity. This 'osmotic stress' strategy, now applied to many very different systems, shows that water plays a significant role, energetically, in most macromolecular reactions. It can be required to fill obligatory space, it dominates nearest non-specific interactions between large surfaces, it can be a reactant modulating conformational change; all this in addition to its more commonly perceived static role as an integral part of stereospecific intramolecular structure.

Keywords: osmotic stress; water, hydration; protein conformation; molecular assembly

1. INTRODUCTION

There is more water in cells than anything else. Molecular life began bathed in it, and has never found a way of getting away from it. Much of evolution has provided mechanisms not only for keeping it there, but also for making sure it does not change concentration, even in the face of drastic environmental conditions. It is necessary to fill the space around macromolecules, to allow spaces to change shape, and to allow spaces to open and close. It bathes practically all interfaces and surfaces of other molecules, and it dominates the closest interaction between them. It has to be removed and supplied when interfaces get covered and uncovered. It often forms an obligatory part of the structure within intermolecular interfaces and macromolecules. It often participates in the energetics of many molecular interactions and macromolecular conformational changes. There is nothing else available that substitutes for it.

All this, and water is usually taken for granted. When not taken for granted, pursuit of its unique and mysterious physical properties in isolation has led to three scientific scandals concerning water in the past few decades (think of polywater! water with memory! cold fusion!). One safe perspective is to treat water 'classically' as one would treat any other small molecule to determine its role in molecular interactions. Such a relatively new perspective of considering water as an energetically significant participant in any biochemical reaction has evolved slowly over the past three decades. It has led to the design of many experiments whose results point to a greater appreciation of the

critical role that water plays in many cellular molecular systems.

In what follows, I describe the relatively new experimental strategy required to probe water's role in a biochemical reaction, a strategy whose deceptive simplicity, paradoxically, has been difficult to appreciate. Selected examples demonstrate a growing appreciation for the role of water in a wide variety of systems of molecular association and macromolecular conformation (Rand *et al.* 2000).

(a) *Solvent or solute?*

A change in perspective required to design experiments to probe the role of water itself, in any system in which it plays a part, is to eliminate the conceptual distinction between solute and solvent (Parsegian *et al.* 1986, 1995). Water is usually considered the 'solvent' largely because it provides the means to dissolve, disperse and dilute 'solutes' in a medium within which their apparently more interesting reactions occur and are investigated. Water is usually ignored as a significant player in biochemical reactions. But to pose the simple question of whether water is a reactant, that is whether it contributes significantly to the energetics of a reaction, is to treat water no differently than any other solute or small molecule. To investigate the role of any molecule in a reaction is, among the simplest of means, to vary its concentration, or activity, and measure how that influences the reaction. To ask the same question about water is no different. Change its activity—no effect, no role—large effect, large role, energetically, perhaps structurally, in the reaction. Water can be viewed as just another small molecule, and can be treated as such. There is a huge concentration of it in living cells, *ca.* 54 M, far outnumbering any other molecular or ionic species; so should it be so easily ignored as a reactant? In those many reactions where the effects of changing water's activity

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have now been measured (Rand *et al.* 2000), water is usually seen as contributing significantly to the reaction. 'Osmotic stress' is the strategy of measuring water's contribution.

(b) *Osmotic stress*

The impetus to focus more on water as a reactant came after a long history of observations showing that a universal, common, exponentially varying force dominates contact between surfaces, in water, within 10–20 Å ($1 \text{ Å} = 1 \times 10^{-10} \text{ m}$) separation (LeNeveu *et al.* 1977; Parsegian *et al.* 1979; Rand *et al.* 1988). This dominant force is independent of the chemical nature of the surfaces, and the solute composition of their medium (Leikin *et al.* 1993). Considering that water was the only common element in these systems and was 'non-structural' water, the near interaction was called a 'hydration force' (Parsegian *et al.* 1986). Because of its ubiquity, there eventually emerged a focus on the effects of such water on single protein molecules (Parsegian *et al.* 1995).

