Water: now you see it, now you don't

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Recent studies of water in and around proteins have made us acutely aware of the critical role that water plays in protein structure and function. They have also highlighted how little we know about the detailed behavior of water molecules. Four general questions can be asked about water molecules interacting with proteins. Where are they? How long do they stay there? How strongly do they interact with the protein? How do they affect protein structure and stability? Although we do not yet have definitive answers, a consensus is emerging, which brings together the three key techniques of modern structural research: crystallography, NMR spectroscopy, and computer methods of analysis and simulation. To reconcile the data produced by these different approaches, it is also necessary to keep clearly in mind what each technique actually measures (or calculates).

Water in crystal structures

Where are the water molecules? Nature abhors a vacuum, and water molecules will try to occupy all space not occupied by protein atoms. X-ray diffraction studies in the crystalline state were the first to provide detailed structural information on the positions of protein atoms. With the advent of refinement methods, the positions of water molecules were also revealed in electron density maps. At first there was skepticism as to the reality of these isolated peaks of density. Confidence in water positions grew as more structures were refined to high resolution and as X-ray diffraction, which reveals the water oxygen positions, was combined with neutron diffraction, which reveals the water hydrogen positions. Between one half and two ordered water molecules are found per residue in protein crystal structures (about 200 water molecules for a typical protein).

Sreenivasan and Axelsen [1] made a careful study of buried water (not contacting bulk solvent) in seven homologous structures of serine proteases. They find that 16 waters are highly conserved, These buried waters, which appear to be an integral part of the folded protein, are surrounded by backbone atoms and satisfy the main-chain hydrogen bonding capacity of peptide groups not involved in hydrogen bonds with other peptide groups. Kossiakoff and co-workers [2,3] in their neutron diffraction study of water structure in and around bovine trypsin, also find that buried waters in different X-ray structures are highly conserved. Furthermore, they note that surface waters are less conserved, and that water molecules involved in crystal lattice contacts are least conserved of all.

In an analysis of 56 high-resolution crystal structures, Kuhn et *al* [4] relate the distribution of surface waters to protein surface topography. They find that ordered waters are three times more likely to be in surface grooves than elsewhere on the surface and, moreover, that non-groove waters show considerably less discrimination between polar and non-polar groups than do groove waters. They suggest that deep grooves in the protein surface are formed by protein/water interactions.

An earlier analysis of 16 high-resolution crystal structures by Thanki *et al.* [5] found that the polar side chains interact more with water molecules than do non-polar side chains, and that ordered water molecules are generally arranged to make hydrogen bonds with polar atoms of the protein. The authors point out that the highly mobile side chain of lysine residues has no preferred orientation for water contacts.

Water in solution structures

Are the ordered water molecules observed in crystal structures representative of the bound water in solution? With the advent of structural NMR methods, it has been shown that the three-dimensional structures of proteins are by and large the same in solution as in the crystal. These same methods have also determined, in solution, positions of the most slowly exchanging water molecules. For pancreatic trypsin inhibitor [6], interleukin-1 β [7], thioredoxin [8] and FK506 binding protein [9], a handful of bound water molecules are observed by NMR. These few water molecules are buried and occur at the same locations as do buried water molecules in the crystal structure. They have very long residence times ranging from 10^{-2} to 10^{-8} seconds.

Otting *et al.* [10] were also able to observe the surface waters of hydration. They find that these waters are in rapid exchange with bulk water and have residence times below 500 picoseconds (ps). The waters of hydration that correspond to an ordered water molecule in the crystal structure have similar residence times.

Simulation and theory

Theory had much to say about protein water structure and dynamics even before experimental evidence became available. As long ago as 1988, short residence times were predicted for surface waters. Molecular dynamics studies of proteins in solution [11,12] predicted that hydrating waters would be extremely mobile, with a diffusion constant little different from that of bulk water. More specifically, Levitt and Sharon [11] showed that the population of water molecules close to any polar atom (within 3.2 Å) decays with a time-constant of about 100 ps, whereas the population close to any non-polar atom (within 4.5 Å) decays twice as quickly.

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A very recent molecular dynamics study of the hydration of pancreatic trypsin inhibitor by Brunne *et al* [13] attempts to reproduce the experimentally determined residence times of waters near specific protein atoms. Their simulation agrees well with experiment, in that average residence times for surface waters are less than 100 ps. The correlation between simulated and experimental residence times for specific water molecules is rather poor, which is hardly surprising considering the difficulty of the experiments. The average simulated residence times of water molecules near backbone and side chain atoms are 39 ps and 24 ps, respectively. Residence times near charged side chain atoms are shorter than those for other polar and non-polar atoms. Brunne *et al.* confirmed that the residence times of crystallographically well-ordered surface waters are indistinguishable from those of other less well-ordered water molecules. These residence times are what one would estimate from diffusion (see Fig. 1).

