Structure, dynamics and reactions of protein hydration water

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The apparent simplicity of the water molecule belies the wide range of fascinating protein phenomena in which it participates. We review recent computer simulation work on buried, internal water molecules, discussing the thermodynamics of water molecule binding and the participation of water in proton transfer reactions. Surface water molecules are also considered, with emphasis on the modification of average solvent structure on a protein surface, the role of water in the protein dynamical 'glass' transition and a simplified description of the protein motions thereby activated.

Keywords: protein hydration; proton transfer; binding thermodynamics; solvent structure

1. INTRODUCTION

A characterization of protein hydration is essential for understanding protein structure, folding and function. This requires elucidating the effects of both the solvent on the protein and the protein on the solvent (Teeter 1991; Schoenborn *et al.* 1995). Concerning the latter effect, detailed information on the ordering and dynamical properties of individual, highly perturbed, strongly bound water molecules has been furnished by high-resolution X-ray crystallography and NMR spectroscopy (Badger & Caspar 1991; Badger 1993; Brunne *et al.* 1993; Bouquiere *et al.* 1994; Badger *et al.* 1995; Bon *et al.* 1999; Nakasako 1999; Venu *et al.* 1999). Other water molecules, not so strongly bound and with only fleeting residence times at binding sites with the proteins, require other techniques for their characterization.

We consider five aspects of water–protein interaction:

- (i) water molecule binding inside a protein;
- (ii) water molecules in protein reactions;
- (iii) solvent structure on a protein surface;
- (iv) solvent role in the protein glass transition;
- (v) the protein motions activated by solvent.

2. WATER MOLECULE BINDING INSIDE A PROTEIN

Understanding the binding of small molecules to proteins is of both fundamental and practical interest and the water–protein interaction can be considered an important special case of this. In principle, the small size of the water molecule should simplify computational analysis of the

underlying thermodynamics. Of particular interest are buried water molecules that fill some of the voids in protein structures. Characterization of the binding structure, dynamics and thermodynamics of well-ordered, strongly interacting, buried water molecules is likely to provide much information that will also be of use in understanding ligand binding in general. Central to this is an understanding of the free energy change on binding, owing to changes in the nature of the degrees of freedom of the ligand. A theoretical framework for determining the free energy of transfer of a water molecule into a given site in a protein has been derived (Roux *et al.* 1996). An example of a well-ordered water molecule is found in the BPTI. This deeply buried water molecule (W122) makes four strong hydrogen bonds with the protein, and has served as a probe for investigating the dynamics and thermodynamics of structural water in proteins. In recent theoretical work (Fischer *et al.* 2001) normal mode analysis was performed to provide an understanding of the entropy changes accompanying the binding of water molecule W122 to BPTI. The binding was shown to be accompanied by an increase in the total vibrational entropy of the complex, relative to the unbound protein, and the origin of the vibrational entropy change accompanying the protein–water interaction was examined. The calculations indicate that the binding of W122 with the formation of four near-optimal hydrogen bonds induces an increase in the protein flexibility for the delocalized vibrations below 50 cm^{−1}. This 'softening' of the structure on binding is due to modification of the force–constant matrix and is not simply a mass effect.

3. WATER MOLECULES IN PROTEIN REACTIONS

Another important role of water molecules is participation in protein reactions. In enzymes, water is frequently present, participating in hydrolysis and/or proton transfer reactions, for example. We present one example of a

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proton transfer reaction that we have studied in detail, and discuss the role of water molecules in facilitating the proton transfer.

BR is a seven-helical light-driven proton pump protein found in the purple membrane of the archaea *Halobacterium salinarum* (for recent reviews, see Haupts *et al.* 1999; Subramaniam *et al.* 1999; Lanyi 2000). The absorption of one photon by the all-*trans* retinal chromophore leads to rotation of the C13–C14 bond into the 13-*cis* conformation. This triggers a photocycle through a series of intermediates distinguished spectroscopically:

The net effect is the transfer of one proton from the cytoplasmic to the extracellular side of the membrane. The resulting electrochemical gradient is used by the cell to synthesize ATP. Understanding, at an atomic level, how BR transfers protons is a key element in elucidating how this protein transforms light into chemical energy.

