Water as an Active Constituent in Cell Biology

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1. Introduction

When Szent-Györgyi called water the "matrix of life",[1] he was echoing an old sentiment. Paracelsus in the 16th century said that "water was the matrix of the world and of all its creatures."[2] But Paracelsus's notion of a matrix—an active substance imbued with fecund, life-giving properties—was quite different from the picture that, until very recently, molecular biologists have tended to hold of water's role in the chemistry of life. Although acknowledging that liquid

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water has some unusual and important physical and chemical properties—its potency as a solvent, its ability to form hydrogen bonds, its amphoteric nature—biologists have regarded it essentially as the backdrop on which life's molecular components are arrayed. It used to be common practice, for example, to perform computer simulations of biomolecules in a vacuum. Partly this was because the computational intensity of simulating a polypeptide chain was challenging even without accounting for solvent molecules too, but it also reflected the prevailing notion that water does little more than temper or moderate the basic physicochemical interactions responsible for molecular biology. What Gerstein and Levitt said 9 years ago remains true today: "When scientists publish models of biological molecules in journals, they usually draw their models in bright colors and place them against a plain, black background".[3]

Curiously, this neglect of water as an active component of the cell went hand in hand with the assumption that life could not exist without it. That was basically an empirical conclusion derived from our experience of life on Earth: environments without liquid water cannot sustain life, and special strategies are needed to cope with situations in which, because of extremes of either heat or cold, the liquid is scarce.[4–6] The recent confirmation that there is at least one world rich in organic molecules on which rivers and perhaps shallow seas or bogs are filled with nonaqueous fluid—the liquid hydrocarbons of Titan[7]—might now bring some focus, even urgency, to the question of whether water is indeed a

unique and universal matrix of life, or whether on the contrary it is just the one that happens to pertain on our planet.

Fundamental to that question is the role that water plays in sustaining the biochemistry of the cell. It has become increasingly clear over the past 2 decades or so that water is not simply "life's solvent" but is indeed a matrix more akin to the one Paracelsus envisaged: a substance that actively engages and interacts with biomolecules in complex, subtle, and essential ways. There is now good reason to regard the "active volume" of molecules such as proteins as extending beyond their formal boundary (the van der Waals surface, say), by virtue of the way they shape and manipulate the shell of water that surrounds them. Moreover, the structure and dynamics of this hydration shell seem to feed back onto those aspects of the proteins themselves so that biological function depends on a delicate interplay between what we have previously regarded as distinct entities: the molecule and its environment. Many proteins make use of bound water molecules as functional units, like snap-on tools, to mediate interactions with other proteins or with substrate molecules or to transport protons rapidly to locations buried inside the protein.

Here I review the case for considering water to be a versatile, adaptive component of the cell that engages in a wide range of biomolecular interactions. In order to provide some basis for assessing water's often-alleged "uniqueness" to life, however, I shall try to highlight throughout this paper the distinctions between generic and specific behaviors of biological water. That is to say, some of its roles and properties may be expected from any small-molecule liquid solvent. Others depend on water's hydrogen-bonding capacity, but not in a way that could not obviously be fulfilled also by other hydrogen-bonded liquids. But some of water's biochemical functions do indeed seem to be quite unique to the H_2O molecule. From an astrobiological perspective, the question is then whether we can regard these latter roles as *optional* or *essential* for any form of life to be tenable.

2. Water as a Liquid and Solvent

2.1. Water Structure

Water is not like other liquids, but neither is it wholly different. The structure of a so-called simple liquid—one in which the molecules can be represented as particles that interact via some spherically symmetric potential function—can be depicted in terms of a radial distribution function (rdf) $g(r)$, which is related to the liquid density $\rho(r)$ around a particle at $r = 0$: $\rho(r) = \rho_b g(r)$, where ρ_b is the bulk density. The rdf for a simple liquid interacting through the Lennard-Jones potential ($V \sim \sigma/r^{12} - \sigma/r^6$, where σ is the molecular diameter) is not very different from that of a "hard-sphere" fluid, in which the particles experience no intermolecular force until they touch, whereupon they act as infinitely hard spheres ($V = 0$ ($r > \sigma$); $V = \infty$ ($r \leq \sigma$)). In both cases, the rdf is oscillatory, with a prominent first peak around $r = \sigma$ and smaller subsequent peaks at separations close to $r = n\sigma$ that decay rapidly until the density reaches the bulk average value (Figure 1). This implies that the oscillatory density profile is dominated by the steep repulsive core of the particles and is related to the geometric aspects of a dense, random packing of particles. These repulsive interactions create short-ranged ordering in the liquid that the inclusion of an attractive potential (without which there is no liquid—gas transition) modifies only slightly.
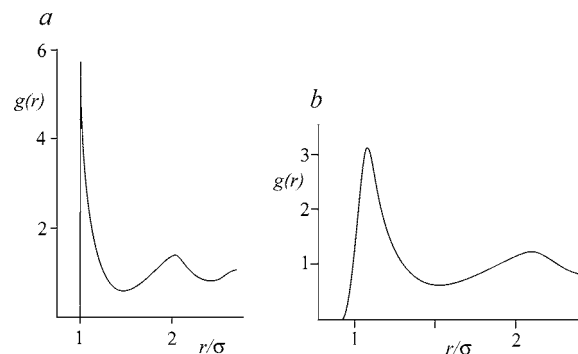

Figure 1. Typical radial distribution functions (rdfs) of simple fluids: (a) hard spheres; (b) Lennard-Jones potential.
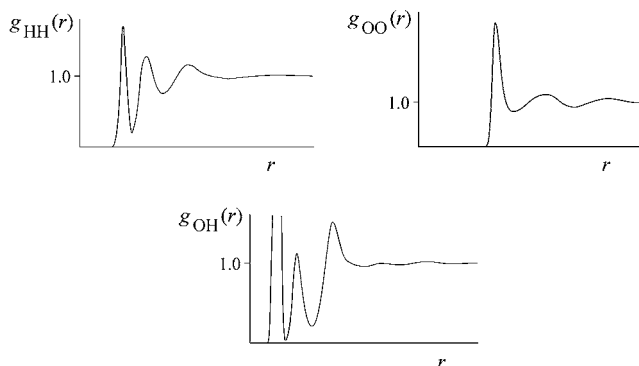

Figure 2. Partial radial distribution functions (rdfs) for water at 298 K. The first (off the scale) peak in $g_{OH}(r)$ is the intramolecular peak. Data courtesy of Alan Soper, ISIS.

Liquid water also has an oscillatory rdf. In this case a single function $g(r)$ will not suffice to fully characterize the liquid structure, because there are two types of atom in the molecules: H and O. So one must define a series of partial rdfs $g_{XY}(r)$, which denote the probability of finding an atom Y a distance r from the center of atom X. For example, $g_{OO}(r)$ indicates the radial distribution of oxygen atoms in other H_2O molecules around any given molecule. The partial rdfs for water, determined by neutron scattering,[8] are shown in Figure 2.

The structure is more complex than the oscillatory profile with decaying amplitude found for simple fluids such as hard-sphere or Lennard-Jones systems or for real liquid argon; but the basic features look qualitatively similar. This local structuring of liquid water has, however, a quite different origin. Whereas hard-sphere repulsion controls the short-range order of simple liquids, the structure seen in Figure 2 is primarily due to the attractive interactions between water molecules: the hydrogen bonds. These generate a peak in $g_{OO}(r)$ at a separation considerably greater than the mean "molecular diameter" (radius of gyration) σ—the peak is at about 1.4σ. In other words, the molecules do not, on average, sit as "close" as do the particles of a simple liquid. They are held apart by the hydrogen bonding, which imposes geometric constraints on the molecular positions: the hydrogen atom in an O···H−O union sits, on average, roughly along the axis between the two oxygen atoms. In other words, the hydrogen bond is linear. If it is "bent", the orbital overlap is poorer and the bond is weaker. Thus, one might say that hydrogen bonding keeps the H_2O molecules at "arm's length", preventing them from packing as closely as they would in a simple liquid. The hydrogen bonds are directional: they bind the water molecules into particular spatial orientations.
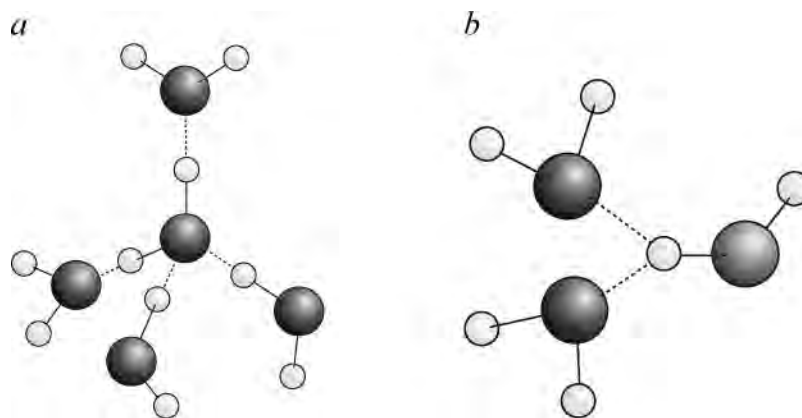
Figure 3. Tetrahedral coordination geometry of water molecules (a) and defects involving bifurcated hydrogen bonds (b). The latter are posited to play a key role in molecular mobility in the liquid state.
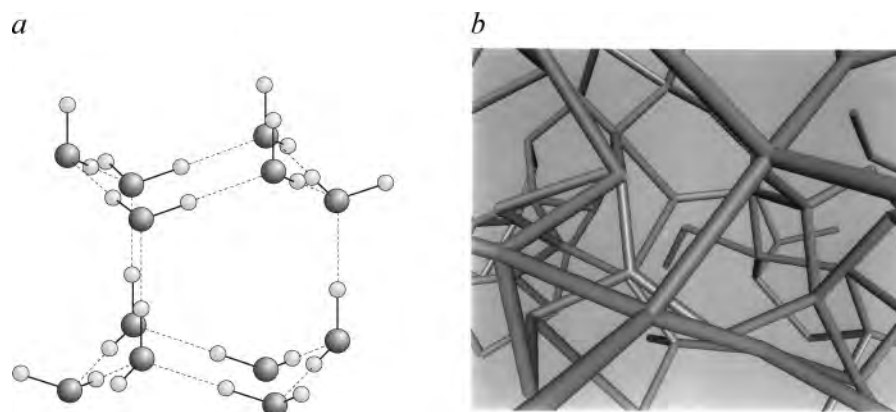

Figure 4. In ice (a), tetrahedral coordination around each water molecule is rigidly imposed. In liquid water this geometry is relaxed so that the network formed by hydrogen-bonded molecules is more disorderly and fluxional (b). Frame b courtesy of H. E. Stanley.

The sp^3 hybridized oxygen atom creates an essentially tetrahedral coordination geometry, with each H_2O molecule coordinated to four others (Figure 3a). We should resist the traditional notion of two "rabbit-ear" lone pairs on each molecule, however: the electron-density distribution shows only a single, broad maximum on the "rear" of the oxygen atom. And since the molecules are mobile in the liquid, the four hydrogen-bonding sites are not necessarily fully occupied. Neutron-scattering measurements of the rdfs, along with computer simulations, have tended to suggest that the number of hydrogen-bonded nearest neighbors for water molecules at room temperature averages about 3.5. Recent EXAFS (extended X-ray absorption fine structure) studies by Wernet et al.[9] challenged this picture, appearing to suggest that only two nearest neighbors are, on average, actually hydrogen-bonded to the central molecule; but this interpretation has been vigorously disputed.[10–14]

Simulations by Sciortino et al.[15] posed a different challenge to the conventional picture of tetrahedral coordination via linear hydrogen bonds, suggesting that the liquid state contains many defects in the form of bifurcated bonds: a single hydrogen coordinates to two oxygen atoms on different molecules (Figure 3b). Sciortino et al. conclude that these bifurcated bonds in fact play a central role in the molecular mobility of the liquid state by lowering the Gibbs energy barrier to diffusion.

Nonetheless, it is clear that the tetrahedral hydrogen-bonded geometry is the reason for water's well-known density anomaly on freezing. The density of the solid state is lower than that of the liquid because in crystalline ice the constraints of hydrogen bonding are rigidly imposed: the

molecules form an essentially perfect tetrahedrally coordinated network, linking them into six-membered rings with much empty space between the molecules (Figure 4a). When the lattice melts, the three-dimensional hydrogen-bonded network becomes more fluxional, distorted, and defective (Figure 4b), enabling molecules to approach one another more closely: the average distance between molecules slightly decreases. Other anomalies of the liquid state, such as the density maximum at 4 °C and the putative existence of a liquid–liquid phase transition in metastable, supercooled water,[16] can be considered to result from the competition between these "icelike" and "disordered" configurations. One might say that as the temperature is lowered below 4 °C, "icelike" configurations become predominant, leading to a decreasing density, whereas at higher temperatures the decrease in density with increasing temperature is simply a reflection of normal thermal expansion. The notion[17,18] that water is best viewed as a two-phase mixture of distinct icelike and liquid-like configurations, with fully formed and partly broken hydrogen-bond networks, respectively, now seems untenable, however; rather, the distribution of hydrogen-bond geometries and energies appears to be continuous.[14]

In pure liquid water, hydrogen bonds have an average lifetime of about 1 ps, so although there exists an extended, essentially infinite (percolating) dynamical three-dimensional network (Figure 4b), there are no long-lived icelike structures. Defects in this hydrogen-bonded network owing to dangling OH bonds seem to be very short-lived (<200 fs)—"broken" hydrogen bonds re-form almost immediately.[19]
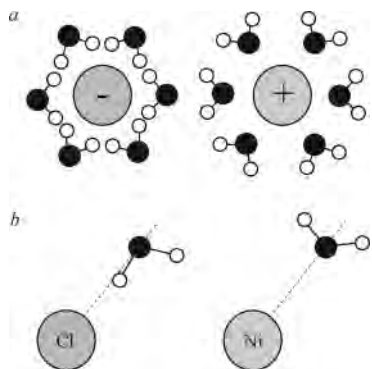
Figure 5. Solvation of cations and anions. (a) The conventional view. (b) Water orientations revealed by neutron scattering.

For water's role as a solvent, particularly in the cell, one of the key questions is how this network structure is affected by the introduction of a solute molecule or proximity to a surface. In each case, the structural response of liquid water depends on whether the foreign body is hydrophobic or hydrophilic. But in more or less all cases the issue has been contentious and is still not well resolved.

2.2. Small-Molecule Solutes: Hydrophiles

Water is an extremely good solvent for ions. In part, this is a result of water's high dielectric constant, which screens the ions' Coulombic potential effectively and prevents the aggregation and crystallization of ions of opposite charge. By the same token, water is an efficient solvent for biomolecular polyelectrolytes such as DNA and proteins, shielding nearby charges on the backbone from one another.

As a polar species, the water molecule can engage in favorable Coulombic interactions with ions and other polar solutes. According to the traditional picture, water molecules will solvate cations by orienting their oxygen atoms toward the ion, whereas they will adopt the opposite configuration for anions (Figure 5a). Such reorientation, however, perturbs the hydrogen-bonded network, and the question is how readily this disruption can be accommodated.

The key technique for investigating this issue is neutron scattering, particularly because of the large scattering cross section of hydrogen and because of the facility for altering the various cross sections by isotopic substitution, enabling the several water–water and ion–water partial rdfs to be disentangled. Pioneering work by Enderby, Neilson, Soper and their co-workers[20] has confirmed the expected picture in which anions such as chloride are coordinated to water in the hydration shell via hydrogen atoms, such that the H–O···Cl⁻ bond is almost linear, whereas for cations like nickel the water molecules are oriented with the oxygen atoms facing toward the ions (Figure 5b). Recent XAS (X-ray absorption spectroscopy) studies of cation hydration have been interpreted as implying little or no geometric distortion of "water structure" over long ranges,[21,22] although divalent cations do seem to induce a redistribution of charge among water molecules in the solvation shell, leading to changes in the X-ray absorption spectra.[22] This very local view of the effects of ions on water structure is supported by first-principles MD (molecular dynamics) simulations, which reveal only some degree of preferential orientation in the first hydration shell for both monovalent and divalent ions,[23] and by femtosecond pump–probe spectroscopy,

which shows that ions have essentially no influence on the rotational dynamics of water molecules beyond the first hydration shell.[24] It is important to note, moreover, that neutron-scattering studies caution against too static a vision of hydration: there is clearly a lot of dynamic variation in these structures.

While, as we shall see later, the hydration of hydrophilic regions of proteins has been investigated extensively, there has been surprisingly little work to date on small, biologically relevant hydrophiles. As a result, the "ground rules" for interpreting the hydration of larger biomolecules have not really been established. What little we do know about such simple "model" species, however, suggests that it may not be possible either to generalize or to reduce the structural and dynamic aspects of hydration to simplistic rules of thumb. A neutron-scattering study of the hydration of L-glutamic acid in alkaline solution[25] suggested a significant disruption of the water structure, with the average number of water–water hydrogen bonds being reduced from 1.8 to 1.4. But this was at high concentrations of both glutamic acid and NaOH (each 2 M), so it is not clear how the results might relate to a physiological context. In contrast, L-proline engages in strong hydrogen bonding with water but in a manner that seems barely to perturb the hydrogen-bonded network at all.[26] Proline is an osmoprotectant, preventing protein denaturation in the face of water stress. One possible mechanism for this invokes the formation of a loosely associated sheath of proline molecules around a protein to "chaperone" it—something that appears feasible without any significant disruption of the protein's hydration.

One of the best studied small hydrophiles is urea, which can be considered a mimic of a hydrophilic residue but is also known for its tendency to denature proteins. A neutron-scattering study shows that the urea molecule can "substitute" quite readily for water in the hydrogen-bonded network: the rdf of urea around water in a 1:4 solution looks remarkably like that of water around water.[27] Although urea has nearly 3 times the molecular volume of water, the structure of liquid water is sufficiently "open" that a urea molecule appears to displace just two waters, offering up to eight hydrogen bonds in place of the displaced pair. The idea that urea "fits" rather easily into the hydrogen-bonded structure of water has been supported recently by dielectric spectroscopy.[28] Yet despite this apparently "easy" substitution, incorporating urea into the network appears to disrupt it, creating a local compression of the second hydration shell around water molecules in a manner similar to the effect of high pressure on the liquid. All the same, the orientational dynamics of the water molecules seem largely unaffected even at urea concentrations high enough for all the water to be part of hydration shells.[29] Only one water molecule per urea, on average, has a significantly slower reorientational time constant (about 6 times that of bulk water), which can be rationalized according to a hydration structure in which one water is complexed to the urea molecule via two hydrogen bonds.

These findings shed light on the action of urea as a protein denaturant. Proposed reasons for this behavior have invoked direct interactions of urea with the protein backbone, for example, via hydrogen bonds[30,31] or electrostatic[32] or hydrophobic contacts,[33] which act to swell the protein as a precursor to denaturation, and indirect effects due to the disruption of "water structure" by urea, making the hydrophobic groups more readily solvated.[34] The experimental findings for the effect of urea on water do not seem to support
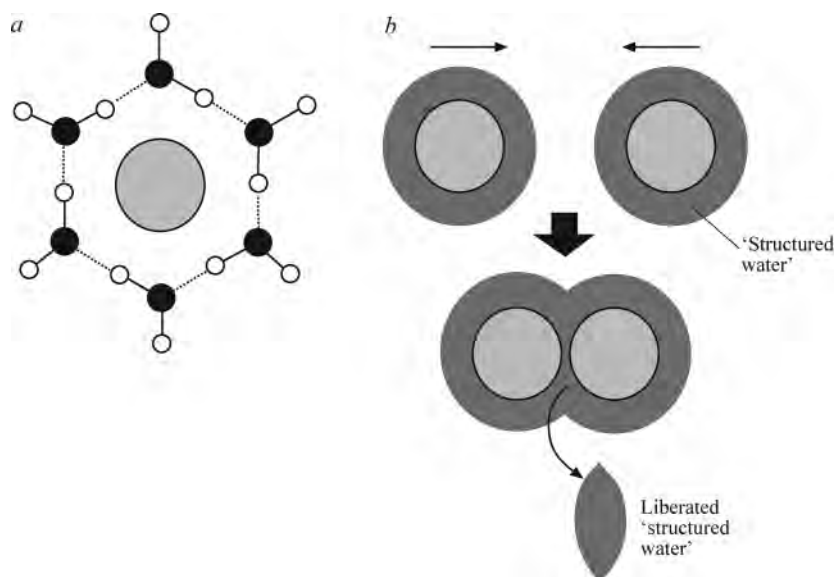
Figure 6. (a) "Iceberg" model of hydrophobic hydration. (b) Kauzmann's explanation of the hydrophobic attraction.

the idea of significant disruption of the hydrogen-bonded network (although one might argue that the mimicking of high pressure in the second hydration shell might encourage something akin to pressure-induced denaturation). Rather, it seems best to consider the nature of specific interactions between the various solutes, instead of regarding the protein as being solvated by "modified water". Indeed, experiments and thermodynamic analysis imply that in general a cosolvent such as urea will be inhomogeneously partitioned around a biological macromolecule—urea tends to expel water from a protein surface, whereas sugars are preferentially excluded from the hydration sphere.[35]

We will see below that the same principle—considering local, direct interactions rather than global "water structure" modifications—holds for hydrophobes too. In any event, one can see that the overall picture of the effect of hydrophiles on water structure is rather complex even for this relatively simple species, demanding a detailed consideration of the structure and dynamics of the hydration shell(s), and so it seems unwise to fall back on popular but simplistic notions about whether solutes such as urea "break" the structure of the hydrogen-bonded network[36,37] (see below).

