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Water–protein interactions from high-resolution protein crystallography

Masayoshi Nakasako

Department of Physics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan (*nakasako@phys.keio.ac.jp*)

To understand the role of water in life at molecular and atomic levels, structures and interactions at the protein–water interface have been investigated by cryogenic X-ray crystallography. The method enabled a much clearer visualization of definite hydration sites on the protein surface than at ambient temperature. Using the structural models of proteins, including several hydration water molecules, the characteristics in hydration structures were systematically analysed for the amount, the interaction geometries between water molecules and proteins, and the local and global distribution of water molecules on the surface of proteins. The tetrahedral hydrogen-bond geometry of water molecules in bulk solvent was retained at the interface and enabled the extension of a three-dimensional chain connection of a hydrogen-bond network among hydration water molecules and polar protein atoms over the entire surface of proteins. Networks of hydrogen bonds were quite flexible to accommodate and/or to regulate the conformational changes of proteins such as domain motions. The present experimental results may have profound implications in the understanding of the physico-chemical principles governing the dynamics of proteins in an aqueous environment and a discussion of why water is essential to life at a molecular level.

Keywords: water; hydrogen bond; hydration structures of proteins; cryogenic protein crystallography

1. INTRODUCTION

Water is a 'mother liquid' indispensable to life. More than 60% of the volume of living cells is occupied by water, and the loss of 10–20% of the volume of water causes the cell death. Biomolecules composing cells are constantly bathed in an aqueous environment and have unique structures for their biological activities (figure 1). Water, for example, is essential for inducing bilayer vesicles of amphiphilic lipids partitioning cells and organelles. When 'heavy water' replaces the 'light water' of living cells, the homeostasis of cells is drastically changed. Although the chemical properties of the two kinds of water are similar, their physical properties are different. Therefore, to answer why water is indispensable to life, we have to understand the physical interactions between water and biomolecules and, in particular, proteins.

The water molecule comprises one oxygen atom and two hydrogen atoms and is tiny, with a molecular size of *ca*. 3 Å (1 Å = 0.1 nm; Eisenberg & Kauzmann 1969). The covalent bonds between the oxygen and hydrogen atoms have an unbalanced distribution of electrons, resulting in hydrogen-bond interaction. One water molecule has four hydrogen-bond arms radiating out tetrahedrally from the oxygen atom, and the arms are the structural basis in forming a three-dimensional network of hydrogen bonds. In addition, the small moment of inertia of a water molecule is advantageous for a rapid response to external perturbations. When many water molecules gather together, liquid water is formed. From the perspective of the physico-chemical theory of liquids, water is a strange liquid exhibiting unusual physical properties, such as high phase-transition temperatures, a large specific heat, a strong surface tension and the reluctance to dissolve non-polar solutes. The rotational and translational motions of water molecules induce the reorganization of networks, ensuring the fluidity of water as a liquid (Ohmine & Tanaka 1993). These properties predominantly come from huge networks of hydrogen bonds among water molecules and must have profound implications in the interactions between water and biomolecules at molecular and atomic levels.

Biomolecules, in particular proteins, correctly fold and exhibit biological functions in an aqueous environment (figure 1). The structures and interaction modes at the interface between water and proteins, so-called 'hydration structures', have been recognized as important targets toward understanding the folding and the biological functions of proteins (Rupley & Careri 1991; Westhof 1993). Many physico-chemical techniques have been applied to visualize hydration structures, and particular contributions have been made using X-ray (Watenpaugh *et al.* 1978; Blake *et al.* 1983; Saenger 1987; Baker *et al.* 1988; Teeter 1991) and neutron crystallography (Finner-Moore *et al.* 1992; Kossiakoff *et al.* 1992). Crystallographic studies provide structural evidence about the predominant contribution of water molecules inside proteins in respect of their biological functions, such as catalytic efficiencies (Zhang *et al.* 1999), molecular recognition (Bhat *et al.* 1994), stabilization of the higher-order structures of

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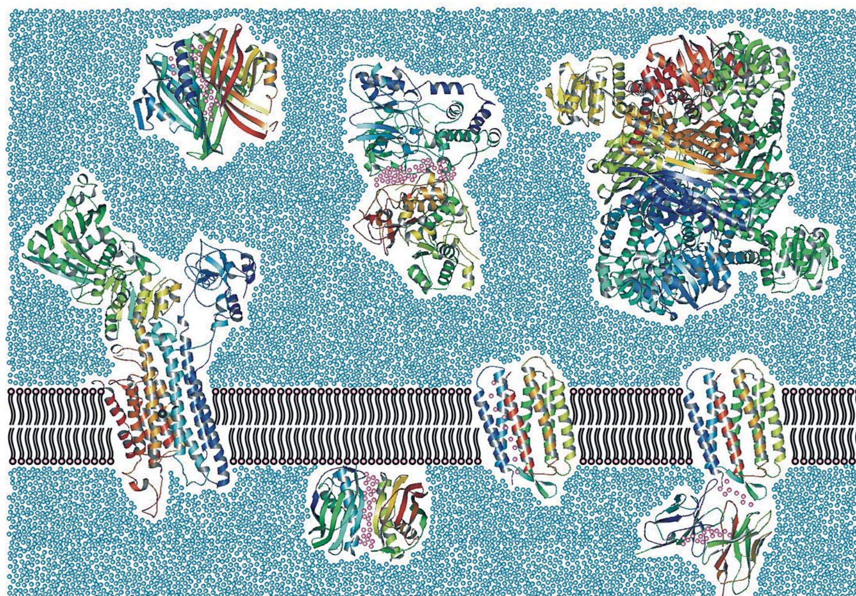

Figure 1. A schematic but realistic illustration of proteins around the cell surface. The cyan-coloured small spheres are water molecules in bulk, and those in pink are located inside proteins. The coordinates of proteins are taken from the Protein Data Bank (accession codes are 2STD, 2AHJ, 1EZU, 1EUL, 1DFV, 1KVD and 1BMB) and their ribbon models are drawn by using MOLSCRIPT (Kraulis 1991). The thickness of the illustrated lipid bilayer is 4 nm.

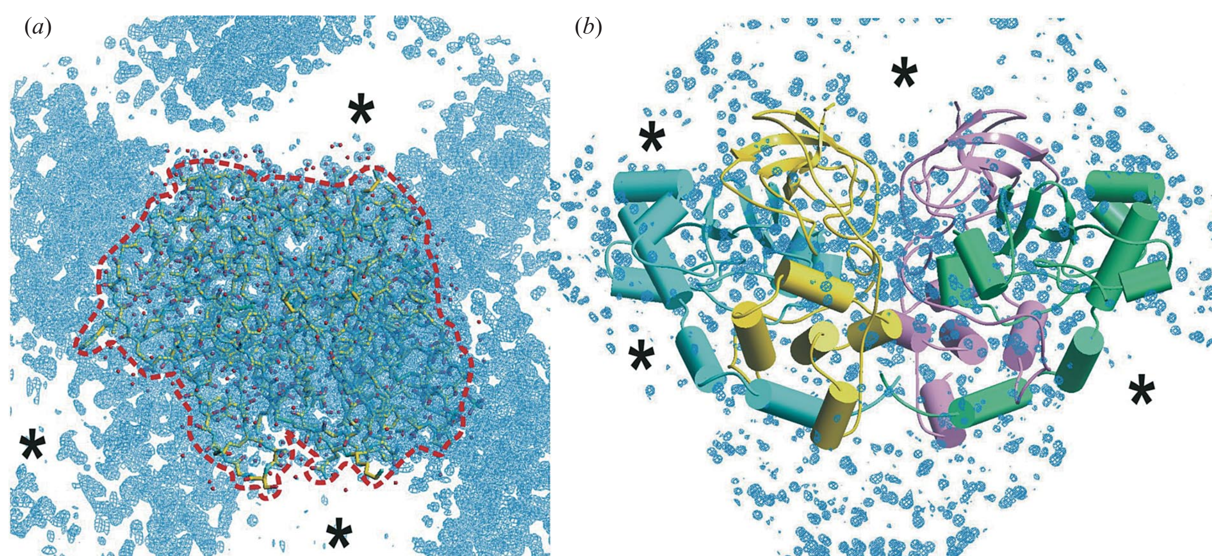

Figure 2. The electron density maps of protein crystals showing solvent channels indicated by asterisks. (*a*) A $2F_0-F_c$ difference Fourier map in a trypsin crystal. The map is calculated with the diffraction data between the Bragg spacing of 8.0 and 1.7 Å and is contoured at 1.5 standard deviation level from the average of the map. Data were collected at 100 K (Nakasako 1999). One trypsin molecule is shown by stick models. (*b*) $F_0 - F_c$ omit-difference Fourier maps of water molecules around nitrile hydratase (Nagashima *et al.* 1998). One $\alpha_2\beta_2$ hetero-tetrameric enzyme (blue and green, α-subunits; yellow and pink, β-subunits) schematically is shown as cylinder-ribbon models. Diffraction data were collected at 37 K using a cooling device (Nakasako *et al.* 2002*a*) at BL41XU of SPring-8.

proteins (Nakasako *et al.* 1999) and proton translocation pathways inside membrane proteins (Luecke *et al.* 1999). It has been demonstrated that the ion selectivity filters of the potassium-ion channels have structures mimicking the hydration pattern of the ion in solution (Zhou *et al.* 2001). In addition, hydration structures obtained by crystallographic experiments provide an appropriate database for extracting statistically significant trends and geometrical characteristics in hydration structures (Thanki *et al.* 1988; Walshaw & Goodfellow 1993).