The first, and arguably best characterized, proton transfer step occurs between the L and M states on a time-scale of *ca.* 10 μs (Ludman *et al.* 1998). In this step, a proton is transferred from the retinal Schiff base NH group to Asp85 over a distance of *ca.* 4 Å. The mechanism of this primary step is much debated, with renewed impetus to the discussion being provided by recent high-resolution X-ray crystallographic and electron microscopic structures of BR and relevant photocycle intermediates (Belrhali *et al.* 1999; Edman *et al.* 1999; Luecke *et al.* 1999; Royant *et al.* 2000; Sass *et al.* 2000; Subramaniam & Henderson 2000; Schobert *et al.* 2002; Lanyi & Schobert 2002, 2003).

An open question is whether the proton is transferred directly from the Schiff base to Asp85, or whether nearby protein residues and water molecules act as intermediate proton carriers. In the latter case, the proton could, in principle, be transferred in two ways. In one, the proton is transferred to the final acceptor group after passing over the intermediate carriers. Alternatively, the intermediate carriers form a proton wire, so that the proton given by the Schiff base is not the same proton accepted by Asp85. This second mechanism is illustrated as follows for the case of a single intermediate (I):

To address the above questions we have undertaken an exhaustive exploration of the different mechanisms of the primary proton transfer by using an efficient method for computing reaction pathways and their transition states, together with a realistic model for the energetics of the proton transfer. Reaction pathways were determined using the Conjugate Peak Refinement algorithm (Fischer & Karplus 1992) as implemented in the TRAVEL module of CHARMM (Brooks *et al.* 1983). The protein was partitioned into QM and MM parts. The QM region comprised retinal and surrounding protein groups that may be involved in proton transfer (86 QM atoms). The link atom approach (Field *et al.* 1990) was used for the QM/MM boundaries at the $\text{C}_\beta\text{--C}_\gamma$ bond in Lys216 and at the $\text{C}_\alpha\text{--C}_\beta$ bond for the other side chains. The QM/MM interactions have been described (Cui *et al.* 2001). Interactions between the MM atoms are given by the potential energy function of CHARMM (Brooks *et al.* 1983) with the

parameter set 22 (MacKerell *et al.* 1998) for aromatic side chains and parameter 19 for the rest of the protein (Neria *et al.* 1996). SCC-DFTB (Elstner *et al.* 1998) was used to describe the QM region for the initial estimations of the possible proton transport pathways. B3LYP/6-31G** energy calls were performed on the SCC-DFTB/MM-optimized pathways.

Minimum-energy paths for more than 30 scenarios of proton transfer were computed, varying elements such as the number and location of nearby water molecules, the initial orientation of the Schiff base, the intermediate proton acceptors involved, and with different protein crystal structures. The calculations yield atomic detail ‘movies’ of the various proton transfer pathways explored together with their associated barriers.

One of the low-energy proton transfer paths found is a two-step mechanism, involving Asp212 and water w402. The proton is transferred down the Asp212 side of the retinal. The pathway begins with a conformational transition involving a significant change in retinal twisting, such that the Schiff base NH group reorients from the cytoplasmic to the extracellular side of the membrane. The subsequent proton transfer steps are shown in figure 1 and take place with little structural rearrangement. First, the Schiff base proton is transferred to Asp212. This is the rate-limiting process, whose transition state (at a value of the reaction coordinate $\lambda = 0.1$ in figure 1) lies 11.5 kcal mol⁻¹ above the initial state. The hydrogen-bonding pattern around Asp212 then reorganizes to form a proton wire from Asp212 to Asp85 via water w402, with the proton still located on Asp212 at $\lambda = 0.6$. Finally, the protonation switches from Asp212 to Asp85 in a concerted manner via this proton wire. The activation barrier of 4.6 kcal mol⁻¹ at $\lambda = 0.7$ is not rate limiting.

The activation energy barrier found for this pathway is in agreement with experimental evidence (Ludman *et al.* 1998), and thus provides evidence for internal water molecules playing a direct role in the primary proton transfer process in BR. Locating water molecules that play a direct role in protein function is a major challenge for crystallographers and computer simulations alike. The quantitative determination of their role along a reaction coordinate remains, however, the terrain of computational studies.

