Protein hydration

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A hydration shell surrounds proteins in solution. The structures of proteins at atomic resolution presently available in the Protein Data Bank (PDB) provide detailed information on the mode of hydration and plausible roles of water molecules in protein structure and function. This article presents an analysis of water structure in proteins determined at atomic resolution. Water molecules bind to proteins by making hydrogen bonds with oxygen and nitrogen atoms with oxygen as the preferred atom. The mean length of hydrogen bonds between oxygen and water is shorter than that of nitrogen and water. However, the mean thermal parameter of water molecules hydrogen-bonded to oxygen atoms is higher than that of water molecules hydrogen-bonded to nitrogen atoms. Water molecules of the first hydration shell are stabilized by hydrogen bonding with protein atoms, while the stability of the second hydration shell is derived from hydrogen bonding with molecules of the first shell. The number of molecules in the second and higher shells is less than half of that of the first shell. As could be expected, the mean thermal parameter of molecules is higher in the second shell compared to that of the first shell. The number of water molecules buried in protein interfaces increases with increasing size of protein. In several proteins, water molecules form hydrogen-bonded networks that could cover a large part of the protein surface. Pentagonal and tetrahedral arrangements of hydrogen-bonded water molecules could be observed in several structures. Strongly bound water molecules with low thermal parameters appear to be essential for maintaining the loops in appropriate positions. Tightly bound water molecules are also found at the active site of some proteins. These molecules may play important roles in protein function.

Keywords: Atomic resolution, hydration shell, hydrogen bond length, protein hydration, water cluster.

PROTEINS are hydrated macromolecules. Purification of proteins by biochemical techniques usually leads to the final purified sample dissolved in a suitable buffer. For storage over extended periods of time, proteins are usually lyophilized to a solid form as most of them are more stable in the solid state. Even in the lyophilized state, proteins remain partially (5–8%, w/w) hydrated. Controlling residual moisture in lyophilized proteins is important for application in pharmaceuticals[1]. Hydration plays a critical role in protein structure and function[2]. Hence, a number of studies on protein hydration have been conducted. These include studies on the geometry of protein hydration[3], molecular dynamics simulations of water molecules bound to proteins[4], hydration of protein secondary structures[5], differences in the hydration of proteins at room and cryogenic temperatures[6], the role of water molecules in protein function[7] and conformational changes induced by dehydration[8]. However, the structural features of ordered water molecules in atomic resolution X-ray crystal structures of proteins that have become available in the Protein Data Bank (PDB) in recent years have not been analysed in great detail. Due to the availability of powerful synchrotron radiation and improvements in structure determination techniques, a large number of atomic resolution structures are now available in the PDB. Here, we present an analysis of atomic resolution crystal structures addressing the degree of proteins hydration, position of water molecules with respect to the polar groups of proteins, interactions that stabilize water molecules, distribution of water molecules in different hydration shells, significance of the water-binding sites for the structure and function of proteins, atomic displacement parameters (temperature factor or *B*-factor) of water molecules and other features. The results reveal some intriguing features of water molecules bound to proteins.

Methods

A number of protein structures determined to atomic resolution (higher than 1 Å) and available in the PDB were selected for analysis of hydration. In high-resolution structures, several residues are observed in dual conformation. In such cases, only the major conformation was retained. Also, occasional residues of the deposited structures that were post-translationally modified were omitted. Results of the analysis suggested that the nature of hydration is similar in these structures. Therefore, the results obtained for only 12 representative structures determined at better than 1 Å resolution are reported here. Table 1 provides a list of these proteins. Hereafter 'waters' is used in place of 'water molecules' for convenience.

For each selected PDB file, the number of protein atoms, number of waters and frequency of amino acids in the polypeptide were counted. The number of waters for

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Table 1. List of proteins selected as representative structures. Their PDB codes, protein names, resolution, number of atoms in the asymmetric unit, number of associated waters and solvent content of the crystal are specified. Resolution and solvent content are from the PDB

