Journal of Biological Physics **28:** 129–137, 2002.

Structural Characteristics in Protein Hydration Investigated by Cryogenic X-ray Crystal Structure Analyses

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Abstract. Cryogenic X-ray crystallography has heen applied to investigate the hydration structures of proteins. The amount of hydration water molecules identified at cryogenic temperature is more than twice those at ambient temperature, and the structural models of proteins with a lot of hydration water molecules have provided much information to elucidate the static and dynamical characteristics of hydration structures of proteins. On protein surface, hydration water molecules distribute non-randomly and still retain the tetrahedral hydrogen-bond geometry as well as in bulk solvent. In addition, water molecules form clathrate-like arrangements to cover the hydrophobic residues exposed to solvent. The standard interaction geometry enables the three-dimensional extension of hydrogen-bond networks around protein molecules and, simultaneously, ensures the concerted reorganization of hydration structures during the dynamical motion of proteins at work. The hydration structure analyses at cryogenic temperatures may contribute to understanding physical principles governing the dynamics of 'molecular machines' in aqueous environment.

Key words: cryogenic X-ray crystallography, hydrogen bond, protein dynamics, protein hydration, water molecule

Proteins fold and work in water, a typical complex fluid. Because water has unusual physical properties, such as the phase transition temperatures, the specific heat and the surface tension caused by the three-dimensional networks of hydrogen bonds [1], it must have great influences on the structures and the dynamics of proteins. In fact, protein molecules correctly fold in aqueous environment, and the association of water molecules with proteins is essential to the structural stability, the catalytic efficiency and the molecular recognition of proteins [2]. The specific heat, the thermal stability and the glass-like transition of proteins correlate strongly with their hydration level [3, 4]. Therefore, the structures and interaction modes at the interface between proteins and water, i.e. the hydration structures of proteins, is the subject of much discussion to understand physical principles governing the folding, dynamics and functions of proteins in aqueous environment.

In recent years, cryogenic techniques [5] have been routinely and widely applied in protein crystallography to overcome radiation damage of protein crystals. In this technique, protein crystals are rapidly cooled to near liquid nitrogen temperature,

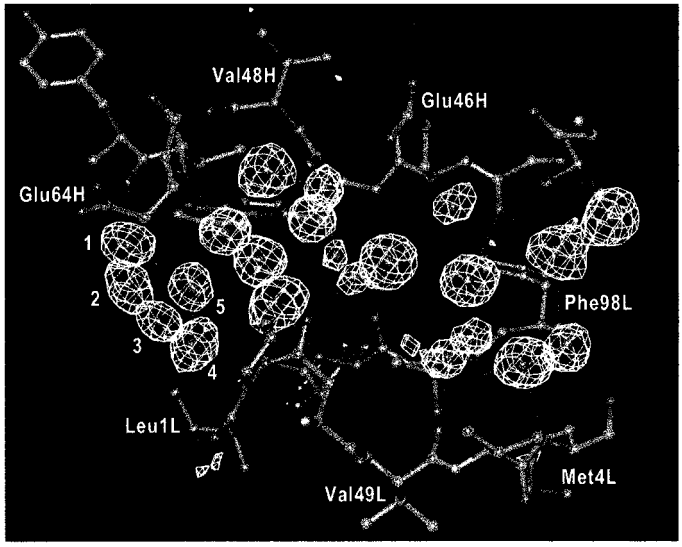

Figure 1. Electron density maps of hydration water molecules associating with the Fv fragment of an anti-dansyl antibody [8]. The electron density maps were calculated with the reflections between the Bragg spacings of 8.00 and 1.45 Å, and were contoured at 4.0 standard deviation level from the average of the map. The small spheres indicate the position of the oxygen atoms of hydration water molecules, and the ball- and stick models represent the polypeptide chains. The dotted lines indicate possible hydrogen bonds between water molecules and polar protein atoms. The water molecules numbered as 1–5 form a planar clathrate-like arrangement shielding a hydrophobic surface from bulk solvent.

and the diffraction data are collected at the cryogenic temperatures. In the cooling process, water molecules loose their kinetic energies, and the molecules in the vicinity of protein surface reside stably in hydration sites. Thus, the hydration structures appear far clearer at cryogenic temperature than at ambient temperature. The space-time averaged hydration structures from cryogenic crystal structure analyses have enabled to approach to hydration-related problems in protein-water system and provided an experimental data base for discussing solvent-solute interactions in complex fluids. Here we summarize our recent hydration structure analyses by cryogenic X-ray crystallography [6–11] and discuss the implication of the results to understand the protein dynamics in aqueous solution.