The amount of hydration water molecules adsorbing the protein surface, i.e. hydration levels of proteins, critically correlates with the thermodynamic properties and biological functions of proteins (Rupley & Careri 1991). Protein

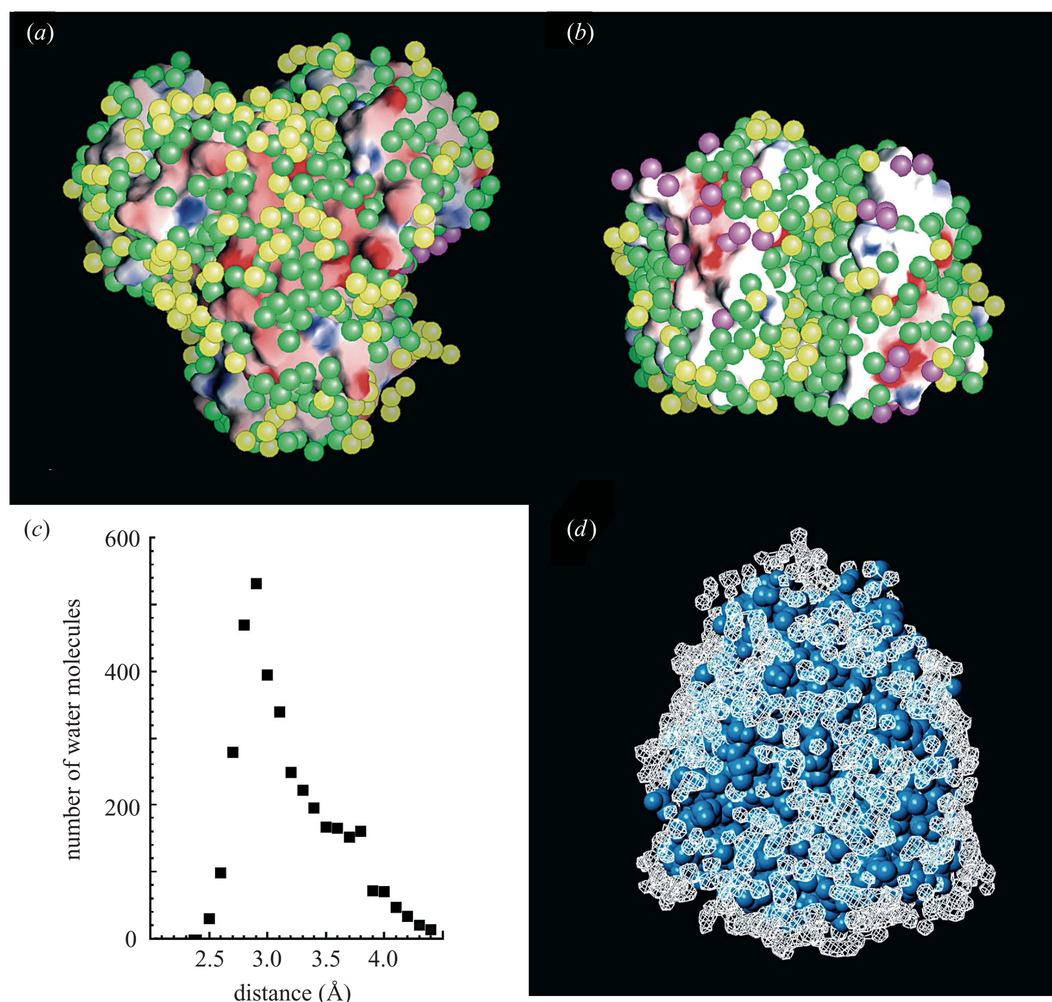

Figure 3. Illustrations showing the distribution of hydration water molecules around (*a*) scytalone dehydratase at 37 K (Nakasako *et al.* 1998), and (*b*) the killer toxin at 110 K (Nakasako *et al.* 2002*b*). The green, yellow and purple spheres (diameter of 3 Å) are the hydration water molecules in the 'first-layer', the 'second-layer' and the 'contact' classes, respectively (see § 3). The proteins are illustrated by their molecular surface with the electrostatic potentials visualized by the program Grasp (Nicholls *et al.* 1991). (*c*) A plot showing the distance distribution of hydration water molecules from atoms on the protein surface. The data were prepared from 15 crystal structures refined using diffraction data collected below 110 K. (*d*) A distribution of mass densities of hydration water molecules (white fishnets) around trypsin shown by a blue-coloured space-filling model. The density is reconstructed by superimposing the 'first-layer' hydration water molecules identified in three crystal forms with different molecular packing of trypsin. The method for the density calculation was described previously (Nakasako 1999). The data shown in the four panels were prepared using the Festkop program suite for hydration structure analyses developed by the author (Nakasako 1999, 2001).

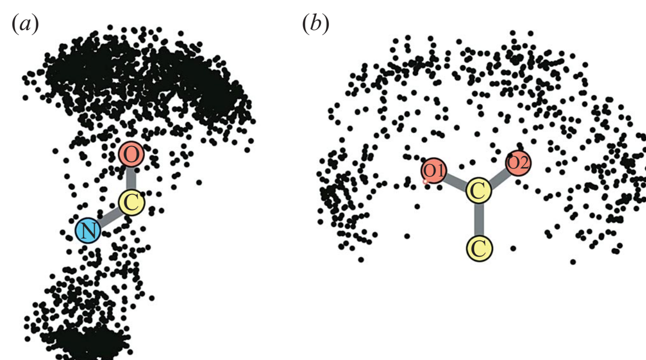

Figure 4. Plots showing the distribution of hydration sites around oxygen and nitrogen atoms of proteins. The dots indicate the position of hydration sites (*a*) around peptide bonds and (*b*) around the tip of the side chains of glutamate and aspartate residues. This figure was prepared using the Festkop program suite (Nakasako 1999, 2001).

specific heats and thermal stabilities are greatly under the influence of their hydration level, and a minimum monolayer hydration of protein seems to be necessary for the realization of the functions of proteins. The so-called 'glass-like transition' of proteins occurring at *ca*. 200 K, where intramolecular anharmonic motions responsible for the functions of proteins appear, is a highly hydration-dependent phenomenon (Ferrand *et al.* 1993; Miyazaki *et al.* 1993). Theoretical simulation studies postulated the influence of hydration, particularly on the collective motions of proteins (Hayward *et al.* 1993). Therefore, the structures and interaction modes at the interface between water and the protein surface are the subject of much discussion in an effort to understand why life requires water at a molecular level.

Recent progress in cryogenic X-ray crystallography (Nakasako 2001) and MD simulations (Karplus & Faerman 1994; Pettit *et al.* 1998) have accelerated studies of hydration structures over the entire surface of proteins. In

this paper, I introduce some progress that we have made in our laboratory in the cryo-crystallography and computation of protein hydration.

2. CRYOGENIC X-RAY CRYSTALLOGRAPHY AS A TOOL FOR INVESTIGATING HYDRATION STRUCTURES OF PROTEINS

Crystallographic methods provide the average locations of water molecules on a protein surface as well as their MSDs. Protein crystals contain more than 27% water (Matthews 1968), and many large solvent channels run through crystals, retaining fluidity and enabling the penetration of small molecules to associate with proteins (figure 2). Thus, the protein surfaces exposed to solvent channels are expected to have an aqueous environment similar to that in living cells.

At ambient temperature, because of the high mobility of water molecules on the protein surface, it is difficult to visualize the hydration structures over the entire protein surface. Previously, some algorithms have been developed and applied to diffraction data collected at ambient temperature to visualize the hydration structures on the protein surface (Badger & Casper 1991; Kossiakoff *et al.* 1992; Jiang & Brünger 1994). However, these methods provide ambiguous results in specifying the hydration sites on the protein surface and it would seem to be advantageous to discuss the dynamics of hydration structures at ambient temperature.

In the past decade, cryogenic techniques in X-ray diffraction experiments have been routinely and widely used to overcome radiation damage to protein crystals, in particular the use of intense synchrotron X-rays (Garman & Schneider 1997). In this technique, protein crystals are 'flash cooled' to near liquid-nitrogen temperature, and their diffraction data collected at *ca.* 100 K (Garman & Schneider 1997) or 35 K (Nakasako *et al.* 2002*a*). In flash cooling of protein crystals, water molecules rapidly lose their kinetic energies and are frozen into the vitreous state. Hydration water molecules 'wandering' around the protein surface at ambient temperature probably reside in hydration sites on the protein surface. Thus, at cryogenic temperature, the electron densities of hydration water molecules appear far clearer than at ambient temperature, and the amount of hydration water molecules identified becomes 1.5–3 times that at ambient temperature (figure 3*a*,*b*).