4. SOLVENT STRUCTURE ON A PROTEIN SURFACE

We now turn to the more global, statistical aspects of protein surface hydration. Although NMR and crystallography provide detailed information on well-ordered water molecules, to furnish the data required for a complete thermodynamic description of protein hydration it is necessary to obtain a more global picture in which the solvent is described in terms of probability distributions. Recent work in this direction has used MD simulation (Lounnas *et al.* 1994; Pettitt *et al.* 1998) and novel crystallographic methods (Burling *et al.* 1996) to enable radial distribution functions of water molecules around protein groups in crystals and related quantities to be obtained.

It is of particular importance to determine how protein surface water is perturbed from the bulk. An intriguing result in this regard was reported by Svergun *et al.* (1998) who combined SAS of X-rays in H₂O with that of

Figure 1. Proton transfer via Asp212 and via water w402. Energies are taken relative to the state with retinal Schiff base in its cytoplasmic orientation. λ is the normalized sum along the path of the change in all atomic coordinates measured as a root-mean-square difference (Fischer *et al.* 1998). $\lambda = 0$ is the reactant state (i.e. protonated Schiff base and negatively charged Asp85) and $\lambda = 1$ is the product state (i.e. deprotonated Schiff base and protonated Asp85). The insets show the transferred protons as small spheres.

neutrons in H_2O and D_2O to show that for lysozyme and other proteins the average density of the first hydration shell (0–3 Å from the surface) is significantly higher than that of bulk water (Svergun *et al.* 1998). In recent work, the physical origin of this effect was explored (Merzel & Smith 2002*a,b*). MD simulation was performed of lysozyme in explicit water to determine the contributions to the hydration density profile.

MD simulation allows the detailed structural properties of the hydration water to be analysed and compared with bulk water. As described in Merzel & Smith (2002*b*), excellent agreement was found between the SAS profiles calculated from the lysozyme simulation and the experimental results of Svergun *et al.* (1998). The next stage is to decompose the observed density increase. Even if the water structure were unperturbed there could be a pure 'geometric' or 'correlation' effect in the distribution of atoms that could lead to a non-uniform density distribution. This is due to the fact that the protein surface is defined to run between the two sets of atoms (water and protein) and therefore is at non-random distances from the water oxygens. Consequently, the structure of the bulk water radial-distribution function will be partly reflected in that calculated normal to the surface. The simulation results indeed show that about two-thirds of the first-shell density increase of *ca.* 15% is due to this geometric contribution that would also be present if the water were unperturbed from the bulk. The remaining one-third (5%) density increase involves significant changes in the average water structure. Approximately half this density increase arises from shortening of the average water–water distances, and the other half from an increase in the coordination number.

Which aspects of the protein surface determine water density variations? Classically, protein hydration structure has often been discussed in terms of the hydrophobicity/hydrophilicity of the surface groups. A series of calculations showed no clear relationship between the hydration

shell water density and the chemical characteristics of the surface atoms or residues, for example, whether polar/non-polar or hydrophobic/hydrophilic. In agreement with this result, in several recent studies (Finney *et al.* 1993; Pertsemidis *et al.* 1996; Kovacs *et al.* 1997) no clear relationship between first-shell water density variations and the hydrophobicity/hydrophilicity of chemical groups has been found. The exceptions are groups with net charges, about which the density is increased. However, these charged groups make up only 5% of the protein surface and therefore have only a small effect on the average density profile properties. We use a simpler description, in which the protein surface is considered to possess a topography and the protein atoms to generate an electric field. The relationship between these two effects and the water density is determined and leads to a simple physical picture of the global surface-density effect.