2.3. Small-Molecule Solutes: Hydrophobes

Hydrophobic solutes in water experience a force that causes them to aggregate. This hydrophobic interaction is responsible for several important biological processes:[38] for the aggregation of amphiphilic lipids into bilayers, with their hydrophobic tails "hidden" from water and their hydrophilic heads at the surface, for the burial of hydrophobic residues in polypeptide chains that helps proteins to fold and to retain their compact forms, and for the aggregation of protein subunits into multi-subunit quaternary structures. There is a tendency for even rather small hydrophobic moieties to cluster—something of the sort seems to happen for methanol dissolved in water, leading to dynamic heterogeneity of the mixture.[39] The question is whether a single mechanism for the hydrophobic interaction can account for all these behaviors, and if so, what is it?

We do not yet know how to answer either point. Part of the reason for that perhaps surprising ignorance may be that there has for some time existed an apparent explanation that

made such seemingly good sense that it has been hard to dislodge and is still routinely cited in biochemistry textbooks. This is Kauzmann's "entropic" origin of the hydrophobic attraction,[40] which draws on the picture of hydrophobic hydration posited in 1945 by Frank and Evans.[41]

The conventional story is as follows. In order to accommodate a hydrophobic species, the hydrogen-bonded network must be disrupted to create a void. But this can be done in such a way as to avoid the enthalpic penalty of losing hydrogen bonds, if the water molecules arrange themselves around the hydrophobe in a relatively ordered fashion (Figure 6a). In the words of Frank and Evans, "when a rare gas atom or nonpolar molecule dissolves in water at room temperature it modifies the water structure in the direction of greater crystallinity—the water, so to speak, builds a microscopic cage around it".[41]

This is the "iceberg" model: the hydrophobe is encased in an icelike shell of water. It has been often suggested[42,43] that, based on a description of water structure due to Pauling,[44] a better model for this pseudocrystalline cage is that found in gas hydrates, where small hydrophobic species such as methane are enclosed in cavities made from pentagonal rings[45] rather than the hexagonal rings of ice. (Hydration structures around the hydrophobic portions of proteins are now commonly described in terms of pentagonal rings.) Kauzmann pointed out that the price of creating any relatively ordered structure of this sort to preserve the integrity of the hydrogen-bonded network is that the rotational and translational freedom of the molecules in the cage wall are compromised: there is an entropy decrease. But if two "caged" hydrophobes were to come together, the "structured" water in the region between them is returned to the bulk, leading to an entropy increase (Figure 6b). Thus, there is an entropically based force of attraction between these solute particles.

The argument seems sound in principle, but the question is whether hydrophobic hydration really has the "semicrystalline" character proposed by Frank and Evans. To date, there is no good reason to suppose that it does, and some evidence indicating that it does not. Reviewing the existing data on hydrophobic hydration—both structural and thermodynamic, and based on both experiment and computer

simulation—Blokzijl and Engberts[46] concluded that there was no reason to suppose that hydrogen bonding is enhanced in the first hydration shell or that the favorable enthalpy of hydrophobic hydration need be attributed to anything more than normal van der Waals interactions between water and a nonpolar solute. Moreover, they found no evidence that hydration involved any enhancement in the ordering of water molecules around the solute; rather, the hydrogen-bonded network seemed simply to *maintain* its structure, in particular by orienting water molecules such that the O—H bonds are tangential to the solute surface. There is no inconsistency between the occurrence of such orientational effects and the lack of any enhancement in structure, since we must bear in mind that the hydrogen-bonded network introduces directional preferences in local water orientation even in the bulk. The importance of attractive solute—water van der Waals interactions, relative to considerations of "water structure" and water—water interactions, was emphasized by Ashbaugh and Paulaitis,[47] whose Monte Carlo simulations show that although water densities in the first hydration shells of clusters of methane molecules are greater than those of the bulk, the corresponding density for hydrated hard spheres (lacking any attractive interactions) of the same size as the methane clusters decreases as the sphere's radius increases and ultimately falls below that of the bulk.

Blokzijl and Engberts suggested that aggregation of hydrophobes results not from Kauzmann's entropic mechanism but from the increasing difficulty in accommodating hydrophobes within the hydrogen-bonded network as their concentration in solution increases: "hydrophobic interactions", they say, "are not so much a result of a structural property of the hydrophobic hydration shell of apolar compounds but rather reflect the limited capacity of liquid water to accommodate the apolar solute and maintain its original network of hydrogen bonds."[46] From the perspective of whether there is anything "special" about water that introduces a hydrophobic interaction, therefore, the message is mixed. Yes, the hydrogen-bonded network seems to be important, but not because it becomes more highly structured by hydrophobes; rather, it is because this network is disrupted by too great an accumulation of cavities.

On the other hand, Lucas[48] and Lee[49,50] have proposed that this disruption is not a function of the hydrogen-bonded network at all but stems merely from the small size of the water molecules, which creates a high free-energy cost to opening up a cavity to accommodate a hydrophobe—an argument based on scaled-particle theory,[51] which Stillinger[52] first adapted to the case of hydration. Hummer, Pratt, and their co-workers[53–58] have argued that models based on the spontaneous formation of cavities through density fluctuations in liquid water can account quantitatively for the thermodynamics of small-hydrophobe solvation—a claim supported by MD simulations.[59] Yet Southall et al.[60] assert that neither water "structure" nor the small-size effect can by themselves fully explain several of the characteristics of the hydrophobic effect, such as its dependence on temperature and on solute shape.

While all of these studies help to decouple the real puzzles of the hydrophobic effect from comfortable but outdated explanations, there is a tendency in discussions of the hydrophobic interaction to regard water as *the* liquid rather than *a* liquid: little comparison has been made with other small-molecule liquids, both associated and simple, perhaps because of the notion that the hydrophobic effect is clearly
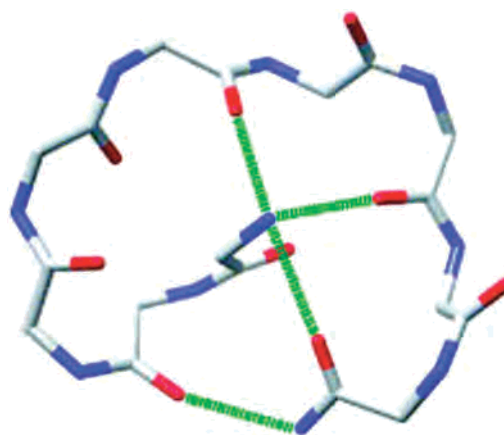

Figure 7. Backbone folded structure of an N-substituted glycine nonamer peptoid in acetonitrile. The dashed green lines indicate hydrogen bonds. Reprinted with permission from ref 61. Copyright 2006 American Chemical Society.

biologically relevant, whereas such solvents are not. As a result, we lack much basis for comparison in deciding how much of water's behavior as a solvent for solvophobic particles is unique (and thus perhaps due to its three-dimensional hydrogen-bonded network) and how much is generic to other, related liquids. Only when such questions are answered can we expect to say much about water's supposed centrality to life.

Evidently, however, there is nothing unique to water about solvophobic aggregation in general: the existence of reverse micelles in nonpolar solvents indicate as much. More strikingly, Huang et al.[61] have shown that non-natural peptide-like molecules with N-linked rather than C-linked side chains (N-substituted oligoglycines or "peptoids"[62]) will fold into well-defined secondary structures in acetonitrile in which the polar units (the carbonyl groups) are buried in the interior (Figure 7): one apparently does not need a strong degree of solvent structure in order for such packing to occur. It will be very interesting to explore the potential complexity of structure and function available to this and other non-aqueous pseudoprotein chemistry.

All the same, one can imagine there being scope for added subtlety in the interactions of a solvophobe and a structured solvent like water. Indeed, Hummer and co-workers[53,54] argue that if cavity-opening fluctuations are the dominant factor in hydrophobic hydration, water's unusual equation of state distinguishes the nature of these effects from those that might be anticipated in other liquids—there *is* then something "special" about water.

But how, if at all, does water structure manifest itself in the hydration environment of hydrophobes? Obviously the best way to deduce the effect of a hydrophobic molecule placed within water's network is to measure it directly; and again neutron scattering is the ideal probe. But that is no simple matter, because archetypal small hydrophobes such as methane and krypton are insufficiently soluble in water to permit an easy detection of the hydration shells above the bulk water signal.

Nonetheless, de Jong et al.[63] used neutron scattering to deduce that methane molecules in water are surrounded by about 19 solvent molecules and that those in the first hydration shell are tangentially oriented. The structure of a hydrophobic hydration shell has been most aggressively pursued by Finney, Soper, and their co-workers. Filipponi et al.[64] used EXAFS to show that krypton is hydrated by

around 20 water molecules. This hydration structure was barely altered by applying pressures of up to 1 kbar.[65] This not only throws into question some proposed explanations for pressure-induced protein denaturation in terms of hydration changes but also cautions against too heavy a reliance on computer simulations for evaluating hydration structure, some of which[66] predict significant pressure-induced changes in the hydration shell of methane.

Molecular dynamics simulations of krypton in aqueous solution[67,68] show no sign of the clathrate-like hydration shells invoked in the classic iceberg model. That is borne out experimentally by Bowron et al.,[69] who used EXAFS to highlight the differences between the hydration shell of krypton in solution and the clathrate cage of krypton in the crystalline clathrate hydrate. The former is unambiguously more disordered, and the tangential orientation of water molecules found in the hydrate is not rigorously maintained in the liquid. Indeed, Finney[70] points out that the first-neighbor water structure in the hydration shell is all but unchanged from that in bulk water: "water in this environment still thinks it is liquid water". And ab initio MD simulations of the diffusion of small hydrophobes in water—which is found experimentally to be anomalously fast[71]—indicate that, far from being icelike, the hydration shell is unusually dynamic and loose.[72] Even if the Frank—Evans iceberg model is no longer interpreted too literally in any case, these results give direct cause to doubt any such picture that places emphasis on enhanced "water structuring" around hydrophobes.

Another model small hydrophobe is benzene, since aromatic groups are common in biological molecules. But while phenyl groups are generally regarded as hydrophobic, it has been known for some time that the π-electron system can act as a weak hydrogen-bond acceptor,[73] creating strong orientational preferences in the bound water molecules.[74] The converse is also possible: the lone pairs on the oxygen may also interact favorably with π electrons. This balance appears to be rather subtle, making it hard to generalize about how aromatic groups are likely to be hydrated. Allesch et al.[75] find in first-principles MD simulations of benzene and hexafluorobenzene in water that both molecules act as hydrophobes equatorially but that the axial hydration structure is very different: water molecules typically point toward the ring hydrogen-first for benzene, but lone-pair-first for hexafluorobenzene.

A particularly attractive minimal model for investigating the role of hydration structure on hydrophobic interactions in biological systems is provided by simple alcohols such as methanol. Not only are they amphiphiles that mimic "shrunken lipids", but the juxtaposition of nonpolar groups and polar, hydrogen-bonding groups shares to that extent the character of a typical portion of a protein's peptide chain. Moreover, they provide an opportunity to study hydrophobic hydration without the problems of low solubility. Dixit et al.[76] find that a concentrated solution of methanol (7:3 methanol/water ratio) is imperfectly mixed and highly structured: water clusters bridge hydroxyl groups on the alcohol to form hydrogen-bonded chains that thread through a "fluid" of methyl groups. Most of the water molecules, however, cluster into groups of 2−20, in which their local structure is surprisingly bulklike, again challenging any idea of iceberg-like ordering. The segregation of methanol and water even persists at high dilution of the alcohol:[77] in a 1:19 mixture of water and methanol, more than 80% of the
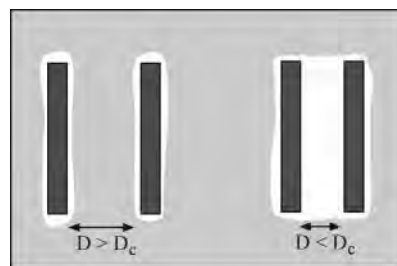

Figure 8. Hydrophobic attraction in the model of Lum et al.[80] The hydrophobic surfaces are surrounded by a thin layer of vapor. At some critical separation D_c, there is a collective drying transition in the space between the surfaces.

alcohol molecules are clustered into groups of 3−8. Again, here neutron scattering shows no evidence for structure enhancement in the hydration shells of the nonpolar portions of the methanol molecules.[78]

What, then, *is* the origin of this clustering effect? Dixit et al.[77] propose that it might indeed have an entropic origin, but subtly so. They find that the hydration shells of the water molecules themselves are slightly altered by the presence of methanol (at a 1:19 concentration, most of the water molecules lie within the hydration shell of at least one methanol): the second hydration shell becomes slightly compressed, and the correlations between second-neighbor waters slightly sharpened, leading to a small reduction in entropy. This small decrease in freedom of the water molecules could promote aggregation of the methanols. "Perhaps it is here", Finney et al.[79] conclude, "in the *water's second* shell rather than the *alcohol's first* hydration shell, that the entropic driving force for the hydrophobic interaction is to be found". If so, this is a small effect, and by no means obvious, and of unproven generality—so it might be argued that explanations based on general factors such as cavity formation[53−58] have a stronger appeal, not least because they are more intuitive. But in any event, these direct structural probes seem to have diminished any argument for the classic "enhanced structuring" model of the hydrophobic interaction along the lines defined by Kauzmann.

2.4. Large Hydrophobic Solutes and Surfaces

Although small hydrophobic species can be accommodated in the hydrogen-bonded network of liquid water without much perturbation of the network, large hydrophobes, for example the hydrophobic surfaces of proteins, are another matter. An attractive interaction evidently exists between such species too, being responsible for protein folding and aggregation. Does this have the same origin as the force that causes small hydrophobes to cluster? That is not obvious.

Close to an extended hydrophobic surface, it is geometrically impossible for the network to maintain its integrity. It has been proposed that this can even lead to "drying" of the interface[43]—the formation of a very thin layer of vapor separating the liquid from the surface. In a highly stimulating contribution, Lum, Chandler, and Weeks (LCW)[80] argue that this difference between small and large hydrophobes should lead to qualitatively different behavior, with a crossover length scale somewhere in the region of 1 nm—about the van der Waals diameter of an α-helix. This idea derives from the notion, proposed by Wallqvist and Berne,[81] that when two hydrophobic surfaces come in close proximity, water can withdraw from between them (Figure 8) and that the

resulting imbalance in pressure would cause the two surfaces to attract. In effect, the confined water undergoes "capillary evaporation". This kind of drying transition has been seen in simulations of hydrophobic plates in water when the separation between them falls to just a few molecular layers.[81−83]

Simulations of hard-sphere solvation by Rajamani et al.[59] support the LCW picture, indicating that the thermodynamics of hydration is entropically controlled for small hydrophobes but enthalpic for larger ones. This is precisely what one would expect if the former case depends on density fluctuations and cavity formation while the latter involves the formation of a liquid−vapor interface and thus introduces the surface tension into the hydration Gibbs energy. This crossover of hydration mechanisms is accompanied by a change in the variation of hydration Gibbs energy ΔG with solute size: ΔG is a linear function of solute volume for small sizes but becomes closer to being proportional to solute surface area for larger sizes. Rajamani et al. find that the crossover occurs for hard-sphere solute radii of the order of a few angstroms under ambient conditions but that this size scale can be "tuned" either by altering thermodynamic parameters (for example, placing the water under negative pressure) or by adding other solutes. Ethanol, for example, decreases the crossover length scale to molecular dimensions. Ashbaugh and Pratt[58] show that this picture of a crossover from entropically to enthalpically dominated hydrophobic hydration as a function of particle size can be rationalized by applying scaled-particle theory[52] to a thermodynamic analysis of cavity formation.

But does hydrophobic collapse induced by cooperative dewetting really play any role in the association of hydrophobic macromolecules, for example, in protein folding and aggregation? Here the picture remains unclear, although it seems fair to say that the LCW model looks increasingly unlikely to provide a general description of macromolecular hydrophobic interactions. Simulations by ten Wolde and Chandler[84] suggest that a hydrophobic polymer acquires a compact conformation in water via a process resembling a first-order phase transition in which the rate-limiting step is the nucleation of a sufficiently large vapor bubble—the classical mechanism of heterogeneous nucleation. But simulations of protein folding show a more complex situation. Berne's group found that collapse of the two-domain enzyme BphC, which breaks down polychlorinated biphenyls, showed no sign of a sharp dewetting transition as the domains came together.[85] Here the drying seen between hydrophobic plates[82] seems to be suppressed by attractive interactions between the protein and water: dewetting was recovered when the electrostatic protein−water forces were turned off and was stronger still in the absence of attractive van der Waals forces. Thus, it seems that the inevitable presence of such interactions in proteins complicates the simple picture obtained from hydrophobic surfaces. Consistent with this view, MacCullum et al. found that simulations of the dimeric association of both polyalanine and polyleucine (A_{20} and L_{20}) α-helices showed no dewetting between the chains until it was induced sterically by a mere insufficiency of space for a water monolayer.[86]

On the other hand, Berne's group found a first-order-like dewetting transition in simulations of the association of the melittin tetramer, a small polypeptide found in honeybee venom.[87] But single mutations of three hydrophobic isoleucine residues to less hydrophobic ones were sufficient to suppress the dewetting. Is melittin a rarity, even a unique case, or might other proteins also exhibit dewetting? Berne's group has performed a survey of the protein data bank to search for other structures that might show similar behavior.[88] They find that dewetting is indeed rather rare but does happen in several other cases: they identify two two-domain proteins, six dimers, and three tetramers that behave this way. It seems that any significant number of polar residues in the hydrophobic core (which is common) is generally enough to suppress dewetting. Using the same tools, Berne's group finds preliminary evidence that dewetting may also sometimes play a role in ligand binding.

These results suggest that even if the LCW mechanism can operate in the collapse of some proteins, nonetheless it is extremely sensitive both to the precise chemical nature of the protein domains involved and perhaps to the geometry of association: melittin subunit association forms a tubelike enclosed space, whereas that for BphC is slablike.

Choudhury and Pettitt have attempted to clarify these issues by returning to the case of two planar, nanoscopic hydrophobic plates.[89,90] They find[89] that the existence or absence of a wetting layer between the plates at separations of less than about 1 nm depends on a fine balance between the plate−water interaction energy, the hydrogen-bonding energy, and the plate size. For example, graphite-like plates measuring 11×12 Å^2 undergo a steric dewetting transition for separation below about 6.8 Å for a Lennard-Jones interaction potential, but dewetting occurs at about 10 Å for a purely repulsive plate−water interaction. This LCW-like behavior vanishes, however, if the plates are smaller.

This finding seems consistent with the view provided by density functional calculations on confined simple liquids,[91] which show that, although unfavorable liquid−surface intermolecular interactions (relative to liquid−liquid interactions) can counteract the usual enhancement of liquid density close to a wall owing to packing effects, and can even lead to a depletion in average density here, it takes a rather extreme set of interaction parameters to induce capillary evaporation in the manner of the LCW model. In other words, the bulk liquid−solid contact angle has to be very low, and it is far from clear that water in contact with a typical hydrophobic surface (an alkyl-covered surface, say, let alone the hydrophobic surface of a protein) represents such an extreme case. Choudhury and Pettitt concur[90] that in general, "chemically reasonable" estimates of the plate−water interaction strength lead to a microscopically wet state and not to plate association triggered by a dewetting transition. That conclusion is supported by MD simulations of Bresme and Wynveen, who have studied the effect on interactions between two hydrophobic solutes of varying their polarizability.[92] The solute polarizability has a strong influence on the water contact angle, and a drying transition occurs only for rather extreme conditions (outside the range of permittivities typical for proteins) in which the contact angle is close to 180°. Otherwise a fluid layer remains between the solute surfaces, albeit with a density significantly lower than that of the bulk liquid. This situation is nevertheless associated with strong hydrophobic forces, showing that complete drying is not essential to promote an attractive interaction.

This perspective leads to the more general question of how water behaves and is structured close to a *single* hydrophobic surface. Although, as Choudhury and Pettitt point out,[90] drying in the interplate region may be a cooperative

phenomenon and thus does not necessarily demand drying of the isolated surfaces themselves, nonetheless the nature of the interface between water and a hydrophobic surface is the precondition, one might say, for any discussion of potential dewetting by confinement. Moreover, this question is central to the more general and much-disputed issue of the hydrophobic hydration of proteins.