Even after flash cooling of the crystals, protein molecules retain their three-dimensional structures as indicated by the r.m.s. differences of less than 0.5 Å, and linear expansion coefficients in the order of $10^{-5}\,K^{-1}$ between cryogenic and ambient temperatures (Nakasako 1999, 2001). Thus, the very small structural changes in protein structures ensure that hydration structures remain similar between ambient and cryogenic temperatures. In fact, almost all hydration sites observed at ambient temperatures are also identified in cryogenic experiments (Nakasako 1999). Thus, the cryogenic method has great potential for investigating hydration structures on the protein surface.

In practice, to experimentally identify the average locations of hydration sites unambiguously, the structural analysis resolution is expected to be better than 2.3 Å, where hydration water molecules are detected as isolated and spherical scattering densities. In addition, we take care when identifying water molecules to consider two factors: the thermal nature of water molecules and the level of apparent electron density. In the stereochemical characterization of hydration structures, the maximum distance of a hydrogen bond is set at 3.4 Å and that in van der Waals contact at 3.7 Å.

3. CLASSES OF HYDRATION WATER MOLECULES

In our cryogenic crystal structure analyses, no hydration water molecules at a distance greater than 4 Å from the protein surface have been observed (figure 3*c*). Water molecules at greater distances from the protein surface distribute randomly, in a manner similar to atoms in glass materials, and probably contribute little to the hydration structures on the protein surface. Before exploring the hydration structures, the hydration water molecules (or hydration sites) identified are divided into four classes dependent upon their relative locations to the SAS of the protein molecules (Connolly 1983) and their interaction modes. The four classes are 'inside', 'contact', 'first-layer' and 'second-layer' (figure 3*a*,*b*).

(a) *The 'inside' class*

The water molecules in the 'inside' class are located inside the SAS and occupy cavities in the protein molecules. They are plausibly confined in the folding process and merely exchange with water molecules in bulk solvent. They have approximately four hydrogen-bond partners and perform as glues for stabilizing the tertiary and quaternary structures of proteins. When measuring MSDs of 'inside'-class molecules in a MD simulation at 300 K, a magnitude of less than 1.4 $Å^2$ is actually advantageous for acting as glues (Higo & Nakasako 2002). In multi-subunit or multi-domain proteins, many hydration water molecules are located at the subunit interface. The molecules mediate the inter-subunit interactions indirectly and arrange the surface shape for inter-subunit interaction to be complementary (Nakasako *et al.* 1999; Nakasako 2001). In some cases, they probably also function as a lubricant, assisting the structural changes of proteins through their three-dimensional four hydrogen-bond arms. From the thermodynamics viewpoint, the 'inside' class may contribute to partial compensation for the entropic cost of folding and molecular association by a small but significant level of the mobility.

(b) *The 'contact' class*

Water molecules in the 'contact' class are located outside the SAS and mediate intermolecular interactions between adjoining molecules related by crystallographic symmetries. The amount of 'contact'-class water molecules is dependent on the crystal contact area of the protein molecules. They have three or four hydrogen-bond partners with an average distance of 2.9 Å from polar protein atoms. The 'contact'-class hydration sites are considered to be artificially formed accompanying the crystallization process, as revealed by a systematic comparison of 'contact'-class sites between the hydration structures of a protein in crystal systems with different molecular packing (Nakasako 1999).

Thus, this class is usually omitted in analysis of the monolayer hydration of proteins.

The 'contact' and 'inside' classes may form a structural database to analyse the behaviour of water molecules confined in a narrow space (Israelachivili 1985; Tarazona & Vicente 1985), and to understand the interactions in molecular recognition occurring in cells, such as protein–DNA interactions, antigen–antibody complexes and protein–protein interactions in signal-transduction pathways.

(c) *The 'first-layer' and 'second-layer' classes*

Water molecules of the 'first-layer' and 'second-layer' classes are located outside the SAS. Water molecules in the 'first-layer' class are defined as those interacting directly with atoms of the protein surface through hydrogen bonds and/or van der Waals contacts. Molecules in the 'second-layer' have no direct interaction with protein.

In many instances of cryogenic crystal structure analyses, the amount of water molecules in the 'first-layer' is greatest among the classes. For instance, this class occupies 70% of the total number of water molecules in a crystal with a solvent content of *ca*. 60% (v/v). Of course, on a protein surface engaged in direct crystal contacts or around residues with a structural disorder, it is difficult to identify the class of water molecules. The 'first-layer' molecules form aggregates connected through hydrogen bonds, and the aggregates cover the protein surface like 'patchwork' (figure 3*a*,*b*). The dense distribution of the molecules and aggregates is prominent not only in the surface grooves but also on the flat surface. In addition, there is little correlation between the distribution and the electrostatic properties of the protein surface. Hydration sites of the 'first-layer' class around a protein are well conserved between crystal systems with different molecular packing (Nakasako 1999). Therefore, it may be possible to reconstruct the monolayer hydration structure of a protein by superimposing the classes obtained from several crystal structures in different crystal systems (figure 3*d*; Nakasako 1999).

Figure 3*c* shows the distance distribution of hydration water molecules in the two classes from atoms of the protein surface. There is one prominent peak at 2.9 Å and an enhancement at *ca*. 3.5 Å. The peak is located at the optimal hydrogen-bond distance between a water molecule and a polar protein atom. Two-thirds of this peak represent the molecules strongly hydrogen bonded with the oxygen atoms of proteins, and the remainder represents those with nitrogen atoms. The enhancement arises from the molecules covering the hydrophobic surface of proteins through van der Waals contacts. The molecules form two to three hydrogen bonds with oxygen, the nitrogen atoms of the protein surface, and/or water molecules.

4. AMOUNT OF HYDRATION WATER MOLECULES IN THE MONOLAYER HYDRATION OF PROTEINS

One water molecule in the 'first-layer' class is expected to cover a protein SAS of *ca*. 20 $Å^2$ as an average (Nakasako 2001). Together, the average value and SAS area of monomeric proteins, and the expected amount of hydration water molecules required for the monolayer hydration of monomeric proteins, is estimated to be 0.34–0.39 g of water per gram of protein. However, the hydration structures presented here are observed in a crystalline state of proteins at cryogenic temperatures. Thus, one question arises: do so many water molecules in crystal structures really bind to proteins in solution at ambient temperature? To answer this question, the amount estimated should be compared with those from other physicochemical measurements estimated for proteins in solution.

The dielectric relaxation method is one of the tools used to estimate the amount of hydration water molecules accompanying proteins in solution (Suzuki *et al.* 1996). The dielectric relaxation signals that appear in the frequency range of 2–20 GHz are thought to include information about the amount of hydration water molecules with dielectric dynamics that differ from that of bulk solvent. The amount of hydration water molecules estimated for several soluble protein molecules ranges between 0.34 g and 0.39 g of water per gram of protein, and agrees well with the estimation from the statistical average in the cryogenic crystal structure analyses described previously.

SAXS measurement also provides an insight into the hydration structures of proteins in solution. When hydration water molecules bind specific sites as observed in cryogenic crystal structures, they contribute significantly to the electron density of the solute. If this proposal is correct, the radius of gyration (R_g) reflecting the molecular dimension, measured by SAXS, deviates significantly from that of protein alone. In fact, the radii of gyration of soluble proteins are frequently larger than those calculated from crystal structures. The difference is thought to arise from the hydration shells of proteins and is explained by introducing a monolayer hydration shell around crystal structures (Fujisawa *et al.* 1994; Svergun *et al.* 1998).

Through these comparisons, we now believe that the amounts of hydration water molecules in cryogenic crystal structure analyses are consistent with those around proteins in solution. In addition, the electron density in the 'first-layer' hydration shell is still greater than that in bulk solvent, and the dielectric properties of hydration water molecules in the 'first-layer' classes must differ from those in bulk solvent.

5. HYDRATION PATTERN AROUND THE HYDROPHILIC AND HYDROPHOBIC PROTEIN SURFACE

(a) *Hydration patterns around polar protein atoms*

Figure 4 illustrates the distribution of hydration water molecules located around peptide bonds and side chains of glutamate/aspartate residues. Water molecules around peptide bonds are densely distributed into three clusters lying in the directions of hydrogen-bond arms of carbonyl oxygen and amid nitrogen atoms. Four clusters around glutamate and aspartate residues reflect the two hydrogen-bond arms in each oxygen atom. The spatial patterns in the distribution are consistent with the statistical trends in hydration structures around polar protein atoms found at ambient temperature (Thanki *et al.* 1988).