THE AMOUNT OF HYDRATION WATER MOLECULES IN MONOLAYER HYDRATION OF PROTEINS

As a typical example, Figure 1 shows the electron density maps of the hydration water molecules adsorbing on a Fv fragment [8]. In X-ray crystal structure analyses, the electron densities of hydration water molecules appear as discrete and spherical peaks beyond a resolution of 2.3 Å, and the positions of water-

oxygen atoms are determined. The amount of hydration water molecules identified at cryogenic temperature is more than twice of those at ambient temperature, being advantageous to discuss the static characteristics of hydration structures in detail [6, 10]. Identified hydration water molecules distribute non-randomly in hydrogen-bonding with oxygen and/or nitrogen atoms of proteins or forming clathrate-like aggregates covering the hydrophobic surface of proteins (see water molecules numbered as 1–5 in Figure 1) [6, 10]. Until now, no hexagonal arrangements of water molecules in ice have heen observed around protein molecules.

As an average, one hydration water molecule covers ca. 19 $Å^2$ of the solvent accessible surface of proteins [6, 10], and the total amount of hydration water molecules estimated for the monolayer hydration of monomeric proteins ranges 0.34–0.39 g water g^{-1} protein. This amount is consistent with those estimated by the dielectric relaxation measurements for the hydration water molecules adsorbing on monomeric proteins in solution [12]. In addition, the non-random distribution and the amount of hydration water molecules explain radius of gyration (Rg) values of proteins observed in small-angle X-ray scattering (SAXS) measurements. In many cases, Rg values of proteins in solution are frequently larger by 1–2 Å than those calculated from protein models alone, and an analytical method assuming hydration shell approximately explains the discrepancy [13].

Very recently, we compared the time-averaged hydration structure of human lysozyme in the crystalline state with that from a molecular dynamics (MD) simulation of 1 nano-second for the enzyme immersed in a numerous number of water molecules (Higo and Nakasako submitted). In the MD simulation, high water-density peaks appear discretely only in the vicinity of proteins and are consistent with the hydration sites identified in the crystal structure. Better correlation is found for the hydration sites near surface residues with smaller conformational fluctuations during the simulations. In addition, the water molecules located at and around those sites exhibit coherent and persistent orientational ordering. The discrete appearance and the orientational ordering of hydration water molecules in the MD simulation may be a good explanation for the consistent results between the cryogenic crystal structure analyses, the SAXS experiments and the dielectric relaxation measurements.

Through the comparison described above, we conclude that the hydration structures observed in the cryogenic crystal structure analyses exist on protein surface in solution. Around protein molecules in solution, the distribution of hydration water molecules is expected to be non-random, and the dielectric properties of the molecules are different from those in bulk solvent.

THE GEOMETRY OF HYDROGEN BONDS FORMED BY HYDRATION WATER MOLECULES

Water molecule has four hydrogen-bond arms radiating out from the oxygen atom in the tetrahedral geometry as shown in Figure 2a, and the four arms are the basis

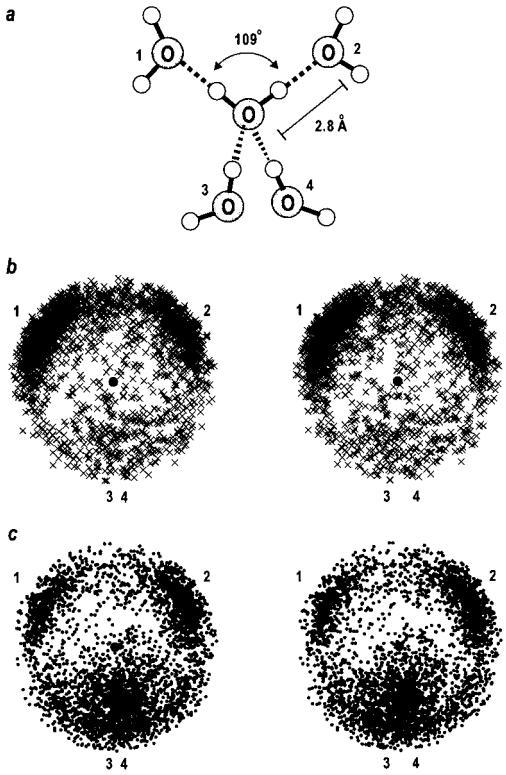

Figure 2. (a) An illustration schematically showing the tetrahedral interaction geometry of water molecules. (b and c) Stereo-plots comparing the geometry of hydrogen bonds in the hydration structures around proteins with the standard tetrahedral geometry in (a). The hydration water molecules having more than three hydrogen bond partners are selected and the constellation of the partners are fitted to the standard model through least-square calculations. (b) The distribution of 2256 polar protein atoms (shown with the cross symbols) within hydrogen bond distance from the hydration water molecules set at the center of the plot. (c) The distribution of 2886 hydration water molecules (shown with the small circles) within the hydrogen bond distance from the water molecule set at the center. The coordinates of hydrated protein were taken from our previous crystal structure analyses [6–11]. All plots are produced by the program suit FESTKOP [6, 10].

