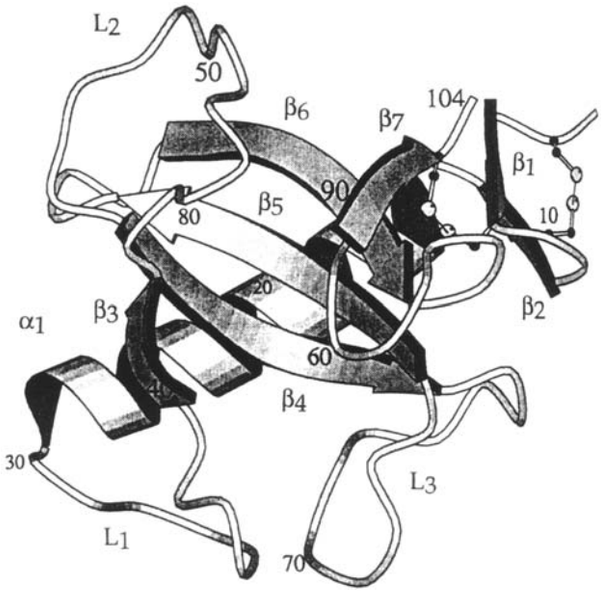


Fig. 23.4.4.10. Three-dimensional structure of RNase T1. Secondary structure is denoted as follows: $\alpha_1$, $\alpha$-helix; $\beta_n$, strands of $\beta$-sheet structure; $L_n$, loops. Drawn using *MOLSCRIPT* (Kraulis, 1991). Residue numbers indicate the beginning and end of secondary-structure elements. Reprinted with permission from Pletinckx *et al.* (1994). Copyright (1994) American Chemical Society.

guanine. Therefore, the information obtained from a study of the solvent structure in ribonuclease A is completely independent from that described above for ribonuclease T1. A collection of ten crystal structures of ribonuclease A, derived from five different crystal forms, were compared pairwise after least-squares superposition (Zegers *et al.*, 1994). 17 conserved water molecules were found to be within a sphere of 0.5 Å of each other in all of the ten structures and are shown in Fig. 23.4.4.11. These water molecules were found in small clusters of two or three or as part of a larger solvent network. Not surprisingly, they form multiple hydrogen bonds with the protein and generally have low temperature factors. Of the 17 structurally conserved sites, 13 are associated with one of the three $\alpha$-helices. Most of these link the helices to one of the $\beta$-strands. Three water molecules are involved in hydrogen bonding with unpaired amido and carbonyl groups on the protein, and one is found on top of the $\beta$-pleated sheet. These interactions result in bringing together elements of secondary structure and in stabilizing distortions within these elements. Conserved water molecules are also responsible for bridging the N-terminal helix to the C-terminal $\beta$-strand, which form the two halves of the active site.

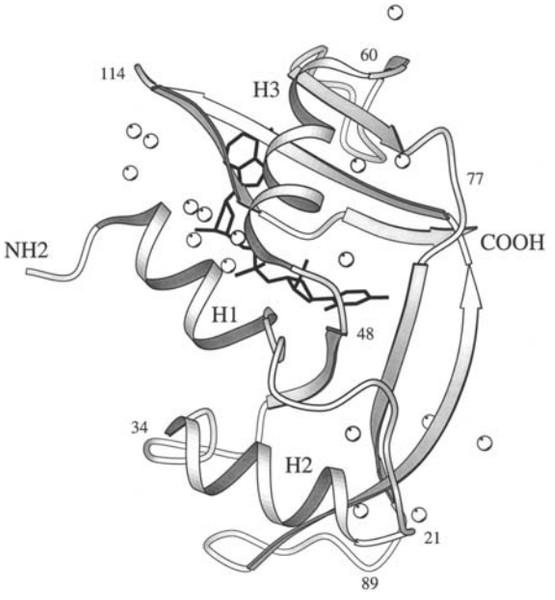


Fig. 23.4.4.11. Overall structure of RNase A. The overall structure of the d(CpA) complex of RNase A is shown as a ribbon drawing using *MOLSCRIPT* (Kraulis, 1991). The conserved water molecules are shown as white spheres and the d(CpA) inhibitor in black. The three helices are labelled H1, H2 and H3. Reprinted with the permission of Cambridge University Press from Zegers *et al.* (1994). Copyright (1994) The Protein Society.

#### 23.4.4.2.5. *Protein kinase A*

The comparative study of water molecules in seven different protein kinase A structures in complex with different ligands focused exclusively on the active site (Shaltiel *et al.*, 1998). All of the structures were solved from isomorphous crystals to resolutions ranging from 2.0 to 2.9 Å. The more lenient cutoff of 1.5 Å for the radius of the sphere within which the conserved water molecules must be found among the different structures is consistent with the relatively low resolutions of the structures included in this study. The group of structures represents the open, the closed and an intermediate conformation of the catalytic kinase domain. There is a set of six conserved water sites in the active site, in addition to the ATP molecule and the magnesium ion. The conserved water molecules coordinate to ATP, the metal ion and a conserved Tyr residue from the carboxyl terminus of the protein. Thus, the active site consists of an extended network of interactions that weave together both domains of the core, with water molecules playing an integral role in maintaining the structural features important for catalysis. Many of these water molecules associate directly with the inhibitors. In addition, five water sites are observed in positions that would be occupied by substrates or substrate analogues. These water molecules are displaced by ligand oxygen atoms that can compensate for the water hydrogen-bonding interaction with the protein.

### 23.4.4.3. *Summary*

Water molecules associated with proteins can be divided between those that are conserved as a result of their functional significance and those that are partially conserved or not conserved at all. The conserved water molecules are generally classified as buried or channel (by a variety of criteria). They tend to be present in the clefts between domains, are critical components of active sites, or bridge between secondary-structure elements. The water molecules that are not conserved occupy hydration sites with favourable hydrogen-bonding characteristics, where the presence of a water molecule is not essential for the structural or functional integrity of the protein.

The displacement of water molecules by organic solvent molecules in the elastase work described above showed that most displaced waters are those classified as surface or crystal-contact waters (Mattos *et al.*, 2000). In the three cases where a buried water molecule was displaced, an alcohol hydroxyl oxygen was found to replace the protein–water hydrogen-bonding interactions. This is analogous to the active-site water molecule in the HIV aspartate protease that gets replaced by a carbonyl group of a potent cyclic urea inhibitor (Lam *et al.*, 1994). In these situations, release of a tightly bound water molecule is entropically favourable, and its enthalpic interactions with the protein are compensated by similar protein–ligand interactions.

The effect of crystal contacts on the water structure was clearly illustrated in the T4 lysozyme work (Zhang & Matthews, 1994).

The internal structurally conserved water molecules are unaffected by crystal contacts. Conversely, any of the surface water sites are potentially available either to be replaced by or to mediate crystal contacts, as 95% of the T4 lysozyme surface is involved in a crystal contact when all ten crystal forms are taken together.

### 23.4.5. The classic models: small proteins with high-resolution crystal structures

Crambin and BPTI are among the handful of proteins for which X-ray crystal structures have been obtained to 1 Å resolution or better. In general, these proteins are relatively small (BPTI, the largest in this group, has 58 amino-acid residues) and often contain at least one disulfide bond. These high-resolution crystal structures have provided structural information beyond that available for larger proteins, particularly with respect to the surface solvent structure. Their small size renders their structures accessible by NMR techniques, making it possible to assess the effect of the crystal environment on the protein and water structure. Finally, the available detail and precision of the structures, as well as their small size, make them ideal models in computational studies of protein energetics and dynamics. Both crambin and BPTI were used during the pioneering years of protein molecular-dynamics calculations. In this section, special attention is given to crambin and BPTI as representative proteins for which very high resolution structures are available. Focus is on the features of solvent structure that are not available for other proteins.

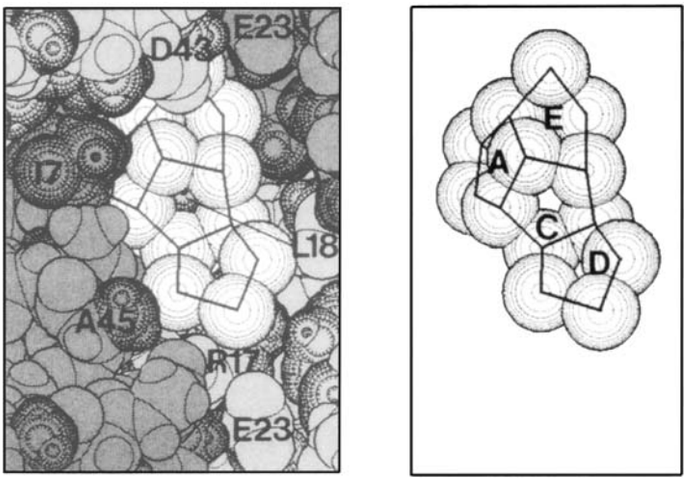


Fig. 23.4.5.1. van der Waals surface diagram of the water pentagons A, C, D and E in crambin viewed in the negative **a** direction. Rings A, C and E form a cap around leucine 18. Hydrophobic atoms are shown as dark circles, and water oxygens are shown as light circles. The methyl group of leucine 18 can be seen through the C ring. Adjacent translationally related molecules are shaded. The van der Waals radii used for the protein C, N and O atoms are 1.7, 1.4 and 1.4 Å, respectively, and for water oxygen, 1.8 Å. The larger radius is used for the water oxygens because hydrogen atoms have been omitted. Reprinted with the permission of the author from Teeter (1984).

#### 23.4.5.1. *Crambin*

Crambin is a plant-seed hydrophobic protein of unknown function. It contains 46 amino-acid residues and was reported to form crystals that diffract to 0.88 Å resolution (Teeter & Hendrickson, 1979). The crystal structure of crambin was determined to 0.945 Å resolution directly from anomalous scattering by the six sulfur atoms involved in three disulfide bonds (Hendrickson & Teeter, 1981). Crambin is an amphipathic molecule in that the hydrophilic components (including six charged groups) are segregated from a mainly hydrophobic surface.

A total of 64 water molecules and two ethanol molecules were located in the electron-density map, despite the fact that the structure was determined in 60% ethanol. The overwhelming number of water molecules compared to ethanol is consistent with the results of the multiple-solvent crystal structures experiments described above for elastase (Mattos & Ringe, 1996).

Most of the 64 water molecules found in crambin interact with polar side chains in the typical manner described previously. The unusual information about solvent structure offered by the crambin model is that the arrangement of water molecules around hydrophobic residues is similar to that observed for clathrate hydrate structures (Teeter, 1991). Pentagonal water rings are observed to cap the C$\delta$2 atom of Leu18 as well as the hydrophobic methylene groups of Arg17 (Teeter, 1984, 1991). The set of five connected water rings is shown in Fig. 23.4.5.1. This ring cluster extends toward the protein, forming heterocyclic rings that are described in detail in the original article (Teeter, 1984).

Although crambin provides the clearest example of pentagonal water rings on a hydrophobic protein surface, it is not the only one. Other high-resolution crystal structures (better than 1.4 Å), such as insulin and cytochrome *c*, have also revealed pentagonal rings, but never to the extent seen in crambin (Teeter, 1984). This is very likely to be a general mode of interaction between water and hydrophobic moieties, be it in inorganic, organic, or biological molecules. The fact that it is not observed in protein structures in general may be related to the lower resolution of most X-ray structures, where it is not possible to model the more disordered areas where these patterns are likely to be found.

#### 23.4.5.2. *Bovine pancreatic trypsin inhibitor*

Bovine pancreatic trypsin inhibitor (BPTI) is a protein of 58 amino-acid residues whose X-ray crystal structure was obtained in the original crystal form to 1.5 Å resolution (Deisenhofer & