International Tables for Crystallography (2006). Vol. F, Section 23.4.3, pp. 625–630.

Park present the state of our understanding of protein–water interactions as it was in 1993, based on the synthesis of results obtained from the various methods discussed above.

23.4.3. Structural features of protein–water interactions derived from database analysis

The location and nature of water interaction with protein atoms are of great interest for understanding the role played by water molecules in the structural integrity and function of macromolecules. Baker & Hubbard (1984) presented an extensive analysis of hydrogen bonding in 15 proteins. A good portion of the study focused on hydrogen bonding with water. They observed that, in general, hydrogen bonds have a certain degree of flexibility, ranging in distance between 2.4 and 3.4 Å, with angular deviation from linear of up to 60°. The authors discussed the hydrogen-bonding geometry of water itself as well as the general aspects of the hydration of protein groups. Along the protein backbone, each carbonyl group is capable of making two hydrogen bonds, while amido groups make only one. Bifurcated hydrogen bonds are relatively rare, comprising only about 4% of the main-chain amido groups and even fewer of the side chains. Baker & Hubbard (1984) observed that of all of the hydrogen bonds made by water molecules, 42% are to main-chain carbonyl oxygens, 14% to main-chain amide groups and 44% to side-chain atoms. In a subsequent review that surveyed protein–water interactions, Savage & Wlodawer (1986) pointed out some of the major problems that hinder the accurate study of the precise hydrogen-bonding geometry and chemical features of protein–water interactions: the size of the biomolecular system, the resolution of the data, and the disorder of both the biomolecule and the solvent. The review was based on a comparison of X-ray and neutron diffraction studies of water interactions in a handful of proteins solved to a resolution of 1.5 Å or better with hydration properties in crystals of small- and medium-sized molecules solved to better than 1.0 Å resolution. Although a great deal had been learned about hydrogen-bonding properties of water in crystals of small molecules that presumably can be transferred to analogous interactions with protein atoms (Savage, 1986), the authors pointed out that for biomolecules there was, at the time, no consistent method being used for solvent analysis (Savage & Wlodawer, 1986). This problem was demonstrated and analysed in a more recent review, where a comparison of three independently solved structures of interleukin-1 reveals a large variability in solvent structure (Karplus & Faerman, 1994).

The growing number of high-resolution protein crystal structures currently available in the Protein Data Bank (Berman *et al.*, 2000) allows for studies that extract statistically significant trends specific to protein–water interactions. The analysis of where and how water molecules bind to protein surfaces can be made at different levels. One can look at general properties of water interacting with each of the 20 amino-acid side chains, as well as with main-chain carbonyl oxygens and amido nitrogen atoms. At a higher level, one can study how these local interactions are modulated by the secondary-structure elements in which the residues are found. At the tertiary-structure level, one can study the location and function of water molecules as they are found in bridging secondary-structure elements and their role in the integrity of the protein architecture. At this level, studies regarding surface shape and hydrophilicity become important components of the analysis. Finally, the role of water molecules can be studied at the level of mediating protein–protein and protein–ligand interaction and their function in the affinity and specificity of these interactions. The remainder of this section summarizes information from database analysis of protein–water interactions at these various levels. The following sections then focus on individual examples to illustrate the classifications and functions of water–protein interactions.

23.4.3.1. *Water distribution around the individual amino-acid residues in protein structures*

The most comprehensive study of water molecules at the local level of binding to the individual types of amino-acid residues in protein structures was published in a series of papers (Thanki *et al.*, 1988, 1990, 1991; Walshaw & Goodfellow, 1993). The initial database consisted of 16 protein structures solved to better than 1.7 Å resolution and refined to an *R* factor of 26% or better (Thanki *et al.*, 1988). It was subsequently increased to 24 proteins using the same selection criteria (Thanki *et al.*, 1990, 1991; Walshaw & Goodfellow, 1993). All equivalent side chains as well as carbonyl or amide groups present in the database were brought to a common reference frame constructed from previously established bond lengths and bond angles (Momany *et al.*, 1975). The distribution of water molecules interacting with each of the 20 types of side chains was studied by focusing on particular atoms. Therefore, water molecules within 3.5 Å of N and O polar side-chain or main-chain atoms or within 5.0 Å of apolar side-chain carbon atoms were appropriately translated to the reference frame.

Fig. 23.4.3.1 shows the results of these superpositions for the polar main-chain amido and carbonyl groups as well as for some representative polar side chains: Ser, Tyr, Asp, Asn, Arg, His, Trp and Ala. The overall results show that despite the complex protein architecture, water molecules interact with hydroxyl, carbonyl and amide moieties, as well as with the sp^3-hybridized and ring nitrogen atoms, as expected from their known stereochemical requirements (Baker & Hubbard, 1984). Thus, there are water clusters in positions that optimize interaction with the lone-pair electrons on oxygen atoms and with the hydrogen atoms of amide and hydroxyl groups. Figs. 23.4.3.1(*a*) and (*b*) show the distribution of water molecules around the main-chain carbonyl oxygen and amido nitrogen atoms, respectively. The stereochemical requirements mentioned above are satisfied, with the distribution around the carbonyl oxygen clustered in two distinct regions peaking at an O–O distance of 2.7 Å. In contrast, there is a single water cluster interacting with the nitrogen, in line with the N—H bond at an N–O distance of about 2.9 Å. This cluster is much tighter than seen for the interactions with oxygen, reflecting a greater flexibility of water interaction with the carbonyl oxygen relative to the amido-group nitrogen atom.