(b) *Hydration patterns around the hydrophobic surface*

In contrast to the patterns around polar protein atoms, there are no clear trends in the distribution of water

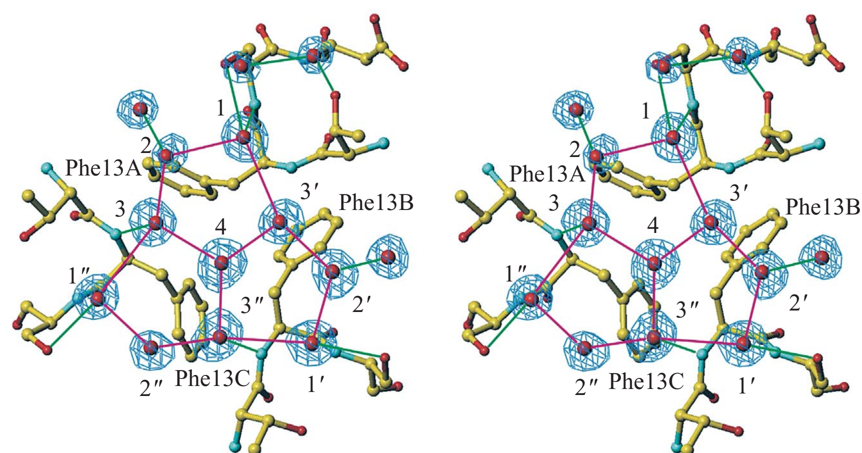

Figure 5. A stereo-plot showing polygonal arrangements of hydration water molecules around the hydrophobic surface of scytalone dehydratase (Nakasako *et al.* 1998). The omit-refined F_0-F_c difference Fourier maps of the hydration water molecules are superimposed on the structural models represented as coloured ball-and-sticks. The density maps were calculated with the reflections between the Bragg spacing of 8.0 and 1.45 Å collected at 37 K and were contoured at 3.5 standard deviation levels from the average of the maps. The pink lines indicate the possible hydrogen bonds within the polygonal cluster of water molecules, and the green line indicates the possible hydrogen bonds between hydration water molecules and polar protein atoms.

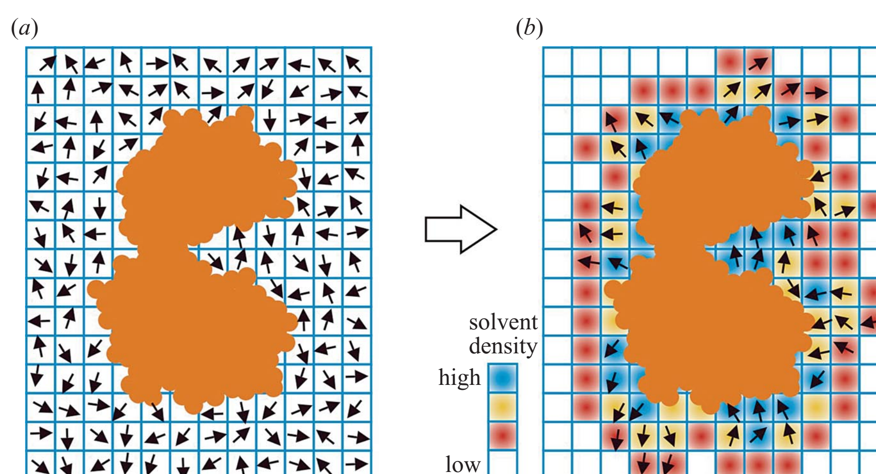

Figure 6. An illustration schematically showing the time averaging (*b*) of MD trajectories (*a*) compared with the crystal structures at cryogenic temperatures. In many cases, the time-averaged solvent density (indicated by the colours of the partitions) and solvent dipoles (arrows) are significant only in the vicinity of the protein surface (Higo & Nakasako 2002).

molecules on the hydrophobic surface at cryogenic temperature as well as at ambient temperature (Walshaw & Goodfellow 1993). One of the reasons is that hydration water molecules on the hydrophobic surface interact, predominantly, with carbon atoms through isotropic van der Waals contacts rather than anisotropic hydrogen bonds.

One of the characteristics in hydration of the hydrophobic surface is polygonal 'patches' of hydration water molecules originally found in crambin (Teeter 1984). The polygonal arrangements are not the result of crystallization and probably originate from the intrinsic interaction mode around hydrophobic molecules as observed in hydrate inclusion compounds (Jeffrey 1984). Figure 5 shows a typical example of the hydration patterns around phenylalanine residues exposed to solvent. On the hydrophobic surface of a trimeric enzyme, hydration water molecules in contact with three phenylalanine residues form three sets of pentagonal arrangements. The polygonal patches are suspended on the hydrophobic surface and are anchored by polar protein atoms surrounding the hydrophobic surface. The shape, size and orientation of polygonal patches do not correlate with the types of hydrophobic residue and are affected by the constellation of polar protein atoms surrounding the hydrophobic surface. Therefore, it appears difficult to estimate the hydration-free energy of hydrophobic residues using only the SAS of residues.

Most of the polygonal arrangements are not visible in the electron density maps at ambient temperature. However, polygonal arrangements probably exist even on the hydrophobic surface at ambient temperature to shield the hydrophobic faces with large degrees of positional fluctuation, because of the slight change in protein structures between cryogenic and ambient temperatures. In fact, MD simulations revealed pentagonal patches around a highly exposed hydrophobic residue (Lounnas & Pettit 1994; Higo *et al.* 2000).

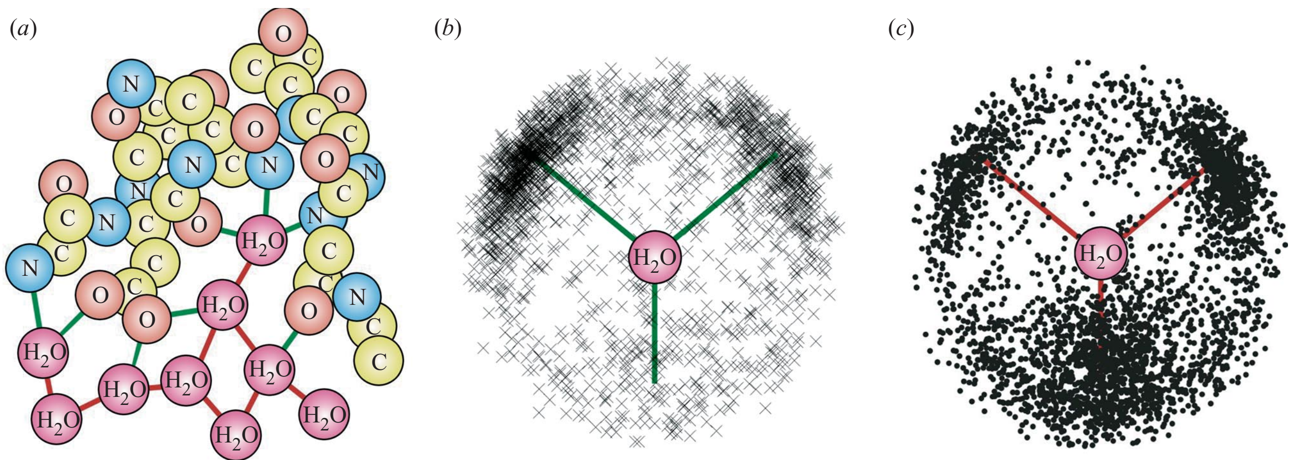

Figure 7. Comparison of hydrogen-bond patterns in hydration structures of proteins with the standard tetrahedral geometry in bulk water. (*a*) A schematic illustration of the hydration structures on the protein surface. The hydrogen bonds between protein atoms and hydration water molecules are indicated by green lines and those between water molecules by red lines. (*b*,*c*) Approximately 2500 hydration water molecules having more than three hydrogen-bond partners in 12 high-resolution crystal structures are selected and compared with the standard model through a least-square calculation. (*b*) Hydrogen-bond patterns between polar protein atoms and water molecules in the 'first-layer' or 'contact' class. The crossed symbols indicate the positions of protein atoms hydrogen bonded to the water molecules set at the centre. (*c*) Hydrogen-bond patterns between hydration water molecules. The dots indicate the positions of water molecules hydrogen bonded to the centre molecule in the 'first-layer' or 'second-layer' class. (*b*,*c*) Prepared using FESTKOP (Nakasako 1999, 2001).