PDB code	Protein	Resolution (Å)	Protein atoms	Waters	Water/ 100 protein atoms	Solvent content
2jfr	Ser–Thr phosphatase	0.83	1705	493	29.8	44.5
1gci	Serine protease	0.79	1881	384	20.4	47.0
1oew	Endothiopepsin	0.90	2376	612	25.8	38.0
1v0l	Xylanase	0.98	2343	641	27.4	35.3
2vxn	Triose phosphate isomerase	0.82	1899	420	22.1	52.6
1us0	Aldose reductase	0.66	2494	613	24.6	28.7
5tda	Ubiquitin protein ligase	0.90	583	107	18.4	28.5
1vyr	Pentaerythritol protein ligase	0.90	2772	784	28.3	44.7
1gwe	Catalase	0.88	3893	878	22.0	52.3
2wfi	Peptidyl prolyl cis-trans isomerase	0.75	1344	433	32.2	36.1
4ua6	B-Lactamase	0.79	3897	854	21.9	38.5
1w0n	Carbohydrate-binding domain	0.80	895	206	23.1	31.6

100 protein atoms was used to represent the degree of hydration of the protein in the crystalline form (Table 1). A hydrogen bond was assumed to be present if the donor–acceptor distance was less than 3.2 Å. For each water, the closest protein atom was found by distance calculation. Waters were grouped into those that are closest to O, N, C and S atoms of the protein. Only a small number of waters was closest to sulphur atoms, as expected from the rare occurrence of sulphur-containing cysteine and methionine residues. Therefore, further examination was confined to waters closest to O, N and C atoms. The mean distances between water and each type of the closest protein atom (O, N, C) and their standard deviation were calculated. The frequency of occurrence of waters in 0.1 Å bins from the closest atom was determined. In each bin, the mean temperature factor of waters was evaluated. Without considering which of the O, N and C atoms is the closest, frequency of waters occurring in 0.5 Å bins from the nearest protein atom was also separately calculated. This frequency distribution is likely to reveal the waters in the first and higher hydration shells. The average temperature factor of waters belonging to each 0.5 Å bin was determined. To examine plausible water networks connected by hydrogen bonds, the distances between waters were computed. Waters that were within hydrogen-bonding distance of each other were merged into the same group. Therefore, waters assigned to a group will be hydrogen bonded to at least one other water of the same group and there will be no hydrogen bond between waters of different groups. The sizes of these hydrogen bonded groups of waters were determined. The total number of hydrogen bonds between waters was determined and the average number of hydrogen bonds made by each water was found. These calculations were aimed at determining how isolated or inter-connected the bound waters are. Waters present in the interfaces of protein molecules were identified by examining the distance of water to atoms of the reference and symmetry/translation-related molecules. Water was assumed to be at the interface if the distance is less than 4.5 Å from one of the atoms of the reference as well as symmetry/translation-related molecule. Those with relatively low temperature factors were grouped as tightly bound waters. These were examined with respect to their involvement in the active site of the protein and their position in the three-dimensional structure of the protein.

Illustrations were prepared using Pymol[9] and the plotting option in Microsoft Excel.

Results and discussion

Ordered waters

An undesirable feature of structures determined by X-ray diffraction technique is that the number of observed waters critically depends on the resolution of the structure. At low resolution, either significant peaks are not observed for waters or water peaks cannot be distinguished from random noise peaks or those arising from systematic error in the diffraction data. However, at the resolution at which the structures used in this study have been determined, it is likely that most of the bound waters have been located. The number of waters bound and detected in structural studies might also depend on the nature of the proteins. For example, it may be related to the number of polar atoms fully or partially exposed to the exterior of the protein, allowing hydrogen-bond formation with a water molecule[10]. Table 1 also shows the number of protein atoms and waters found in each of the selected structures. From these values, the number of waters bound for 100 protein atoms was found and are listed in Table 1 along with solvent content of the crystal form as reported in the PDB file. In the structures analysed, the least and maximum number of waters bound per 100 protein atoms were 18.4 (5tda) and 32.2 (2wfi) respectively. Most proteins contain around 25 waters for every 100 protein non-hydrogen atoms. Waters cover