The symmetry and reciprocal coupling among small molecules, 'solute' and 'solvent', means that if the activity of one is changed the activities of the others can also be changed. Consequently, in observing the effects of changing the activity of one solute molecule, the activity of the solvent has, necessarily, also changed. Thus, are such observed effects a result of solute or solvent? Sorting out this difficult issue and dealing with it linguistically, thermodynamically and experimentally has been treated in considerable detail (Timasheff 1998, 2002; Parsegian *et al.* 2000). I proceed with examples where care is taken to endeavour to separate solute and solvent effects, and focus on water effects.

The essence of osmotic stress is to control the activity of water by using solutes or 'osmolytes', which themselves do not otherwise interact with the system of interest. Solutes, excluded from aqueous compartments, act osmotically on them. Figure 1 illustrates two basic regimes or classes of compartments that exclude solute or osmolyte. The first, conceptually easier to see, is steric exclusion where the osmolyte, for example a large polymer, is sterically excluded from spaces within molecular arrays or from cavities or channels in proteins. Importantly and usefully, the size of the space that excludes the osmolyte varies with the size of the osmolyte, thus allowing different spaces to be probed with different size osmolytes. Less obvious is the exclusion of a solute, compared with water, from the surface of proteins. For example, the ubiquitous 'preferential hydration' of proteins in a solution of PEG (Timasheff 1993), is an example of a solute-depleted compartment that can be acted on osmotically.

Osmotic stress can result in two kinds of reaction, illustrated in figure 2. Withdrawal of water from molecular arrays 'shrinks' them to a new equilibrium packing whose internal stresses balance the applied osmotic pressure. Quantitative osmotic stress, used to gently, systematically and quantitatively stress and strain systems, is combined with measuring the structural response by independent means. Such an osmotic tool, combined with X-ray diffraction for example, has been used to deform molecular arrays such as membranes (Rand & Parsegian 1989), polysaccharides (Rau & Parsegian 1990), proteins (Leikin *et al.* 1994), DNA (Rau *et al.* 1984) among others, and has

provided a detailed quantitative characterization of the forces of interaction between the structural elements.

A second kind of response to osmotic stress, and one more relevant to the question of protein conformation and activity, is changing the equilibrium between conformational states, or perturbing the kinetic activity of an enzyme. For any system of equilibrium between two states of unequal 'hydration', the application of osmotic pressure has the consequence of shifting the equilibrium toward the less 'hydrated' state. A measure of that shift as it varies with osmotic pressure gives a measure of the difference in hydration between the two states (Parsegian *et al.* 1995, 2000).

In the simplest equilibrium reaction scheme, $A \leftrightarrow B$, between two states, the probabilities of their occurrence depend upon the difference ($G_B - G_A$) in work needed to go between the two states. In reaction language, the ratio of probabilities (as concentrations) of A and B is related to the equilibrium constant (K_{eq}), as $\ln(K_{eq}) = -(G_B - G_A)/kT$, where k is Boltzmann's constant and T is absolute temperature.

If these two states have different volumes, V_A and V_B , of solute-excluding water, an added osmotic pressure Π of the excluded solute creates additional work $\Pi(V_B - V_A)$ needed to go between the two states. Then K_{eq} changes with Π as

$$\ln(K_{eq}) = -(G_B - G_A)/kT - \Pi(V_B - V_A)/kT.$$

The slope of a plot of $kT \ln(K_{eq})$ versus Π gives the difference ($V_B - V_A$) in aqueous volumes that are inaccessible to the added solute. To determine the number of waters rather than a volume of waters, divide $(V_B - V_A)$ by the volume of a water molecule, nominally 30 Å^3 .

(i) *'Is life possible without water?'*

The strategy of osmotic stress is a very direct way of measuring the dependence of any molecular system on water activity. Where a large number of waters is involved, extremely mild osmotic stress alters the system so much that it appears inconsistent with the normal and usually highly coordinated molecular functions of the cell. Where a small number of waters is involved, such systems appear at least to tolerate low water activity. Few systems have shown no dependence on water activity.

2. AN EXAMPLE: WATER IN ENZYME CONFORMATION AND ACTIVITY

Hexokinase is the first enzyme of glycolysis. It binds glucose in a cleft and phosphorylates it. We ask whether glucose binding involves a change in water according to:

where HK is the hexokinase molecule and N_w indicates several osmolyte-excluding water molecules associated with it in the unbound/open (o) and glucose-bound/closed (c) conformations, and ΔN_w any difference between those numbers.