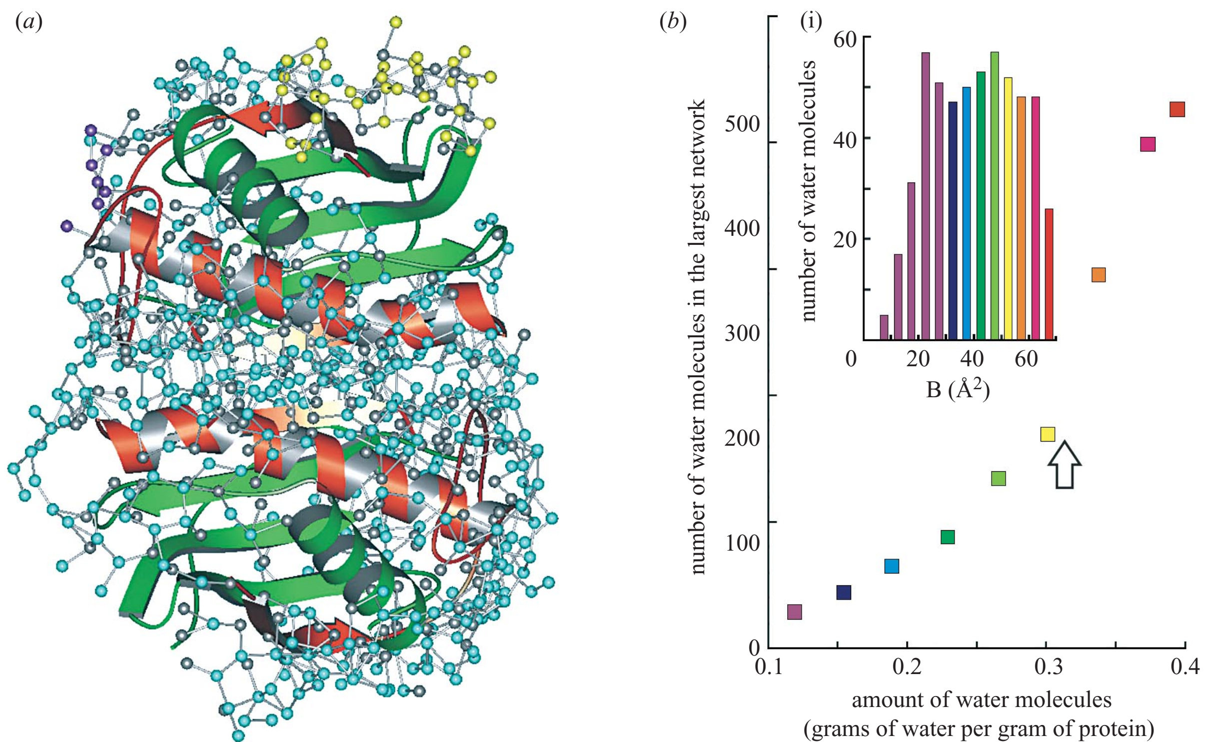

Figure 8. (*a*) An illustration demonstrating the network of hydrogen bonds around a hetero-tetrameric killer toxin molecule (the ribbon model of the secondary structures; Nakasako *et al.* 2002*b*). Water molecules forming different networks are coloured differently (cyan, yellow and purple), and polar protein atoms are represented by grey spheres. The sticks represent possible hydrogen bonds among protein atoms and hydration water molecules. This figure was prepared using the programs FESTKOP (Nakasako 1999, 2001) and MOLSCRIPT (Kraulis 1991). (*b*) A plot showing the correlation between the number of hydration water molecules on the killer toxin and that comprising the largest network (for instance, the cyan-coloured network in (*a*)). The correlation is analysed as follows. First, the threshold level of B-factors of water molecules to be analysed is defined from the histogram shown in (i), and the amount of the water molecules is calculated in units of grams of water per gram of protein. A network search is then performed to find the largest network formed only by the selected water molecules with B-factors less than the defined value.

6. MD SIMULATION TO INVESTIGATE PROTEIN HYDRATION

MD simulation is an alternative approach for studying the hydration structures of proteins (Karplus & Faerman 1994; Cheng & Rossky 1998; Pettit *et al.* 1998). When averaging MD trajectories on the coordinate system fixed on a protein molecule, MD simulations also provide time-averaged characteristics of hydration structures to be compared with cryogenic crystal structure analyses. Recently, we introduced two physical quantities, 'solvent density' and 'solvent dipole' (Higo *et al.* 2000; Higo & Nakasako 2002). Solvent density quantitatively measures the probability of residence of water molecules on the protein surface, and solvent dipole measures the orientational ordering of water molecules, as illustrated in figure 6.

We briefly describe the comparative studies on hydration structures of human lysozyme between MD calculation and cryo-crystallography (Higo & Nakasako 2002). The solvent density calculated from MD trajectories of 1 ns appears as many discrete peaks with densities greater than 0.04 $Å^{-3}$ within a distance of *ca.* 4 Å from the protein surface. By contrast, there are no peaks further than 6 Å from the protein surface, and the average solvent density value is 0.033 $Å^{-3}$. This result indicates that water molecules flying across the protein surface stay in specific hydration sites. The location of high solvent density peaks in the vicinity of the protein surface is consistent with the hydration sites found in cryogenic crystal structures.

Solvent dipoles exhibit spatially coherent patterns over sites with high solvent density peaks, as schematically illustrated in figure 6. The orientational order of water molecules on the protein surface in a MD simulation has also been reported by Merzel & Smith (2002). The effective distance range of the solvent dipole was *ca.* 6 Å from the enzyme surface. The ordering is prominent only around polar protein atoms, and water molecules around the hydrophobic surface exhibit large rotational diffusion. In addition, the results are partly consistent with recent neutron crystallography at high resolution, which experimentally revealed orientational ordering of water molecules (Niimura *et al.* 2003). The agreement between the amount of hydration water molecules in both cryogenic crystal structure analyses and dielectric relaxation measurements also supports the present computational results.

It should be noted that the spatial patterns of the solvent dipole are different from that expected for water molecules set in the electrostatic field obtainable from the ordinary Poisson–Boltzmann equation used for discussing the electrostatic interactions of biomolecules (Higo & Nakasako 2002). In a MD simulation on a system consisting of two amino acid molecules, the interaction mediated by solvent dipoles is stronger and of longer range than that calculated by the Poisson–Boltzmann equation (Higo *et al.* 2001). When a space is filled by orientationally ordered water molecules, a dielectric constant smaller than 80 is expected in the space. Thus, the electrostatic field in the space is stronger than that expected from the Poisson–Boltzmann equation, assuming a uniform dielectric constant. In the vicinity of the protein surface, water molecules interact mutually by dipole–diople interactions and are under configurational restraints owing to the networks of hydrogen-bond water molecules as described in § 7. Thus, the Poisson–Boltzmann equation must be carefully applied in obtaining the orientational ordering of water molecules near the protein surface.

7. THE GEOMETRY OF HYDROGEN BONDS OF HYDRATION WATER MOLECULES

A water molecule has four hydrogen-bond arms radiating out from the oxygen atom in the tetrahedral geometry, and the arms are the basis for forming and reorganizing the three-dimensional network of hydrogen bonds in water (Eisenberg & Kauzmann 1969; Ohmine & Tanaka 1993). Figure 7 compares the statistical trends in hydrogen-bond patterns of hydration water molecules with the standard tetrahedral geometry. In water molecules hydrogen bonding with polar protein atoms (figure 7*b*), two clusters of polar protein atoms lie in the directions of two arms in the standard geometry, and the other two less-ordered clusters probably correspond to the sites expected from the remaining two arms. The statistical trends in interaction patterns between water molecules are also consistent with the standard geometry. The average value of the angle between any pair of hydrogen bonds in the plots is 110°, and the half-width of the variation is 30°, reflecting the flexibility of hydrogen bonds.

The tetrahedral interaction geometry retained on the protein surface strongly suggests that the arrangement of polar protein atoms on the protein surface must satisfy the interaction geometry of water molecules. When the geometry is satisfied throughout the folding process of polypeptide chains in aqueous solution, the number of possible conformations of chains is expected to be reduced drastically. So far, the folding funnel model (Dill & Chen 1997) is proposed as a conceptual mechanism for understanding the self-organization of a protein and to avoid the Levinthal's paradox (Levinthal 1968), which postulates the large difference in time-scale of folding between the actual *in vitro* experiment and the theoretical calculation, assuming a random conformational search. From the point of view of interaction geometry of water molecules with polypeptide chains, water molecules may work as inducers to transform the energy surface of polypeptide chains from the Levinthal-like model into the funnel shape.

Hydration with tetrahedral interaction geometry may provide a novel microscopic interpretation of the unfolding process of proteins in the presence of urea, guanidine and methanol at high concentration. The reagents possess planar hydrogen-bond arms and are two-dimensional rather than tetrahedral. Their hydrogen-bond geometry probably causes drastic and cooperative reorganizations of the hydrogen-bond network including protein atoms, when a large number of the reagent molecules surround protein molecules. The reorganization of the hydrogen-bond network probably initiates deformation of the protein structure into those proteins distinctly different from those in an aqueous environment, the so-called denatured state.

8. NETWORK OF HYDROGEN BONDS FORMED BY HYDRATION WATER MOLECULES

Hydration water molecules of the 'first-layer' and 'second-layer' classes form aggregates through hydrogen bonds as seen in figure 3. The aggregates link mutually together and are indirectly further connected by polar protein atoms having bifurcated hydrogen-bond arms (see figure 4). As a result of the chain connections of hydrogen bonds, a network of hydrogen bonds appears on the protein surface (Nakasako 1999, 2001). Figure 8*a* illustrates a typical example of a hydrogen-bond network found in the hydration structure of a killer toxin molecule (Nakasako *et al.* 2002*b*; figure 3*b*). The largest network, covering *ca.* 50% of the SAS of the molecule (indicated by cyan-coloured water molecules), starts at the tip of the upper subunit and runs through approximately 250 polar protein atoms and 400 hydration water molecules. Several hydration water molecules filling the cleft between the two subunits form a complicated network like that of covalent bonds in glass materials. The shape and the size of network probably depend on the surface topology, the secondary structures and the surface electrostatic potential of proteins.

Such networks are common characteristics in the hydration structures of proteins so far analysed at cryogenic temperatures. The expected amount of hydration water molecules around proteins from the cryogenic analysis is consistent with those measured on proteins in solution. Thus, when proteins are in monolayer hydration, hydration water molecules must be distributed at a density that the chain connections of hydrogen bonds naturally occur among water molecules and polar protein atoms. In fact, a MD simulation predicted the existence of such networks on hydrated myoglobin (Lounnas & Pettit 1994). In crystal structure analyses at ambient temperature with density modification algorithms, there are tubular and disordered scattering densities of water molecules in the vicinity of the protein surface (Kossiakoff *et al.* 1992; Jiang & Brünger 1994). These computational and experimental results must correlate with the network of hydrogen bonds found in cryogenic diffraction experiments.