Ser and Thr residues present a wide distribution of water molecules around the hydroxyl groups, presumably due to the freely rotating side chain. Fig. 23.4.3.1(*c*) shows the water-molecule distribution around Ser, which is only slightly different from that for Thr and can be representative of both. In contrast, the Tyr hydroxyl group is involved in resonance stabilization with the aromatic ring and, consequently, water molecules are clustered in the plane of the ring in well defined positions (Fig. 23.4.3.1*d*).

Fig. 23.4.3.1(*e*) shows the clustering of water molecules around the Asp side chain into four distinct groups, corresponding to the four available lone-pair electrons. The distribution around Glu is similar. Most water molecules interact with a single carbonyl oxygen, although about 11% (for Asp) and 15% (for Glu) of water molecules around these side chains interact with both oxygen atoms of a single carboxyl group. Water molecules that interact with Asn and Gln also show four clusters, with the two clusters around the carbonyl group (C=O) less distinct than those around the amido (NH_2) group. Fig. 23.4.3.1(*f*) shows the distribution of water-molecule sites around Asn. In the case of Gln, the difference in water clustering around the carbonyl and amido groups is much less pronounced, possibly due to a greater degree of confusion in placing this longer side chain in the correct orientation. About 6% of the

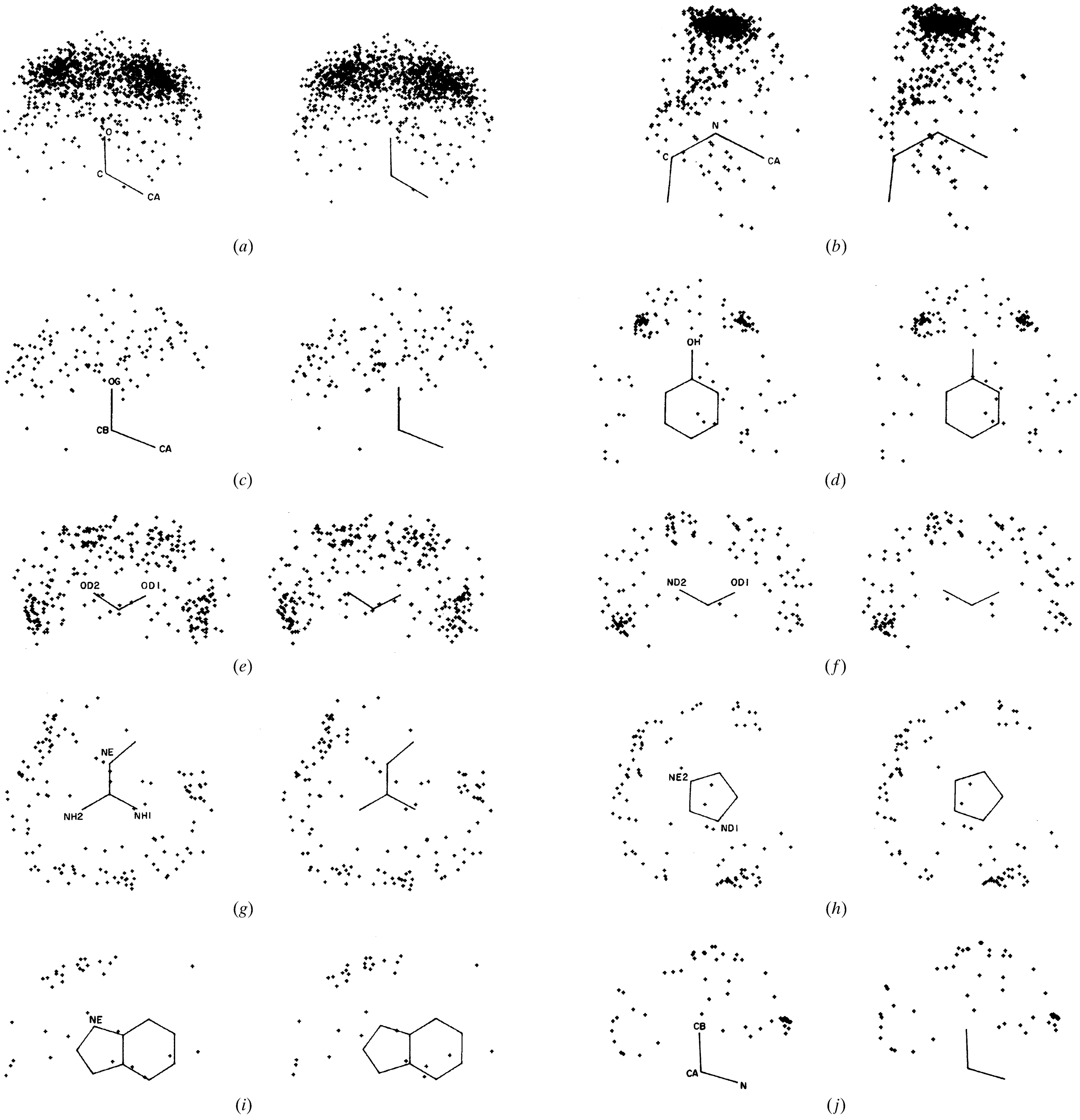

Fig. 23.4.3.1. Distribution of water-molecule sites in stereo around: (*a*) main-chain O, (*b*) main-chain N, (*c*) Ser OG, (*d*) Tyr ring, (*e*) Asp OD1 and OD2, (*f*) Asn OD1 and ND2, (*g*) Arg NH1, NH2 and NE, (*h*) His ring to 3.5 Å, (*i*) Trp ring to 3.5 Å, (*j*) Ala CB. Reprinted with permission from Thanki *et al.* (1988). Copyright (1988) Academic Press.

water molecules that interact with Asn or Gln are involved in hydrogen bonding to both the carbonyl oxygen and the amido nitrogen atoms.

The clustering of water molecules around the planar guanidyl group of Arg is distinctly positioned around the Nε atom and on either side of the NH1 and NH2 atoms. This is shown in Fig. 23.4.3.1(*g*). The clusters peak at a distance of about 3.0 Å from the nitrogen atoms. 7% of these water molecules are shared between NH1 and NH2, and only 3% are shared between the Nε and NH1 atoms. The distribution around the Lys side chain is much broader and is qualitatively similar to the one shown for Ser in Fig. 23.4.3.1(*c*), with no particular orientational preferences, mainly due to the freely rotating nature of the Cε—Nζ bond.

His and Trp are the two residues that contain ring nitrogen atoms, which comprise the main site of interaction with water molecules for these side chains. The distributions of water molecules within 3.5 Å of these residues are shown in Figs. 23.4.3.1(*h*) and (*i*). The clustering around His shows a peak at 2.7 Å and a larger peak at