According to the general scheme above, one seeks the osmotic dependence of the equilibrium constant for glucose, $\ln(K_{glu})$, in this equilibrium as it changes with osmotic pressure or lowering water activity. The

Figure 1. Illustration of aqueous compartments around a variety of cellular/molecular systems, which can be osmotically stressed as a result of the exclusion of solute (the large 'squiggly' molecules) from either cavities or channels, as a result of size (steric exclusion), or from surfaces, as a result of 'preferential hydration' of those surfaces. Such exclusion depends on the combinations of solute size, the nature of the surface and the convolutions of the surface. The excluded solutes act osmotically on the compartment from which they are excluded by lowering the activity of water outside them.

Figure 2. Two kinds of reaction to increasing osmotic stress, i.e. to lowering water activity. (a) Molecular assemblies respond by taking up a structure shrunk by water removal. (b) States at equilibrium respond by increasing the probability, i.e. concentration, of the more dehydrated state.

consequence of lower water activity, using PEG molecules of MW 1000–10 000, is *increased* affinity of the enzyme for glucose (Reid & Rand 1997). It is as if glucose and water compete for the binding site. To what extent?

Figure 3 shows the dependence of $\log K_{\text{glu}}$ on osmotic pressure. The slope translates into a ΔN_w of *ca.* 325. That is, 325 fewer polymer-inaccessible waters are associated with hexokinase when one glucose molecule has bound compared with the unbound enzyme.

The osmotic dependence of glucose binding also occurs for the active enzyme that binds and then phosphorylates glucose in its turnover cycle (Rand *et al.* 1993). The osmotic dependence of the intrinsic Michaelis–Menten constant for glucose, which in this case equals the binding constant for glucose, shows that water acts as a competitive inhibitor for glucose to exactly the same extent as in equilibrium binding. The same osmotic dependence has been shown for other polymers used to lower water activity, methylated PEG and polyvinylalcohol (Bradford 2002). At the same time the enzyme's rate of turnover as measured by V_{max} is unchanged in all osmotic environments.

At high osmotic pressures the numbers of waters 'displaced' upon glucose binding, measured in the same way, decrease considerably. At the highest osmotic pressures used, ΔN_w falls to a minimum of *ca.* 25, a number that

Figure 3. Dependence of the glucose-binding constant, K_d , as it varies with osmotic pressure of the medium, controlled with PEGs of MW 1000–10 000. No difference is seen with MW. The slope of the best-fit linear line to this data suggests that *ca.* 325 fewer water molecules are associated with the glucose-bound conformation of hexokinase (Reid & Rand 1997).

Figure 4. Schematic representation of the effects of osmotic stress on glucose binding to hexokinase. Only glucose binding induces the fluorescence change associated with the conformational change A–B and C–D. For PEG of MW 1000–10 000 and of low osmotic pressures, the decrease in numbers of waters associated with glucose binding, ΔN_w , is *ca.* 325. ΔN_w decreases at high osmotic pressure to *ca.* 25. These larger MW PEGs, excluded from the same size aqueous compartment around the protein, apparently act to osmotically shrink that compartment (A–C) by *ca.* 300 water molecules in the absence of glucose binding. (There are no data to indicate what ΔN_w for the step B–D might be; we assume here that it is negligible for the two closed glucose-bound states.)

matches very well the difference between the crystallographic structures of the open and the closed conformations of hexokinase (Bennett & Steitz 1980). Figure 4

Figure 5. Measured ΔN_w as it varies with the MW of the PEG osmotic probe. The constant number at high MW (greater than 1000) shows that these larger probes are excluded from the same size aqueous compartment around the protein. The sharp decrease in ΔN_w suggests that smaller probes are excluded from an ever-decreasing size of aqueous compartment around the protein.

summarizes the number of water molecules that are involved in the glucose-binding reaction at high and low osmotic pressures. Assuming that the closed, glucose-bound states (B and D) are equally hydrated, then apparently, raising the osmotic pressure alone ‘dehydrates’ the protein by *ca.* 300 ($325 - 25$) water molecules (step A–C; figure 4) even before glucose is bound. That is a great deal of water. Is it energetically significant?