Experiments have tended to give a rather confused and contradictory picture of this situation,[93] although a consensus now seems to be emerging. X-ray reflectivity measurements[94−96] suggest that, although there is a depletion in liquid density adjacent to alkane monolayers, it is far less pronounced than what would be observed for complete drying and happens only within a few molecular diameters of the surface. Measurements of water density adjacent to a crystalline paraffin monolayer floating on the surface of water suggest that the depletion region extends about 1.5 nm into the liquid phase and that it corresponds to a deficit of about one water molecule for every $25−30$ $Å^2$ of the paraffin surface.[94] On the other hand, MD simulations in the same study indicated the formation of a very thin (about 1 Å) layer of "vacuum" between the water phase and the surface—something beyond the resolution of the experiment itself. This latter result might be considered broadly consistent with the simulations of Pertsin et al.,[97] which indicated only a very small reduction in water density close to an alkylated surface. Other MD simulations suggest that, contrary to what would be expected in the presence of a vapor-like film, there is significant penetration of water molecules into a layer of tethered n-C_{18} chains.[98]

A more recent high-resolution X-ray reflectivity study[95] corroborates the existence of a "hydrophobic gap" for a monolayer of n-C_{18} chains on silica but suggests that it extends no further than $1−6$ Å from the surface and that it corresponds to an integrated density deficit of 1.1 Å g cm^{-3}. Similarly, Poynor et al.[96] find a density deficit of more than 40% extending about $2−4$ Å from the surface.

In contrast, some neutron reflectivity studies of the water density adjacent to a self-assembled monolayer of alkylthiols[99,100] have apparently indicated a density depletion extending for several nanometers. But recent results make this now seem unlikely. Doshi et al.[101] found a reduced density extending only 1 nm or less from the surface, the distance depending on the amount and chemical nature of dissolved gases (see below; at this stage the influence of dissolved gases is by no means clear—the smaller hydrophobic gap reported by Mezger et al.[95] was unaffected by a wide range of such gases). And Maccarini et al. report a depletion layer of no more than about 2 Å.[102]

From a theoretical perspective, even if hydrophobic surfaces do not induce anything like complete drying, the existence of a depletion layer extending over distances of $2−5$ nm would be very perplexing, since there is no obvious physical interaction in the system that could introduce such a length scale. That recent results seem to be converging on a depletion layer with a thickness of the same order of the water molecule itself is therefore reassuring.

It is important to know whether similar effects are seen for other liquids so that one might elucidate the role (if any) of water's hydrogen-bonded network. Maccarini et al.[102] do find that depletion layers of a similar order, that is, just a few angstroms thick, appear to be present at the interface of hydrophilic surfaces and nonpolar liquids, showing that we should be wary here of attributing anything "special" to water.

How water dynamics might be affected within a hydrophobic depletion layer is another matter, which has received little attention. Dokter et al.[103] found that nanodroplets in reverse micelles, where the interface is not hydrophobic but is thought nonetheless to have decreased hydrogen bonding, have slow orientational dynamics and relatively immobile water molecules in the interfacial layer. Jensen et al. also found retarded dynamics in simulations of water next to a hydrophobic surface.[104] As we will see below, a significant change in rotational dynamics in this region could have implications for the hydrophobic interaction itself.

Any change in the nature of "water structure" close to a hydrophobic surface can be expected to alter its solvating characteristics, leading to the possibility of segregation of small solutes such as ions at or away from such interfaces.[105] But it seems likely that this view is putting the cart before the horse. There is now strong evidence that some ions do indeed segregate preferentially at, or away from, the air−water interface.[106−112] Yet this is not because the water is "different" there, but because there is an intrinsic thermodynamic driving force for this segregation, and if anything we might expect an excess of ions to alter the properties of the solvent rather than vice versa. Since one might expect the interface of water with a hydrophobic surface to mimic in many respects that with air, this inhomogeneity of solutes at a surface could have significant implications for the solvation of proteins, as we will see below.

The same applies to the finding that hydronium ions seem to have a preference for the water surface.[113−118] This result, which was predicted theoretically[113,114,118] and confirmed by thermodynamic analysis,[111] by surface spectroscopy,[114,116,117] and by deuterium exchange at the surface of ice nanocrystals,[118] apparently has a rather different origin from the surface segregation of other ions. H_3O^+ may form three donor hydrogen bonds to neighboring water molecules, but because most of the positive charge resides on the oxygen atom, it can no longer act as a good hydrogen-bond acceptor. Indeed, this makes the oxygen somewhat hydrophobic, so that hydronium acts as an amphiphile.[113,115] Both that and the reduced hydrogen-bond capacity encourage the surface accumulation of hydronium, oriented with the oxygen atom outermost. It has been estimated[113,118] that this effect shifts the surface pH of pure water to around 4.8 or less (although the applicability of this bulk parameter on a localized scale is not entirely clear). As much the same behavior might be expected at hydrophobic surfaces, this finding could have significant implications for biomolecular hydration that have yet to be investigated; for example, one might expect to see a shift in the dissociation of protonatable residues close to hydrophobic patches and perhaps even a stabilization of hydrophobic species by a kind of surfactant behavior of hydronium.

2.5. The Influence of Ions: Structure-Making and Structure-Breaking

The coexistence of ions and hydrophobes in aqueous solution has some puzzling consequences. Hofmeister noted in 1888 that some salts tend to precipitate albumin from solution (salting-out), whereas others enhance its solubility (salting-in).[119,120] The Hofmeister series ranks ions in order of their "salting-out" tendency for proteins:

$$H_2PO_4^- > SO_4^{2-} > F^- > Cl^- > Br^- >$$
$$NO_3^- > I^- > ClO_4^- > SCN^-$$

$$Mg^{2+} > Li^+ > Na^+ \sim K^+ > NH_4^+$$
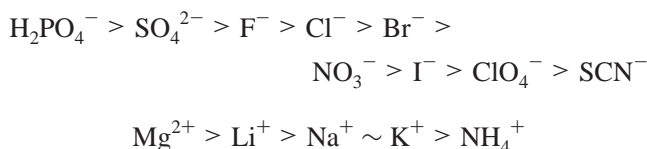
Similar sequences are observed for the solubility of amphiphiles.

The traditional explanation for the Hofmeister series introduces another of the tenacious myths of hydration: the concept of "structure-making" and "structure-breaking" ions. The basic idea is that large, low-charge ions such as I^- and NH_4^+ disrupt "water structure"—they are structure-breakers—whereas small or highly charged ions such as F^- and Mg^{2+} are structure-makers, imposing order on the hydrogen-bonded network. Then salting-out and salting-in of proteins are explained on the basis of entropic changes induced in their hydration shells by the addition of ions or of a reduction in the strength of hydrogen bonding of water molecules complexed to dissolved ions. The classical hypothesis is that salting-out arises from a competition for solvation between the salt and the protein, in which an ion's ability to sequester waters of solvation is somehow connected to its effect on water structure. Thus, the structure-making effect of small or highly charged ions depletes proteins of hydration water and causes precipitation.

Yet there is little consensus—indeed, sometimes outright contradiction—about what structure-making and -breaking actually entails. For example, one suggestion holds that although an ion always induces ordering of the water molecules in its first hydration shell, because of the tendency for the molecules to orient themselves either oxygen-first or hydrogen-first, subsequent layers in the hydration sphere may become more or less ordered as a result, depending on the commensurability with the hydrogen-bonded network. Moreover, although some interpret "structure-making" as an enhancement in the ordering of the hydrogen-bonded network, Franks[121] insists that the local structure enforced by small and/or highly charged ions is not commensurate with the tetrahedral pattern of bulk water and so may disrupt that.

Such ideas have been extended to produce "two-state" models of liquid water in which different solutes are classified in terms of their ability to enhance or degrade the short-range order in the liquid: they are called either kosmotropes or chaotropes, respectively. Kosmotropes, by increasing the local order, render the water more icelike, with a consequent decrease in its ability to dissolve electrolytes. Chaotropes promote a denser, more "liquid-like" water structure. Thus, chaotropes and kosmotropes may set up gradients in chemical potential and solute concentration: a hypothesis that has been advanced to account for a range of cell functions extending well beyond the original Hofmeister effects.[105]

Monte Carlo simulations using a simple two-dimensional model of ion hydration, in which water molecules are represented as disks interacting via a Lennard-Jones potential on which directional hydrogen-bonding interactions are superimposed, present an opposite picture.[122] That is to say, small ions with high charge densities act as kosmotropes insofar as they enhance the first peak of the rdfs—but this increase in density, although in some sense representing an enhancement the local liquid structure, is produced by stronger electrostatic interactions, at the cost of *breaking* hydrogen bonds. The hydration environments of large ions

(chaotropes), meanwhile, are more hydrogen-bonded. Thus, the peculiarity of water, whereby enhanced ordering of the hydrogen-bonded network leads to a decrease in density, seems to have introduced further confusion even about what "structure-making" and "structure-breaking" imply. One has to concur with Franks,[121] who says that "much has been written about structure-making and structure-breaking, and much of it is misleading."

The fact is that all of the conceptual scaffolding of "structure-making" and "structure-breaking" has been erected without any real evidence from experimental studies of the structure of electrolyte solutions that significant changes to the bulk hydrogen-bonded network of water really do occur. Indeed, as we saw in section 2.2, the available evidence is to the contrary: simple ions seem to have little or no effect on "water structure", at least beyond the first hydration shell.

So instead of trying to understand the Hofmeister series on the basis of "global" changes in solvent structure induced by ionic solutes, it seems far more logical to consider the effects that these ions have on the local hydration of protein residues or other hydrophobes. The fact is that ions do not, in general, simply disperse homogeneously throughout the solution so as to create a kind of "mean-field" solvent for other large solutes such as macromolecules; rather, as noted above, many ions tend to segregate preferentially at either hydrophilic or hydrophobic surfaces.[106−112,123] It now seems likely that Hofmeister effects must be understood in terms of these specific and often rather subtle interactions between ions and proteins or other biomolecules.

For example, although the ability of so-called chaotropic anions to inactivate enzymes has been rationalized on the basis of their tendency to disrupt water structure and, in consequence, enzyme structure, Ninham and co-workers[124,125] have shown that the effect of adding sodium salts of various anions to buffer solutions of lipase enzyme is to alter the enzymatic activity systematically in a manner that cannot be explained this way but, rather, according to the specific interactions of the anions with the enzyme surface. And Sachs and Woolf[126] found that large anions seem to penetrate deeply into lipid bilayers, becoming partially stripped of their hydration shells as they do so. Thus, whereas chloride ions penetrate to within 17.5 Å of the center of palmitoyl-oleoylphosphatidylcholine bilayers, anions with a van der Waals radius 37.5% greater reach to within 12.5 Å of the center. This can help to explain how membrane structure might be disrupted by different ions again without any need to invoke "chaotropic" disruption of the general water structure in the hydration of the lipid head groups.

So far as the original Hofmeister effect—the ion-specific changes in solubility of proteins—is concerned, the phenomenon is now generally interpreted in terms of the tendency of ions of different size and charge to modify the hydrophobic interaction. Direct probing of the effect of ions on hydration structure of organic solutes has been conducted only for simple model systems such as alcohols, using neutron scattering.[127−129] These, as indicated previously, are model amphiphiles, in which the polar hydroxy group can engage in hydrogen bonding while the nonpolar alkyl tail models the hydrophobic residues of a protein. Adding sodium chloride to solutions of *t*-butyl alcohol alters the hydration environment of the alcohol molecules due to a direct interaction with chloride ions. At the concentrations studied (alcohol/water ratios of 1:50), in the absence of salt the alcohol molecules engage in "head-to-head" contacts between
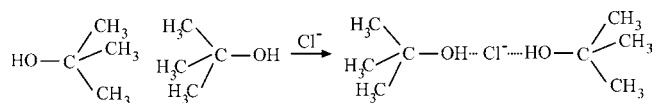
Figure 9. Chloride bridging of *t*-butyl alcohol in solution.

the nonpolar *t*-butyl regions: a classic instance of hydrophobic clustering. But for NaCl/water ratios of 1:100, about half of the chloride ions form salt bridges between the polar OH groups of two alcohol molecules. The other chlorides remain fully hydrated, as do the sodium ions. (Although many of the cations lie close to the nonpolar regions of the alcohol molecules, they do not significantly perturb the hydration structures here: there is no sign of any generalized structure-making or structure-breaking in the alcohol hydration layer caused by sodium.) Thus, the interactions of alcohol molecules change dramatically: from nonpolar–nonpolar to polar–polar, bridged by chloride (Figure 9). This, of course, exposes the hydrophobic regions to the solvent, and it would be expected to make the alcohol less favorably disposed to the aqueous environment.

Zangi and Berne have considered the more general question of how in ions interact with small hydrophobic particles.[130] In simulations of hydrophobic Lennard-Jones particles of diameter 0.5 nm, they found that ions with high charge density (q) produce salting-out, inducing stronger hydrophobic interactions that promote particle aggregation. But low-q ions could have either a salting-out or a salting-in effect, depending on their concentration (low or high, respectively). These effects were related to preferential absorption or exclusion of the ions at the particle surfaces, but not in any simple, monotonic fashion. High-q ions tended to be depleted at the surface of the hydrophobic particle clusters, but are tightly bound to water elsewhere, thereby decreasing the number of water molecules available for solvating the particles. Low-q ions are absorbed preferentially at the particle surfaces, and at high ionic concentrations this can lead to salting-in in a subtle way: the hydrophobic particles form clusters surrounded by ions, a micelle-like arrangement that keeps the aggregates stably dispersed (Figure 10a). At lower concentrations, the ions are unable to solubilize aggregates in this way but can nonetheless still act as pseudosurfactants that stabilize the interface formed on phase separation and precipitation of large aggregates (salting-out). Such a concentration-dependent switch from an enhancement to a reduction of hydrophobic association of *t*-butyl alcohol in aqueous solution induced by urea has been observed in NMR studies,[131] and Zangi and Berne suggest that this polar molecule may be acting in the same manner as a low-q ion.

The interaction of ions and protein molecules is, however, perhaps often better viewed as comparable to that between the electrolyte and a *surface*. As noted, a hydrophobic surface might be expected to induce effects similar to those of the air–water interface, particularly if there is water depletion, or indeed complete drying, at the interface as suggested above. Traditionally, ions have been considered to be excluded from the air–water interface because electrolytes increase surface tension. But recent studies show that the picture is not so simple.[106–112] For example, Jungwirth and co-workers[108,109] find that although this picture may hold for hard (nonpolarizable) ions such as sodium and fluoride, large soft ions such as iodide (and to a lesser extent, bromide and chloride) may accumulate preferentially at the surface. At the same time, these latter ions seem to be depleted in the

subsurface layer, where there is an enhanced concentration of cations.

Zangi et al. have considered what the analogous partitioning of ions at the surfaces of nanoscale hydrophobic plates might do to the hydrophobic interaction between them.[132] Again, changes in the strength of this interaction appear to be related to adsorption or exclusion of ions at the interface. High-q ions are once again excluded (Figure 10b), but the consequent salting-out is here identified as an entropic effect related to the formation of tight ion–hydration complexes and thus a decrease in configurational entropy of all of the species in solution. Medium-q ions induce salting-in because of a different entropic effect: they are adsorbed by the plates, and their expulsion into solution when the plates associate leads to a reduction in water entropy owing to the formation of hydration complexes. But low-charge-density ions cause salting-in enthalpically, since they bind to the surfaces and lower the surface tension of the plate–water interface, a mechanism analogous to the surface stabilization of large aggregates of small hydrophobic particles.[130] These results imply that Hofmeister effects may have a different origin, and thus a different character, for small and large hydrophobic particles: whereas in the former case there is an increase in hydrophobic aggregation for both high-q and low-q but not medium-q ions (except at high concentrations), for hydrophobic plates the relationship is monotonic, with an increasing tendency toward salting-in as the ion charge density decreases. But in both cases the mechanism is somewhat subtle and dependent on the direct ion–hydrophobe interaction, and need not (indeed, should not) invoke the deus ex machina of "water structure".

2.6. Long-Range Hydrophobic Interactions and the Role of Bubbles

As though this picture were not complicated enough, there seems to be a further type of hydrophobic interaction. In the early 1980s, measurements using the surface-force apparatus (SFA) revealed that there is an attractive interaction between hydrophobic surfaces that seems to extend over very long distances, exceeding the range of the normal hydrophobic interaction.[133,134] This puzzling observation was anticipated by the measurements of Blake and Kitchener in 1972,[135] who inferred the action of such a force by looking at the rupture of a water film at a hydrophobic surface as it was approached by an air bubble. This attraction is measurable even at separations of about 300 nm—several thousand molecular diameters.[136] What could be the source of such a long-ranged interaction? Speculations that it might be connected to some kind of extreme ordering or structuring of liquid water between the two surfaces[137] never quite shrugged off an air of implausibility. It has suggested that correlated charge or dipole fluctuations on the two surfaces might lead to a long-ranged electrostatic interaction, in a manner that makes no direct appeal to water structure per se.[138–140] Recently, Despa and Berry[141] have refined this perspective by considering the effects of the apparent orientational ordering of water molecules at hydrophobic surfaces[46,104] and the concomitant retardation of relaxation dynamics.[142] They suggest that the resulting slow reorientation of water dipoles at the surface will give rise to strong coupling and correlations between them, creating a polarization field that will induce a dipole at the hydrophobic surface. In this view, that the long-range attraction between two such surfaces is thus electrostatic, arising from induced dipole–dipole interactions.
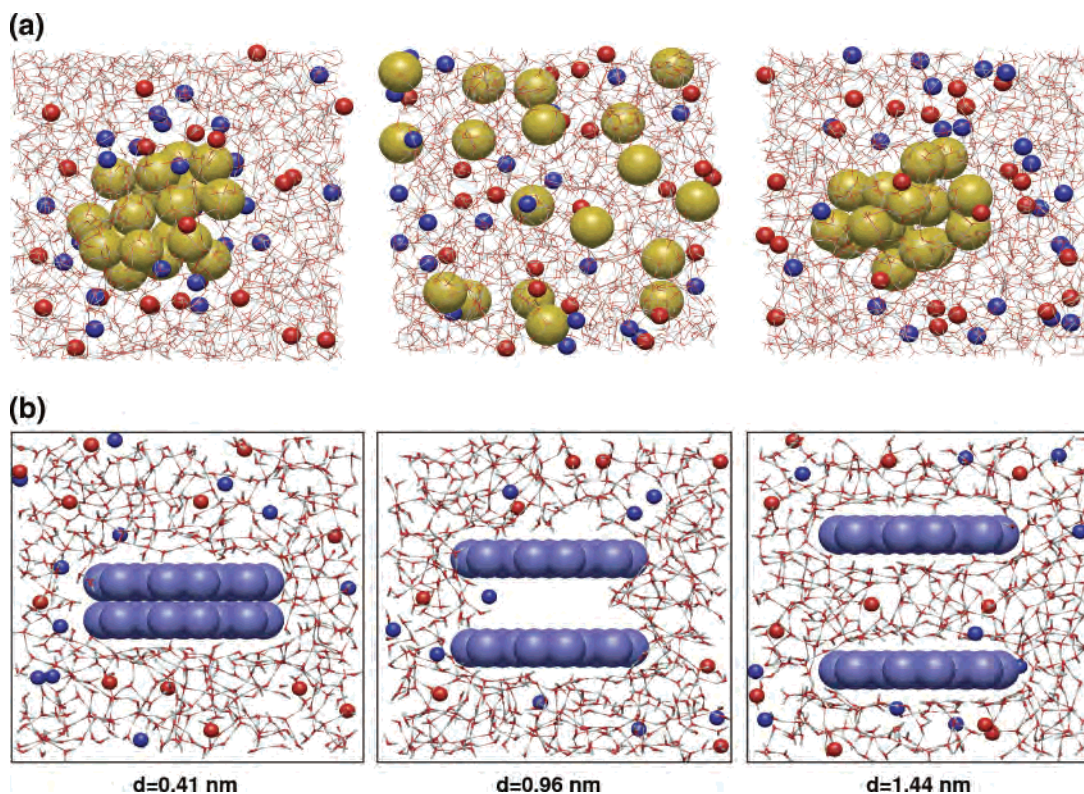
Figure 10. (a) Distribution of ions around hydrophobic (Lennard-Jones) particles in water. The hydrophobes are yellow, positive ions are red, and negative ions are blue. Low-q ions (left) are adsorbed preferentially at the particle surfaces, leading to micelle-like clusters of hydrophobic particles surrounded by ions, which prevents further aggregation and precipitation. High-q ions (right) tend to be depleted at the particle surfaces, which again leads to the formation of clusters. In the intermediate-q case (center) there is neither adsorption nor depletion, and the hydrophobes remain individually dispersed. Reprinted with permission from ref 130. Copyright 2006 American Chemical Society. (b) The distribution of high-q ($|q| = 1.00\ e$) ions around hydrophobic (LJ) plates at varying plate separations d. The ions are preferentially excluded at the surfaces and in the intervening water film, which retreats in a drying transition at around $d = 0.96$ nm. Reprinted with permission from ref 132. Copyright 2007 American Chemical Society.