Figure 2 illustrates the variation with water density of topographic and electrostatic properties of the protein surface, together with probability distributions of the properties examined. We define the surface topographic perturbation, $h(\theta)$, as the difference between multipolar expansions of the protein surface at two different resolutions, one low (smooth) and one high (rough). This function is negative for depressions (grooves or cavities) in the surface and positive for protuberances, with the magnitude determined by the depth of the depression or height of the protuberance. Figure 2*a* shows that $h(\theta)$ is clearly correlated with the local water density, the denser regions occurring in the surface depressions. Figure 2*b–d* shows how the water density varies with the electrostatic field generated by the protein atom partial charges at 1.5 Å from the protein surface. Figure 2*b* shows the dependence of the density on the angle between the electric field vector and the surface normal. Higher density appears at larger angles for which the electric field points towards the surface. Lower density occurs where the field is parallel to the normal or pointing away from the surface. Figure 2*c*

Figure 2. Density increase in the first hydration layer as a function of: (a) surface topographic perturbation, $h(\Theta)$; (b) angle between electrostatic field and surface normal; (c) component of electrostatic field parallel to the surface normal; and (d) component of electric field tangential to the surface. The average increase in density is denoted by a dashed line at 5.1%. In the lower part of each graph are shown the populations (in percentages) of the corresponding properties. (From Merzel & Smith (2002b).)

shows that high density occurs in fields for which the component parallel to the surface normal is negative. In this field, the hydrogen atoms are closer to the surface than the oxygen atoms. The strength of the field tangential to the surface is also correlated with the density over the most highly populated field values, with higher tangential field strengths accompanying higher density (figure 2d).

Thus, we find clear density effects if a simple view of the protein surface is taken, in which it is considered to be an envelope with an associated electrostatic field generated by the protein atom partial charges. A relationship between peptide hydration structure and surface topography has been demonstrated in computer simulations of melittin in water (Cheng & Rossky 1998; Cheng *et al.* 1999). Moreover, analysis of the corresponding densities of some crystallographically resolved water molecules revealed that concave cavity regions are of higher water density (Harpaz *et al.* 1994; Gerstein & Chothia 1996). The present results are in agreement with these studies in that surface topography is found to be strongly related to water density and depressions in the protein surface contain higher-density water. However, the present results generalize this effect to the whole protein-solvent interface rather than just a small proportion of crystallographically

resolved solvent molecules and, moreover, a clear electrostatic effect is demonstrated here. In depressions on the surface the water molecules have a stronger tendency to align with the electrostatic field generated by the protein atoms.

This can be simply understood, because in a depression, water molecules are less exposed to the randomizing, disorienting effect of neighbouring water molecules. They align more tangentially to the surface than if they were unperturbed, and more parallel to each other than in bulk water, leading to higher-density packing.

The above results present a coherent picture of electrostatically driven protein surface-water-density effects. In depressions in the protein surface, water molecules are less exposed to the disorienting effect of bulk solvent. Therefore, they tend to orient themselves to follow the electrostatic field lines generated by the protein atoms.

5. SOLVENT ROLE IN THE PROTEIN-GLASS TRANSITION

We now turn to the effect of solvent on protein dynamics. A variety of experiments have demonstrated the existence of a dynamical transition in hydrated proteins at *ca.*

Figure 3. Mean-square fluctuations, $\langle u^2 \rangle$, of the protein non-hydrogen atoms for different sets of simulations. (a) Filled squares, control set with protein and solvent at same temperature; filled diamonds, protein held at 80 K; filled circles, solvent held at 80 K; open diamonds, protein held at 180 K; open circles, solvent held at 180 K. (b) Solvent held at 300 K. (c) Protein held at 300 K. Standard errors were estimated by calculating the mean-square fluctuations for 10 bins each 20 ps long.

180–220 K, characterized by deviation from linearity of the temperature dependence of the mean-square displacement, $\langle u^2 \rangle$ (Cohen *et al.* 1981; Parak *et al.* 1981; Knapp *et al.* 1982; Doster *et al.* 1989, 1990; Rasmussen *et al.* 1992; Tilton *et al.* 1992; Ferrand *et al.* 1993; Green *et al.* 1994; Fitter *et al.* 1997; Daniel *et al.* 1998, 1999; Reat *et al.* 2000; Bicout & Zaccai 2001; Lee & Wand 2001; Teeter *et al.* 2001). The protein transition has dynamical aspects in common with the liquid–glass transition. For example, as in glass-forming liquids, proteins exhibit diffusive motions above the transition and are trapped in harmonic potential wells below. Experiments have shown that in several proteins biological function ceases below the dynamical transition (Parak *et al.* 1980; Rasmussen *et al.* 1992; Ferrand *et al.* 1993).