An energetic calculation requires the integration of the pressure–volume relation in the reaction involved. The reaction A–C in figure 4 gives the remarkably small value of *ca.* 1 *kT*. In other words hexokinase *in solution* and *in the ‘open’ conformation without glucose bound* can readily occupy all the hydrated conformational states between states A and C. The ‘open’ crystal structure conformation, without glucose bound, is consistent with only the substantially ‘dehydrated’ state, C, as might be expected given the osmotic character of crystallization conditions.

Figure 5 shows that as the MW of the PEG osmotic probe decreases from 1000, so too does the measured ΔN_w even at low osmotic pressures. This suggests that all the larger probes, MW 10 000–1000, are excluded from and act osmotically on the same maximum size aqueous compartments around the protein. The smaller probes are probably excluded from an ever-decreasing size of aqueous compartment around HK, as illustrated in figure 1.

The nonlinearity of the osmotic sensitivity of glucose binding, which becomes apparent at high osmotic pressures, warns that a strictly osmotic interpretation, acting on only two identical states of differing hydration, might not be valid. The aqueous compartments may themselves be expected to be deformed under osmotic stress, as interpreted here. Alternatively, the osmolyte may not be acting in the ideally osmotic way that this formalism assumes.

One important consideration (Timasheff 2002) is whether there is a difference in osmolyte–protein interaction between the open and closed HK conformations. Differences in PEG–protein interaction can be detected between native and unfolded conformations of proteins. However, one does not expect differences between the two very closely related native conformations. That PEG alone is causing the effects makes little sense (Reid & Rand 1997). Further, the osmotic dependence seen with PEG is identical for methylated PEG and for the polymer polyvinyl-alcohol (Bradford 2002), and thus it is unlikely that identical results come from specific interactions of different osmolytes. Finally, any binding does not detectably affect the maximum rate of enzyme turnover. All this points to a dominant osmotic effect.

3. SYSTEMS THAT HAVE BEEN INTERROGATED WITH OSMOTIC STRESS

Analogous osmotic stress experiments have been performed on a large variety of enzyme systems. Among an ever-growing list of water-soluble enzymes are, for example, adenosine deaminase (Dzingeleski & Wolfenden 1993), aspartate transcarbamylase (LiCata & Allewell 1997), even oxygen binding to haemoglobin (Colombo *et al.* 1992). Osmotic responses have been demonstrated for membrane-bound enzymes, including several membrane channels (Zimmerberg & Parsegian 1986; Rayner *et al.* 1992; Vodyanoy *et al.* 1993; Bezrukov & Vodyanoy 1993); the membrane enzyme cytochrome *c* oxidase (Kornblatt & Bon Hoa 1990) and the receptor rhodopsin (Mitchell & Litman 1999). The numbers of workers using osmotic stress and the emerging numbers of systems showing water dependence are growing (LiCata & Allewell 1998). A Web site that consequently remains permanently out of date is provided to collate this work. More importantly, it provides empirical osmotic pressure data for many osmolytes that can be used directly for performing osmotic stress experiments. It can be found at http://www.brocku.ca/researchers/peter_rand or <http://dir.nichd.nih.gov/Lpsb/docs/OsmoticStress.html>.

Even with a quantitative measure of water's participation in a reaction, one can be left with little knowledge of the location or molecular role of the participating water. For channels, the numbers of water correlate well with channel size. The large number measured for hexokinase bears little relation to the crystal structure conformations. Changing haemoglobin hydration on oxygenation appears to be related to its quaternary change in water-exposed area, both changes being absent in the case of myoglobin. One can view the many empirical measurements of osmotic effects as demonstrating different roles for water.

(a) *Water filling space*

Many molecular assemblies, to achieve a configuration of lower free energy, require water to fill otherwise empty spaces. Without such a space filler, or enough of it, the assemblies will be stressed or, with increasing probability, form other higher energy structures. All configurations with space that is at least 2–3 water molecule diameters from any molecular boundary fall into this category.

One example, figure 6, describes how many phospholipids, assembled into their spontaneous or intrinsic molecular

Figure 6. One common assembly of diacylphospholipids is this reverse hexagonal phase, made up of water-filled cylindrical micelles seen in cross-section. Most membrane lipids form this structure spontaneously under conditions of excess water and in the presence of alkane, both of which are required to fill space the molecules cannot reach. Under these circumstances the monolayers are assembled into their stress-free packing geometry and express their intrinsic curvature. Without such solvent space fillers the molecules must assemble into a higher energy packing, often unrelated even to this geometry, usually stressed bilayers. This system is meant to illustrate that bulk water is required as a space filler in any molecular structure that contains space more than 2–3 water diameters from the molecular surface.

packing arrangements, require water to fill large cylindrical spaces. Without that water the lipids assemble into stressed states, often flat bilayers, unrelated to the hexagonal symmetry of this lower free energy. Interestingly, these particular structures also require another, hydrophobic, solvent to fill hydrophobic spaces unreachable by the hydrocarbon tails of the assembled lipid molecules. It is only on this dual solvent basis that the lower free energy packing configurations of many different membrane lipid assemblies have been detected. By using osmotic stress to quantitatively measure the work of removing water from these spaces, the intrinsic curvatures and bending moduli of lipid layers have been measured (Rand & Parsegian 1997).

Other examples of systems that require space filling would be large membrane channels. For all channels that have been investigated, the lower the water activity, the less likely the channel will open. Channel gating requires the central space of a large open channel (Zimmerberg & Parsegian 1986) to be filled, independent of water that may hydrate its molecular boundaries.

In principle, a space filler, and the most likely candidate in aqueous systems is water, is required to occupy large spaces within molecular conformations and assemblies, all space that is 2–3 water diameters distant from any molecule surface. If only a space filler is required, any molecule might do. However, the particular molecular conformation, or molecular assembly, itself is dependent on its surface hydration properties. Therefore the near-range water binding and resultant long-range space-filling requirements are coupled. By contrast, if another solvent were used the resultant conformation and consequent space-filling requirements may differ. Certainly in the case of phospholipid assembly into multibilayers, other solvents have been used (Simon & McIntosh 1989) and changes

in bilayer interactions observed and correlated with some non-specific properties of those solvents.

(b) *Water hydrating surfaces*

The interaction and resulting forces between all large surfaces within 10–20 Å of each other are dominated by the work required to remove water. Such ‘hydration forces’ are universal and have a common decay length: lipid layers (Rand & Parsegian 1992), polysaccharides (Rau & Parsegian 1990), polypeptides (Leikin *et al.* 1994) and DNA (Rau & Parsegian 1992), all show such forces. Their origin is buried deep in the physics of water (Leikin *et al.* 1993). Such water can be viewed as precluding spontaneous molecular contact between large surfaces, making possible, for example, cellular compartmentalization by membranes, and stabilization of macromolecular assemblies. However, it also makes necessary specialized mechanisms to regulate membrane contact and fusion, and macromolecular contact.

(c) *Water determining macromolecular conformation*

The case of hexokinase has been summarized above. Many other enzymes and macromolecules have been examined. A surprising, relatively recent, example was the demonstration that the binding of four oxygens to haemoglobin requires the binding of 60 additional water molecules to the oxygenated form. The activity of many enzymes in solution, membrane-bound enzymes and membrane channels, has been shown to be osmotically sensitive. The separate but probably interdependent roles of filling space and hydrating surfaces appear to be critical in determining and modulating molecular conformation and activity.

(d) *Water as a less mobile part of molecular structure*

In DNA–drug (Sidorova & Rau 1995) and protein–DNA interactions, the stability of the complexes are osmotically sensitive. Even the specificity of the restriction enzyme *EcoRI* is dependent on its osmotic environment (Robinson & Sligar 1993; Sidorova & Rau 1996). Contacts within the tryptophan repressor–DNA operator complex are largely water mediated (Brown *et al.* 1999). In such examples, supported by higher structural resolution, the number of waters mediating the effects is small and the osmotic pressure required to detect them is large. They appear to fall into the realm of water that can be seen in protein crystals. Such relatively immobile water, detectable by crystallographic and NMR methods (Kornblatt 2002), contrasts with the unperturbed bulk water of simple space filler, and the extremely mildly perturbed water hydrating most surfaces.

Although distinct roles for water can be envisaged, it is convenient but unwise to think of classes of water, bulk, bound, etc. The use of osmotic stress, a finely tuned way of varying water activity, especially in characterizing hydration forces, has taught us that a continuum of water activities exists that has a continuum of effects on macromolecular interactions and conformations.

4. LESSONS LEARNED FROM MEASURING WATER'S ROLE

In higher multicellular organisms water is important, usually because of its large numbers rather than its range of activity. Although osmotic stress can be used as a tool to measure water's role and to stress systems structurally, it is not easy to envisage significant intracellular water activity gradients as playing a major role in regulating molecular activity. Given the widespread sensitivity of many structures and systems to changing external water activity, it is difficult to imagine normal molecular life working in anything but a narrow extracellular range of water activity. A large part of evolution and of homeostatic mechanism is devoted to rigorously maintaining that level of activity.

By contrast, one must acknowledge the growing recognition and importance of micro-, transient, intracellular gradients of small molecules and ions, such as calcium. A fusogenic mechanism in synaptic vesicles is ‘sprayed’ and activated with a high concentration of calcium supplied from an adjacent local set of calcium channels, calcium that quickly disperses in the cell. Similarly, even with water's extremely high permeability to membranes, evolution has found it necessary to design water channels for its even faster transmembrane equilibrium in some cells. One can ask what an osmotically sensitive macromolecule experiences when ‘splashed’ with the water, released from hexokinase, or coming from a water channel. As with all small molecules it is still an open and elusive question whether local transient intracellular water gradients play any role in intracellular molecular function.

In contrast to higher life forms, for lowly micro-organisms water activity is a hugely variable environmental and unavoidable cellular parameter. Soil micro-organisms have to regulate their intracellular osmotic environment in the face of the desiccating baking sun one moment, and the flooding bath of rainwater the next. They have evolved mechanisms to survive largely through the regulation of solute transport systems, which rapidly change their internal osmolarity to follow the environmental. Remarkably, these cells have been shown to have membrane proteins that function both as osmosensor and transport system built into the same molecule (Wood 1999; Racher *et al.* 2001; Rubenhagen *et al.* 2001). Such micro-organisms might be a source of proteins capable of functioning at low water activity.

Water is important because what is called ‘hydration’ depends upon the competitive affinity of a macromolecule (or a region of a macromolecule) for water compared with its affinity for the many other solutes in the bathing solution. This competition is realized in many ways. It is the basis for a complex functional interaction of solute–water–protein.

Waters near the surface of a protein are not available to dissolve some other solutes. This may be because the protein strongly perturbs the water so that it cannot dissolve other molecules; or it may be that some solutes are too big to enter the thin surface region. In either case, there is a ‘preferential hydration’ of the protein relative to that particular solute. The cost of forming that protein surface will go up with added solute, in direct proportion to the number of water molecules that can no longer act as a solvent to that solute. These waters have been drawn away

from the solution by the protein. The extra cost of drawing away that water is proportional to the extra osmotic pressure from the added solute.

Waters in a cavity may be available to smaller solutes but unavailable to sterically excluded large species. Added small solute will not act osmotically on that cavity, but large solutes will. The extra cost of forming the cavity in the presence of large solute will be the extra osmotic pressure imparted by it multiplied by the volume of water from which the large solute is prevented access. If the cavity is a membrane channel, the gating properties of that channel can bear a complex relation to the full complement of solutes surrounding it (Bezrukov & Vodyanoy 1993; Bezrukov *et al.* 1994, 1996).

In this context, 'osmotic' takes on a complex and variable meaning. Any specific macromolecule will be subject to a variety of osmotic pressures depending on the variation of its surrounding solutes and their access to its various boundaries and cavities. The response of a different macromolecule to these same solutes will be different.

Discrimination between solutes becomes a means whereby the cell, the macromolecule, or the experimenter, can use these solutes to control different kinds of change in conformation. Cells do regulate, and vary widely, the set of small molecules that control macromolecular association and reaction. That wide variety of small molecules implicates water by acting osmotically on a variety of macromolecular aqueous compartments.

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Discussion

M. W. Ho (*Institute of Science in Society, London, UK*). How do you know it was glucose that made the water come off from hexokinase, and not ATP?

R. P. Rand. The equilibrium binding studies for glucose use no ATP in the binding reaction mixture. More recent studies (Bradford 2002) show that in the enzymatic turnover of the enzyme, the Michaelis–Menton constant for ATP binding is independent of osmotic pressure. So glucose appears to be the culprit.

M. W. Ho. Have you done osmotic stress reactions with glycerol?

R. P. Rand. We have not used glycerol *per se*. The osmotic pressure dependence for small osmolytes is very small in this case and our approach has been to use large osmolytes that are excluded from large regions and show the larger effects.

J. A. Littlechild (*School of Biological Sciences and Chemistry, University of Exeter, Exeter, UK*). With regard to your experiments with hexokinase and removal of water in the cleft on glucose binding, have you considered the effect of salt on these experiments? Salt affects the solubility of proteins and affects the water around the protein. In relation to the crystal structure determination, high salt in crystallization conditions results in an ‘open cleft’ structure in this type of hinge-bending enzyme.

R. P. Rand. We have not yet studied the effects of salt on this osmotic dependence. Our current difficulty of accounting for the large numbers of waters measured is

making us cautious about introducing more complicating factors or new variables. Perhaps it would help!

J. L. Finney (*Department of Physics and Astronomy, University College London, London, UK*). You emphasize the universality of the hydration force in that it operates between *all* surfaces. You also give a qualitative explanation that relates to the removal of, for example, a ‘layer’ of water. But such a mechanism would operate for *any* liquid, so perhaps we should note the universality of a *solvation* force? The only change with changed solvent will be a quantitative change—the solvation force itself depends only on there being a liquid—*any liquid*—between the surfaces.

R. P. Rand. I certainly agree—my emphasis on water derives from how I have spent most of my life! And thinking about the mechanism of hydration forces has dominated the area. However, other solvents have been used in the case of phospholipid multilayers, where hydration forces were first seen. McIntosh *et al.* (1989) and Simon & McIntosh (1989) have shown that multilayers form in other solvents and that an empirically measured repulsion forces exists, although modified, and which they have attempted to correlate with various properties of the solvents. So the universality of a general ‘solvation force’ you suggest seems entirely reasonable. What that tells us about mechanism, however, leaves me behind.

A. Cupane (*Department of Physical and Astronomical Sciences, University of Palermo, Palermo, Italy*). In view of your data, one expects that proteins themselves lower water activity; therefore, one would also expect a pronounced dependence of protein conformational equilibria and of protein dynamics upon protein concentration. Are such effects observed? And what is the physiological role of osmotic stress?

R. P. Rand. We tried to look for such effects, using bovine serum albumin as an osmolyte for example. Proteins, even at the highest concentrations obtainable, exert little osmotic pressure, i.e. they lower the activity of water by trivial amounts, especially when compared with the pressures needed in these studies. One must of course be aware of the possibility of protein- or osmolyte-concentration dependence of dimerization or such effects as you suggest. We have not seen such effects.

We have addressed the question of any physiological role for osmotic stress in the text. Briefly, for most multicellular organisms, extracellular osmotic pressure is highly regulated, but the intriguing question of intracellular microgradients is always an interesting speculation. For unicellular organisms external osmotic stress can be a continuing and huge variable for which many regulatory means have evolved.

J. B. F. N. Engberts (*Physical Organic Chemistry Unit, University of Groningen, Groningen, The Netherlands*). There appears to be another factor that may contribute to hydration forces: recently there is rather compelling evidence that OH[−] ions bind to the hydration layers of hydrophobic groups on surfaces, even at pH *ca.* 7. The group or surface then gets a *negative* zeta potential (for a recent account, see Johnsson *et al.* (2003)). A recent molecular dynamics simulation (R. Zangin, A. E. Mark and J. B. F. N. Engberts, unpublished data) is consistent with this effect. So it might well be that under appropriate

conditions, electrostatic forces contribute to hydration forces.

R. P. Rand. We distinguish between intrinsic hydration forces and intrinsic electrostatic forces. Hydration forces derive from some perturbation, perhaps ‘polarization’, of water by the surface and the propagation of that ‘polarization’ into adjacent water layers. Completely neutral surfaces show hydration forces of universal characteristic decay length. You are certainly correct, charged surfaces can polarize water and give rise to hydration forces as well as electrostatic forces. However, the hydration force and the electrostatic force can be distinguished in that the former dominates the latter at close separation and shows the characteristic universal hydration force decay length and not the electrostatic. So in our view charged groups contribute to both electrostatic and hydration force components of the total net force.

Additional references

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GLOSSARY

MW: molecular weight
PEG: polyethylene glycol