for forming and reorganizing the three-dimensional networks of hydrogen bonds in water [1]. Figure 2b shows the interaction geometry of hydration water molecules with polar protein atoms after superimposition with the standard geometry. Two clusters of polar protein atoms lie in the directions corresponding to the two arms in the tetrahedral interaction geometry, and the other less ordered clusters likely do the sites expected from the rest two arms. This standard interaction geometry is well conserved, in particular, in the surface grooves of proteins, where 'trains' of water molecules (see Figure 1) mediates indirect interactions between polar protein atoms forming the wall of the grooves. In the interaction between hydration water

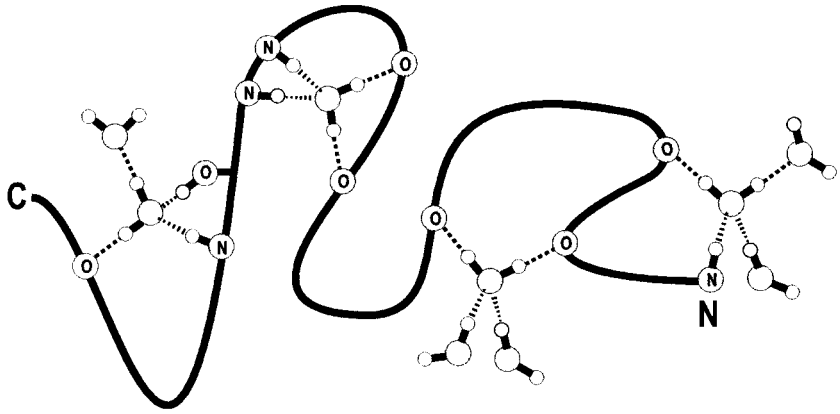

Figure 3. A schematic drawing of the expected roles of hydration water molecules in the folding process of proteins in aqueous solution. Thick line shows a polypeptide chain, and some oxygen and nitrogen atoms in the chain form hydrogen bonds with hydration water molecules in the standard tetrahedral interaction geometry.

molecules (Figure 2c), the four clusters of water molecules are very clear. The average value in the angle between any pairs of interaction arms in the plots is 110°, nearly equal to the standard geometry (Figure 2a). The half width of the variation is ±30°, reflecting the flexibility in the hydrogen bonds between the molecules and polar protein atoms, and probably ensuring the reorganization of hydrogen-bond networks on protein surface as described below.

Thus, it is now clear that the tetrahedral interaction geometry of water molecules still retains even in the vicinity of protein surface. In other words, the experimental results strongly suggest that protein folds to satisfy the tetrahedral interaction geometry of water molecules as schematically illustrated in Figure 3. In addition, from the current knowledge on the hydration structures, it is also suggested that protein must fold into the constellation avoiding the formation of the 'ice' arrangement of water molecules near protein surface [10].

Cyrus Levinthal postulated the large difference between the actual and the theoretical time-scale required in protein folding, when assuming a random conformational search in the theoretical estimation [14]. In the random conformational search, there are too many possible conformations to find the native structure in conformational space. However, when the interaction geometry of hydration water molecules (Figure 2) retains throughout the folding process of polypeptide chains in aqueous environment as illustrated in Figure 3, water molecules restrict the conformations of polypeptide chains. As a result, the area in conformational space to be searched during the folding process may be reduced drastically.

The tetrahedral interaction geometry in the hydration structures may also provide a novel microscopic interpretation on the unfolding process of proteins under the presence of urea, guanidine and methanol having the hydrogen bond arms of planar