An important physical question concerns the environmental effect on the dynamical transition. MD simulations (Smith *et al.* 1990; Hayward & Smith 2002) and neutron-scattering experiments (Paciaroni *et al.* 2002) have shown that isolated or dehydrated proteins present dynamical transition behaviour. However, several experiments and simulations have indicated that when a protein is solvated the dynamical transition is strongly coupled to the solvent (Ferrand *et al.* 1993; Steinbach & Brooks 1993, 1996; Fitter *et al.* 1997; Cordone *et al.* 1999; Fitter 1999; Reat *et*

Figure 4. Decomposition of the mass-weighted protein $\langle u^2 \rangle$ into contributions arising from the harmonic (dark grey), quasi-harmonic (hatched) or multimimum (open) classes of PCA. Vertical arrows indicate the number of multimimum modes. The number of quasiharmonic modes at certain temperatures is indicated by the hatched area. The $\langle u^2 \rangle$ calculated at 300 K from the normal mode analysis using the same model system and potential function is indicated by filled squares. The value of this concurs with that calculated from the 'harmonic' principal component modes.

al. 2000; Tarek & Tobias 2000, 2002; Teeter *et al.* 2001; Caliskan *et al.* 2002; Paciaroni *et al.* 2002). Highly viscous solvents, such as trehalose, suppress dynamical transition behaviour (Hagen *et al.* 1995; Cordone *et al.* 1999; Walser *et al.* 2000), and neutron-scattering experiments on enzymes in a range of cryosolvents showed that the dynamical transition behaviour of the protein solution resembles that of the pure solvent (Bizzarri *et al.* 2000; Reat *et al.* 2000; Daniel *et al.* 2003).

In recent work (Tournier *et al.* 2003), dual heat-bath methods were used to probe the role of solvent in the dynamical transition. In dual heat-bath methods two subsystems (here the protein and its solvent) are connected to a different heat-bath characterized by a different reference temperature. Thus, simulations can be performed with one subsystem at one temperature and another at a different temperature.

Figure 3a presents the protein fluctuations calculated from a control set of hydrated myoglobin simulations (in which in each simulation the protein and solvent are at the same temperature), together with those obtained by fixing the temperature of one component (solvent or protein) at a temperature below the dynamical transition while varying the temperature of the other. In the control set the experimentally known dynamical transition is reproduced, with nonlinearity starting at *ca.* 220 K. Fixing the solvent temperature at 80 K or 180 K suppresses the dynamical transition, the protein $\langle u^2 \rangle$ increasing linearly with temperature up to 300 K. Therefore, low-temperature solvent cages the protein dynamics.

Figure 3a also shows that holding the protein temperature constant at 80 K or 180 K and varying the solvent temperature also abolishes the dynamical transition behaviour in the protein. Thus, in summary, figure 3a demonstrates that holding either component at a low temperature suppresses the protein dynamical transition.

Figure 5. Motion of myoglobin along the first principal mode calculated at 200 K. The structures of myoglobin are shown in the two minima (black and white) of the mode. The helices are represented as cylinders. For ease of viewing the amplitude of the motion is multiplied by a factor of 6. In (a) the side chain of Asp126 is also shown.

Figure 3*b,c* shows the effect of holding one component above the transition temperature while varying the temperature of the other. Holding the solvent temperature at 300 K (figure 3*b*) leads to increased protein fluctuations at most temperatures relative to the other simulation sets. However, there is again no clear deviation from linearity, i.e. no dynamical transition behaviour. By contrast, fixing the protein at 300 K and varying the solvent temperature (figure 3*c*) recovers dynamical transition behaviour in the protein, incipient at *ca.* 200 K, a slightly lower temperature than in the control set.

The above results are interpreted as follows. When fixing the solvent at 300 K, only effects due to changes with temperature in the sampled region of the protein energy landscape appear. The absence of a dynamical transition indicates, then, that these changes do not control the transition. However, when the protein is held at 300 K, variations with temperature in the sampled solvent landscape trigger the protein transition.

Further work showed that there is a qualitative transition in the temperature dependence of the water translational diffusion constant at the dynamical transition. Thus, water translational diffusion is seen to drive the protein dynamical transition. By contrast, the water rotational reorientation is completely decoupled from the transition in the translational diffusion seen in this simulation.