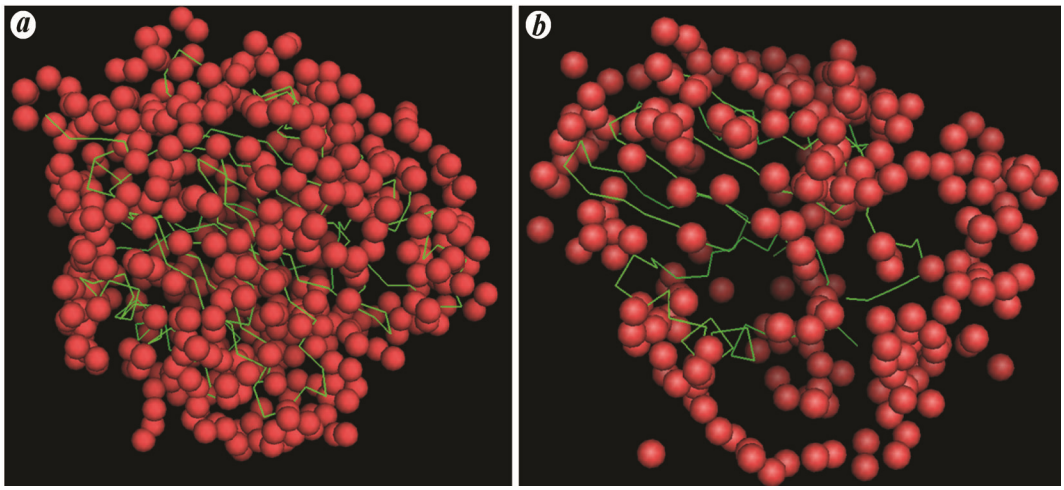

Figure 1. Hydration of proteins: (***a***) heavily hydrated 2jfr with 32.2 waters for every 100 non-hydrogen atoms and (***b***) poorly hydrated 4ekf with 17 waters per 100 protein atoms.

most of the solvent-accessible surface of 2jfr, which has 32.2 waters per 100 protein atoms (Figure 1 *a*). In contrast, a number of patches not covered by waters are observed in 4ekf (Figure 1 *b*, adenovirus proteinase determined at 0.98 Å resolution; it is not one of the proteins included in Table 1, but is in the larger list of proteins analysed), in which 17 waters were present for every 100 protein atoms. Neither the amino acid composition of these two proteins nor the solvent content of their crystal forms appears to account for the difference in the number of well-ordered waters in these structures (calculation not shown). The patches that are not highly solvated may correspond to the apolar regions of the solvent-accessible surface of the protein or to unmodelled solvent.

Since most of the proteins examined have 18–32 waters for 100 protein atoms, if the number of waters is much less than 18 per 100 protein atoms, it is plausible that all waters have not been detected. Similarly, if the waters bound far exceed 32 per 100 protein atoms, some noise peaks might have been identified as waters. This analysis might therefore be useful as a check on the validity of the proposed hydration model based on X-ray crystal structure determination.

Waters closest to different protein atoms (O, N and C)

For every bound water, the closest protein atom was identified by calculating the distance between waters and every protein atom. The distance of the water to the closest protein atom was noted. For water positions closest to O, N and C atoms of the protein, the mean distance between water and the closest protein atom, and the standard deviation of the distance were calculated. The mean temperature factor of these waters was also calculated. Table 2 shows the results. The table reveals many interesting features. The protein atom that is most often close to water positions is O. The next atom closest to water positions is most often C, which is unlikely to be involved in hydrogen bonding. The atom that occurs least frequently (ignoring sulphur atoms) is usually N, although in some cases it is C. The distances of 2.91–3.08, 2.96–3.29 and 3.67–4.00 Å between water position and closest O, N and C atoms respectively, were observed in the 12 structures analysed. Thus, the mean distance of waters from N when N atom is the closest is about 0.1–0.2 Å more than the mean distance from water to O when O is the closest atom. These distances represent the hydrogen bond lengths of O–water and N–water respectively. Thus, O–water hydrogen bond length is shorter than N–water hydrogen bond length. The more surprising result in all the structures is the higher thermal parameter values observed with waters hydrogen-bonded to O atoms when compared to waters hydrogen-bonded to N atoms. This observation could be rationalized as follows. Proteins tend to have a universal amino acid composition due to the number of codons that code for different amino acids modulated by evolutionary constraints. If f_i is the fractional composition of the ith amino acid, the number of C, N and O atoms expected in proteins will be in the ratio of $\Sigma f_i C_i : \Sigma f_i N_i : \Sigma f_i O_i$, where summation is over the 20 standard amino acids, and C_i, N_i and O_i are the number of C, N, O atoms in the ith amino acid. Evaluating these sums, the expected ratio of C, N and O atoms in proteins is found to be about 3 : 1 : 1. Table 2 shows that the number of O atoms of the protein that are closest to waters is 2–3 times greater than the number of N atoms that are closest to waters. This clearly shows that waters have a high tendency to form hydrogen bonds with O atoms of proteins. Apart from main chain