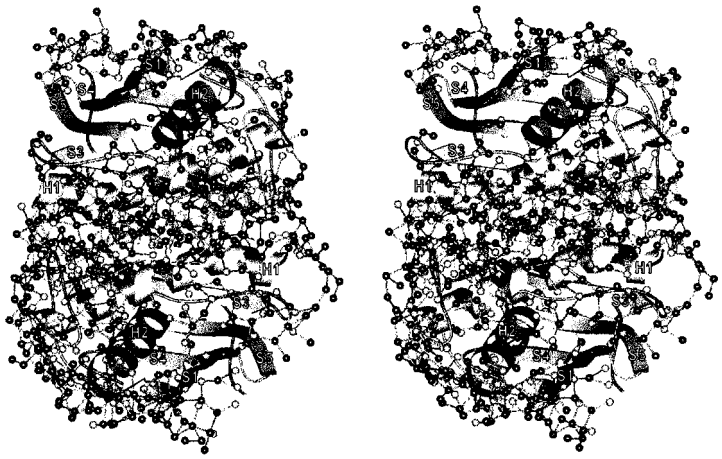

Figure 4. A stereo-plot demonstrating the large-scale networks of hydrogen bonds around the killer toxin molecule form *Yeast* [11]. This plot present only networks containing more than 10 hydration water molecules (black circles) and polar atoms of the enzyme (white cireles). The sticks represent possible hydrogen bonds between polar protein atoms and hydration water molecules. The structures of proteins are schematically shown with ribbon diagrams of the secondary structures. Names of secondary structures indicated as H1-H2 and S1-S5. This figure is prepared using the programs FESTKOP [6, 10] and MOLSCRIPT [23].

and two-dimensional. The interaction geometry largely different from that of water molecule probably cause drastic and cooperative reorganizations of hydrogen-bond networks including protein atoms, when a large number of the reagent molecules surround hydrated protein molecules. The reorganization of networks probably deforms the structures of proteins into those distinctly different from that in aqueous environment.

NETWORKS OF HYDROGEN BONDS INDUCED BY WATER MOLECULES AROUND PROTEINS

As seen in Figure 1, hydration water molecules form aggregates *via* hydrogen bonds. The aggregates link together and are indirectly connected further by polar protein atoms having two hydrogen bond arms. As a result of the chain connections of hydrogen bonds, large-scale networks of hydrogen bonds cover the large proportion of protein surface [6, 10]. Figure 4 illustrates an example of hydrogen-bond networks found around the killer toxin molecule from *Yeast* [11]. One large network runs through about 250 polar protein atoms and 400 hydration water molecules covering more than the half of the solvent accessible surface area of the molecule. Such networks are likely common characteristics in the hydration structures of proteins [6, 10]. The shape and the size of networks depend on the surface topology, the secondary structures and the surface electrostatic potential of proteins [10]. The networks connecting distant secondary structures and do-

mains of proteins may contribute to over-damping low energy collective motions in proteins as postulated in a simulation study [15].

As discussed in the previous section, the amount of hydration water molecules found in the cryogenic analyses is consistent with those measured on proteins in solution. When proteins are in monolayer hydration in solution, hydration water molecules distribute so dense that the chain connections of hydrogen bonds naturally occur between hydration water molecules and polar protein atoms. Therefore, networks must exist on the surface of proteins even at ambient temperature in solution. A theoretical simulation study has predicted the existence of such networks on hydrated myoglobin [16], and the tubular and disordered scattering densities of water molecules observed in crystal structure analyses at ambient temperatures [17, 18] may reflects the hydration-induced networks on the surface of proteins.

The pattern in the extension of those networks [10] is very similar to the percolation networks found in amorphous solids [19]. In this regard, the percolation phenomenon observed in the direct current conductivity of hydrated protein powder [20] is very interesting. The experimental result strongly suggests the existence of proton-translocation networks in the hydrated protein powder, and may be explained by the percolation-like networks of hydrogen bonds found in the cryogenic crystal structure analyses (Figure 4) [6, 10].

In water, the dynamical reorganization of hydrogen-bond networks ensures the fluidity of water [21]. Therefore, the networks around proteins are also expected to reorganize dynamically in coupling with the conformational changes of proteins. In fact, in the case of glutamate dehydrogenase, it has heen revealed that the reorganization of hydration structures in the active site cleft coupled strongly with the spontaneous hinge-bending domain motion [9]. In that case, the tetrahedral interaction geometry of hydration water molecules seems to be essential for the three-dimensional reorganization of networks as well as in bulk solvent. In addition, the flexibility of the networks is responsible to transmit the dynamical motion of water molecules in bulk directly to the exposed residues. As a result, the residues may acquire liquid-like dynamics as reported [22].

In the biological point of view, the roles of networks in molecular association are interesting. In the molecular association between protein molecules, networks may split into small patches through their dynamical reorganization and are confined in the interface of protein complexes. The confined water molecules work as glue, make up surface shape and electrostatic potential, and compensate the entropic cost lost in the association [7, 10].

In conclusion, based on the current experimental results on the hydration structures of proteins, the interaction geometry of ‘tiny’ water molecule may have great influences and essential roles for the folding, dynamics and functions of proteins in aqueous solution. Of course, further experimental and theoretical studies are necessary for describing the physicochemical roles of water molecules in life.

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