Interestingly, Despa and Berry suggest that the orientational ordering of water at a hydrophobic surface provides a vindication of Frank and Evans' "iceberg" model[41]—a contrast with what Blokzijl and Engberts[46] concluded from the same basic observation, indicating how little consensus there is about what precisely is implied by "water structure" in this context.

An alternative explanation for the long-range hydrophobic attraction invokes the formation of submicroscopic bubbles between the surfaces, whereupon the meniscus pulls them together.[136,143] Such bubbles are hard to visualize directly—they would be too small to be seen in optical microscopy—and moreover it was not clear how the highly curved interfaces could be viable, since they would generate a high internal gas pressure (via the Laplace equation) that should lead to bubble dissolution.

Nonetheless, there is now some evidence that such bubbles may be formed. Using high-resolution optical microscopy, Carambassis et al.[144] saw bubbles about 1 μm in diameter in water in contact with a glass surface coated with fluorinated alkylsilanes. They observed jumps to contact between the surface and a similarly coated glass microsphere as it was brought toward the surface on the tip of an atomic force microscope (AFM). These jumps occurred at different separations—typically 20—200 nm—in different experimental runs, suggesting the abrupt appearance of bubbles of various sizes. Tyrrell and Attard[145,146] have also imaged submicroscopic bubbles, about 100 nm in radius and flattened against the surface, in AFM studies of hydrophobic surfaces in water. This flattening might explain why the bubbles are not

ruptured by the high pressures that would be inferred if they were assumed to be spherical with a radius equal to the jump-to-contact distance as the surface is approached by another hydrophobic object. More recently, such bubbles were seen also by Simonsen et al.[147] and Zhang et al.[148] using the AFM. The latter report flat gas bubbles about 5—80 nm thick and 4 μm across that remain stable at such a hydrophobic interface for over 1 h. But the bubbles form only when a particular protocol is followed for introducing the gas layer (carbon dioxide): in other words, the presence of the gas phase depends on the previous history of the interface.

A possible objection remains, however, that the bubbles imaged this way might be nucleated by the AFM probe tip itself, rather than pre-existing. Doshi et al.[101] argue that a dynamically fluctuating water density depletion owing to (or at any rate enhanced by) the adsorption of dissolved gases at a hydrophobic surface[149] could act to help nucleate bubbles heterogeneously when two such surfaces are brought together, as suggested in ref 99, rather than there being any stable bubbles already present at such surfaces.

Thus, it has been proposed that there may be distinct regimes for the interaction between hydrophobic surfaces: a long-ranged attraction created by bridging bubbles (either pre-existing or nucleated as the surfaces come together) and a medium-ranged interaction felt at separations of less than 20 nm or so where the attraction is of the same type as that involved in protein aggregation and folding—whatever that might entail. If the latter is due (at least for nanometer length scales) to the capillary-evaporation mechanism of Lum et al.,[80] any such distinction is at risk of becoming blurred: the
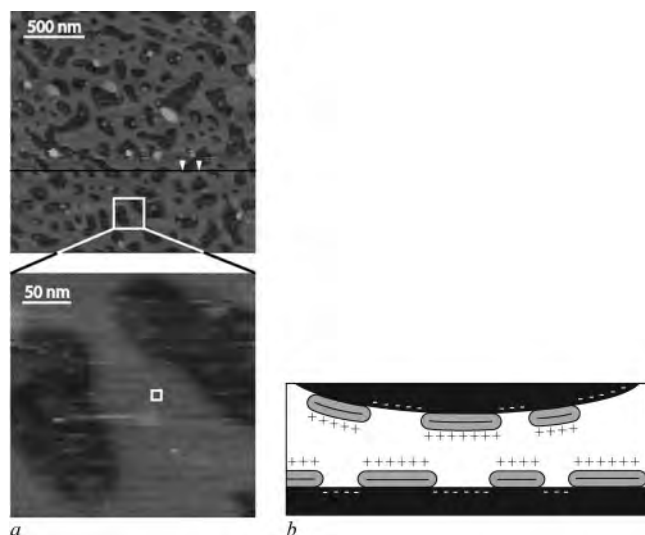
Figure 11. Patchy structure of surfactant films on mica, as revealed by AFM (a), has been explained in terms of the delamination and folding over of the monolayer to form bilayers separated by bare mica (b). (a) Reprinted with permission from ref 157. Copyright 2005 National Academy of Sciences.

force is then "bubble-driven" in both cases. But it is important to maintain the distinction between "bubble formation" induced by the solvent's liquid−gas equilibrium (cavitation/drying/capillary evaporation) and that induced by dissolved gas. It is strictly the latter that has been proposed as a mechanism for the long-ranged hydrophobic interaction.

If bridging bubbles are truly responsible for the long-ranged interaction, one would then expect the removal of dissolved gas from the liquid to influence the effect. Degassing does indeed seem to decrease the range and magnitude of the attraction[150−152] and, consistent with that effect, to increase nanoparticle adsorption on surfaces[153] and to enhance the stability of colloids[154,155]—although it is hard to differentiate between bulk and surface effects here.[150] Doshi et al.[101] found that removal of dissolved gases decreased the width of the depletion layer observed by neutron reflectivity, which could make the nucleation of bubbles less likely. None of this is inconsistent with the observation of Meyer et al.[156] that, although deaeration altered the force curves between two hydrophobic surfaces in the SFA for separations greater than about 25 nm, the short-ranged "jump-in" behavior was essentially identical for aerated and partially deaerated solutions—supporting the idea that there are indeed two distinct attractive hydrophobic mechanisms involved. It is only fair to conclude, however, that we are still not sure how either of them operates.

Indeed, Meyer et al.[157,158] have proposed that the principal source of the long-ranged hydrophobic interaction may have yet another origin. This force has generally been observed to operate between two surfaces rendered hydrophobic by monolayer coatings of surfactants: in the SFA, these films are typically adsorbed onto sheets of mica. AFM images of a mica surface coated with the cationic surfactant dimethyldioctadecylammonium bromide (DODAB), however, show that once immersed in water, the monolayer becomes patchy on a scale of about 100 nm. The film delaminates and forms bilayer patches separated by bare mica (Figure 11). Meyer et al. argue that as the two surfaces are brought together, bilayer patches will migrate by a rolling mechanism to bring them opposite bare patches on the opposing surface. This places regions of opposite charge facing one another—the

cationic head groups of DODAB against the negatively charged mica surface—giving rise to an electrostatic attraction. In this picture, then, there is no real "hydrophobic interaction" at all: the long range of the attraction is due simply to the Coulombic force.

Bubble coalescence should be influenced by a long-ranged interaction between hydrophobic surfaces. It appears to be suppressed by ions,[159] but in a selective manner: certain combinations of anions and cations have this effect, whereas others do not.[160] This is deeply perplexing, and there is no known explanation for it. Since salts in general decrease the surface tension of water, they would be expected to reduce coalescence; indeed, this has been proposed as the explanation for the foaminess of seawater relative to pure water. But the fact that some electrolytes apparently do not have this effect is truly strange. Craig et al.[160] suggested that coalescence might be somehow mediated by the long-ranged hydrophobic attraction, which salts might modify in an ion-specific way related to Hofmeister effects. But if this attraction is itself to arise from bubble formation or coalescence, then the argument becomes circular, and one might instead elect to invert the argument and explain the reduction of the long-ranged attraction in the presence of salts such as KBr and $MgSO_4$ by the salt effect on bubble coalescence.[161,162] The phenomenon provides another reminder of how poorly understood the influence of salts is on water structure and behavior. Nonetheless, Craig et al.[160] propose that this suppression of bubble coalescence might be physiologically useful, in that the coincidence of the salt concentration for maximum suppression and the concentration in blood suggests a role in the avoidance of decompression sickness.

2.7. Hydrophilic Surfaces

We must note with some resignation that the interactions between two hydrophilic surfaces are equally mired in uncertainties and controversy. Measurements with the SFA have suggested that there is a monotonically repulsive interactions between such surfaces.[163−165] But van der Waals interaction between surfaces would be attractive, and so once again "structuring effects" unique to water are among the explanations proposed to account for the difference.[166−168] Israelachvili and Wennerström dispute that idea,[169] arguing that in fact the hydration force between two hydrophilic surfaces is indeed either attractive or, because of the layering effects experienced by any liquid close to a sufficiently smooth solid surface, oscillatory. They suggest that the steep repulsion often measured between hydrophilic particles and surfaces at small separations is instead due to the characteristics of the surfaces themselves, for example, an entropic effect caused by increasing confinement of mobile surface groups such as silicic acid protrusions on the surface of silica, or the constraints imposed on the fluctuations of bilayer membranes. "As a suspending medium", they argue, "water should be seen as an ordinary liquid".[169]

In the cell, this situation is commonly encountered when two bilayer membranes come into close proximity, sandwiching a layer of water between the sheets of hydrophilic head groups. A repulsive force is indeed experienced by the bilayers when they are 1−3 nm apart.[170,171] Whatever its origin, this force is clearly of fundamental importance to the membrane dynamics. Simulations suggest that water molecules within 1 nm of the bilayer surface might have enhanced orientational order,[172,173] and Cheng et al.[174] have confirmed this picture experimentally by using coherent anti-
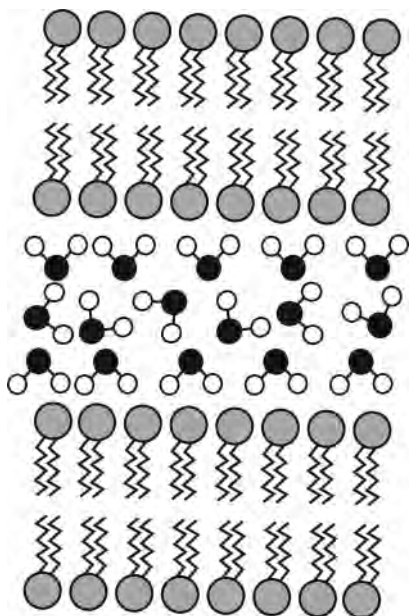
Figure 12. Orientational ordering of water molecules between lipid bilayers, as proposed in ref 174.

Stokes Raman scattering (CARS) microscopy to study water at lipid bilayer surfaces. They found that these superficial water molecules are preferentially oriented with their dipoles opposed to those of the lipids (Figure 12) and that they are more weakly hydrogen-bonded than in the bulk. It is tempting to conclude that in this instance water is not such an "ordinary" solvent after all. But one should not leap too far with that inference. No one should expect water adjacent to a surface to be bulklike: even simple liquids are layered in that circumstance by packing effects, and indeed models that include laterally ordered surfaces predict such lateral ordering of the liquid too.[175] Both densification induced by molecular packing and the lateral ordering due to surface structure—universal effects expected for any liquid—would be expected to disrupt the hydrogen bonding in the water layer. So here as elsewhere, before concluding that a change in "water structure" is a consequence of its unique hydrogen-bonded network, we must remember to ask not just whether that structure is different from that in the bulk but whether the differences go beyond those we might expect from the theory of simpler fluids.

3. The Aqueous Environment of the Cell

In that same spirit, whatever else we do and do not know about water structure and hydration, experience with simple liquids shows that we cannot expect much if any of the water in the cell to behave as it does in the bulk. The cytoplasm typically contains up to 400 g L^{-1} of macromolecules, which may occupy 5−40% of the total volume of the cell,[176] and as a result the cell is extremely crowded (Figure 13): macromolecules are typically separated by only 1−2 nm. Such narrow confinement would be expected to alter the structure of any liquid; for water in hydrophilic pores (Vycor glass, where the surface is covered with OH groups that may engage in hydrogen bonding), the hydrogen-bonded network appears to be significantly perturbed at these scales,[177] and the average coordination number is reduced from about 3.6 in the bulk to about 2.2. If such a structural change is widespread in the cytoplasm, the implications for hydration and hydrophobic association (for example, in terms of the

free-energy costs of displacing bound water) could be profound, indeed rather fearsomely so.

Moreover, molecular crowding means that diffusion rates are considerably lower—typically by a factor of 3−8 in the mitochondrion and endoplasmic reticulum—than in the bulk. NMR studies suggest that in fact confinement of water even within spaces several hundreds of nanometers wide can lower the molecular mobility of water significantly below that of the bulk,[178] whereas force-microscopy experiments reveal that water confined between two hydrophilic surfaces less than about 2 nm apart has a viscosity several orders of magnitude greater than that of the bulk, apparently owing to greater tetrahedral "ordering" of the liquid and (in this case) a large number of hydrogen bonds to the surfaces.[179,180] There is evidence[181] that crowding in the cytoplasm may make water molecular motions subdiffusive: the typical time t for a molecule to travel a distance l scales not as the Brownian $t \sim l^2$ but as $t \sim l^{2/\alpha}$, where $\alpha = 0.74$. This means that molecules take longer to reach their "target" but then stay in its vicinity for longer.

It seems nature may put this crowding to good effect: since processes that reduce the crush are entropically favorable, both the native stability and the refolding rate of globular proteins seem to be enhanced in a crowded environment relative to dilute solutions.[182] In addition, some protein functions may be optimized to the dynamics of the crowded environment in which they will operate.[183]

As well as being crowded, it seems likely that the cytoplasm is inhomogeneous: there is increasing evidence that even soluble proteins in such concentrated solution form relatively long-lived clusters. Small-angle X-ray and neutron scattering shows that lysozyme forms clusters of about 3−10 molecules at volume fractions of between 0.05 and 0.2,[184] whereas light scattering from concentrated solution of bacterial lumazine synthase reveals metastable clusters with lifetimes of around 10 s and a mean radius of about 350 nm (the individual molecules are about 15.6 nm in diameter).[185]

If water in the cell is evidently *not* like bulk water, the question that remains unanswered is whether those differences are sufficiently pronounced to matter. Pretty much all extremes of opinion, and everything in between, can be found represented in the literature. Despite the existence of a well-developed thermodynamic theory of small chemical systems,[186] biochemistry texts have tended to employ bulk descriptions of the thermodynamics and kinetics of the cellular environment—and generally to surprisingly good effect. On the other hand, Pollack has argued that the cytoplasm is like a gel (without the macromolecular network that makes ordinary gels cohere), which maintains its integrity if relatively large sections of the cell membrane are removed.[187,188] It has been claimed that the proton NMR relaxation times of water in cells differ from that in the bulk and, moreover, that the relaxation becomes more "bulklike" in diseased, such as cancerous, cells.[189,190] (The recent observation that most of the cell water in the halophilic Dead Sea extremophile *Haloarcula marismortui* has a translational diffusion coefficient more than 2 orders of magnitude lower than that of bulk water[191] seems to be exceptional and somehow due to the high salt concentration, although this remains as yet unexplained.) The notion often advanced here is that the cell somehow "tames" bulk water and thereby renders it "biophilic". This superficially appealing idea tends to blur together the many different things that can happen to water close to ions and surfaces and in confined spaces
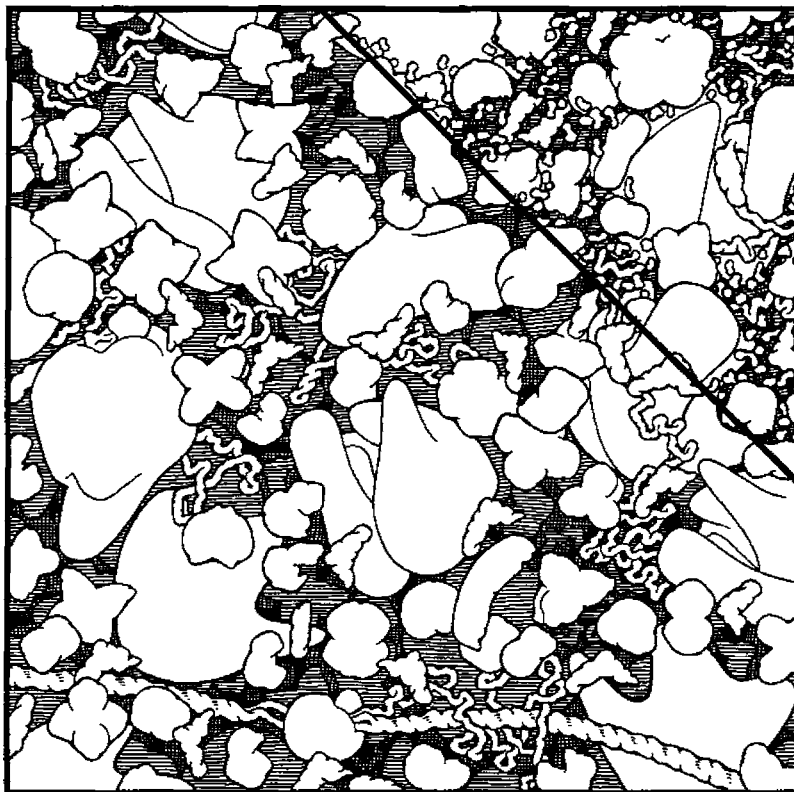
Figure 13. Cytoplasm is a crowded environment, as revealed in this scale drawing. For clarity, the small molecules are drawn only in the upper right corner. Reprinted with permission from D. Goodsell, *The Machinery of Life*. Copyright 1993 Springer-Verlag.

within a cell—some of them perhaps specific to water, some generic to any associated liquid, and others to any liquid. I have discussed some of the general phenomena that might be involved in this putative "taming" (one should really say modification) of water. Now I shall look at some of the specific roles that solvation (in general) and hydration (in particular) play in the structure and behavior of biological macromolecules.

4. Protein Hydration: Nonspecific Effects

4.1. The Hydration Shell

The pioneering early studies of protein secondary structure by Pauling[192,193] have, perhaps understandably, something of the in vacuo mentality about them insofar as they stress intra- and intermolecular hydrogen bonding in the polypeptide chains without much consideration of the role of the solvent. Thus Pauling's iconic α-helices and β-sheets are held together by hydrogen bonds between polar residues, and the apparent implication is that there is an enthalpic penalty to breaking up these structures, without consideration of the hydrogen bonds that can then form between the peptide residues and water. That picture would actually argue for an enhanced stability of these secondary structural motifs in nonpolar solvents, where there would be no such competition for hydrogen bonding from the solvent molecules. Indeed, completely unsolvated polyalanine oligomers in the gas phase remain helical up to at least 450 °C, where the peptide is almost entirely dissociated: the noncovalent interactions exceed the strength of a covalent bond.[194] The role of water, then, appears to be not to stabilize the α-helix but to loosen it and allow flexibility of the peptide chain.

What Pauling's model lacked, of course, is the existence of hydrophobic interactions between the nonpolar protein

residues—a feature proposed by Langmuir[195] and promoted by Bernal[196] in the late 1930s. Crudely speaking, this notion invokes the burial of hydrophobic residues in the protein interior. It is now generally accepted that protein folding is driven primarily by a balance between these two factors—intramolecular hydrogen bonding and hydrophobic interactions—although there is no consensus on precisely where the balance lies. Certainly it is an oversimplification to present globular proteins as a kind of "polymer micelle" that is wholly hydrophobic inside and wholly hydrophilic on the surface: the two types of residue are in any case not sufficiently segregated along the chain to make such a separation possible. It is true that on average 83% of nonpolar side chains are buried in the interior of folded proteins—but so are 63% of polar side chains and 54% of charged side chains.[197] And as we shall see in section 5.6, it seems unwise to assume that the hydrophobic interior of a folded protein is "dry".

We have seen that the precise mechanism of the attraction between hydrophobic groups in water remains imperfectly understood. And it is as well to point out now, before expanding on it below, that any attempt to understand protein function cannot rely on statics (structure) alone but must consider also the dynamical behavior. With those provisos, what can be said about the role of water in producing the characteristic folded structures of proteins in general? And how does water hydrate and decorate those three-dimensional forms?

A key point is that it does not seem sufficient to incorporate water into protein structure prediction merely via some heuristic potential that acknowledges the existence of hydrophobic interactions. This approach has been common in attempts to simulate protein folding, because an explicit, atomistic representation of the solvent in MD simulations

remains computationally very expensive, restricting the timescales and the conformational space that can be accessed. But atomistic simulations of the folding of the SH3 protein domain have revealed that the process may depend on a rather gradual, molecule-by-molecule expulsion of water from the collapsed interior.[198−200] These studies characterize folding as a two-stage process: first, collapse to a near-native structure that retains a partially hydrated hydrophobic core, followed by slower expulsion of the residual water. This water might play the part of a lubricant to enable the hydrophobic core to find its optimally packed state. Moreover, some water molecules typically remain in the core, hydrogen-bonded to the peptide backbone. NMR evidence suggests that the SH3 domain of the drk protein of *Drosophila* indeed may adopt a collapsed but loosely structured conformation.[201,202] This challenges the picture presented by Lum et al.[80] and ten Wolde and Chandler[84] of an abrupt, collective drying transition as the water is confined between basically hydrophobic surfaces.