3.1 Å. The closer peak corresponds to interactions with deprotonated nitrogen (Nδ), where the lone pair of electrons renders the deprotonated nitrogen more negatively charged than the corresponding protonated nitrogen (Nε) and, therefore, the deprotonated nitrogen pulls the water molecule closer. The peak at 3.1 Å is due to water interactions with the protonated nitrogen (Nε) of His. There is a strong preference for the water molecules to lie in the plane of the ring. Relatively few water molecules exist within 3.5 Å of Trp. They mostly cluster around the Nε nitrogen at varying distances. The number of water molecules interacting with His and Trp within 5.0 Å of the ring increases greatly and peaks at a distance of about 4 Å, as discussed below for hydrophobic residues in general (Walshaw & Goodfellow, 1993).

Overall, there seem to be weaker geometric constraints on oxygen acceptors compared to nitrogen donors. Furthermore, the water interaction with oxygen atoms peaks at a distance of about 2.8 Å, while the interactions with protonated nitrogen atoms occur at a somewhat longer distance of about 3.1 Å. This is possibly due to the larger van der Waals radius of nitrogen (1.8 Å) *versus* that of oxygen (1.7 Å) (Thanki *et al.*, 1988). A more recent study of hydration around polar residues is based on seven proteins solved to better than 1.4 Å resolution (Roe & Teeter, 1993). The authors used cluster analysis to derive a predictive algorithm to locate water sites around polar side chains on protein surfaces, given the atomic coordinates of the protein alone. These more precise results confirm the general conclusions outlined above. The authors find that the water–oxygen distance is less than that of water–nitrogen by 0.07 Å and suggest the difference to be due to a van der Waals radius of 1.5 Å for nitrogen and 1.4 Å for oxygen (Roe & Teeter, 1993). Although the two groups cite different atomic radii for nitrogen and oxygen, this does not have an effect on the statistical analysis of the data. Roe & Teeter (1993) also find that the clusters associated with nitrogen atoms are approximately two times denser than those around oxygen atoms.

The analysis of the local water structure around the apolar side chains Ala, Val, Leu, Ile and Phe was extended to a distance 5.0 Å from the atom of interest, since these residues show only a few water molecules within the 3.5 Å cutoff used to analyse interactions with polar residues. The most noticeable observations from the analysis of apolar side chains are the water peak at a distance of 4 Å from the carbon atoms of interest and the presence of a polar protein atom within a hydrogen-bonding distance for 75% of these water molecules (Walshaw & Goodfellow, 1993). Phe prefers in-plane interactions and has peaks corresponding to the direction of the Cε1, Cε2, Cδ1 and Cδ2 atoms from the centre of the ring. Otherwise, any clustering observed for water molecules near apolar side chains is due to interactions with polar protein atoms and, consequently, is modulated by secondary structure.