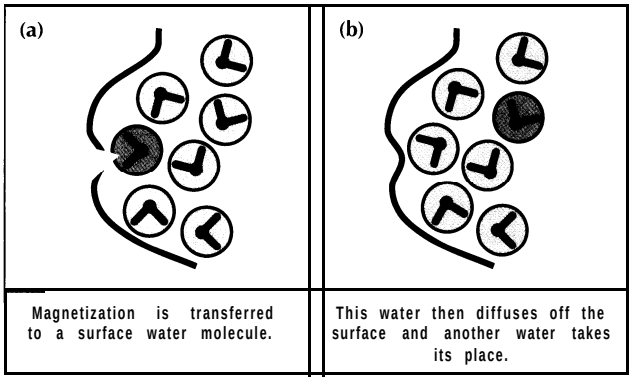

Fig. 1. **NMR experiments transfer magnetization from the protein to a water molecule and then measure how long it takes for that water to move away from the protein surface. A particular water molecule can move away from the protein surface without affecting the time-averaged arrangement of water molecules. By rough calculation based on diffusion, a water molecule moves a root mean square distance r in time t, given by $6Dt = r^2$. In bulk water at room temperature, the diffusion constant, D, is 2.2 $\times 10^{-5}$ $cm^2 sec^{-1}$ or 0.22 $Å^2 ps^{-1}$. Thus, a water molecule can diffuse 5 Å in time 19 ps [$t = 25/(6 \times 0.22)$] psl. Molecular dynamics simulation indicates that the first layer of water diffuses more slowly by a factor of two [11,12], increasing this time to about 40 ps.**

Other theoretical work also relates to the interaction of water with proteins. Using a simple geometrical model, Nicholls *et al* [14] conclude that water molecules will be less tightly bound in narrow crevices: strong surface tension forces pull the crevice water towards the other water molecules. In a Monte Carlo simulation of water around a rigid a-helix, Gerstein and Lynden-Bell [15] recently found that the water-water hydrogen bonds pull the water molecule away from hydrophobic surface.

In a thermodynamic pertubation theory calculation of the affinity of buried water for cavities in sulfate-binding protein, Wade *et al.* [16] show that the free energy of binding to one cavity is very favorable at 10 kcal mol^{-1}, whereas for a second cavity the free energy is slightly unfavorable at 0.2 kcal mol^{-1}. In the crystal, a water molecule is observed in the first cavity but not in the second. The water molecule in the favorable cavity makes four good hydrogen bonds with the protein, whereas a water molecule placed in the unfavorable cavity would be surrounded by non-polar side chains. It is interesting that the interaction of a water with the non-polar cavity is almost as favorable as that of a water molecule in liquid water.

Different surfaces, different surface waters

How can waters be detected in X-ray diffraction experiments, if NMR measurements and simulations suggest residence times of nanoseconds or picoseconds? These experimental and theoretical results make us pause to consider what it means when a surface water is found in a crystal structure. The natural assumption has been that crystallographically well-ordered waters are those whose positions are particularly energetically favored, which is true but misleading. The electron density map derived from X-ray diffraction is a density map averaged over a time measured in hours, which is extremely long relative to the characteristic periods of atomic motions. When discrete water density is found in a crystal structure, it indicates simply that the potential of mean force for a water molecule at that point in the structure has a local minimum; that is, the free energy of a water at closely neighboring regions (within say 2–3 Å) is relatively high, forming an energy pocket. If the potential of mean force did not have a local minimum at that point, high electron density would not be found since, on average, waters would be located relatively uniformly in that region.

It is not surprising that Kuhn *et al.* [4] more commonly find crystallographic waters in crevices than elsewhere. A water-sized crevice on a protein surface is indeed likely to be a local energy minimum, since by sitting in a crevice a water is circumscribed in its motion. Add hydrogen-bonding partners to the crevice and the likelihood of an energetically favorable pocket increases greatly. In this regard, it is important to realize that the visibility of a water molecule depends strongly on the extent of its motion (Fig. 2). A water molecule vibrating 1.5 Å about a fixed mean position will have a peak electron density of 10 % that of a water molecule vibrating 0.5 A; its temperature factor will be 60 $Å^2$ as opposed to 4 $Å^2$. A consequence of this is that the protein environment in which a water molecule is located can affect whether it is detectable (Fig. 3). Fig. 3 focuses on the steric nature of a protein surface, but the charge, as well as the shape, of the protein surface is important. Polar atoms, which can be in crevices or at the ends of side chains, interact through specific hydrogen bonds to water molecules and limit water motion. Non-polar atoms interact less specifically and may not order the water molecules sufficiently to be visible in electron density maps.