Figure 8*b* shows the correlation between the amount of hydration water molecules and the number of water molecules comprising the largest network on the surface of killer toxin. In the plot, the hydrogen-bond network is searched for hydration water molecules having thermal factors less than a certain value as shown in the inset. The size and extension of the network increase drastically beyond a threshold level of *ca.* 0.3 g of water per gram of protein. Because the amount required in a drastic extension of the hydrogen-bond network is close to that required for the maturation of thermodynamic properties of proteins (Rupley & Careri 1991), the formation of a hydration network in the presence of that amount of water molecules may influence the vibrational state of proteins, which directly correlates with the thermodynamic properties. The correlation seems to be described approximately by the percolation theory used for amorphous solids (Cusack 1987). In this regard, the measurement of the direct current conductivity in lysozyme powder (Careri *et al.* 1988) is interesting. The conductivity is highly dependent upon the hydration level of the sample, and the dependence is also described as a percolation of the proton-translocation network.

Figure 9. (*Overleaf.*) (*a*) A schematic illustration of the hydration structural changes accompanying domain motions of a protein. (*b*) A stereo-plot demonstrating the metastable conformations of domain motions found in hexameric glutamate dehydrogenase. The enzyme is composed of six identical subunits of 420 residues. One subunit has the N-domain for NADP- or NADPH-binding and the C-domain for the hexamer formation. A large active-site cleft is formed between the two domains. In the crystal structure, one hexamer of the enzyme occupies a crystallographic asymmetric unit (Nakasako *et al.* 2001). The plot shows Cα-traces of subunit 1 in the 'open' conformation (cyan), subunit 3 in the 'intermediate' conformation (green) and subunit 5 in the 'close' conformation (red) after optimally superimposing the C-domains. The blue spheres indicate the residues composing the hinge connecting the two domains. The coloured stick models are the side chains of the residues forming the active site in the 'open' conformation. (*c*) An enlarged stereo-view demonstrating the distribution of hydration water molecules in the active-site cleft of the three conformations in the domain motion in (*b*). The polypeptide chains forming the cleft are shown by stick models of the backbone atoms coloured as in (*b*). Blue, yellow and pink spheres indicate the positions of hydration water molecules in the 'open', 'intermediate' and 'close' conformations of the cleft, respectively. The orange fishnets are omit-refined difference Fourier electron density maps of the three hydration water molecules located in the depth of the cleft in the 'open' conformation. The maps are calculated using the diffraction data in the Brag spacing of 8.0 and 2.25 Å. (*d*) Magnified views of the region indicated by the arrow in (*c*). The electron density maps of polypeptide chains (blue fishnets), hydration water molecules and a sulphate ion (orange fishnets) are calculated as in (*c*). In the area indicated by the dotted circles, drastic changes in hydration structures occur between two successive conformations. The coloured bars shown between the panels indicate the positions of hydration water molecules approximately located at the left side of Arg187.

9. REORGANIZATION OF HYDRATION STRUCTURES ACCOMPANYING DOMAIN MOTIONS

As shown in figures 3 and 8, proteins are wrapped in shells of hydration water molecules connected by hydrogen bonds. When hydration structures are retained, as in figures 3 and 8, water molecules must hinder the conformational changes of proteins required for their function. Thus, hydration structures must reorganize flexibly and dynamically in coupling with the conformational changes of proteins as schematically shown in figure 9*a*.

Figure 9*b* shows the structures of the three subunits of hexameric glutamate dehydrogenase in its non-liganded state (Nakasako *et al.* 2001). The structures of subunits in figure 9*b* correspond to the three metastable conformations ('open', 'intermediate' and 'close') described as a hinge-bending domain motion caused by the two flexible loops connecting the upper and lower domains. The motion rotates the upper N-domain *ca.* 7° and shifts the tips of the domain (residue 275) *ca.* 10 Å between the

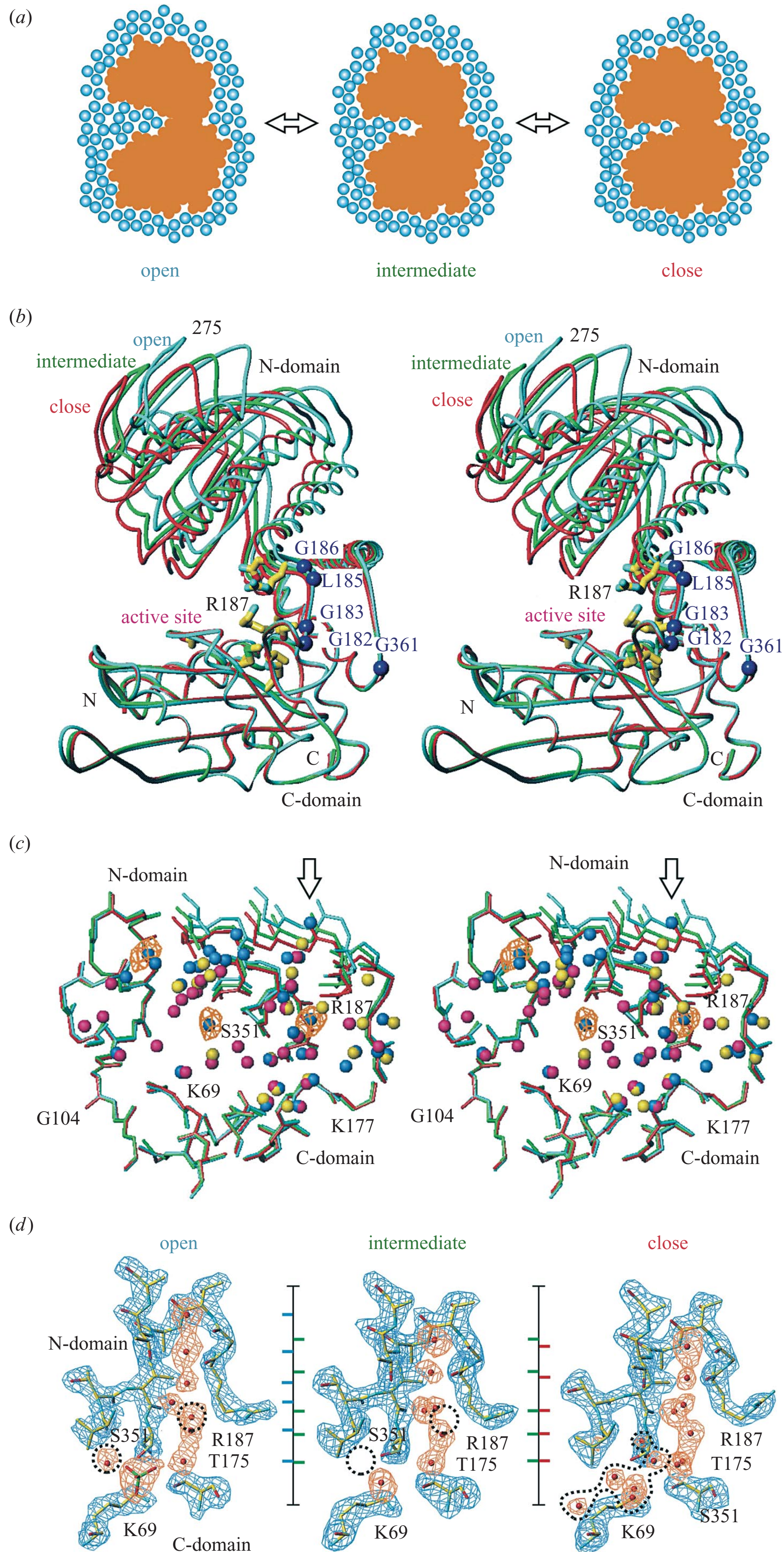

Figure 9. (*Caption p. 1199.*)