Papoian and co-workers[203,204] have modified a typical Hamiltonian used for protein structure prediction to incorporate the possibility of water-mediated interactions between residues. This allows for the formation of relatively long-ranged (6.5−9.5 Å) connections between hydrophilic parts of the folding chain through bridging water molecules—something that is not permitted by the "dry" Hamiltonian. Such water bridges can be "squeezed dry" in the later stages of the folding process: the water acts as a temporary, loose glue that holds the folded chain together until it is ready for final compaction—in effect constraining the conformational freedom, reducing potential topological frustration, and generally "smoothing" the funnel in the folding energy surface.[204] There is a substantial improvement in the simulation structure predictions for several proteins when these water-mediated contacts are included.[203] Thus, whereas the classical Kauzmann model of interactions between hydrophobic species postulates an attraction resulting from the liberation of "bound" or "ordered" hydration water, the contrasting picture that emerges here in the interactions between *hydrophilic* residues is one in which the attractions are promoted enthalpically by water molecules that are constrained at the macromolecular surface, despite the entropic cost.

Harano and Kinoshita argue that excluded-volume effects can play a significant role in offsetting the entropic cost of protein compaction.[205,206] This idea, familiar from the theory of colloidal interactions, explains how large particles (protein chains and side chains) and small particles (water molecules) tend to phase separate to minimize the volume of space surrounding each of the large particles that is inaccessible to the small particles. This exclusion results in a reduction of translational entropy of the small particles. Harano and Kinoshita use simple Lennard-Jones potentials to estimate that, for a peptide of the order of 50 residues or more, the gain in translational entropy can equal or exceed the loss of configurational entropy of the compact chain. Here, the fact that water is a small solvent molecule with a relatively dense liquid state under ambient conditions plays an important role in the relatively large gain in translational entropy.

Studies like those above raise the issue of how highly resolved a model of protein hydration needs to be in order to capture its important features. The classical picture favored in biochemistry posits two distinct classes of water molecule in the hydration shell.[207] "Bound" waters are hydrogen-bonded to the protein at specific locations and typically remain in position even in an anhydrous environment (nonaqueous solvent, vacuum, or "dry" powder). They can thus be identified crystallographically and are regarded as in some sense an intrinsic part of the protein structure, to all intents "frozen" in place and not liquid-like at all. Typically 10% of the "dry mass" of proteins consists of such bound water. "Free" water, meanwhile, is deemed to remain not only mobile but essentially bulklike even in the immediate hydration layer of the protein. There is no doubt now that this is a highly oversimplified picture. Certainly, some water molecules in the hydration shell have dynamics very different from those in the bulk, with residence times of up to several hundred picoseconds.[208,209] (Water molecules in protein interiors can be even slower to exchange with the bulk, with residence times measured in milliseconds.[210,211]) But there is a continuum between these "bound" molecules and those that behave dynamically as though they are indeed bulklike. "Bound" and "free" are categories that are so ill-defined as to be positively misleading.

The existence of discrete water-binding sites on a protein surface may not necessarily imply that they are occupied by water molecules. Makarov et al.[212] found that, even though several hundred such sites could be identified in simulations of myoglobin, only about half of them were typically occupied at any time. This led them to propose that an atomistic representation of tightly bound waters on a protein surface may not in general be appropriate and that instead a continuous density distribution model of the hydration waters is more suitable. Moreover, the residence times of water molecules in specific hydration sites does not seem to depend in any simple way on the nature of the residue to which they bind—its polarity or hydrogen-bonding ability, for example.[212,213] In the simulations of Makarov et al., these residence times seemed to depend almost exclusively on the geometry of the protein surface: hydration waters with long residence times were those within clefts and cavities, implying that steric hindrance to diffusion is the key factor. And yet it appears, in simulations at least, that the diffusion rate *normal* rather than parallel to the protein surface is in general the most strongly perturbed component.[214] As these studies indicate, part of the problem of describing the hydration structure of proteins is that there is no agreed or obvious framework to use: continuum or discrete, static or dynamic, scalar (density) or vector (orientation).

There seems to be considerable variation even in crude measures of hydration structure such as the average water density in the first hydration shell. Smolin and Winter[215] see an enhancement of only 0.3−0.6% relative to the bulk density for simulations of staphylococcal nuclease (SNase), whereas a simulation of hydrated lysozyme[216] yields a hydration layer 15% denser than the bulk. X-ray and neutron-scattering studies indicate that a density enhancement of this latter magnitude might be quite typical for proteins.[217] Smolin and Winter describe the hydration layer of SNase in terms[215] of water rings, which form structures somewhat reminiscent of clathrate cages around convex nonpolar residues. As seen above, however, this kind of "crystallographic" picture of hydration may not be the most appropriate way of thinking about the way the hydrogen-bonded network accommodates hydrophobes. The upshot, according to Makarov et al.,[214] is that "in general, the current hydration description does not provide us sufficient information about the solvent structure around the protein in its natural aqueous environment. Hence,

such descriptions can rarely be relied upon in accurate studies of molecular docking and ligand design and folding".

Rand[218] has suggested that binding and release of water molecules may play a quite general role in the energetics of protein function and that in this context water should be thought of as a ligand as well as a solvent. For example, the thermodynamic measurements of Colombo et al.[219] indicate that about 60 extra water molecules bind to hemoglobin during the transition from the fully deoxygenated to the fully oxygenated state, whereas Kornblatt and Hui Bon Hoa[220] show that the addition of an electron to cytochrome *a* bound to cytochrome oxidase is accompanied by the binding of 10 water molecules to the oxidase. This solvation-energetic contribution to protein function should apply regardless of the precise nature of the "bound" water, but as we can see, the structural details may in fact make a considerable difference to the quantitative effects of such processes.

4.2. Dynamics, Cooperativity, and the Glass Transition

Any notion that proteins "only work in water" has now been thoroughly dispelled by evidence that enzymes can retain some functionality both in nonaqueous solvents and in a vacuum.[221,222] But neither of these environments is truly nonaqueous in the sense of being devoid of water: in both cases, some water molecules remain tightly bound to the protein. Although this "bound water" may not be enough to fully cover the protein surface with a monolayer "aqueous sheath", nonetheless proteins seem to require about 0.4 g of water/g of protein to achieve their normal functionality.[223]

This is a rather nonspecific effect, suggesting that there is some general property of this hydration water that activates the protein. As indicated earlier, it seems likely that water lubricates the protein dynamics, giving the peptide chain the mobility it needs to conform to and bind its substrate. But precisely how this occurs is a complex matter, in which it seems insufficient to regard hydration water as a kind of all-purpose plasticizer. Broadly speaking, one can consider the problem from three aspects: how does the protein modify water dynamics, how does the water modify protein dynamics, and how are they coupled?

It seems clear that one cannot generalize about the dynamical behavior of water in the hydration layers: as we have seen already, there is a very wide range of residence times at different sites on the protein surface, and equally there are big variations in the rotational and translational relaxation times. Modig et al.[224] find from magnetic relaxation dispersion measurements of hydrated bovine pancreatic trypsin inhibitor that on average 95% of the water molecules in the hydration layer have rotational and translational dynamics retarded by only about a factor of 2. So the dynamics of the hydration shell may be dominated by just a small number of hydration sites in deep surface pockets and clefts, where the motions of water are much more strongly perturbed.[212] That picture is supported by ultrafast spectroscopic studies of bovine pancreatic α-chymotrypsin,[225] which show that 90% of the hydration shell has more or less bulklike relaxation, while the remainder is appreciably slower (tens of picoseconds). In both experiments and simulations, Li et al. find slow relaxation on timescales of 5−87 ps in the water dynamics around a tryptophan group Trp7 of apomyoglobin.[226] Bhattacharyya et al. have suggested that such slow dynamics are due to the effect of the protein's potential field on the hydration water,[227] but Li et al. argue

that the protein and water motions are strongly coupled: if either the water or the protein is frozen in the simulations, the slow component disappears. Russo et al.[228] suggest that the dynamics of hydration water are most strongly perturbed at the interfaces between hydrophilic and hydrophobic regions on a protein surface, as a result of the frustration that may arise between the differing hydration structures involved.

How does hydration affect protein dynamics? It is generally considered that a protein needs to maintain a delicate balance between rigidity and flexibility of structure: the specificity of the folded shape is clearly central to an enzyme's substrate selectivity, but it must also remain able to adapt its shape by accessing a range of conformations without getting stuck in local energy minima.[229] In water, hydrogen bonds that might otherwise form between donor and acceptor groups in the protein side chains, rigidifying the structure, may instead be transferred onto solvent molecules, providing greater mobility of the backbone;[230] in other words, the kind of intramolecular hydrogen bonding that is often assumed to be central to protein structure can potentially inhibit protein function.

For example, Olano and Rick[231] find that for both bovine pancreatic trypsin inhibitor (BPTI) and barnase, which have polar and hydrophobic cavities, respectively, the addition of a water molecule into the cavity makes the proteins more flexible by weakening intramolecular hydrogen bonds. Dwyer et al.[232] find that water molecules buried in the hydrophobic interior of mutant staphococcal nuclease, in which a hydrophobic valine residue is replaced by a glutamic acid residue, increase the dielectric constant in the interior markedly. Such solvent penetration would shield charge−charge interactions in the protein chain, increasing its flexibility as well as significantly affecting pK_a values of ionizable residues.

But as well as merely loosening structure by breaking hydrogen bonds, the solvent might also play a more active role in protein dynamics. It is been suggested that this role is to "inject" fluctuations into the protein to boost its conformational flexibility—an effect that would involve cooperative aspects of the molecular motions. Simulations of scytalone dehydratase indicate that water molecules in the protein's binding pocket seem to play a part in the conformational flexibility that is necessary for binding of the substrate and that there is cooperativity between the motions of the "bound" water molecules and the ligand-free protein.[233] These cooperative motions assist in the binding event by arranging for water molecules to be expelled through a "rear gate" as the ligand enters through the binding site's "front gate".

If indeed one of the roles of the hydration water is to actively excite protein dynamics via liquid-state fluctuations, we might ask whether there is anything special about water that enables this—after all, fluctuations occur in any liquid. That is to say, are there features of water that make the dynamics of the hydration shell qualitatively different from those of a solvation layer in a simple liquid, for example because of cooperative effects stemming from the hydrogen-bonded network? Are these fluctuations any different from those that take place in the bulk liquid? And do hydrogen bonding or other aspects of water structure promote coupling to the protein?

Both simulations[234] and experiments[228,235] show that water dynamics in the hydration layer of a peptide are anomalous with respect to the bulk. Quasi-elastic neutron scattering

(QENS)[228,235] suggests that the translational dynamics of hydration water for model peptides at room and physiological temperatures are non-Arrhenius-like, with slow and nonexponential relaxation dynamics reminiscent of those seen in supercooled water below −20 °C. The hydration water seems to adopt a state akin (albeit not necessarily equivalent) to that of a glass, with a very rough potential-energy landscape and slow hopping between local potential minima. Thus, the water molecules no longer diffuse independently: their motion is dependent on that of their near neighbors. It is tempting to regard this as a result of the interconnected nature of the hydrogen-bonded network, which is highly constrained close to the protein surface and so might develop an enhanced degree of cooperativity relative to the bulk. That is indeed suggested by QENS studies[228] of solutions of the model hydrophilic peptide *N*-acetyl glycine-methylamide: at concentrations corresponding to a single hydration layer shared between solute molecules, the water dynamics seem to be strongly coupled to those of the peptides, whereas at lower concentrations equivalent to 2−3 hydration layers per solute, there appears to be dynamical coupling between inner and outer hydration layers.

By impressing a pseudoglassy character on its hydration sphere, the protein may suppress crystallization at low temperatures, which might afford freeze-tolerance. After all, a common physiological response of freeze-tolerant organisms is to manufacture glass-forming compounds such as glycerol and trehalose, which might further suppress crystallization of the solvent when the intrinsic "glassiness" of hydration water is not sufficient.

But if the protein induces pseudoglassy dynamics in its hydration layer, how then might the hydration dynamics feed back on the behavior of the protein? It seems that this kind of anomalous dynamics is just what a protein needs to attain the kind of conformational flexibility that is intrinsic to its function. One is tempted to suggest that, if the protein needs to "feed off" the dynamics of its solvation layer, then water is the ideal solvent because its hydrogen-bonded network makes it ideally suited to being "molded" by the protein into a pseudoglassy state.

There is some evidence to support the idea that the dynamics of a protein can be "slaved" to those of the solvent—that the large-scale fluctuations of the protein chain reflect those of the solvent.[236] Below about 200−220 K, proteins seem to "freeze" into a kinetically arrested state that has genuine analogies with a glass:[237−240] the protein atoms undergo harmonic vibrations in local energy minima but no diffusive motion. Both experiment[241] and simulations[242,243] imply that this glasslike transition of a protein coincides with dynamical changes characteristic of a true glass transition in the solvent. It is suggested[244,245] that the solvent and protein motions are intimately coupled so that as a protein is warmed through its glass transition temperature the dynamics of the hydration shell "awaken" motions in the protein. Although simulation of proteins in vacuo show evidence of low-temperature dynamical changes,[246] there is no sharp glasslike transition around 200 K unless the protein is hydrated. Bizzarri and Cannistraro speculate that the dynamics of the protein and solvent are so strongly coupled that they "should be conceived as a single entity with a unique rough energy landscape." In other words, the protein motions are not simply "slaved" to those of the solvent, but "the very topological structure of the protein energy landscape could be deeply altered by the spatial organization, as well as by

the dynamical behavior of the hydration water".[234] In support of that idea, Tarek and Tobias[247,248] use MD simulations to argue that relaxation in the coupled protein−water hydrogen-bonded network close to the glass transition is governed by translational diffusion of water molecules. On the other hand, the dynamical transition at 200−220 K does not appear to affect the rate-limiting step in enzyme catalysis,[249,250] and Fenimore et al.[251] have suggested that protein function can be divided into processes that are and are not "slaved" to the solvent dynamics.

Chen et al. have proposed, however, that the dynamical change around 200 K is not a true glass transition at all but a crossover from "fragile" to "strong" behavior in the hydration water.[252] "Strong" liquids follow the Arrhenius law in the variation of viscosity with temperature as the liquid is cooled toward its glass transition temperature, whereas fragile liquids (which is most of them) show significantly faster increases in viscosity. A strong-to-fragile crossover has been proposed for bulk water near 228 K[253] and was reported at around 225 K for water confined in nanopores.[254,255] This behavior is expected to be characteristic of strongly associated "tetrahedral" liquids such as water and silica. Meanwhile, Kumar et al.[256] have linked the dynamical transition to the first-order phase transition between high- and low-density liquid states of water that is predicted at around 200 K at high pressure.[16] These interpretations, which are not in fact inconsistent, hinge on aspects of water's low-temperature behavior that are highly unusual, making at least this aspect of biomolecular solvation unlikely to be observed in another solvent system. That the dynamical transition is intimately linked to the intrinsic properties of supercooled water is further implied by the observation of a similar crossover between strong and fragile dynamics in the hydration shell of DNA[257] and RNA[258] at around 220 K.

Something analogous to the effect of temperature on solvation dynamics might occur as the degree of hydration is altered.[247,248,259] Pizzitutti and Bruni[259] find that the dielectric relaxation time for rapid proton motions along chains of water molecules at the surface of lysozyme—a measure of the collective dynamics of the water network—diverges at the same hydration level as that required for lysozyme to become functional (about 130 water molecules per protein molecule). Further support for this picture comes from MD and neutron-scattering studies of water dynamics as a function of protein concentration,[260] which suggest that the water behavior changes qualitatively for low degrees of hydration: the translational and rotational dynamics are markedly slower below the "critical" level of hydration needed for proteins to be fully functional. Only when a secondary hydration layer is present might molecular diffusion between the inner and outer hydration shells promote "plasticity" of the hydration network and thus catalyze the motions of protein side chains.

Oleinikova and co-workers[261,262] suggest that this behavior depends on the formation, at a critical "water coverage" on the protein surface, of a fully connected hydrogen-bonded network of water molecules. In other words, the collective dynamics become "activated" in a two-dimensional percolation transition. This threshold for a single lysozyme molecule appears to require about 50% of the protein surface to be covered with water, which would correspond to about 66% coverage of the purely hydrophilic regions. This is essentially identical to the percolation threshold for clusters formed on two-dimensional square and honeycomb lattices. The typical

lifetimes of percolating hydration networks are comparable with the (picosecond) lifetimes of a single water−water hydrogen bond, and protein side-chain dynamics can become slaved to these network dynamics if their characteristic timescales coincide.[262]

The idea of a "critical hydration threshold" below which proteins cease to function has, however, recently been questioned by Kurkal et al.,[263] who find using incoherent neutron scattering that anharmonic, diffusive motions of the protein chain—generally taken to be a signature of "glassy" dynamics—in pig liver esterase are present even for strictly zero hydration at room temperature. Thus, even though enzyme activity does appear to increase with increasing hydration, it is not clear that the "loosening up" generally thought to be induced by water is always essential for function.

These considerations prompt the pragmatic question of how much water needs to be included explicitly in a hydration shell in order to simulate a protein realistically. Since this is computationally expensive, one might like to get away with including as little water as possible. Hamaneh and Buck[264] find that a shell just two or three layers thick (using the CHARMM22/CAMP potential function) will suffice, which implies, perhaps, that the cooperativity of water dynamics in the hydration shell does not extend very far, at least for the case of lysozyme considered in that study.

The heterogeneity of a protein surface, which is typically 50−60% hydrophobic, means that there are significant lateral variations in water dynamics in the hydration layer: Russo et al. find that water motions are about an order of magnitude faster near hydrophobic side chains than near hydrophilic residues.[235] They hypothesize that these variations in water mobility might serve a functional role by producing fast-moving "slip streams" on the protein surface, in a manner analogous to a "nanofluidic" technology, that direct water molecules to active sites where they mediate recognition events[260] (see below).

A further, subtle feedback between solvation dynamics and protein structure and function and has been proposed by Despa et al.[142] They suggest that if hydrophobic units on a peptide chain elicit a locally "structured" hydration shell, this slows the dynamics, and thus the dipole reorientation and the dielectric susceptibility, of the water molecules in that shell. This in turn increases the Coulombic interactions between nearby polar groups. Thus, they conclude, "hydrophobic residues play an active role in mediating intramolecular interactions between the polar side-chain residues of a protein." Despa et al. propose that slaving of protein dynamics by solvent motions may in fact be mediated primarily by this effect, which can lead to strong dipole fluctuations in some parts of the hydration layer.

Even while the details of the dynamical coupling between protein and hydration water remain to be clarified—and that may depend on, among other things, an ability to move beyond a reliance on simulations in probing local dynamics on a heterogeneous protein surface and a better understanding of the issue of "water structure" around polar and nonpolar residues—it seems clear that there are strong arguments for regarding proteins as fuzzy-edged entities that not only influence their solvation environments but are in turn "fine-tuned" and modified by these environments in ways that affect their biological function and their behavior at extremes of temperature and dehydration, and quite possibly at elevated pressure too.
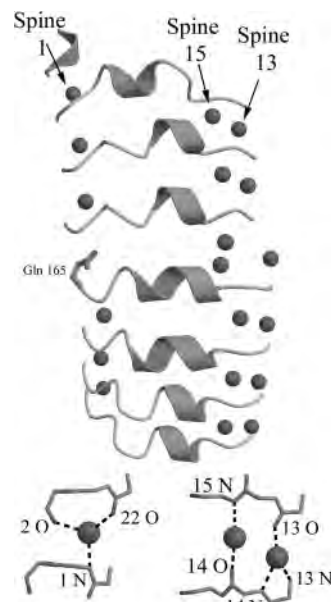

Figure 14. Water "spines" in the secondary structure of the LRR domain of Inl B. The water molecules are the dark gray spheres, and the lower frame shows the typical hydrogen-bonding pattern. Reprinted with permission from ref 266. Copyright 1999 Elsevier.

Thus, we can see already that there are apparently various ways in which proteins might make functional use of local variations in hydration structure.[265] It is important to recognize, particularly in the context of the astrobiological debate over whether "nonaqueous life" is feasible, that this sort of utility goes beyond merely exploiting the characteristics that a particular molecule happens to have in a particular solvent, such as the clustering tendency of amphiphiles. Rather, it amounts to a functionally motivated reconstruction of the environment—something that may be especially (if not uniquely) feasible in aqueous solution.

5. Protein Hydration: Specific Roles of Water in Structure and Function

5.1. Secondary Structure

Given that water molecules may evidently mediate the folding of proteins,[203,204] it is scarcely surprising to find that they may sometimes apparently get "frozen" in place in the folded structure—not as a kind of lubrication that has been imperfectly expelled but as an element of the secondary structure in their own right. This seems to be the case in internalin B (Inl B), a bacterial surface protein found in *Listeria monocytogenes* that helps to activate the bacterium's phagocytotic defense against the mammalian immune system. The leucine-rich repeat motif of Inl B, which is common to all proteins of the internalin family, contains a series of stacked loops that are held together by water molecules bridging the peptide chains.[266] These waters are organized into three distinct "spines" through the stack and are an integral part of the secondary structure (Figure 14).