A somewhat more puzzling point made by Kuhn *et al.* [4] is that water molecules only prefer hydrophilic groups that are in crevices. We must conclude that ordered waters, with the exception of those in crevices, are essentially an accident of the geometry of the crystal lattice. The fact that surface waters are not in general conserved across different crystal forms of the same structure supports this conclusion. Of 60 waters observed in the crystal structure of pancreatic trypsin inhibitor, only six are conserved in all three crystal forms [10].

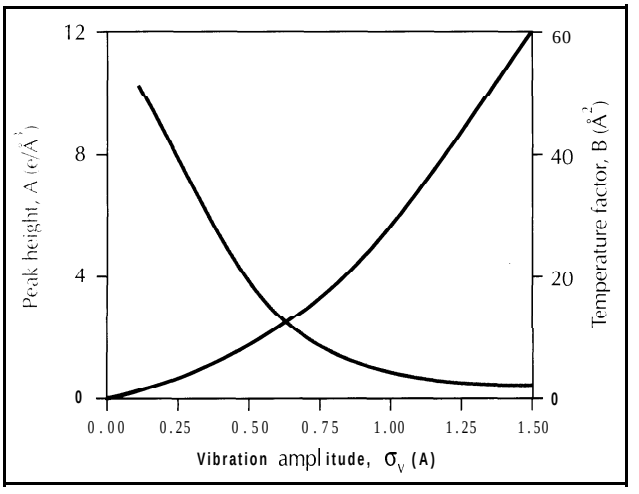

Fig. 2. The effect of atomic motion on the electron density peak height (blue line) and temperature factor (red line) depends sensitively on the extent of atomic motion. The electron density, $\rho(r)$, varies with distance, r, from the atom center as $\rho(r) = A \exp(-r^2/\sigma^2)$, where $\sigma^2 = \sigma_a^2 + \sigma_v^2$ is the sum of squares of the intrinsic atom size, σ_a, (0.50 Å for oxygen) and the amplitude of vibration, σ_v. The peak height, A, given by $Z/(\sqrt{\pi}\sigma)^3$ (where Z is the number of electrons), decreases rapidly as σ_v increases. The temperature factor, B, defined as $B = (8/3)\pi^2\sigma_v^2$, is a crystallographic measure of disorder.

Thanki *et al.* [5] do not distinguish crevice water and non-crevice water and find that water binds to polar side chains as expected from hydrogen bond stereochemistry. These same interactions will operate whether or not the side chain is in a crevice. Waters may not be seen interacting with side chains that protrude from the protein surface for two reasons. Firstly, a polar atom in the side chain is exposed to solvent over a large part of its surface. A water molecule has no inherent reason to prefer one orientation relative to the polar atom of another. Secondly, the side chain may bind the water tightly but then itself be moving enough to make the water molecule appear disordered in the electron density map (Fig. 3).

Bound water

The energetics of bound water are less clear-cut. While Kuhn et *al.* [4] find more ordered water molecules in crevices, Nicolls et *al.* [14] argue that surface tension should make these same crevice waters bind less strongly. NMR experiments [10,13] and simulations [11,13] find that even those water molecules that are well-ordered in the crystal, and presumably in surface grooves, have very short residence times. Clearly, in solution almost all surface water molecules are in rapid exchange with bulk water. Such exchange is interesting in that it cannot involve any change in free energy: all water molecules are equivalent and the system must be unchanged after exchange. Residence times depend only on the free energy of the transition state, which may depend on both the strength of binding and the geometry of the site. When a particular water molecule leaves the protein surface, its place will be taken by another identical water molecule. The rate of exchange depends on the energy of the transition state (Fig. 4), a state in which neither water is in the site. The height of this energy barrier may be expected to correlate with the strength of binding but another important factor is access: if water molecules can enter and leave the site by different routes then exchange will be facilitated. The residence times of bound waters are not known in the crystal state. The fact that water molecules are well-resolved in electron density maps that are time averaged over hours does not mean that waters cannot be exchanging as rapidly in the crystal as in solution. As long as there are distinct sites of favorable interaction, individual water

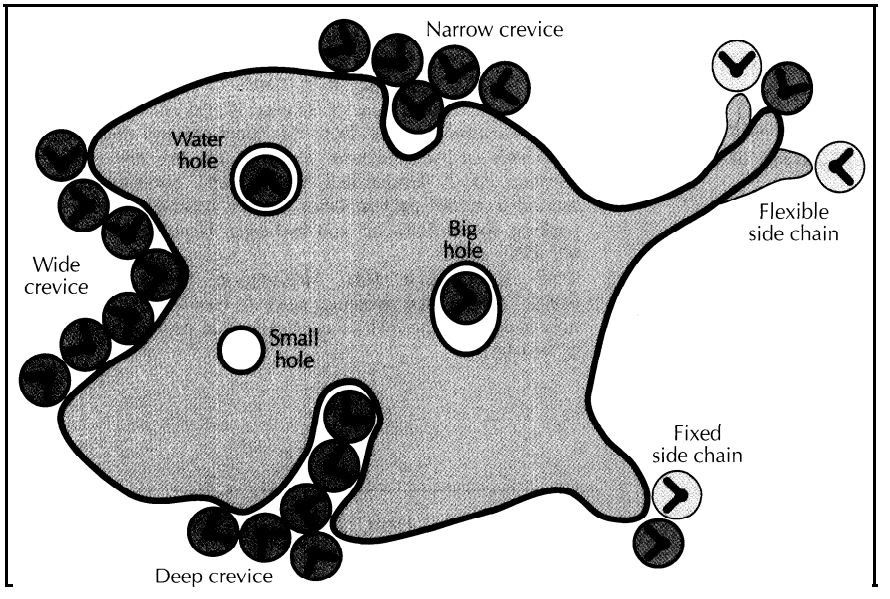

Fig. 3. A cross-section through a hypothetical protein molecule illustrates how water molecules are arranged in different environments. Water molecules cannot occupy holes inside the protein that are too small, whereas a water molecule in a large hole may be able to move enough to be invisible in an electron density map. Both theory and NMR measurements indicate that buried waters exchange slowly (0.01 seconds to 10 nanoseconds). Water molecules will be well-localized in narrow and deep crevices that are big enough to accommodate a single water. Surface tension forces will pull water molecules out of narrow crevices but have less effect on waters in deep crevices. A water molecule that is tightly bound to a flexible side chain protruding into the solvent will not be seen in the electron density. A water that can bind to a rigid side chain in several equally favorable ways will move between these states and will also not be seen in the electron density.

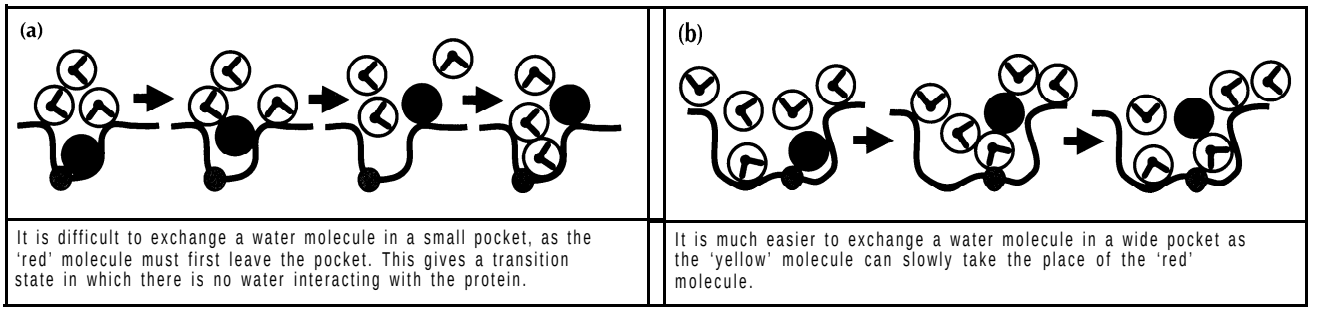

Fig. 4. The effect of pocket geometry on residence times. The time for which a particular water molecule remains associated with a protein group is not a direct measure of the strength of the interaction. Residence times will be longer if there is a high energy barrier to the exchange of water molecules.

molecules will be resolved. Given that water molecules have high mobility, are indistinguishable, and occur at high concentration, we may expect short residence times of bound water in protein crystals.

In conclusion, we have a composite picture of water in which almost all space not occupied by protein atoms is filled with water. Many of these water molecules, particularly those in narrow crevices, are sufficiently ordered (< 1.5 Å vibration) for electron density to be resolved. Most of the water molecules, including those that appear to be fixed in the crystal, are in rapid motion in solution with exchange times of less than 100 ps. Water molecules in interior cavities are generally conserved in different homologous structures and are seen both in crystals and in solution; these buried water molecules exchange much more slowly (10 nanoseconds to 0.01 seconds). Simple geometrical considerations indicate that surface tension forces pull water molecules out of narrow crevices; this should strengthen the interaction of substrates with concave binding sites. Simulations show that the free energy of waters in cavities depends critically on the size and polarity of the cavity; some cavities will not be filled with water.

The broader role of water in stabilizing protein structures is still controversial. Water molecules appear to be both the cement that fills crevices between amino acid building blocks, and the lubricant that allows motion of these building blocks. This allows a protein chain to fold without being trapped in local minima and compensates for poor steric fit of side chains in protein interiors and of substrates in binding sites.

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