A study of protein hydration based on atomic and residue hydrophilicity presents general results consistent with those discussed above, but also adds information that can be correlated with various experimentally and computationally derived hydrophilicity–hydrophobicity scales (Kuhn *et al.*, 1995). The authors used 10 837 water molecules found in 56 high-resolution protein crystal structures to obtain the average number of hydrations per occurrence over each amino-acid type and specific atom types. The hydration of the various amino-acid residues has already been discussed above. The atomic hydrophilicity values calculated for the different protein-atom types are of interest. Fig. 23.4.3.2 and Table 23.4.3.1 show that, regardless of where these atoms are found, neutral oxygen atoms exhibit the greatest hydration level per occurrence, closely followed by negatively charged oxygen atoms, which in turn are followed by positively charged nitrogens and neutral nitrogens, in that order. Carbon and sulfur atoms are indistinguishable in terms of hydration per occurrence and are grouped together as the least hydrated atoms (Kuhn *et al.*, 1995).

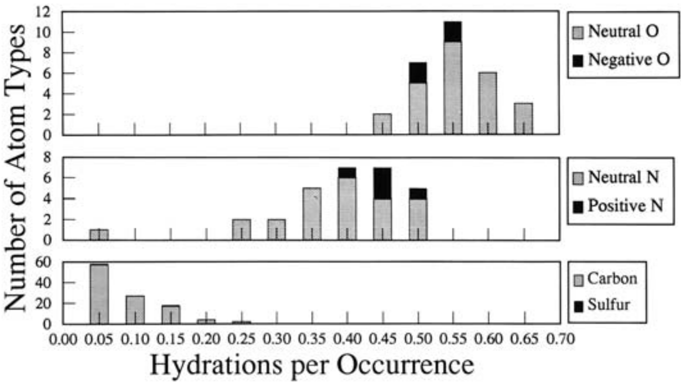

Fig. 23.4.3.2. Distribution of atomic hydration values. To determine which atoms are similar or distinct with respect to water binding, we plotted the number of atom types (*e.g.* Ala amide nitrogen, Ala Cα, . . .) at each hydration per occurrence value. Each atom type contributed one vertical unit to the graph. Oxygen atoms were the most hydrated (top graph), with negatively charged oxygen (black bars) slightly less hydrated on average than neutral oxygen (grey bars). Nitrogens (middle graph) were the next most hydrated, overlapping the oxygen distribution, and positively charged nitrogens (black bars) were somewhat more hydrated than neutral nitrogens (grey bars). Proline's amide nitrogen, with no hydrogen-bonding capacity, had the lowest nitrogen hydration value (leftmost bar). Carbon and sulfur atoms (bottom graph; note change of y-axis scale) were the least hydrated, with sulfur values at 0.05 and 0.15 hydrations per occurrence. Reproduced from Kuhn *et al.* (1995). Copyright (1995) Wiley-Liss, Inc. Reprinted by permission of Wiley-Liss, Inc., a division of John Wiley & Sons, Inc.

23.4.3.2. *The effect of secondary structure on protein–water interactions*

The main effect of secondary structure is on the hydration of main-chain carbonyl oxygens and amido nitrogen atoms. The clustering of water molecules around the small aliphatic apolar side chains (Walshaw & Goodfellow, 1993) and the Ser and Thr side chains (Thanki *et al.*, 1990) were also found to be guided by interactions with main-chain atoms belonging to a specific secondary structure. Other side chains are too large to have their hydration significantly affected by secondary structure. The broad solvent distribution around Ser and Thr side-chain hydroxyl oxygen atoms results from the combination of complex, but distinct, patterns that emerge when hydration around these side chains is examined separately in α-helices and β-sheets. Preferential hydrogen-bonding positions around Ser and Thr residues result from water molecules bridging between the hydroxyl group and another polar protein atom within the α-helix or β-sheet. These positions are dependent both on the χ_1 torsion angle and the type of secondary structure within which these residues are found (Thanki *et al.*, 1990).

Table 23.4.3.1. *Specific hydrophilicity values for protein atoms*

Atom type	Hydrations per occurrence *
Neutral oxygen	0.53
Negative oxygen	0.51
Positive nitrogen	0.44
Neutral nitrogen	0.35
Carbon, sulfur	0.08

* The average number of hydrations per occurrence was calculated over all atoms within each group.