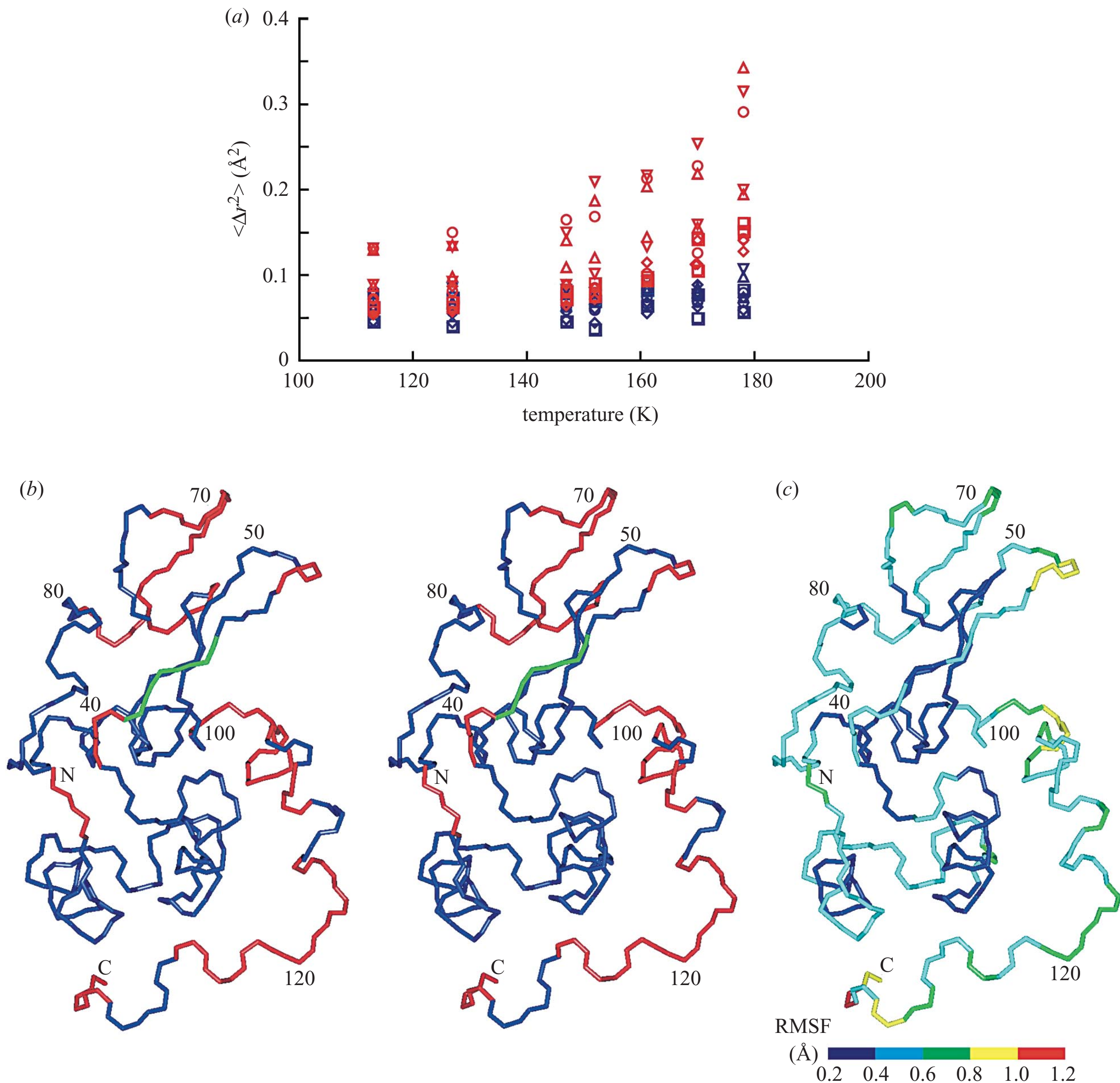

Figure 10. (*a*) A plot showing typical examples of the two types of dynamic response of internal fluctuations of main chain atoms in human lysozyme. The blue symbols show the temperature dependence of main-chain MSD of residues exhibiting the 'harmonic' behaviour, and red symbols residues exhibiting the 'glassy' behaviour. (*b*) A stereo-plot mapping the three types of dynamic response of internal fluctuations onto the main-chain-trace of the enzyme structure. The residues in the 'harmonic' response are coloured in blue, those in the 'glassy' response are coloured red and those in the 'shifted' response are coloured green. (*c*) A plot showing r.m.s. fluctuation of main-chain atoms in the MD simulation for human lysozyme immersed in an explicit water system (Higo & Nakasako 2002). Residues are coloured as in the key.

'open' and 'close' conformations. Cryogenic X-ray crystal structure analyses provide snapshots of transient hydration structures as well as the trapped metastable conformations in the domain motion.

The most drastic hydration structure changes accompanying the domain motion occur in the depth of the active-site cleft formed between the two domains, despite small structural changes in the residues forming the cleft (figure 9*c*,*d*). The numbers of water molecules observed in electron density maps in the cleft are 36 in the 'open' conformation, 23 in the 'intermediate' and 30 in the 'close'. Because the interior of the cleft is completely free from crystal contacts in any subunit, the observed hydration structures in the clefts must be intrinsic in the domain motion of the enzyme in solution. By contrast, the remaining surface area, including the hinge regions, remains almost the same throughout the three conformations. For instance, the hydration water molecules binding to the upper jaw of the cleft move together with the N-domain movement, and those on the lower jaw are retained throughout the domain movement (figure 9*c*).

In the depth of the cleft in the 'open' state, there are three hydration water molecules with relatively large positional fluctuations and one sulphate ion occupying the expected binding site of the glutamate molecule (figure 9*c*,*d*). In the 'intermediate' state, the three water molecules and the sulphate ion disappear in the electron density maps, indicating that the molecules and the ion are squeezed out

accompanying the closing movement of the N-domain. The three cavities, in which the three water molecules reside in the 'open' state, become narrow to prevent the access of water molecules, and the sulphate ion is replaced by one water molecule. At the right side of the cavity indicated by an arrow in figure 9*c*, a column of water molecules moves together with the N-domain movement, and one hydration water molecule located at the lower edge of the column is probably squeezed out into the cleft together with the water molecule residing behind Arg187 in the open state. As shown in figure 7, water molecules on the protein surface retain the tetrahedral interaction geometry, enabling the formation of a three-dimensional network of hydrogen bonds. In the migration of the four hydration water molecules in the depth of the cleft (figure 9*c*,*d*), the interaction geometry must be essential for the three-dimensional reorganization of the network. In addition, flexibility of the network is responsible for transmitting the dynamical motion of water molecules to exposed residues possessing liquid-like dynamics as in Arg187.

As shown in MD simulations and nuclear magnetic resonance experiments (Brunne *et al.* 1993), the residence time of hydration water molecules in hydration sites are *ca*. 10–100 ps, and water molecules frequently enter and leave hydration sites. Because the three hydration sites are distant from each other, the dynamics of water molecules in the cavities are expected to have little correlation with each other. In the closing movement of the N-domain from the open to the intermediate conformation, at least four hydration water molecules (figure 9*c*,*d*) must migrate simultaneously into the cleft. Therefore, the four water molecules seem to work as four independent flexible latches, which appear/disappear randomly. The time of the simultaneous migration of the water molecules must be greater than the residence time of each water molecule in the hydration site, and the domain motion must be described as a stochastic, diffusive and anharmonic process rather than as a harmonic motion. Thus, it might be possible to say that the time-scale of the domain motion of this enzyme is predominantly regulated by a small number of water molecules.

Because of the present limit in the resolution of the structure analysis, the positions of hydrogen atoms in a water molecule are still invisible. The application of modern neutron crystallography is the next step in an attempt to describe completely the dynamical reorganization of hydration structures in the domain motions in glutamate dehydrogenase.

10. CORRELATION BETWEEN THE INTERNAL MOTIONS OF PROTEIN AND HYDRATION AT LOW TEMPERATURE

The internal motions of proteins have been investigated by measuring the MSDs of protein atoms over a wide temperature range (Parak *et al.* 1982; Doster *et al.* 1989; Rasmussen *et al.* 1992; Ferrand *et al.* 1993; Daniel *et al.* 1999). At very low temperatures (below 150 K), the MSDs of protein atoms increase in proportion to temperature, indicating that the internal motions of proteins are approximated as harmonic vibrations. Two transition-like inflections of MSDs are found in the temperature-dependent variations at *ca*. 150 K (Daniel *et al.* 1999) and 200 K (Parak *et al.* 1982; Doster *et al.* 1989; Rasmussen *et al.* 1992; Ferrand *et al.* 1993). It has been argued that the inflections are the result of a transition in protein dynamics from harmonic to anharmonic diffusive motions. Rasmussen *et al.* (1992) observed that crystalline RNase A lost the binding activity to an inhibitor at *ca*. 220 K. The correspondence between the dynamic transition and the onset of the activity leads to a hypothesis that the substrate-binding activity of RNase A requires diffusive anharmonic motions acquired above the transition temperature.

Figure 10*a* shows the temperature-dependent variation of atomic MSD arising from the internal degrees of freedom in human lysozyme measured at seven different temperatures ranging from 113 to 178 K by X-ray crystal structure analyses (Joti *et al.* 2002). In the present study, the normal mode refinement (Kidera & Go 1992) decomposes the mean-square fluctuations of protein atoms from their average position into the contributions from the internal degrees of freedom, which change the shape of the protein structure, and those from the external degrees of freedom, which generate rigid body motions in the crystal. The MSD obtainable from X-ray diffraction experiments is a time- and space-averaged quantity and is intrinsically different from that measured by neutron spectroscopy. The advantage of X-ray crystallography is, however, to visualize the variation of MSDs in individual non-hydrogen atoms over the entire protein structure. In the crystalline state, three types of variation are found. In 87 residues, the temperature variations of MSDs are approximated as harmonic oscillators. We refer to their dynamic responses as 'harmonic'. In 40 residues located on the surface area of the enzyme, while MSDs vary in proportion to temperature below *ca*. 150 K, apparent inflections in the dependence appear above 150 K, indicating that anharmonic motions occur above the inflection point ('glassy' response). In four residues, the variations of MSDs are directly affected by conformational changes ('shifted' response). Figure 10*b* maps the three types of response on the structure of the enzyme, and it is clear that the residues with a 'glassy' response are found mostly in the flexible part exposed to the solvent and in the loop region rather than in rigid secondary structures, particularly at the edges of the two lobes forming the cleft of the enzyme. In addition, the residues face the large solvent channels in the crystal.