Table 2. Number of waters that are closest to N, O and C atoms of the protein, mean distance between waters and N, O, C atoms, and mean temperature factors of waters of the three groups

Protein	*N*	<*d*>	<*B*>	*O*	<*d*>	<*B*>	*C*	<*d*>	<*B*>
2jfr	96	3.10	24.6	271	2.94	39.7	125	3.76	59.0
1gci	97	3.11	15.2	216	2.91	20.1	71	3.86	35.0
1oew	76	3.29	14.0	388	3.08	29.4	148	4.00	45.4
1v0l	158	3.23	15.0	352	2.99	17.5	131	3.74	27.9
2vxn	79	3.07	13.2	215	2.89	18.9	126	3.78	30.1
1us0	135	3.13	12.7	333	2.91	16.1	144	3.67	24.7
5tda	19	3.28	15.3	70	2.95	22.3	16	3.88	34.4
1vyr	143	3.21	23.2	440	3.03	31.5	201	3.85	45.2
2gwe	191	3.04	10.9	534	2.93	16.5	153	3.81	28.5
4ua6	197	3.18	15.34	451	2.99	17.5	206	3.77	26.0
2wfi	104	3.35	23.7	233	3.09	25.2	95	3.73	36.8
1w0n	46	3.05	14.4	129	2.91	23.5	31	3.71	44.0

Protein, PDB code atoms; N, Number of waters closest to protein N atoms; O, Number of waters closest to protein O atoms; C, Number of waters closest to protein C atoms. The two columns following N, O and C list the mean distance between waters and the closest protein atom, and the mean temperature factor of the three groups of waters.

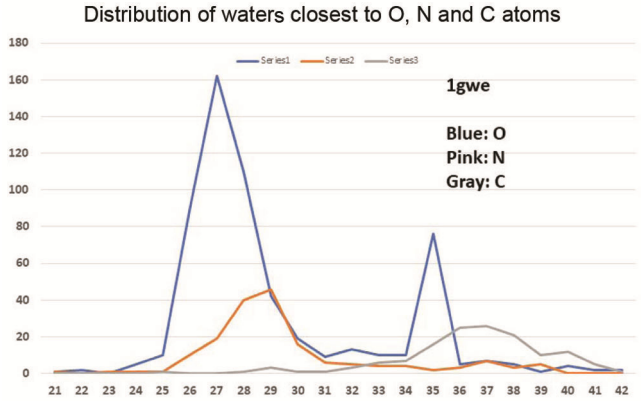

Figure 2. Frequency of waters in 0.1 Å bins starting from the closest protein atom when the closest atom is O (pink curve), N (blue curve) and C (grey curve). The peaks represent the preferred hydrogen bond lengths in the case of O and N and van der Waal's contact distance in the case of C.

N atoms, the N atoms of six residues, viz. lysine, arginine, histidine, glutamine, asparagine and tryptophan could form hydrogen bonds with waters. The O atoms of seven residues besides the main chain O, viz. glutamate, aspartate, glutamine, asparagine, serine, threonine and tyrosine could be involved in hydrogen bonding with waters. Of these, glutamine and asparagine have both N and O atoms in their side chain. Considering the frequency of other amino acids that have N and O atoms in their side chains, the fraction of O atoms that occur in the side chains is greater than the fraction of N atoms that belong to the side chains. Hence a larger fraction of O atoms involved in hydrogen bonding with waters is from the side chains. In all proteins, the temperature factor associated with side-chain atoms is significantly higher than that associated with main-chain atoms due to greater flexibility of the side chains. The temperature factor of bound water tends to be correlated with that of the atom with which it is hydrogen bonded. Hence, the temperature factor associated with waters hydrogen bonded to O atoms is larger. This is consistently observed in all the structures. The number of waters that are closest to C atoms is usually midway between the corresponding number for O and N atoms, although the abundance of C atoms in proteins is three times that of O and N atoms. C atoms that are closest to waters belong mostly to side chains. Therefore, the temperature factor of waters for which C atoms are closest is of the order of the mean temperature factor of side-chain atoms and is substantially higher than the *B*-factor associated with waters closest to O and N atoms. However, as there is no hydrogen bonding between C atoms and waters, the mean distance corresponds to van der Waals contact. In the proteins examined, the mean distance between the closest C atoms and waters is in the range 3.74–4.00 Å (Table 2).

Distribution of waters closest to O, N and C atoms as a function of distance

In all structures examined, there was no water that was not hydrogen bonded to either a protein atom or to another water molecule. With the view of examining waters in the first and second hydration shells, they were assigned to bins of 0.1 Å starting from the closest protein atom separately for waters closest to O, N and C atoms of the protein. Figure 2 shows the frequency in different bins of waters closest to the O, N and C atoms. It is surprising that the closest atom to many waters is C, although it does not form a hydrogen bond with water. Without hydrogen bonding, the bins are empty till about 3.5 Å (Figure 2, grey curve), as shorter distances correspond to sterically repulsive short contacts. Largest number of

Table 3. Number of waters in the first and second hydration shells, their mean temperature factors, waters at the interface of protein subunits, waters that are hydrogen bonded to a protein atom only, size of three largest clusters of waters connected by hydrogen bonding, total number of hydrogen bonds between waters and number of water–water hydrogen bonds per water

PDB code	Waters in the first shell	<*B*>	Waters in higher shells	<*B*>	Interfacial waters	Isolated waters	Three largest clusters	Total hydrogen bonds	Bonds/ water
2jfr	299	36.8	194	49.1	137	88	95, 61, 30	397	0.81
1gci	264	18.9	120	27.5	100	70	43, 24, 20	287	0.75
1oew	359	25.5	253	39.7	203	92	42, 39, 29	475	0.78
1v0l	400	16.4	241	23.3	203	69	81, 59, 45	632	0.99
2vxn	261	18.5	159	25.7	116	50	59, 45, 31	362	0.86
1us0	482	15.3	211	21.4	206	71	129, 117, 55	621	1.01
5tda	74	20.9	33	27.3	80	19	11, 6, 1	63	0.59
1vyr	463	29.0	321	39.9	167	91	130, 88, 78	695	0.89
1gwe	613	16.2	265	20.0	323	113	101, 47, 25	896	1.02
4ua6	522	16.7	332	22.6	268	160	48, 46, 35	645	0.75
2wfi	254	22.4	179	34.4	152	70	29, 25, 23	378	0.87
1w0n	149	22.0	57	31.4	77	38	15, 11, 10	137	0.67

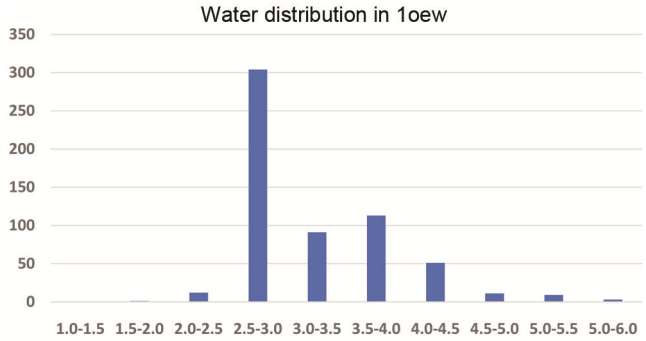

Figure 3. Frequency of waters in shells of 0.5 Å starting from the nearest protein atom for endothiapepsin (1oew). The two peaks in the histogram correspond to the first and second hydration shells.

waters is in the bin corresponding to the 3.7–3.8 Å shell and the frequency slowly reduces with increasing bin numbers. Thus, there is only a single hydration shell of waters closest to C atoms of the protein. With waters closest to O atoms, two peaks in the frequency distribution were observed – at 2.7 Å and 3.5 Å (Figure 2, pink curve) respectively. These waters correspond to the first and second hydration shells. The second shell waters (at 3.5 Å from protein O) do not form direct hydrogen bonds with the protein atoms. They are bonded to protein atoms via other waters that are hydrogen bonded directly to protein atoms. Although waters with closest distance to N atoms revealed a similar distribution with a peak at 2.9 Å (Figure 2 blue curve) and a minor peak at 3.5 Å, the two hydration shells were not well resolved in all structures due to the low number of waters hydrogen bonding with protein N atoms.

Water molecules in the first and higher hydration shells

Examination of the frequency of waters in 0.5 Å bins starting from the nearest atom of the protein without consideration of which is the nearest atom (N, O or C), suggested that the waters occur most frequently in the 2.5–3.0 Å bin. The frequency distribution had a second peak corresponding to 3.5–4.0 Å bin. Figure 3 shows the distribution observed for 1oew. The results with other proteins were similar. The two maxima correspond to peak occupancy of the first and second hydration shells. Based on these observations, a limit of 3.2 Å from the closest protein atom was used as the criterion for identifying the total number of waters in the first hydration shell. Waters that were more distant were assigned to the second or higher hydration shells. Table 3 lists the number of waters in the first and second hydration shells and their average thermal parameters. The number of waters in higher hydration shells was 40–75% of the waters found in the first hydration shell. The mean temperature factor of waters in the second hydration shell was about 1.5–2.0 times that of the first shell (Table 3).

Waters at molecular interfaces

Of the waters bound, those that may belong to the interface of molecules were identified using the criterion that the water should be within 4.5 Å from an atom of both reference and symmetry/translation-related molecules. Table 3 shows the number of waters observed at the interfaces. Between 20% and 50% of the waters bound to the protein were in inter-subunit interfaces. The general trend observed was that the fraction of waters in the interfaces is correlated with protein size (Figure 4).

Hydrogen bonding between waters

With the view of examining water networks connected by hydrogen bonds, the distances of each water from other waters were evaluated and hydrogen bond was assumed to be formed if the distance is less than 3.2 Å. Several

waters were hydrogen bonded only to protein atoms and were not involved in water–water hydrogen bonds. The total number of water–water hydrogen bonds was determined for each protein. From this, the average number of hydrogen bonds made by each water was determined (Table 3). In different structures, each water is involved in 0.75–1.19 hydrogen bonds with other waters. The waters that are hydrogen bonded were grouped into clusters, as explained earlier in the text. In all the structures examined, there were several clusters of size zero, suggesting that these waters are held by a single hydrogen bond with a protein atom and are not involved in water–water hydrogen bonding. However, clusters in some proteins are very large. In 1vyr, 130 waters are joined by hydrogen bonds leading to a very large cluster. There are two clusters of size 129 and 117 waters in 1uso. An even larger network of 159 waters connected by hydrogen bonding was observed in 1pwm (aldose reductase determined at 0.92 Å, not included in the analysis). Some of the very large hydrogen-bonded water clusters could cover up to 25% of the surface area of the protein. Tetrahedral and pentagonal arrangement of hydrogen-bonded waters could be observed in these clusters. Table 3 lists the total number of water–water hydrogen bonds, average bond per water atom and size of the largest three clusters. In different structures each water is involved in 0.59–1.02 hydrogen bonds with other waters. Among the structures analysed, the most heavily hydrated structure of 2jfr and most sparsely hydrated structure in 4ekf are shown in Figure 1 *a* and *b*, respectively. It is plausible that clusters of waters are related to the structure or function of these proteins. In several proteins, the clusters partially cover the active site. In order to identify waters that might be important for structure or function of the protein, the distribution of their *B*-factor was analysed and waters with *B*-factors lower than a specified factor of their mean *B*-value were selected. These were considered as tightly bound waters. Examination of the binding sites of these waters suggested that most of them were partially or fully buried. It is possible to conclude that they could play different but important roles. In many proteins, they appear to stabilize extended loops (Figure 5 *a*). The waters appear to be important for maintaining the distance between loops. If the residues hydrogen bonding with these waters are mutated to abolish the hydrogen bond, the loops may collapse triggering conformational changes in the structure of the protein and affecting its function[10]. In some cases, they were close to the active site. An example is triose phosphate isomerase (Figure 5 *b*). These waters appear to be integral components of the active site and hence may play a key role in protein function.

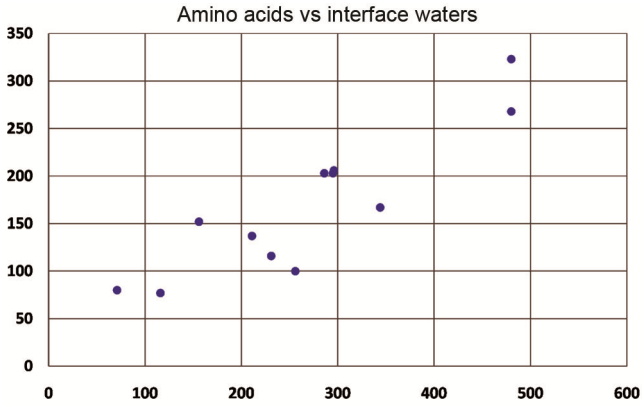

Figure 4. Correlation of the number of interface waters and protein size. The number of amino acids in proteins is shown on the *x*-axis, while the number of interface waters is shown on the *y*-axis.

Conclusion

Although some details regarding hydration are invariably reported in publications on atomic resolution structures of

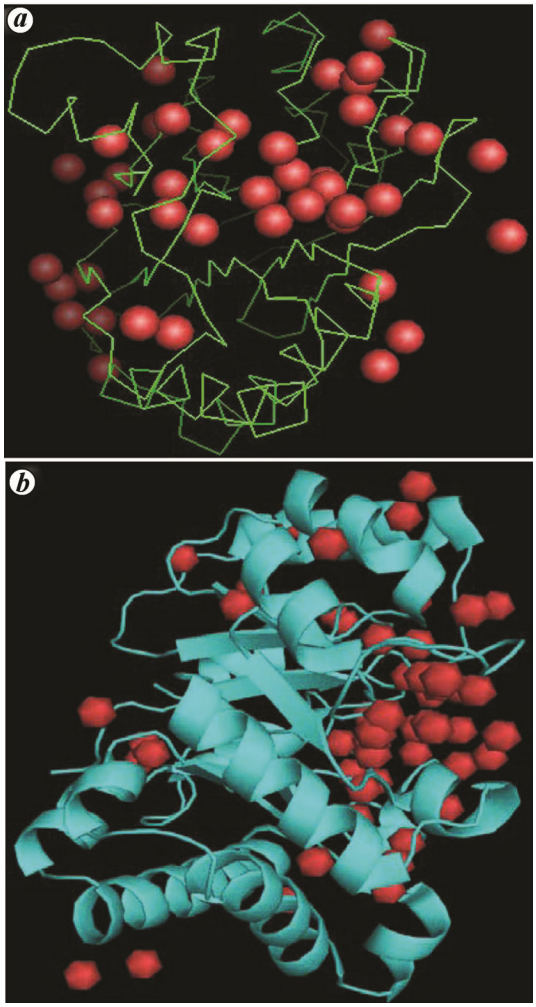

Figure 5. ***a***, Strongly bound water molecules between loops. These appear to stabilize the loops with appropriate geometry. ***b***, Water molecules at the active site of triose phosphate isomerase (2vxn). They seem to be an integral part of the active site and may therefore be important for the catalytic activity of the enzyme.

proteins, most often the discussions are case-specific and confined to the protein of interest only. Systematic analysis of water structure in selected high-resolution protein structures presented here has led to several interesting observations which appear to be generally valid. All waters were found to be hydrogen bonded either to protein O or N atoms, or to another water. On an average, for every 100 non-hydrogen protein atoms, about 25 waters are bound to the protein. Many waters are held by a single hydrogen bond with a protein atom. The preferred atom for hydrogen bonding of waters is protein O. The frequency of waters making hydrogen bonds with O is 2–3 times the frequency of bonding with N, although the abundance of O and N is roughly equal. The mean hydrogen bond length of water with protein O is about 0.1–0.2 Å shorter than the length with protein N atoms. The temperature factor associated with waters hydrogen bonded to protein O was significantly higher than that of waters hydrogen bonded to N atoms. This could be rationalized in terms of the partner atom of the protein that forms hydrogen bonds with waters. A larger fraction of hydrogen bonds between waters and O atoms involve O from the side chains, when compared to hydrogen bonds between waters and N atoms. The number of waters in the second and higher hydration shells is about 50–75% of the waters bound in the first hydration shell. The mean temperature factor of the second shell waters is significantly higher than the mean *B*-factor of the first hydration shell waters. On the average, each water is involved in one hydrogen bond with another water. Ordered waters were occasionally organized into tetrahedral or pentagonal clusters. Waters with low temperature factor, which could be considered as tightly bound, were often partially or fully buried. These waters appear to stabilize loops. In some cases, they were close to the active site. These observations suggest that while considering stability or function of proteins, we need to take into account the structure of the hydrated molecule.

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