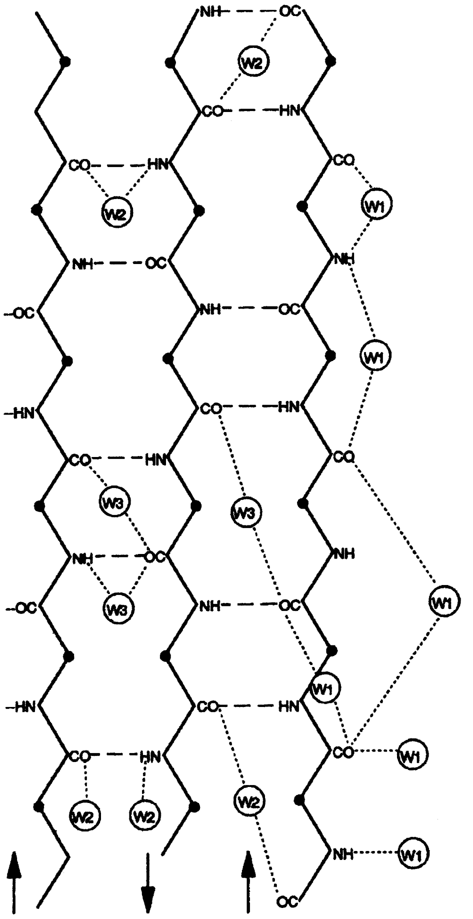

Fig. 23.4.3.3. Diagram of edge (W1), end (W2) and middle (W3) categories of interactions of water molecules with main-chain atoms in antiparallel β-sheets. Reprinted with permission from Thanki *et al.* (1991). Copyright (1991) Academic Press.

The analysis of main-chain hydration focused separately on hydration of β-sheets, α-helices and turns (Thanki *et al.*, 1991). In general, more water molecules were found to interact with carbonyl oxygens than with amide groups, due primarily to the fact that carbonyl oxygen atoms can accept two hydrogen bonds, whereas amido groups can donate a single one. Thus, free carbonyl oxygen atoms have the potential to interact with two water molecules, whereas those already involved in a secondary-structure interaction with the protein still have a lone pair of electrons that can accept a hydrogen bond from a water molecule. Of the free carbonyl oxygen atoms within secondary-structure elements, 45% of those in α-helices and 68% of those in β-sheets interact with water molecules. Of those that are involved in secondary-structure interactions within the protein, 21% of those in α-helices and 17% of those in β-sheets also interact with solvent. The free amide groups are well hydrated, with 38% of those in α-helices and 54% of those in β-sheets interacting with water molecules. However, virtually none (2% in helices and 6% in sheets) of the amides already involved in secondary-structure hydrogen bonding also interact with a water molecule.

Three types of interactions were observed for water molecules in the context of β-sheets (Fig. 23.4.3.3). Most (68%) of these interactions are with the edge of the β-sheet, in an extension of the secondary structure. The second most prominent type of interaction, comprising 23% of the total, is at the ends of the β-strands with either free amido or carboxyl groups. Finally, only 10% of the water molecules are found to bridge between two strands in the middle of the β-sheet.

Interactions of water molecules with α-helices are also found in three distinct positions relative to the secondary structure (Fig. 23.4.3.4): at the carbonyl terminus of the helix, at the amido terminal end and in the middle. Of those interacting at the carbonyl terminus, 48% interact with the carbonyl oxygen alone, 11% also interact with a nearby main-chain atom and 41% are involved in a water-mediated C cap, bridging a small polar side chain (Ser, Thr, Asp, or Asn) to a free carbonyl group at the end of the helix. Of water molecules interacting at the amido terminus of the helix, 25% interact with free amido groups alone, 45% bridge to local main-chain atoms and many of the remaining mediate in N-cap interactions with small polar side chains such as Ser and Asp.

In general, turns have a high exposure to solvent and therefore are found to be well hydrated. The pattern of hydration varies both with the type of turn and the location of the atoms within the turn. Not surprisingly, there are about twice as many hydrogen bonds to carbonyl groups as there are to amide groups in turns. Although the majority of the water interactions with turns are to single carbonyl oxygen or amido nitrogen atoms, bridging water molecules do appear, especially within more open turns. They occur in a variety of different patterns, bridging between two main-chain atoms in the turn or between a main chain and a small polar side chain.

Clearly, water molecules play a functional role in maintaining the integrity of the secondary-structure elements of proteins. They are often seen to extend α-helices or β-sheets, serving as an interface between these secondary-structure elements and the bulk solvent. Water molecules are also found to mediate the