5.2. Protein−Protein Interactions

Interactions between proteins and aggregation of their subunits are commonly discussed in terms of the same guiding forces that govern the folding of the primary protein chain: hydrogen bonding, polar interactions, and hydrophobic interactions. Just as water-mediated intramolecular

contacts may assist in protein folding,[203,204] so these contacts can serve to facilitate selective recognition in protein−protein interactions.[267] In other words, it is not simply the case that water molecules can bridge two proteins: such contacts can be imbued with significant information content that allows the interactions to be discriminating. Thus, protein surfaces in a sense extend the range of their influence via their hydration shells. These water-mediated interactions may be optimal when they involve two oppositely charged groups, such as an acid−base pair, where the apparently favorable Coulombic interaction of a direct contact is offset by a large Gibbs energy penalty to the complete desolvation of the charges that would be required to make such a contact.

Fernández and co-workers[268,269] have proposed a further way in which hydrogen-bonding groups on the backbone can mediate the intra- and intermolecular contacts involved in these processes. They postulate the existence of units called dehydrons, which have the key property that it is energetically favorable to remove water from their vicinity, making them "adhesive" sites for hydrophobic regions. In contrast to the conventional adhesion of two hydrophobic groups via the hydrophobic interaction, dehydrons are (paired) *polar* groups that engage in hydrogen bonding. Any hydrogen bond between peptide chains (inter- or intramolecular) is stabilized by removal of nearby water, which decreases the screening and increases the Coulombic interaction between the polar moieties. That is why most backbone hydrogen bonds in proteins are in fact dehydrated by being "wrapped" in surrounding hydrophobic groups, which "dry" the hydrogen-bonded region. Dehydrons are hydrogen bonds that are rather poorly wrapped in this way, making removal of water, for example, by the formation of a new peptide−peptide contact, energetically favorable. Fernández has used the atomic force microscope to measure directly the attractive "dehydronic force" exerted by a "dehydron" monolayer (a hydroxylated alkylthiol) on an AFM tip carrying a hydrophobic patch of alkylthiols.[270]

Many different proteins possess dehydron units[268]—human myoglobin has 16, for instance, and human ubiquitin has 12. These units appear to be concentrated at sites that engage in complexation with other proteins, and may play an important role in protein−protein interactions such as the association of capsid assemblies in viruses.

Fernández et al.[271] point out that underdehydrated hydrogen bonds seem to be a common feature of proteins with a propensity to form amyloid aggregates, which are associated with neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases. Such proteins appear to undergo a conformational change from a soluble, globular form to an insoluble form that aggregates via β-sheet formation into fibrils. Prion diseases such as scrapie and Creutzfeld−Jakob disease may be caused by such a conformational change in the prion protein PrP from its soluble cellular form PrPC to the insoluble PrPSc. Fernandez et al. propose that destabilization of the globular fold, and consequent amyloidogenic capacity, is related to the tendency of dehydron units to promote β-sheet aggregation. MD simulations of the hydration of native human and sheep PrP confirm the presence of underwrapped hydrogen bonds[272] but show that the hydration structure here is surprisingly fluid, without well-localized water molecules.

Studies of protein−protein interfaces have shown that most of the binding free energy comes from just a few residues, called hot spots.[273−275] Typically, these hot-spot residues are

defined as those for which the binding free-energy difference on alanine mutation is at least 4.0 kcal/mol, whereas "warm spots" have a binding free-energy difference of 2.0−4.0 kcal/mol. Hot spots are often clustered together and surrounded by a ring (a so-called O-ring) of residues that do not seem to have a role in binding but shelter the hot-spot residues from water.[274] MD simulations of hot and warm spots in the binding of hen egg lysozyme to antibody FVD1.3 seem to confirm this O-ring hypothesis, showing that these residues are significantly less accessible to water molecules.[276] Moreover, the water molecules that do penetrate to the hot and warm spot sites have unusually long residence times and seem to play essential roles in binding via the formation of water bridges.

5.3. Mediation of Ligand Binding

The binding of small molecules to protein receptors is generally discussed in terms of the replacement of water in the binding site by ligand groups that are cognate to the adjacent protein surface, through either the juxtaposition of hydrophobic regions or the formation of hydrogen-bonded contacts. In either case, the notion is that there may be both entropic and enthalpic gains that contribute to the ligand binding affinity.

Although this picture is probably correct in broad outline, it ignores many subtleties. For one thing, some water molecules are often retained in the binding site. Renzoni et al.[277] point out that hydration water can potentially serve distinct and in fact divergent purposes in the mediation of ligand binding by proteins. On the one hand, it can make the binding surface highly adaptable and thus somewhat promiscuous; on the other hand, there is evidence that water molecules occupying crystallographically defined sites in a protein structure through hydrogen bonding to polar residues can act as removable "tools" or extensions to the peptide chain for assisting in the specificity of substrate binding.

The former role is illustrated in the mechanism by which oligopeptide binding protein OppA binds very small (2−5 residue) peptides with more or less any amino acid sequence. This lack of specificity is made possible by the fact that all interactions between the protein and the peptide side chains are mediated by water: hydration of the voluminous binding site creates a highly malleable receptor matrix.[278,279] Rather than acting as a plastic medium that can be arbitrarily manipulated to accommodate a substrate, this water seems to constitute a well-defined, "bricklike" filler: some of the water molecules adopt the same positions for different substrates (Figure 15). In this sense, they are not so much a "filler" at all, but rather an extension of the protein surface that bears much the same information content, making highly specific hydrogen-bonding interactions with the cavity walls.

In contrast, biology also uses water to achieve selectivity. Chung et al.[280] found that a network of water molecules in the peptide-binding site of the SH2 domain of tyrosine kinase Src, which plays an important general role in cell biology by mediating protein interactions in tyrosine kinase signaling, dictates the specificity of these interactions. Water molecules play a similar role in the binding of some protease inhibitors to their target enzymes,[281] and in the binding mechanism of some antibodies,[282,283] indicating that nature has mastered the "rules" of incorporating water into the binding site sufficiently to use them for essentially ad hoc challenges of molecular recognition.

A combination of these seemingly contradictory roles of water—specificity and plasticity—was revealed in a crystal-

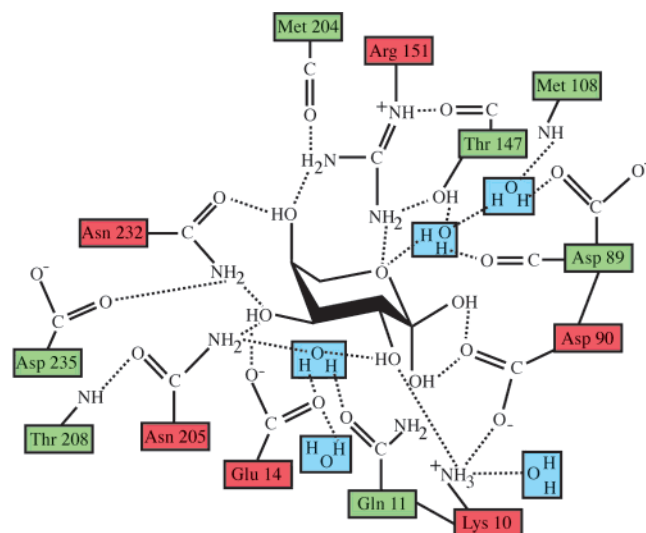**Figure 15.** Schematic picture of water "bricks" in the binding site of OppA.

Figure 16. Active site of arabinose binding protein. Red and green show the first and second binding-site shells.

Figure 17. Concanavalin A binds these two sugars with different specificity.

lographic study of the binding specificity of the bacterial L-arabinose binding protein (ABP) for various related sugars.[284] ABP also binds D-fucose and D-galactose, and in all cases the sugars sit in identical positions and form nine hydrogen bonds with the residues in the binding site. But whereas D-galactose has a binding constant comparable to that of ABP's "natural" substrate L-arabinose, the binding constant of D-fucose is an order of magnitude smaller. The tight binding of L-arabinose can be attributed to the presence of two water molecules in the binding site that "fill in" a potential void, bridging between the protein and the ligand (Figure 16). These favorable interactions are not available to D-fucose. For D-galactose, one of the water molecules is displaced by a −CH$_2$OH group on the sugar, and some of the "freed" hydrogen-bonding capacity is taken up by this group while some of the "lost" hydrogen-bonding interactions are redirected toward the other water molecule. By liberating one of the bound waters, binding of D-galactose also incurs an entropic benefit. Thus, the water molecules here serve as flexible adhesive filling that contributes a degree of selectivity of binding while also allowing the ADP binding pocket to adapt to a different substrate.

Rearrangement of bound water molecules has been implicated as a significant factor in protein−carbohydrate interactions,[285,286] but has generally been discussed in terms of the displacement of loosely bound, disordered water from the protein surface, again providing both enthalpic and entropic benefits in returning these molecules to the liquid phase. Clarke et al.,[287] however, found that displacement of ordered water from the binding site can have subtle effects on substrate selectivity in such interactions that depend on a delicate balance between entropic and enthalpic effects.
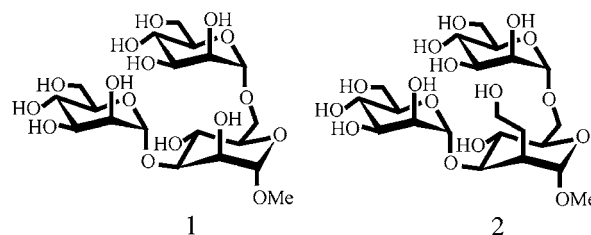
They studied the affinity of the legume lectin protein concanavalin A (Con A) for the two trimannosides **1** and **2** (Figure 17). A water molecule helps to anchor **1** to the protein via hydrogen bonds. This ordered water molecule is displaced by the −CH$_2$OH group in **2**, but this substituent can in principle form the same hydrogen bonds with the protein as those mediated by water in the complex with **1**. Nonetheless the enthalpy of binding for **2** is 2.3 kcal mol^{-1} lower than for **1**, which Clarke et al. explain by a detailed consideration of the number of hydrogen bonds formed by the complexed water relative to the typical hydrogen-bond occupancy in the liquid. This enthalpic factor offsets the favorable entropy term for binding of **2** relative to **1**.

Talhout et al.,[288] meanwhile, have shown that the binding affinity of several synthetic inhibitors of the serine proteinase trypsin can depend not just on the factors commonly enumerated in drug design, such as hydrophobic interactions and steric hindrance involved in ligand binding, but also on more subtle considerations such as the free-energetic cost of dehydration of the active site.

What these examples illustrate is that, despite the entropic advantage of expelling bound water from a binding cleft, one cannot generalize about the consequent Gibbs energy change and, thus, about the role of water in protein−substrate interactions and specificity.[265] As a general rule, the question of whether or not it is advantageous to incorporate a water molecule at the binding interface hinges on a delicate balance. Confining a water molecule clearly has an entropic penalty, but this might be repaid by the enthalpic gains of hydrogen-bond formation—an issue that must itself be weighed against the average number of hydrogen bonds that a bulk water molecule engages in. Dunitz[289,290] estimates that transferring a water molecule from an ordered binding site where it is bound by an "average" hydrogen bond to the bulk involves an overall Gibbs energy change that is close to zero. So it is not obvious which way the scales will tip in any instance.

This message is illustrated in the binding of various inhibitors of HIV-1 protease, one of the key targets in AIDS therapies. Crystal structures show that some of these, such as KNI-272, bind to the enzyme via a bridging water molecule.[291−294] Other inhibitors, such as DMP450,[295] have been designed specifically to exclude this water molecule, while mimicking its hydrogen-bonding capacity, and have
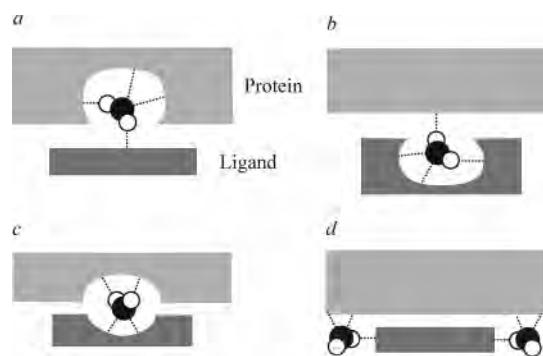
Figure 18. Schematic representations of the ways in which water molecules could be used for designed protein−substrate binding. The water molecule could be bound largely to the protein (a), largely to the ligand (b), or approximately equally to both, either in the binding site itself (c), or at its periphery (d).

Figure 19. Hydrogen-bond network around the Schiff base of retinal (shown in purple) in rhodopsin. Two water molecules, shown as light blue spheres, play a crucial role in this network. Reprinted with permission from ref 304. Copyright 2002 National Academy of Sciences.

found to bind more strongly. Li and Lazaridis[296] have calculated that displacement of the bound water by DMP450 is in itself unfavorable relative to KNI-272 but that this cost is outweighed by the lower cost of desolvating DMP450 to form the bound complex. So the consequences of eliminating the water molecule are both highly specific and not obvious.

The roles of water in protein−substrate binding are further complicated by the fact that the solvent molecules are not always sufficiently localized to be evident in structural studies. Thermodynamic measurements, however, can reveal them. For example, changes in hydrogen bonding and in local packing of water molecules can give rise to changes in the thermally accessible "soft" vibrational and rotational states that will be manifested in changes in heat capacity. Bergqvist et al.[297] have used measurements of heat capacities to study the binding of the TATA-box binding protein of the thermophile *Pyrococcus woesei* to its cognate DNA. Binding produces a large release of hydration water, as well as a small uptake of ions, and changes in the heat capacity for mutations of the protein can be rationalized in terms of changes in the hydration environment of the bound complex.

Despite such evidence of water's role as a moderator and mediator in protein−substrate interactions in a manner that can increase binding affinity and selectivity, the difficulty of predicting and interpreting this role means that there has been little effort to date to make use of the versatility of hydration water in drug design.[277,286,298] The strategy for designing target binding sites is thus generally that of eliminating hydration water and replacing the corresponding hydrogen bonds with protein−ligand interactions. Indeed, Renzoni et al.[277] concluded that "it may be that the very versatility that enables water to bind between interacting molecules makes the design of purpose-built water binding sites an impossibly complex problem". Nonetheless, they propose a strategy for the rational inclusion of water molecules at the binding interface. One can imagine several general situations: the water molecule could be bound primarily to the ligand, to the protein, or approximately equally to both; or it could be situated at the periphery of the binding site[299] (Figure 18). Barillari et al. have sought to facilitate the judicious use of water molecules in drug binding sites by classifying them according to how easily displaced they are by ligands.[300] By studying the thermodynamics of six proteins complexed with a variety of ligands, they say that the water molecules can be apportioned into two classes: those that are readily displaced (by at least some ligands) and those that never are. Although they admit that
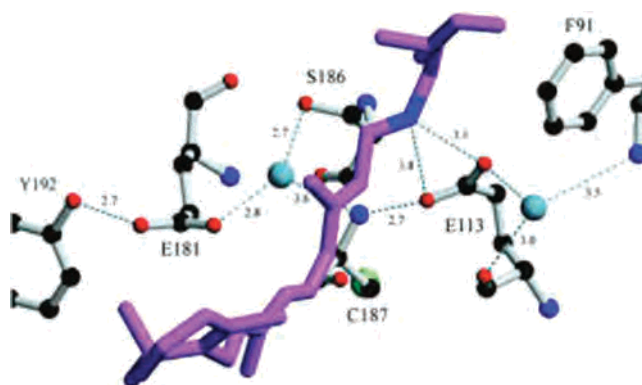
no linear correlation exists between the binding free energies of waters and the change in binding affinity of ligands that displace them, they conclude that the latter class of "conserved" water molecules may be usefully used in the design of drug docking: in effect, they serve as "part of the protein", available for hydrogen bonding to the ligand.

More generally, the release of bound water on ligand binding is often assessed via scoring functions that aim to evaluate the effect of particular recognition motifs on the binding affinity. Friesner and co-workers have pointed out, however, that such scoring procedures seem inadequate for describing the contributions of two motifs in particular: strongly hydrophobic cavities enclosing water clusters and the formation of hydrogen bonds at sites enclosed by hydrophobic groups.[301] Young et al. have shown that the hydration of the binding cavities is particularly perturbed in such cases, imposing unusually large entropic and enthalpic penalties and thereby stabilizing the protein−ligand complex.[302] This accounts, for example, for the large binding affinity of the streptavidin−biotin complex. Young et al. propose that these motifs are attractive targets for drug design.

5.4. Functional Tuning

An indication of the active role of hydration in determining protein function was provided by Ohno et al.,[303] who have used quantum-chemical methods to calculate how the reactivity of ribonuclease T_1 is influenced by the solvent. They find that the electronic state of the enzyme in vacuo is quite different from that in solution and that, in particular, hydration shifts the spatial distribution of the frontier orbitals of the protein into its active site. Thus, hydration not only helps to maintain the native structure but also "tunes" the catalytic behavior.

Further evidence of the "tuning" of protein function by crystallographic water is provided by the light-sensitive transmembrane protein bovine rhodopsin. The central chromophore of rhodopsin, retinal, is tuned to different wavelengths in the red, green, and blue cone cells of the retina. This wavelength selectivity is achieved by small alterations of the retinal conformation owing to its protein environment. Okada et al.[304] showed that two water molecules seem to play a central part in this spectral tuning by participating in a hydrogen-bonded network stretching between polar residues in the retinal site (Figure 19).

5.5. Allostery

Protein−protein contacts mediated by water molecules can not only serve to assist in recognition and docking but may also play a mechanistic role in function. Autenrieth et al.[305] have studied the changes in structured water molecules at the interface between the cytochrome c_2 redox protein (cyt c_2) of *Rhodobacter sphaeroides*, which facilitates photosynthetic electron transfer, and the photosynthetic reaction center (RC), as cyt c_2 switches between its two redox states. The docking of cyt c_2 to RC is finely tuned to be a relatively weak interaction, since the association needs to be transient: the reduced form of cyt c_2 docks to the RC, releases an electron, and detaches. MD simulations of the crystal structure of the complex shows that the primary hydrophobic docking domain contains water molecules bridging contacts between small polar residues in this region. In switching from the reduced to the oxidized form of cyt c_2, the binding interface undergoes only very slight reorganization limited primarily to a change in the interfacial water from a fairly structured arrangement to a less tightly bound, more fluctuating structure. This change could facilitate the undocking of the cytochrome once it is oxidized: in this sense, the interfacial water acts as a kind of latch.

Something similar is observed in the allosteric regulation of oxygen binding to hemoglobin. The hemoglobin of the mollusk *Scapharca inaequivalvis* is dimeric, and the interface of the subunits contains a cluster of 17 well-ordered water molecules. In contrast to mammalian hemoglobin, where the cooperativity of oxygen binding is due to marked changes in the quaternary structure of the hemoglobin complex, in the hemoglobin of *Scapharca* this cooperativity seems to stem from more subtle structural changes. In particular, oxygenation is accompanied by loss of six of the ordered interfacial water molecules. Royer et al.[306] found that these waters have a central role in cooperative oxygen binding, enabling allosteric interactions between the subunits by acting as a kind of transmission unit. The water cluster helps to stabilize the low-affinity form of the protein, whereas a mutant form that lacks two of the hydrogen bonds from this cluster tends to adopt the high-affinity conformation instead. Thus, loss of interfacial water occasioned by oxygen binding to one of the wild-type subunits helps to promote the transition to the high-affinity conformation of the other subunit. In human hemoglobin, as we noted above, solvation also seems to play a role in allostery, but perhaps more in terms of the general energetics of hydration changes than in specific behavior of ordered, "bound" water molecules.[218]

5.6. Hydrophobic Cavities

Many proteins contain hydrophobic cavities in their cores. But these are not necessarily dry, or even water-repelling. In a high-pressure crystallographic study of a mutant of T4 lysozyme containing a highly hydrophobic cavity, Collins et al.[307] find that only modest pressure will enable four water molecules to enter this space and that the free-energy penalty of filling such cavities can be small or even zero. They suggest that this might help to account for the pressure-induced denaturation of most proteins.

The X-ray structure of the tetrabrachion protein of the hyperthermophile *Staphylothermus marinus* reveals several hydrophobic cavities in the 70 nm long "stalk" segment that are all filled with water at 100 K.[308] Simulations of this structure by Yin et al.[309] suggest that the two largest cavities,

containing 7−9 and 5 water molecules at room temperature, are close to switching to a dry state at the organism's optimal growth temperature of 365 K, which may offer a docking mechanism for the binding of the two proteases present in the active form of the protein: emptying of the cavities, as nonpolar anchors on the proteases plug in, would provide a thermodynamic driving force for binding.

As indicated in section 2.3, conventionally "hydrophobic" groups can even engage in hydrogen bonding. Ordered, hydrogen-bonded clusters have been reported in hydrophobic cavities of small supramolecular assemblies.[310,311] Yoshizawa et al.[311] found a cluster of 10 water molecules encapsulated in a self-assembled hydrophobic cavity with tetrahedral symmetry, in which the water molecules form an icelike adamantoid cluster that they call "molecular ice", with the oxygen atoms oriented toward the aromatic groups of the cage. Here, it seems, the constraints of geometry and intermolecular interactions have conspired to produce a genuine case of what is literally a sort of inverse Frank−Evans "iceberg". Although the high symmetry and good fit of the cavity here is likely to represent a rather special case, such an extreme degree of water ordering would be expected to provide a large entropic driving force for the displacement of the water cluster by a suitably sized guest molecule.

Open-ended hydrophobic pores are "cavities" of a quite different nature, potentially allowing throughflow of water, which are discussed in section 5.10.

5.7. Electron Transfer

Electron transfer between proteins and other biomolecules plays a central role in several important biological processes, including photosynthesis and respiration. It was once considered sufficient to regard water as an essentially homogeneous dielectric medium that might intervene between the electron donor and acceptor species and thereby lead to an exponential decay of the transfer rate with increasing separation. But in recent years it has become clear that the story is not so simple. For example, two ordered water molecules bound at the interface between the redox centers of cross-linked azurin proteins appeared to facilitate electron transfer considerably.[312] Similarly, the rate of electron transfer between molecules in crystalline tuna cytochrome c was increased by three bridging water molecules,[313] and water between the two copper centers of peptidylglycine α-amidating monooxygenase appears to facilitate electron transfer over distances of 10−11 Å.[314] Lin et al.[315] have proposed that there are two distinct ways in which water can mediate the coupling of redox groups in proteins (in addition to the situation in which these groups are close enough to be in direct van der Waals contact without intervening solvent). At separations of around 10 Å, structured water at the interface can establish facile electron-transfer pathways that increase the rate and reduce the decay constant. At larger separations (more than about 12 Å) the intervening solvent is bulklike, and the coupling is relatively weak, as seen in earlier work. One might expect the intermediate regime, involving "structured" water, to be highly sensitive to the dynamics of both the protein chain and the hydration water− the issue of the coupling between them, discussed above, then becomes critical. Such a dependence on conformational fluctuations is expected[316,317] but remains to be studied experimentally.
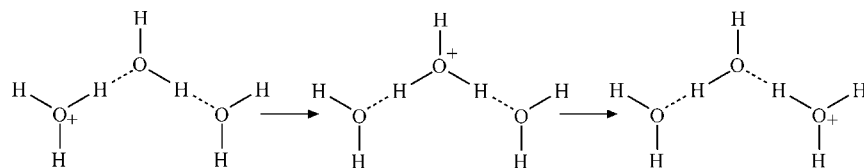
Figure 20. Grotthuss mechanism.

5.8. Involvement of Bound Water in Catalytic Action

Water in the active site of a protein can play more than a purely structural role: as a nucleophile and proton donor, it can be a reagent in biochemical processes. A few examples may suffice to illustrate this very general role.

A water molecule in the bacterial enzyme zinc lactamase, which is involved in resistance to lactam antibiotics, apparently acts as a nucleophile to initiate splitting of the lactam ring.[318] Hydrogen bonding between this water molecule and a zinc-bound aspartate group increases its polarity and nucleophilicity, while the carboxylate group of the aspartate potentially provides a source for the proton that reacts with the cleaved ring.

Erhardt et al.[319] find that the protein-degradation enzyme bovine lens leucine aminopeptidase (*bl*LAP) seems to function by "drip-feeding" water molecules to the active site, where they engage in nucleophilic hydrolysis of peptide bonds. Here too the active site contains two zinc ions (Zn1 and Zn2), coordinated to a glutamate residue; they sit next to a "water channel" that penetrates the protein. Water molecules are delivered in a coordinated fashion to Zn2, where they can be converted to nucleophilic OH by a simple proton transfer to an oxygen atom coordinated to Zn1. The active site looks rather like an automated mechanism in which moving parts (due to molecular rotations) transfer the "sticky" reagent H_2O in a sequential fashion through successive hydrogen-bond making and breaking.

In an ab initio molecular mechanics study of the mechanism of DNA polymerase IV in the thermophilic archaeon *Sulfolobus solfataricus*, Wang et al. found that water molecules in the coordination sphere of the catalytic Mg^{2+} ion appear to play two important roles.[320] The enzyme adds a nucleotide to a growing DNA chain by catalyzing the reaction of the terminal 3′-OH group with the α-phosphate of the new deoxyribonucleoside triphosphate, eliminating pyrophosphate. The initial, and rate-limiting, step is proton transfer from 3′-OH to phosphate, which happens via a bridging water molecule. And the cleaving of pyrophosphate following linkage of the polynucleotide chain and the deoxyribonucleoside involves another water-mediated proton relay that protonates the γ-phosphate and partly neutralizes its negative charge.

Proton transfer facilitated by a bridging water molecule also seems to occur in horseradish peroxidase, where it enables the transfer of a proton from iron-coordinated H_2O_2 to a His residue in the active site[321]—the first step in cleavage of the O—O bond. Ab initio simulations without this bridging water arrive at an energy barrier considerably greater than that found experimentally, because of the large separation of the proton source and sink.

5.9. Proton Wires

One of the most striking consequences of the extended hydrogen-bonded structure of liquid water is the rapid diffusion rate of protons, which is considerably higher than that of other monovalent cations. The traditional explanation, the so-called Grotthuss mechanism,[322,323] invokes the fact that protons moving through the network do not, like other cations, have to "drag" a solvent shell with them. Rather, the water molecules solvating a hydronium (H_3O^+) ion can actually facilitate proton transport by shuttling it to another molecule (Figure 20). In this way, a specific proton does not itself diffuse through the medium; rather, there is a cooperative transfer of protons between successive molecules.

The real picture seems not to be quite so simple, however, because the hydronium ion appears to have a significantly different hydration state to that of a water molecule: MD simulations indicate an average hydration number of about 3, rather than about 4. So intermolecular transfer of the proton entails a significant rearrangement of the hydrogen-bond network. Agmon[323] has proposed that this reorganization *precedes* proton transfer, leading him to call it the "Moses mechanism" by analogy with the parting of the Red Sea before the Israelites could cross it. Day et al.[324] reported support for this mechanism from MD simulations using the empirical valence bond (EVB) methodology. Kornyshev et al.[325] argue from a comparison between MD simulations and experimental data that motion of the proton may involve not only transfer between a hydronium ion and a neighboring water molecule, accompanied by the necessary rearrangements of hydrogen bonds, but also what they call "structural diffusion" of the more complex protonated species $H_5O_2^+$, the so-called Zundel ion, which involves the concerted displacement of two or three protons:

$$(H_2O\cdots H^+\cdots H_2O)\cdots H_2O \rightarrow H_2O\cdots(H_2O\cdots H^+\cdots H_2O)$$
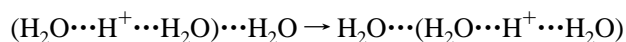
In fact, the rearrangements of the hydrogen-bond network that accompany proton transfer appear to be even more complex than this.[326] Using a multistate EVB approach, Lapid et al. find that although the Zundel ion is indeed the intermediate in proton-hopping between adjacent water molecules, this process is coupled to hydrogen-bond reorganization over at least three hydration shells, with bond making and breaking in the second hydration shell possibly representing the rate-determining step.[327] Within this picture, there are two classes of hydrogen bond that contribute to the process: those emanating from the protonated molecule stabilize it, whereas those pointing toward this center are destabilizing. The "Red Sea" then has two complexions: the "red" (destabilizing) bonds do part in front of the proton and close up behind it, but the "blue" (stabilizing) bonds do the opposite. The cooperativity involved is thus both extensive and complex.

Mezer et al.[328] suggest that proton-hopping involving $H_5O_2^+$ and other protonated water clusters can transfer protons between nearby residues on the surface of proteins, via the network of "rigidified" water molecules in the hydration shell. Deprotonation of an acidic surface residue creates a Coulomb cage which hinders the diffusion of the proton into the bulk for a sufficiently long time that it can become bound by another acceptor site on the surface. Such

a mechanism is supported by observations of proton transfer between two sites a few angstroms apart on the fluorescein molecule.[328] Suzuki and Sota[329] find distinct loops of hydrogen-bonded water molecules between hydroxyl groups at the surface of the sugar β-ribofuranose, using ab initio MD simulations. Such "circular hydrogen-bond networks", first identified in crystal structures by Saenger,[330] persisting at least for several picoseconds in solution, may increase the dipole moments of the water molecules concerned, and could also act as proton-conducting pathways.

Proton-hopping in a hydrogen-bonded chain, along the lines of the original Grotthuss mechanism, remains a viable process for water molecules in confined geometries in which the formation of a bulklike, three-dimensional network is not possible. The existence of such "proton wires" has been postulated in a variety of proteins, where they provide proton-conduction channels connecting the interior and exterior of the molecules. For example, there is a 23 Å water wire in the photosynthetic reaction center of *R. sphaeroides*,[331] disruption of which by site-directed mutagenesis disturbs the functioning of the protein complex.[332] Water chains also seem to play an important proton-conduction role in the catalytic activity of carbonic anhydrase[326,333,334] and of some cytochromes,[326,335−340] and in proton motion through the pore-forming membrane peptide gramicidin A. A chain of three water molecules has been posited to exist in the ammonia channel AmtB of *E. coli*, although its role here remains unclear.[341] Pomès and Roux[342] argue that even for one-dimensional proton-conducting water wires there is some element of a "Moses mechanism"—a degree of reorganization of the chain is needed for proton translocation, and this reorientation of water dipoles might constitute the rate-limiting step of the process.

These one-dimensional proton wires have been modeled by considering the water-filled hydrophobic channels of carbon nanotubes.[343−347] Dellago et al.[344] calculate using ab initio MD that the proton mobility along one-dimensional water chains inside a nanotube can be enhanced by the ordering imposed by confinement and may be about 40 times greater than that in bulk water, if no hydrogen-bonding defects are present in the chain. This conclusion was anticipated in calculations by Brewer et al., who found enhanced proton diffusion within narrow, smooth, cylindrical hydrophobic pores.[348] Increasing confinement here promotes mobility by organizing the water molecules into a one-dimensional proton wire and by preventing stabilization of the protonated center through hydration.

Simulations and experiment suggest that water in larger nanotubes may adopt relatively ordered structures not found in the bulk: Byl et al.[347] found stacks of rings in which the intra-ring hydrogen bonds are comparable to those in the bulk, whereas the inter-ring bonds are weaker. Mashl et al.[346] found a state with structural and dynamical characteristics of both the liquid state and of hexagonal ice. The proton conductivity of these water "tubes" depends sensitively on their structures, making it possible that the proton mobility could be switched "on" or "off" by small and subtle perturbations of the confining environment.

A water wire in the transmembrane proton pump bacteriorhodopsin (bR) has been studied extensively. Crystallographic studies[349,350] and MD simulations[351,352] have shown that there are several ordered water molecules within the internal cavity of the extracellular half of bR. Following light absorption and photoisomerization of the chromophore
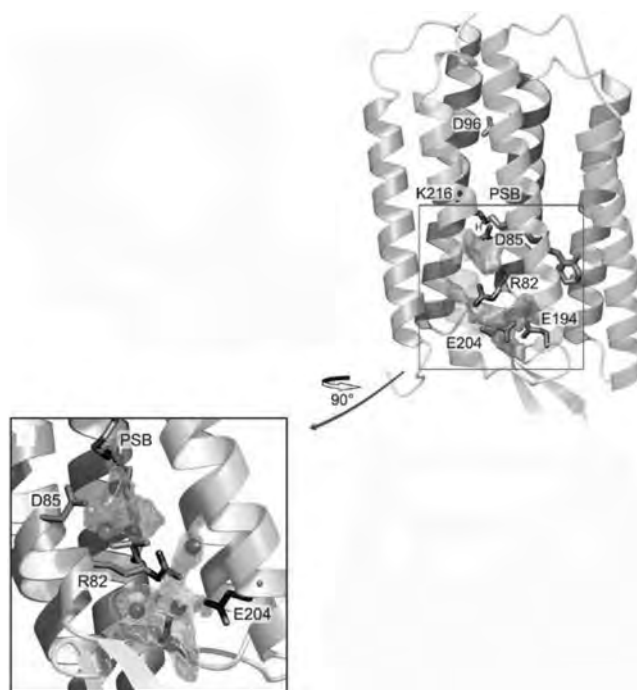

Figure 21. "Proton wire" in bacteriorhodopsin connects the chromophore to the outer surface of the membrane, providing a proton-conducting channel. The water molecules are shown as gray spheres in the left-hand frame. Courtesy of K. Gerwert.

retinal, bR undergoes a transition between so-called L and M conformations, in which a proton is transferred from a Schiff base on the chromophore to an aspartate residue (Asp85), accompanied by the release of a proton to the extracellular surface. Before this event, the latter proton is stored on some group X that has not yet been definitively identified. FTIR spectra[353,354] are consistent with the idea that the proton from the Schiff base moves, via bound waters, to Asp85 (Figure 21), protonation of which induces a movement on Arg82 that triggers proton release from X.

Several studies have pointed toward a hydrogen-bonded network of internal water molecules as the most likely candidate for X. Such a one-dimensional network spans the distance from the Schiff base on the buried chromophore to the external surface of the protein. Two glutamate groups near the extracellular surface (Glu204 and Glu194) have also been proposed as candidates for X, and indeed at face value it seems unlikely that a proton would reside in the hydrogen-bonded chain rather than in the carboxylate groups of the glutamate residues. But it seems that an $H_5O_2^+$ cluster—the Zundel cation—may indeed be the preferred proton storage site.[355,356] The $H_5O_2^+$ group is stabilized by the delocalization of charge across both water molecules and by favorable interactions with the two glutamate residues.[355] Quantum-chemical simulations[354] show that the $H_5O_2^+$ group may have a symmetric hydrogen-bonding arrangement, with the proton shared equally between the two water molecules. This suggests the possibility that proton migration involves transformations between a hydrated Zundel cation $H_5O_2^+ \cdot (H_2O)_4$ and the "Eigen" complex $H_3O^+ \cdot (H_2O)_3$, where the proton is asymmetrically hydrogen-bonded.[357] Garczarek and Gerwert[354] suggest that the movement of Arg82 triggered by protonation of Asp85 destabilizes the $H_5O_2^+$ complex in such a way as to create a connection between this water wire and the protonated Asp85. This enables the water cluster to be reprotonated following release of a proton at the extra-
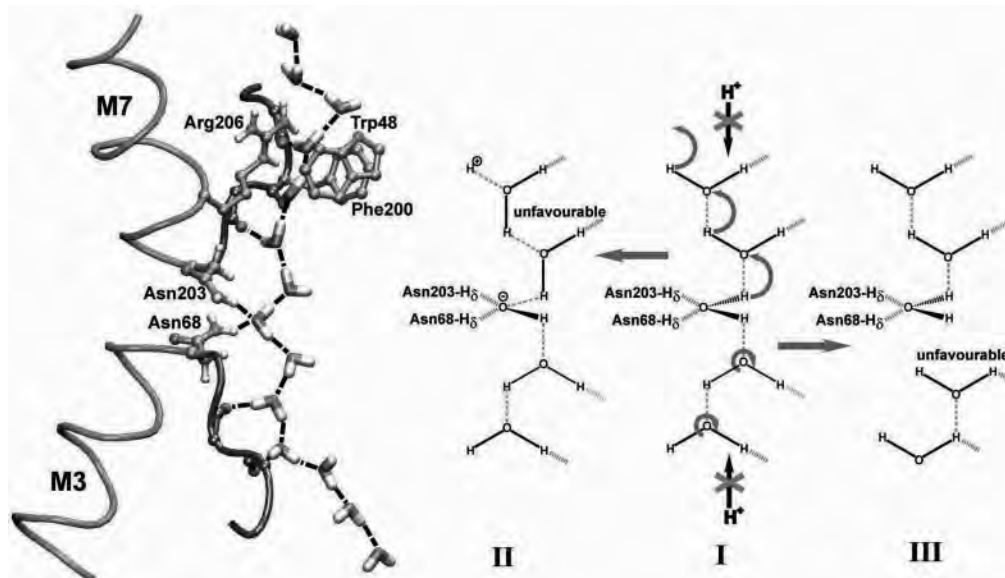
Figure 22. One possible mechanism for the proton impermeability of aquaporin invokes a defect in the chain of water molecules threading through the protein pore (left), such that Grotthuss-like proton transport can only take place toward the defect from either direction (right). Reprinted with permission from *Science* (http://www.sciencemag.org), ref 365. Copyright 2002 American Association for the Advancement of Science.

cellular surface. Mathias and Marx[358] have refined this picture still further using hybrid density functional and force-field simulations to calculate the IR spectra of the proton-release complex, which they compare with experiment.[359] They suggest that the water cluster that facilitates proton storage and release is in fact best not viewed in terms of either of the classical Zundel or Eigen complexes but involves a water wire, stabilized by Glu194 and Glu204, in which the protonic defect is highly delocalized. They propose that such one-dimensional water chains might serve quite generally as "proton sponges" in proteins.

Lee and Krauss[360] suggest that a water wire is also involved in proton transport *to* the (initially deprotonated) Schiff base from the cytoplasmic side of bR, which occurs during the M−N transition of the photocycle. Here the proton motion is initiated at the Asp96 residue in the cytoplasmic region, which is connected to the Schiff base in the M state via a narrow channel. Part of this channel is hydrophobic, but water molecules have been identified in this region, near the Schiff base, in an X-ray structure of the N state.[361] Lee and Krauss[360] show that a water wire threading linking Asp96 to the nitrogen of the Schiff base can effect extremely fast proton transport—within about 0.05 ps if the Asp85 residue hydrogen-bonded to the Schiff base is deprotonated. Thus, they postulate that the rate-limiting step of this part of the photocycle is likely to be the formation of the water wire itself.

The participation of water wires in the function of bR and related proton pumps is consistent with the finding of Furutani et al.[362] that only rhodopsins that contain strongly hydrogen-bonded water molecules are able to act as proton pumps. Moreover, adding such molecules to a mutant of the chloride pump halorhodopsin converted it to a proton pump.[363]

5.10. Function of Protein Channels

The water-conducting protein channel aquaporin provides an interesting contrast to these proton wires. The function of aquaporin proteins is to mediate water transport,[364] which

it appears to do via a chain of nine hydrogen-bonded molecules. But if this chain were to permit rapid transmembrane proton motion, that would disturb the delicate charge balance across the membrane. So aquaporin must somehow disrupt the potential proton wire that threads through it. In fact, aquaporin proteins achieve water conductance rates of around 10^9 molecules s^{-1} while preventing transmission of all ions, protons included.

It has been proposed that this is achieved by the introduction of a defect into the hydrogen-bonded chain.[365−367] Simulations by de Groot and Grubmüller[366] of water permeation through human aquaporin-1 and the closely related bacterial glycerol factor GlpF suggest that the "proton barrier" occurs at a conserved arginine residue in both channels, whereas another conserved region, the asparagine−proline−alanine (NPA) motif, provides a size-selective filter that prevents passage of other small molecules. Tajkhorshid et al.,[365] on the other hand, suggested that, in GlpF at least, it is the interaction of one of the water molecules with surrounding asparagine residues in the NPA region that introduces the defect responsible for proton-blocking. These interactions enforce opposite orientations of water molecules in the two halves of the chain to either side, so that proton transfer from either end becomes arrested in the middle, where a water molecule acts as a hydrogen-bond donor to both of its neighbors (Figure 22). This defect in a water wire, called an L defect, was also found to disrupt proton transport along one-dimensional water chains in carbon nanotubes.[344]

But the proton impermeability of aquaporin may be instead due to electrostatic rather than structural barriers, for example being induced by oppositely oriented α-helical domains that converge in the NPA region where, in addition to arranging the proton-shuttling paths in opposite directions as described above, they produce a bipolar, positively charged electrostatic field.[368,369] Voth and co-workers have now shown that electrostatics, rather than water-wire defects, seem to predominate.[326,370,371] Point mutations in the seemingly crucial, narrow NPA region of the aquaporin channel[372] have helped to resolve the matter here: Beitz et al. found that a

double mutation of aquaporin-1 with the Arg-195 residue removed has an enhanced ability to conduct cations.[373] Chen et al.[370] show that this can be explained by invoking three contributions to the free-energy barrier for proton transport: the bipolar field,[372] electrostatic repulsion by Arg-195, and the dehydration penalty imposed by the narrow neck of the pore. The first of these contributes about half of the Gibbs-energy barrier to proton permeation, and this barrier does not seem to be significantly lowered for protons, relative to other cations, by their unique ability to delocalize their charge in Zundel-type configurations.[371] This dominant influence of electrostatics on gating is supported by simulations of water permeation through a carbon nanotube.[374] When a positive charge is introduced at the midpoint of the nanotube and just outside of the tube wall, it may induce reorientation of water molecules in a single-file chain threading through the tube so as to produce an L defect. Dellago and Hummer have found that proton desolvation in a single-file water chain within a carbon nanotube is by itself sufficient to suppress proton transport significantly, despite the high proton mobility along such water wires.[375]

Voth and co-workers[326,376−378] have proposed that rather similar effects may operate in the M2 proton channel of the influenza A virus, where again a constriction blocks the passage of non-proton ions because of the desolvation this would entail. In principle, a continuous chain of hydrogen-bonded water molecules could thread the pore to produce a proton wire; but a histidine residue (His-37) in the constriction forces the water molecules on either side of it to adopt opposite orientations, breaking the wire. Rotation of His-37 by about 60° can open the pore by allowing a single water molecule to bridge the constriction, completing the proton-conduction pathway while still excluding larger ions.[378]

Disruption of water channels through transmembrane pores has in fact been proposed as a rather general mechanism for gating behavior. Beckstein et al.[379] find that a hydrophobic pore with a funnel-shaped entrance switches from a "closed", dry state to an "open", water-filled state rather abruptly once a critical pore radius is exceeded and that this "opening" radius can be tuned by varying the length of the pore or by adding a few polar groups to its lining. Similarly, Sriraman et al.[380] show that tuning of the hydrophobicity of a carbon nanotube interior can alter its water occupancy between filled and empty states; at intermediate values of hydrophobicity there are more or less long-lived fluctuations between these metastable states.

MD simulations by Wan et al.[381] suggest that gating can be induced by deformation of a hydrophobic pore. They find that an indentation of the wall of a single-walled carbon nanotube caused by a relatively modest external force (of the order of 2 nN) can induce a sharp open-to-closed transition once the displacement exceeds a critical value by just 0.6 Å. Zimmerli et al.[382] have found that the dipole moment induced by curvature in the graphitic wall of a short carbon nanotube can reorient water molecules threading through it so as to create an L defect (see above) and thereby block fast proton transport.

Mechanically induced open−closed transitions do indeed seem to occur in biological hydrophobic pores. Several gated channels contain constrictions lined with hydrophobic residues, where a water channel could quite easily be pinched off by a conformational change. Jiang and co-workers[383,384] have suggested that such a gating process operates in potassium channels, and Sukharev et al.[385] propose a similar

mode of action in the bacterial mechanosensitive channel MscL. Hydration of the hydrophobic gate region contributes critically to the energy of channel opening and shapes the rate-limiting kinetic barrier for gating. Hydrophilic substitutions in this region lead to a permanently hydrated pre-expanded channel that is easy to open.[386]

MD simulations of water inside the mechanosensitive channel MscS of *E. coli*,[387] based on a crystal structure which revealed a pore 7−15 Å across at its narrowest point,[388] revealed that despite the considerable width of this opening, the pore was probably in a closed state in the protein crystal. It is lined with highly hydrophobic residues, and the simulations indicated that this caused cooperative drying within the constriction (Figure 23a). A narrow water bridge was repeatedly formed and dissipated during the simulations, but the constriction was water-free, at physiological ionic strengths, for about 83% of the time. This presents a considerable energetic barrier (about 10−20 kcal mol^{-1}) to the passage of ions through the channel, since they cannot pass through the constriction without being stripped of much of their hydration shell (Figure 23b). A note of caution is added by Spronk et al., however, who find that when the transmembrane potential is included in the simulations, the MscS channel becomes hydrated and conducting.[389] They suspect in consequence that the crystal structure reported by Bass et al.[388] may correspond to an open rather than a closed state.

6. Water and Nucleic Acids

In comparison with the attention given to hydration in determining protein structure and function, the role that water plays in the properties of nucleic acids has been surprisingly neglected. Indeed, it is often overlooked that the famous double-helical structure of DNA is not intrinsic to that molecule but relies on a subtle balance of energy contributions present in aqueous solution. Without water to screen the electrostatic repulsions between phosphate groups, the classic, orderly helix is no longer viable. Thus, DNA undergoes conformational transitions, and even loses its double helix, in some apolar solvents;[390,391] and even though both experiments[392] and MD simulations[393] suggest that the double helix is not lost entirely in the gas phase, it has none of the elegance and order familiar from DNA's iconic representations. On the basis of elasticity measurements of single-stranded DNA in water and nonaqueous solvents, Cui et al.[394] find that in the former case the strands are shorter, which they attribute to water bridges between bases in the chains.[395] These hydrogen bonds appear to be relatively weak (of the order of $0.58kT$), leading Cui et al. to speculate that if the hydrogen bonding of single-stranded DNA with water were much stronger, this might inhibit the formation of the double helix. If that is so, water seems here to function in a "Goldilocks" mode: some hydration is essential for a stable double helix, but not too much.

As with proteins, DNA in the crystalline state[396] preserves a pronounced degree of ordering in its hydration shells. Dickerson and co-workers have reported[397,398] that in the solid state, A−T segments of DNA have a "spine of hydration" in which one layer of water molecules bridges the nitrogen and oxygen atoms of bases in the minor groove, while a second layer bridges water molecules in the first layer. This "spine" seems also to persist in aqueous solution, where NMR measurements[399] show water residence times in the minor groove of more than 1 ns: orders of magnitude larger
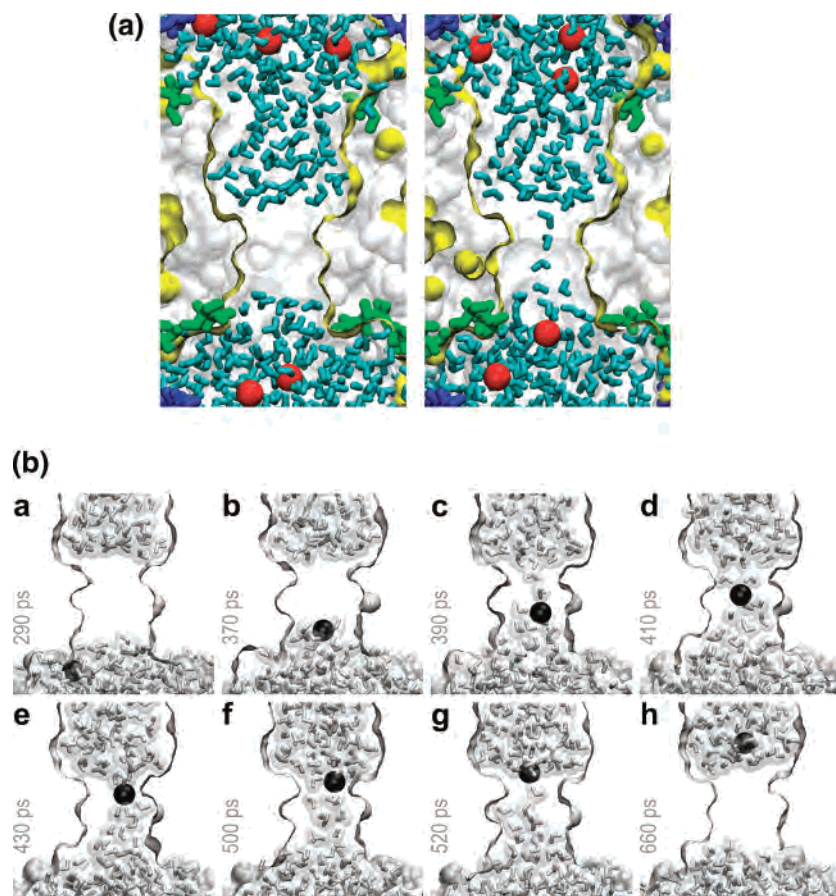
Figure 23. (a) Water dynamics in simulations of the protein channel MscS, showing snapshots in which the pore neck is "dry" (left) and partly water-filled (right). The red spheres are chloride ions, and polar residues are shown in green. (b) Passage of an ion through the pore. It can pass through the narrowest part of the constriction only by being largely stripped of its hydration shell. Reprinted with permission from ref 387. Copyright 2004 Biophysical Society.

than the residence times in the major groove, and comparable to those of "buried" water molecules in globular proteins.

Shui and co-workers[400,401] and Tereshko et al.[402] have identified a further two crystallographically defined hydration layers, which produce a series of fused hexagonal rings of water molecules in the minor groove (Figure 24). But this structure seems to be rather sensitive to the base sequence. Not only is the spine of hydration specific to A-tracts (short sequences of A bases), but Liepinsh et al.[403] find that, although the central AATT tract of GTGGAATTCCAC dodecamers has this hydration structure in solution, it does not appear to be present in the TTAA segment of GTGGT-TAACCAC dimers. The minor groove of the latter is slightly wider, which apparently disrupts the hydration spine. In contrast, Woods et al.[404] find that modifying one of the thymines in each strand of CGCGAATTCGCG dimers to remove keto oxygens thought to play a central role in the hydration structure does not destroy the spine of hydration, although it becomes less stable.

This sensitivity of B-DNA hydration to sequence suggests that the arrangement of water molecules might effectively transmit sequence information to locations remote from the bases themselves. There now seems to be good evidence that hydration structures are indeed used by DNA-binding proteins as part of the recognition process. Specifically, the energetics of water release from sequence-specific hydration structures might be expected to influence the binding strengths. Ha et al.[405] found that the interaction of the *lac* repressor protein with the lac operon site on DNA in the presence of glutamate (which is known to influence protein–DNA interactions) differs between specific and nonspecific binding primarily in that the former incurs release of bound water from the DNA. Robinson and Sligar[406] suggested that the loss of sequence specificity of the restriction enzyme *Eco*RI in the presence of certain solutes could be explained by the fact that water mediates the protein–DNA interaction and that this influence is suppressed under conditions of decreased water activity. They concluded that "water mediation may constitute a general motif for sequence-specific DNA recognition by restriction enzymes and other DNA-binding proteins".

Sidorova and Rau[407] found that, for relatively low osmotic stress, the binding specificity of *Eco*RI increased rather than decreased with addition of osmolytes. They estimated that under these conditions the nonspecific *Eco*RI–DNA complex sequesters about 110 more water molecules than the specific complex. Sidorova and Rau propose that this water is organized into a hydration layer at the protein–DNA interface, from which solutes are excluded, whereas crystal structures of the specific complex show direct contacts without intermediate water. Increasing the osmotic stress would then be expected to enhance specific binding, which minimizes the volume of solute-excluding water. This is not necessarily inconsistent with Robinson and Sligar's observations of decreased specificity at high osmotic stress, if this is considered to influence an equilibrium between water-mediated and direct contacts for nonspecific binding.
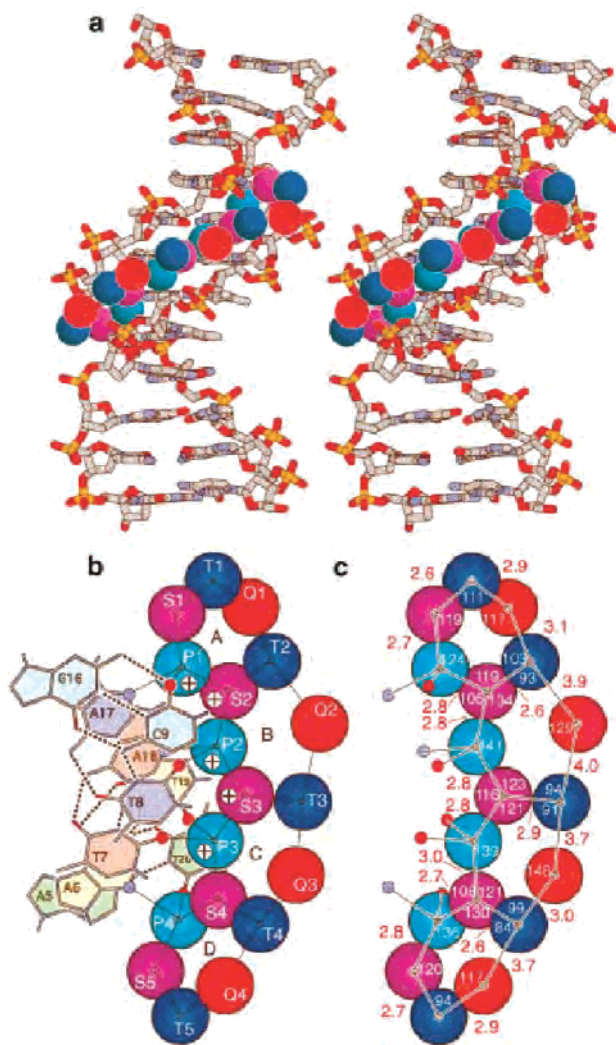
Figure 24. Hydration layers of DNA. The primary layer of water molecules is colored light blue, the second layer magenta, the third layer blue, and the fourth layer red: (a) shows a stereoview into the minor groove, while (b and c) show the view across the groove, illustrating the fused-hexagon motif. The sites that may be occupied by potassium ions are indicated with plus signs. Reprinted with permission from ref 400. Copyright 1998 American Chemical Society.

How these effects depend on the particular sequence-specific hydration structures of DNA has not been clear in general. But Fuxreiter et al.[408] found that these structures influence the water release on binding of the restriction enzyme *Bam*HI to its cognate sequence GCATCC and to similar but noncognate sequences. The entropic and enthalpic changes due to water release from the protein−DNA interface are one of the key driving forces of the interaction, and Monte Carlo simulations showed that this release was highly dependent on sequence so that a given DNA sequence has a "hydration fingerprint" that determines the binding energetics.

The hydration structure of DNA can also play a functional role by determining its conformation. The conformational state of double-stranded DNA in solution is very sensitive to hydration: at low hydration, the most biologically relevant B form undergoes conformational transitions to other forms.[409] The stabilization of the B form occurs very close to the hydration level at which water clusters in the primary hydration shell link up to form a fully connected (percolating)

cluster in the major groove.[410] There is an almost identical percolation threshold for A-DNA, but in that case it corresponds to the appearance of a spanning water network in the *minor* groove.[411] It is not yet clear whether this near-coincidence of thresholds arises from chance or from some deeper physical cause. In any event, these hydration structures may hold the key to transitions between the A and B conformations, particularly insofar as these are governed by the presence of ions, which may alter the hydration structures and thus the relative stabilities. For example, a fully connected water network in the major groove of B-DNA allows counterions such as Na$^+$ to become fully mobile along the length of the chain, and this may prevent the accumulation of such ions in the major groove, which is thought to govern the B-to-A transition.[412]

Some of the water molecules in the minor groove can be substituted by cations, which induce electrostatic effects that can influence DNA curvature,[413] alter the width of the groove,[414] and affect the duplex melting temperature.[415] Shui et al.[400] affirm the presence of cations in the primary hydration layer and suggest that these "extrinsic" influences far outweigh any "intrinsic" contributions to DNA deformation owing to sequence-specific base−base interactions. They conclude that the hydration structure, and the presence of monovalent cations within it under physiological conditions, are essential for stabilizing the native B configuration of DNA, which is broadly consistent with the suggestions of Brovchenko et al.[410] above. Moreover, McFail-Isom et al.[416] suggest that the fused-hexagon motif of the hydration shell may act as a conformational switch in which DNA-binding molecules such as spermine expel sodium ions.

Although the presence of cations ions in the minor groove seems to be supported both by experiment[417,418] and by theory,[419,420] Chiu et al.[421] have questioned the notion of sequence-specific ion-binding sites and their role in modifying DNA structure, arguing instead that short-ranged interactions between the nucleotide bases account for sequence-dependent variations in local structure. The issue remains unresolved; the MD simulations of Hamelberg et al.[422] do seem to show that interactions of ions and water in the minor groove narrow its width, whereas those of Ponomarev et al.[423] show no significant correlations between the width of the groove and the presence of ions.

RNA appears to be more highly hydrated than DNA.[424,425] As in DNA, G−C pairs are more hydrated than A−U pairs, and the hydration structures around the former are better defined.[426] The folding of RNAs into their functional forms resembles in many ways that of proteins: both macromolecules have hydrophilic and hydrophobic segments in their chainlike structures, and both may engage in intramolecular hydrogen bonding in the folded state. But the distribution of the two types of component is more regular in RNA—all the bases are, aside from their hydrophilic substituents, hydrophobic, whereas the sugar−phosphate backbone is uniformly polar. Sorin et al.[427] find that this regular structure leads to correlated collapse of RNA strands into a compact form, which is more likely to trap water molecules between hydrophobic bases than is the less cooperative collapse of proteins, where hydrophobic residues are more sparsely distributed. Their simulations suggest that this trapped water is expelled late in the folding process so that there remains considerable potential for water-mediated interactions as compaction proceeds. In this respect, the results argue

that explicit water molecules buried within the folding macromolecule can play an important role in mediating compaction, as proposed for proteins by Cheung et al.[199] and Papoian et al.[203]

7. Conclusions

Water plays a wide variety of roles in biochemical processes. It maintains macromolecular structure and mediates molecular recognition, it activates and modulates protein dynamics, it provides a switchable communication channel across membranes and between the inside and outside of proteins. Many of these properties do seem to depend, to a greater or lesser degree, on the "special" attributes of the H_2O molecule, in particular its ability to engage in directional, weak bonding in a way that allows for reorientation and reconfiguration of discrete and identifiable three-dimensional structures. Thus, although it seems entirely likely that *some* of water's functions in biology are those of a generic polar solvent rather than being unique to water itself, it is very hard to imagine any other solvent that could fulfill all of its roles—or even all of those that help to distinguish a generic polypeptide chain from a fully functioning protein. The fact that fully folded proteins moved from an aqueous to a nonaqueous environment may retain some of their functionality does not alter this and does not detract from the centrality of water for life on earth.

That, however, is not the same as saying that all life must be aqueous. At least with our present (incomplete) state of knowledge about pivotal concepts such as the hydrophobic interaction, it is not obvious that any one of the functions of water in biology has to stand as an irreducible aspect of a "living system". It is certainly possible to imagine, and even to make,[428] artificial chemical systems that engage in some form of information transfer—indispensable for inheritance and Darwinian evolution—in nonaqueous media. Those properties of water that do seem extremely rare, if not unique, in a solvent—such as rapid proton transfer via Grotthuss or "Moses" intermolecular hopping—are clearly exploited by terrestrial cells but do not seem obviously indispensable for life to exist.

Moreover, creating and sustaining life in water faces some significant obstacles[429]—perhaps most notably the solvent's reactivity, raising the problem of hydrolysis of polymeric structures and of fundamental building blocks such as sugars. How the first pseudobiological macromolecules on the early Earth avoided this problem is still something of a puzzle. It is also unclear whether a solvent capable of engaging in hydrogen bonding might initially help or hinder the use of this valuable, reversible noncovalent interaction for defining complex structures in macromolecules and their aggregates. Certainly, there is now reason to believe that such molecules can utilize both hydrogen bonding and solvophobic effects in acquiring well-defined structures without needing water as their solvent.[61]

Attempts to enunciate the irreducible molecular-scale requirements for something we might recognize as "life" have so far been rather sporadic[429,430] and are often hampered by the difficulty of looking at the question through anything other than aqua-tinted spectacles. From the point of view of thinking about nonaqueous astrobiological solvents, a review of water's roles in terrestrial biochemistry surely raises one key consideration straight away: it is not sufficient, in this context, to imagine a clear separation between the "molecular machinery" and the solvent. There is a two-way exchange

of behaviors between them, and this literally erases any dividing line between the "biological components" and their environment. Water is an extraordinarily responsive and sympathetic solvent, as well as being far more than merely a solvent. If living systems depend on that kind of exchange, for example so that molecular information can be transmitted beyond the boundaries of the molecules that embody it, one is tempted to conclude that these systems would need to make use of water.

It is not just because of its molecular-scale structure that water has been characterized as "biophilic"—when Henderson first raised the intriguing notion of water's "fitness" as life's matrix in 1913,[431] he had in mind the unusual macroscopic properties such as its high heat capacity and density anomalies. Nonetheless, even these have their origins in water's more or less unique set of molecular characteristics. Barring some unforeseen revelation from the exploration of Titan, however, it is likely that we will have to rely on experiment rather than discovery to put Henderson's hypothesis to the test. Rather excitingly, with the advent of synthetic biology,[432−434] along with chemical and biological systems for exploring "alternative biochemistries",[435−439] it is now far from inconceivable that this test can be arranged.

8. Acknowledgments

9. Note Added in Proof

Significant new findings in this field are arriving so frequently that a review cannot be anything more than a snapshot. While the paper was in production, for example, there has been a thorough study of the structure of aqueous salt solutions[440] (which provides further evidence for the lack of utility of the picture of structure-making and structure-breaking), an exploration of the effect of hydrophobic interactions on pressure- and temperature-induced protein denaturation,[441] a beautiful example of hydration as a tuning parameter in the redox behavior of iron−sulfur proteins,[442] a study of the reliance of DNA hybridization on an aqueous environment,[443] an illustration of decoupling of protein and hydration dynamics for membrane proteins,[444] and a review of water structure at hydrophobic and hydrophilic surfaces.[445] Such advances will continue to be reported in an informal and no doubt highly incomplete way on the blog "Water in Biology" (http://waterinbiology.blogspot.com).

10. Note Added after ASAP Publication

This paper was published on the Web on December 21, 2007, with errors in Figure 1 and section 2.1. The correct version was posted to the Web on December 28, 2007.

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