Figure 10*c* shows the atomic fluctuations in MD simulation of the enzyme at 300 K (Higo & Nakasako 2002). Residues having relatively large fluctuations in the simulation exhibit a 'glassy' response at *ca*. 150 K. Thus, the present observation suggests, indirectly, the influences of protein–water interactions on the 'glassy' responses. Although the inflection point is lower than that reported in X-ray crystallography on RNase A (Rasmussen *et al.* 1992), the point is close to that reported on the hydrated powder of glutamate dehydrogenase by neutron spectroscopic measurement with a wide time-window (Daniel *et al.* 1999). Because, at *ca*. 150 K, cubic ice starts to form in water (Mishima & Stanley 1998), the transition-like behaviour at *ca*. 150 K may have been enhanced by the early stage of cubic ice formation in the solvent channel. It is difficult to deny the possibility that the cubic ice formation in the solvent channel induces small changes in

protein conformations and increased fluctuation. This idea is supported by a report that cell expansions are observed in various protein crystals having large solvent channels, and that the magnitude of expansion correlates with solvent channel size (Weik *et al.* 2001).

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Discussion

J. L. Finney (*Department of Physics and Astronomy, University College London, London, UK*). You have a lot of details about the water–protein interactions. With this information, can you think of replacing the water by another solvent and still maintaining the structure and dynamics?

M. Nakasako. In my experimental observation, most hydration water molecules form tetrahedral hydrogen bonds in the vicinity of the protein surface. This finding strongly suggests that polar atoms, oxygen and nitrogen atoms, at the surface of proteins are arranged to satisfy the tetrahedral geometry of water molecules. The three-dimensional interaction arms in the geometry help the extension of the network of hydrogen bonds between polar protein atoms and hydration water molecules. Hydrophobic surfaces of proteins are covered by polygonal arrangements of water molecules like those observed in gas hydrates. Thus, when water in cells is replaced by another solvent, the solvent molecule should have three-dimensional tetrahedral hydrogen-bond arms. From the physico-chemical point of view, the small moment of inertia of the water molecule is important to dynamically rearrange water molecules in maintaining the network of hydrogen bonds. So, in my impression from the experimental results, water is the only solvent on the Earth enabling the structure and dynamics of proteins supporting the fundamental physico-chemical stages of life.

P. Rand (*Department of Biological Sciences, Brock University, Ontario, Canada*). What is the number ratio of immobile (crystallographic) waters to surface-perturbed mobile water (that cannot be seen crystallographically)? Do you think movement of the mobile water during conformational change is energetically significant? You spoke only of the crystallographic water as regulating conformational change.

M. Nakasako. When your question concerns the protein hydration at ambient temperature, I can answer through

comparing the number of water molecules crystallographically identified at ambient temperature and cryogenic temperature. The ratio is not strongly constant and ranges between 1.5 and 3, depending on the surface properties such as electrostatic potential and topology so far measured (Nakasako 1999). Because I have no crystallographic data on glutamate dehydrogenase at ambient temperature, it is difficult to answer without ambiguity whether mobile water during conformational change is energetically significant or not. I think only water molecules confined within the interior of protein molecules are persistently immobile. The water molecules on the surface of proteins are mobile as shown in MD simulations (Higo & Nakasako 2002), and hydration sites identified in crystal structure analyses are visited by several water molecules with various periods of residence. The energies required for incoming to and outgoing from hydration sites will be within the level of thermal fluctuations. So, the energies required for the movement of all water molecules in the vicinity of a protein surface may be comparable. For the dynamics of proteins in solution, the most important is movements of water molecules residing in specific hydration sites closely correlating with dynamical motions of proteins.

J. B. F. N. Engberts (*Physical Organic Chemistry Unit, University of Groningen, Groningen, The Netherlands*). Recent MD simulations indicate that proteins are more dynamic in solution compared to their crystals. Should this not have consequences for protein hydration?

M. Nakasako. I think crystal structures of proteins reflect some stable or metastable conformations in crystallization medium and are stabilized through interactions with crystallographic neighbours. So, the structures observed in crystals reflect only a few samples in the energy landscape of the conformational space of proteins. I think the flexibility of protein conformations observed in MD simulations using a correct set of force-field parameters are quite reasonable without influences of stabilization forces by crystal field and crystallization medium.

J. B. F. N. Engberts. For many proteins, cold denaturation is a common phenomenon. It has been explained by the increased peptide bond–water interactions, bringing buried peptide group to the surface of the protein. Because folding and defolding of proteins might be quite fast, could this have consequences in your studies of the hydration of proteins?

M. Nakasako. When comparing the crystal structures at room and cryogenic temperatures, we found only very small conformational changes. Of course, in solution, proteins undergo cold denaturation near the freezing point of water. However, it is difficult to answer on correlations between hydration and cold denaturation, because I have no structural evidence to discuss the problem. Why does cold denaturation not occur in crystalline proteins? I can suggest two possible causes. The first is the intermolecular contact in the crystalline state. Several van der Waals interactions and hydrogen bonds including hydration water molecules stabilize the conformation of proteins and may hinder the cold denaturation. The second is the large difference in the cooling rate between the cold denaturation experiment and flash cooling in cryogenic crystallography. The cooling rate of a protein crystal with dimensions of 0.1 mm × 0.1 mm × 0.1 mm by cold nitrogen gas at 100 K is less than *ca.* 100 ms, and by liquid ethane is *ca.* 10 ms measured so far. These periods are too short for proteins in crystals to reach thermal equilibrium. So, temperatures of protein molecules in crystal may reach 100 K before cold denaturation in flash-cooling treatment. As shown by neutron spectroscopy and X-ray crystallography, motions of proteins can be approximated as a group of harmonic oscillators (normal modes) below the glass transition temperature at *ca.* 150–200 K. At 100 K, proteins cannot undergo large anharmonic motions that are necessary for their functions and for cold denaturation. The rapid cooling and interactions in the crystalline state may effectively suppress the destruction of the three-dimensional structures of proteins.

R. McKendry (*London Centre for Nanotechnology & Department of Medicine, University College London, London, UK*). Your very nice work, showing the rearrangement of hexameric glutamate dehydrogenase, raises the interesting question of water trapped in confined geometries. There is some evidence from physical measurement techniques such as surface force apparatus and the atomic force microscope that water trapped between two surfaces has quite special properties that differ from bulk water. Can you comment on these findings? And have you seen any evidence of these effects in your measurements or simulations?

M. Nakasako. In the depth of the active site-cleft of glutamate dehydrogenase, three hydration water molecules are confined in narrow hydration sites. As you suggest, the upper and lower faces of the sites may be roughly approximated as two walls. When solvent molecules are sandwiched between two walls, the pressure between the walls is approximated by an exponentially decaying cosine function depending on the distance of the walls and the size of the solvent molecule. The most interesting fact is that the strongest attractive force appears when the distance is the diameter of solvent molecule. Based on the theory, we may speculate the correlation between the hydration and the domain motion of the enzyme. When a water molecule in the narrow hydration site migrates into the active site cleft, the vacant site becomes unstable and readily moves into the closest contact state as observed in the intermediate state. When considering three hydration sites in the open conformation, it seems to take a long time for the simultaneous migration of three hydration water molecules into the cleft. So, the closing motion from the open to the intermediate state is probably a rare event. In addition, the close contacts of the walls are expected to occur immediately after the migration of water molecules; the domain motion from the open to the intermediate state is a stepwise and quick motion rather than a smooth and slow motion. However, this is still an idea, and I have no experimental evidence to support it. However, the theory seems to be helpful in studying the interactions of protein molecules in crystals or molecular association in cellular signalling processes. For instance, around the contact area in the crystal, the surfaces of protein molecules are roughly approximated as walls. When analysing the distance between atoms engaged in water-mediated contacts of protein molecules in crystals, we observe a statistically significant tendency for the atoms to be separated by a distance of one or two water molecules as reported previously (Nakasako *et al.* 2002*b*).

G. Zaccai (*Institut de Biologie Structurale, Grenoble, France*). From neutron diffraction experiments, 7–12 water molecules are in the proton channel in bacteriorhodopsin. Only the well-ordered ones of these are seen in the crystallographic structure (a few).

M. Nakasako. Your comment may correlate with the differences in the resolution of crystal structure analyses and neutron diffraction experiments. In a neutron diffraction experiment, you mainly used the reflections located in the region of Bragg spacings larger than 7 Å. In particular, reflections in very low-resolution region (for instance (11), (20) and (21) reflections of purple membrane, a two-dimensional array of bacteriorhodopsin trimers) have information on the shape of bacteriorhodopsin including the hydration shell of D_2O on the surface exposed to solvent. In contrast, in cryogenic X-ray crystal structure analyses of bacteriorhodopsin, the refined structural model does not simulate the diffraction data in the low angle region. When including low-resolution data and taking into account the contrast of scattering densities in the X-ray analyses, channels of disordered water molecules may be appear in the scattering density maps and the amount of water (hydrogen atoms) may become comparable with the neutron experiments.

GLOSSARY

MD: molecular dynamics
MSD: mean-square displacement
r.m.s.: root mean square
RNase A: ribonuclease A
SAS: solvent accessible surface
SAXS: small-angle X-ray scattering