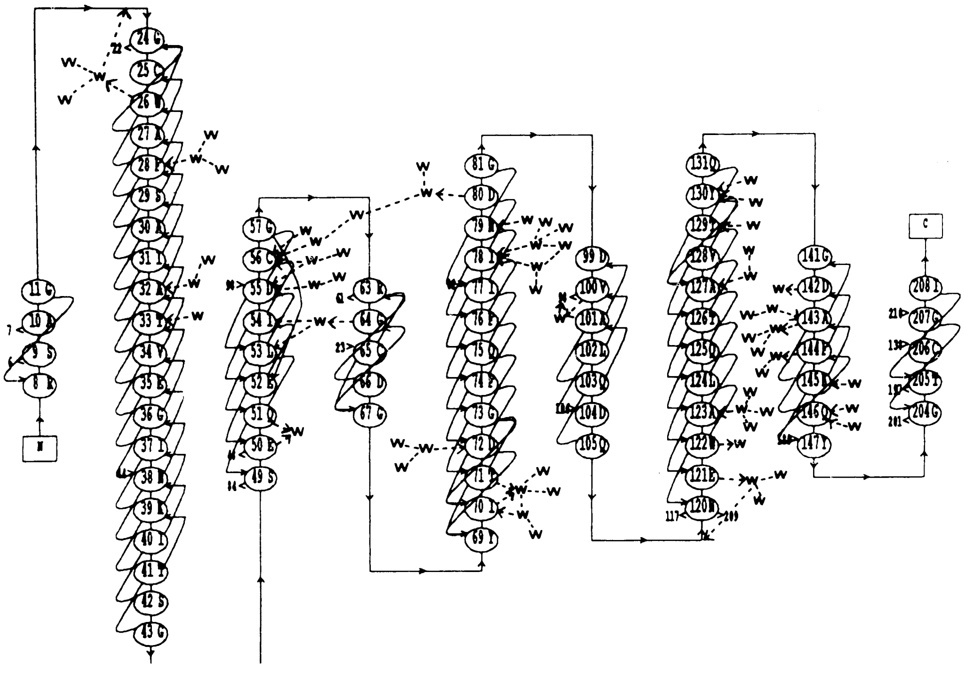

Fig. 23.4.3.4. Diagram of the hydrogen bonds in the α-helical structure in actinidin. Reprinted with permission from Thanki *et al.* (1991). Copyright (1991) Academic Press.

interaction between two protein atoms within a given secondary structure that may be too far from each other to interact directly. This may be of great importance in turns, particularly the more open ones where the protein atoms are not in ideal positions to form a tight two-residue β-turn.

23.4.3.3. *The effect of tertiary structure on protein–water interactions*

At the tertiary level, there is an interdependence between protein surface shape and the extent of water binding (Kuhn *et al.*, 1992). Kuhn *et al.* (1992) studied the binding locations of 10 837 water molecules found in 56 high-resolution crystal structures using fractal atomic density and surface-accessibility algorithms. They found strong correlations between the positions of water molecules and protein surface shape and amino-acid residue type. A probe sphere with the radius of a water molecule revealed that, in general, protein surfaces exhibit convex groove areas and concave contact surfaces. Although grooves account for approximately one quarter of a given protein surface, they bind half the water molecules. Furthermore, only within grooves was hydration found to be dependent on residue type, with charged and polar residues as well as main-chain nitrogen and oxygen atoms exhibiting a greater degree of hydration than the non-polar residues. Outside the grooves, there was a low residue-independent hydration level, with no distinction between main-chain and side-chain atoms (Kuhn *et al.*, 1992). Levitt & Park (1993) discuss the paradox between the experimental observation that water molecules are crystallographically observed primarily in crevices (Kuhn *et al.*, 1992) and the results from theoretical calculations that argue that surface tension should make crevice waters bind less strongly (Nicholls *et al.*, 1991).

While the majority of the crystallographically observed water molecules appear on the outer protein surface, the internal protein packing is not perfect, so that the three-dimensional fold usually results in a number of internal cavities that can accommodate buried water molecules. The first analysis of such cavities was based on a small set of 12 proteins for which the authors characterized such sites by their size and area, as well as by whether or not they were occupied by crystallographically observed water molecules (Rashin *et al.*, 1986). More recently, two methodologically distinct studies of intramolecular cavities used much larger databases to provide extensive and mutually consistent conclusions regarding the properties of these sites (Hubbard *et al.*, 1994; Williams *et al.*, 1994). Hubbard *et al.* (1994) analysed 121 protein chains, with no two possessing a pairwise identity greater than 40%. This study is based on a systematic method of determining the shape as well as the size of the internal cavities and categorizes each cavity as either 'solvated' (with crystallographically visible water molecules) or 'empty' (with no crystallographically visible water molecules), noting the amino-acid-residue preferences in each type. Hydrogen-bonding patterns were also noted within the solvated sites. The second study (Williams *et al.*, 1994) selected 75 non-homologous monomeric proteins, solved at 2.5 Å resolution or better. Although the authors noted the general shape, size and location of cavities, the focus of this study was on the buried water molecules and the hydrogen-bonding patterns that they form within these sites.

In general, larger proteins are able to tolerate larger cavity sizes than small proteins, and nearly all proteins with more than 100 amino-acid residues are found to have at least one cavity. These cavities are found in the protein interior at a variety of distances from the surface and reflect the difficulty of perfect packing within the core. In the database of 121 proteins (Hubbard *et al.*, 1994), 265 cavities were found to be 'solvated' and 383 were 'empty'. The solvated cavities tend to be nearer to the protein surface than the empty cavities. Nearly 60% of the solvated cavities are occupied by a single water molecule and are of spherical shape. About 20% accommodate two water molecules, and 20% more are found to contain larger clusters (Williams *et al.*, 1994). These tend to have an elongated cigar shape. The cavity volume can be as large as 216 $Å^3$ (an elastase cavity containing seven water molecules). The solvated cavities tend to be larger than the empty ones, with average volumes of 39.4 and 20.7 $Å^3$, respectively (Hubbard *et al.*, 1994). The mean volume per water molecule in a cavity is 27 $Å^3$, as compared to 30 $Å^3$ in bulk water, suggesting that a water molecule is not favourably squeezed into a volume comparable to its own (11.5 $Å^3$), but rather occupies similar volumes upon transfer from the bulk into the protein interior.

Solvated cavities differ from empty ones not only in location and size within the protein, but also in the constitution of the amino-acid residues lining the cavity and the secondary-structure elements that are nearby. While 50% of the total cavity molecular surface is provided by polar atoms in solvated cavities, this fraction reflects only 16% of the empty cavity surface. Polarity, not size, is the predominant factor in determining the solvation state of a cavity. Interestingly, solvated cavities have more surface area provided by coil residues than the empty cavities, often found to be lined by residues in secondary structure (Hubbard *et al.*, 1994).

There is on average one buried water molecule per 27 amino-acid residues, although there is great variation between individual proteins. These water molecules most commonly form at least three hydrogen bonds with protein atoms or other buried water molecules. Only 18% of buried water molecules make two or fewer polar contacts. Of all of the hydrogen bonds made by buried water molecules, 53% are to protein backbone atoms, 30% to protein side-chain atoms, 17% to other buried water molecules, and 3% make no visible polar contacts at all (Williams *et al.*, 1994).

The appearance of cavities in the protein core is a consequence of the optimal packing of the protein polypeptide chain as it folds into the native, functional state. Where these cavities expose polar atoms to the hydrophobic protein core, one or more buried water molecules effectively become part of the structure, serving to maintain the protein integrity by fulfilling the hydrogen-bonding potential of atoms which are more favourably solvated.

23.4.3.4. *Water mediation of protein–ligand interactions*

A series of three papers presents the results of an analysis of water molecules mediating protein–ligand interactions in 19 crystal structures solved to better than 2.0 Å resolution and refined to an *R* factor of at least 23% (Poormina & Dean, 1995*a*,*b*,*c*). The studies focus on hydrogen-bonding features of water molecules bridging protein–ligand complexes (Poormina & Dean, 1995*b*), on the surface shape of the protein and ligand molecules at the water-binding sites (Poormina & Dean, 1995*c*), and on the structural and functional importance of water molecules conserved at the binding sites in five sets of evolutionarily related proteins (Poormina & Dean, 1995*a*). This study was largely motivated by an attempt to distinguish between properties of water-binding sites where water molecules are displaced by ligands and those where water molecules must be considered as part of the protein surface. This type of understanding has direct implications for drug and ligand design.

In general, there is a strong correlation between the number of water molecules found to bridge any given protein–ligand complex and the number of hydrophilic groups associated with the ligand. Within this context and in agreement with the conclusions of Kuhn *et al.* (1992), the authors found that the protein shape is important in determining the location of water-binding sites at the protein–ligand interface. Fig. 23.4.3.5 illustrates the different types of grooves observed in this study. Figs. 23.4.3.5(*a*) and (*b*) represent binding of

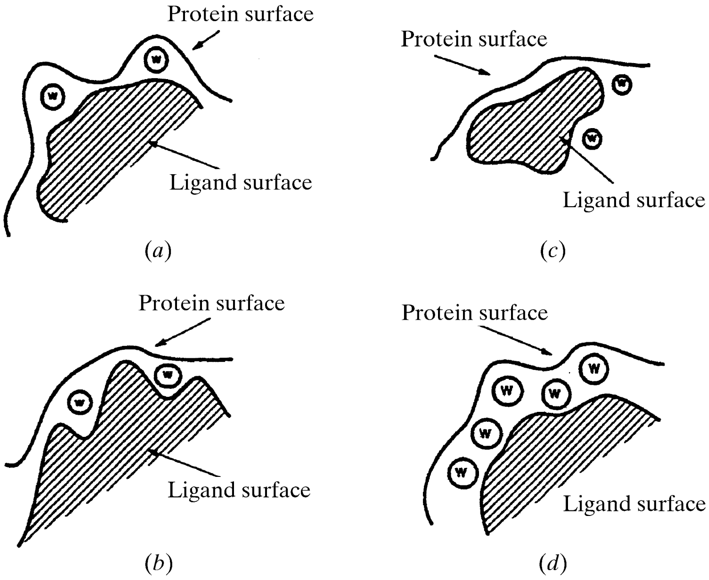

Fig. 23.4.3.5. Schematic illustration of water molecules bound in different types of grooves between protein and ligand. The hatched surfaces represent the ligand surface. (*a*) Water molecules bound in an indentation on the protein surface, where the protein surface area exposed to the water molecules is far larger than the ligand surface area; (*b*) water molecules bound in indentations on the ligand surface, where the ligand surface area exposed to the water molecule is larger than the protein surface area; (*c*) water molecules bound in shallow grooves at the protein–ligand interface and on the ligand surface; and (*d*) water molecules bound in clusters in elongated grooves with micro-grooves. Reprinted with permission from Poormina & Dean (1995*c*). Copyright (1995) Kluwer Academic Publishers.

bridging water molecules in deep grooves on the protein or on the ligand, respectively. The most common situation is illustrated in Fig. 23.4.3.5(*a*), with that in Fig. 23.4.3.5(*b*) occurring very rarely. Fig. 23.4.3.5(*c*) shows the situation where water molecules are found to interact with the ligand alone or at the periphery of the protein–ligand interface. Finally, Fig. 23.4.3.5(*d*) illustrates the situation where clusters of water molecules occupy elongated grooves, mediating the protein–ligand interaction. A striking example of this is given by the complex between chloramphenicol acetyl transferase and chloramphenicol, where two clusters of water molecules are found to form a layer between the enzyme and the ligand (Poormina & Dean, 1995*c*).

For the purposes of analysis, the authors distinguish between water molecules that interact with both protein and ligand, forming a bridge between the two, and water molecules that interact with either the protein or the ligand, but not with both. There is also a group of water molecules that interact with neither protein nor ligand, but are thought to contribute to the stability of the network of water molecules at the protein–ligand interface.

Of the 58 water molecules found to bridge between protein and ligand, 38 (nearly 80%) make three or more hydrogen bonds and satisfy tetrahedral geometry. Furthermore, they bind in deep grooves, generally interacting more strongly with the protein (Fig. 23.4.3.5*a*). The *B* factors of these bridging water molecules are comparable to those of the protein atoms with which they interact. They can, in effect, be considered an integral part of the protein structure and binding site. Many of these bridging water molecules are conserved throughout homologous proteins, even when different ligands are considered, and are clearly structurally significant in maintaining the properties of the protein binding sites.

Water molecules found to bind in shallow grooves do so either at the ligand surface or at the periphery of the protein–ligand interface. For many of these water molecules, the surface areas of the protein and the ligand exposed to the same water molecule are nearly equal. Water molecules binding in shallow grooves are found to have zero to two polar contacts with the protein and are not particularly well conserved within families of homologous proteins.

In general, the authors conclude that water molecules that are to be considered as part of the protein binding site during the design of a new ligand are those that bind in deep grooves, making multiple hydrogen bonds to protein atoms. These water molecules tend to be conserved through families of homologous proteins. The amino-acid residues that interact with deep-groove water molecules tend to be more conserved compared with other residues interacting with the ligand. Conversely, the binding of water in shallow grooves does not seem to be influenced by any special general feature of the protein or ligand surface, and it would be difficult to select water molecules *a priori* for inclusion as part of the protein structure during the process of ligand design.

23.4.4. Water structure in groups of well studied proteins

The analysis of general features of protein–water interactions derived from large databases provides an important context for the study of solvent structure in individual proteins. The number of crystallographically visible water molecules in any one X-ray structure depends on the resolution of the data, the degree of refinement of the model, the criteria used for placement of the less well defined water molecules, and on the experience of the crystallographer. Therefore, to differentiate between water molecules that have functional roles and those that associate randomly with the protein, it is desirable to determine commonalities between several independently solved structures of the protein of interest. There are different types of functional roles that can be determined at several levels. At the global level, one can find a small number of water molecules that are essential for the structural architecture common to a given family of homologous proteins. There are also those water molecules that are structurally important for a specific protein, being present in all independently solved structures of that protein, regardless of the crystal form in which the water molecule was determined or of its interactions with ligands. Water molecules that consistently appear in crystal structures of the protein solved in a specific space group but in no others may be important for crystal packing, but not to the integrity of the protein itself. Finally, a given water molecule may be essential for mediating in a protein–ligand complex, but never appear in the native protein. At this level, all of the independently solved structures of the complex would have the water molecule present. In the examples that follow, comparative analysis between carefully selected groups of structures reveals conserved water molecules at all of these different levels and shows how they carry out particular functional roles in specific examples.

23.4.4.1. *Crystal structures of homologous proteins*

There are two families of homologous proteins for which extensive solvent-structure comparisons have revealed water molecules important in maintaining structural features common to all members of the family. In the first study presented here, 35 crystal structures of eight members of the serine protease family were analysed (Sreenivasan & Axelsen, 1992), while the second study comprises a similar analysis of 11 independently solved structures of six members of the legume lectin family (Loris *et al.*, 1994).

23.4.4.1.1. *Serine proteases of the trypsin family*

The serine proteases have an especially large number of buried water molecules. Using a probe sphere of radius 1.4 Å, an iterative

references