6. PROTEIN MOTION ACTIVATED BY SOLVENT

The final question we address concerns the motions in proteins that are induced by the activation of solvent translational diffusion. MD simulation was used to determine the principal sub-nanosecond dynamical modes of hydrated myoglobin as a function of temperature (Tournier & Smith 2003). To do this PCA is employed to characterize the MD trajectories.

PCA is a convenient method for analysing the conformational space explored in an MD trajectory (Karplus & Kushick 1981; Kitao *et al.* 1998). PCA determines the essential motions present in the simulation: the principal component modes. The set of principal components is the solution to the eigenvalue problem of the second-moment matrix, **A**, of the mass-weighted internal atomic displacements. The elements of the second-moment matrix, $A_{i,j}$, are given by $A_{i,j} = \sqrt{m_i m_j} \langle (\mathbf{r}_i(t) - \mathbf{r}_i^m)(\mathbf{r}_j(t) - \mathbf{r}_j^m) \rangle$, where $\mathbf{r}_i$

is the position of atom *i*, $\mathbf{r}_i^m$ is the mean position of atom *i* and the average is taken over the different time-frames of the trajectory. The diagonalization of **A** yields the eigenvectors, $\mathbf{w}_k$, i.e. the principal components and their associated eigenvalues, ζ_k . ζ_k is the mass-weighted mean-square fluctuation along $\mathbf{w}_k$, and can be used to calculate the averaged mass-weighted fluctuation in the protein:

$$\zeta = \frac{1}{N} \sum_{i=1}^N \langle m_i (\mathbf{r}_i(t) - \mathbf{r}_i^m)^2 \rangle = \frac{1}{N} \sum_{k=1}^{3N} \zeta_k, \quad (6.1)$$

where *N* is the number of atoms in the protein. To optimize computer efficiency, only protein heavy atoms were included in the present PCA. For comparison with the principal component results, normal modes were calculated from the energy-minimized conformation of the model system using the harmonic approximation to the potential function. In a normal mode analysis, the Hessian matrix is diagonalized, yielding the normal modes $\mathbf{w}_k^{NM}$ with their corresponding angular frequencies ω_k^{NM} .

Analysis of the time-dependent myoglobin PCA modes allows the classification of the principal components into three types.

- (i) *Harmonic* modes are those that exhibit approximately Gaussian probability distributions (and for which the anharmonicity is low).
- (ii) *Quasiharmonic* modes exhibit approximately Gaussian probability distributions but possess considerable anharmonicity.
- (iii) *Multiminimum* modes cannot be well fitted by a Gaussian and are also anharmonic.

The contributions made to the mean-square displacement, $\langle u^2 \rangle$, by these three types of principal component are shown in figure 4. The increase in $\langle u^2 \rangle$ signalling the incipient phase of the transition (*ca.* 180–210 K) is seen to arise from a very small number of principal components. By 210 K, only four PCA modes are not harmonic—two of these are multiminimum and two quasiharmonic. At 210 K, 75% of the increase over the linear harmonic $\langle u^2 \rangle$ (the ‘excess’ $\langle u^2 \rangle$) is due to multiminimum dynamics. As the temperature increases,

more principal components become multimimum or quasiharmonic. However, at 300 K still only 20 modes deviate from harmonic behaviour, i.e. only 0.3% of the total number (7530) of modes in the protein. Seventy per cent of the excess $\langle u^2 \rangle$ at 300 K arises from seven multimimum modes, with the remaining 30% originating from 13 quasiharmonic principal components. This is consistent with previous work on lysozyme at 300 K which demonstrated that 90% of mean-square fluctuation can be described with fewer than 1% of the principal coordinates (Amadei *et al.* 1993; Kitao *et al.* 1998).

Graphical examination of modes 1–5 over the temperature range 180–210 K shows that these principal components all involve collective dynamics distributed over most of the myoglobin molecule. In mode 1, the transition between the free energy minima at 180 K comprises the relative motion of two large rigid-body blocks of approximately 900 atoms. One of these blocks contains helices A, H and F and the other B, C, D and G. The relative motion involves helices G and H rotating in opposite directions around the axis of the stationary helix E. Figure 5*a,b* illustrates the myoglobin structures in the two free energy minima. Rigid-body helix translations and rotations are apparent. Owing to rotations around the helix axes the side-chain displacements are generally larger than those of the main-chain atoms, as illustrated by Asp126 on helix H in figure 5*a*.

7. CONCLUSION

This review has examined several aspects of water–protein interactions at an atomic level. The examination of structural buried water in terms of ligand-binding thermodynamics provides a detailed view of non-bonded effects on binding entropy can be obtained. Water molecules participating directly in chemical reactions are also of clear functional relevance, as illustrated here for the case of BR.

Further understanding of the global hydration properties of proteins will require still more detailed decomposition of the various forces competing to determine surface water structure and dynamics. Parallel to this will be detailed analysis of the much-debated dynamics of surface water and the effect of surface water on protein dynamics. Water plays a key role in many of life's processes. It influences protein stability, plays a direct role in protein reactions, and an indirect role by controlling protein fluctuations. The present review demonstrates how computer simulations can directly reveal these phenomena in detail.

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Discussion

A. Kornyshev (*Imperial College London, London, UK*). How many molecules are in the bacteriorhodopsin interior, *in vivo*?

J. C. Smith. Bacteriorhodopsin has several internal water molecules. Some of these were identified by Zaccai and colleagues using neutron diffraction and more recently several X-ray crystallographic studies have reported solvent density in the proton transfer channel. However, there is still uncertainty as to exactly how many water molecules there are in different intermediates in the photocycle and their exact positions.

R. M. Daniel (*Department of Biological Sciences, University of Waikato, Hamilton, New Zealand*). The evidence is that the motions activated at the dynamic transition are not important for enzyme activity, presumably because they are too fast in relation to enzyme activity. Nor are they coupled to the motions required for activity.

J. C. Smith. The appearance of anharmonic motion on the picosecond time-scale at 220 K in glutamate dehydrogenase solution does not lead to an appreciable deviation from Arrhenius behaviour of the rate-limiting step for this enzyme. See Daniel *et al.* (1999).

B. Halle (*Department of Biophysical Chemistry, Lund University, Lund, Sweden*). If you compute a local density by integrating over the first peak in the particle distribution function for a hard sphere fluid next to a hard wall, you will find that it is higher than the bulk fluid density even though there are no attractive interactions in this system. To what extent, do you think, can the excess water density in pockets on the protein surface be accounted for by a two-dimensional version of this purely geometrical correlation effect?

J. C. Smith. The geometric density effect that you mention was explored in Merzel & Smith (2002). It was found that about two-thirds of the density increase came from this effect, and the other third from a modification in the water–water interactions.

B. Halle. The mean square displacement, $\langle u^2 \rangle$, in Doster's neutron-scattering experiment is presumably a non-equilibrium quantity reflecting the quenching of certain degrees of freedom during the rapid cooling of the sample. In your principal component analysis, was the simulated system subjected to a similar quenching and, if so, what is the influence of the cooling rate?

J. C. Smith. Non-equilibrium and hysteresis effects on the protein–glass transition are very interesting and insufficiently explored. They may explain some of the differences we are seeing in X-ray B-factor results. In the simulations mentioned here the system was energy minimized (corresponding to near-instantaneous quenching!) and then 'slowly' heated up on the 100 ps time-scale. Interestingly, at low temperatures, below the glass transition, proteins become trapped in structurally inhomogeneous conformational substates and this leads to noticeable differences between simulation results.

K. Wilson (*Department of Chemistry, University of York, York, UK*). How are the X-ray studies on cyclodextrins relevant? The flip-flop hydrogen bond disorder and the water sites 'tidy up' around 180–200 K. Is this relevant to the phase change observed in proteins?

J. C. Smith. Hydrogen bond ‘tidying up’ in cyclodextrins may well have something in common with the protein–glass transition, in which translational water diffusion plays an important role.

GLOSSARY

BPTI: bovine pancreatic trypsin inhibitor
BR: bacteriorhodopsin

MD: molecular dynamics
MM: molecular mechanics
NMR: nuclear magnetic resonance
PCA: principal component analysis
QM: quantum mechanics
SAS: small-angle scattering
SCC-DFTB: self-consistent-charge-density-functional
tight-binding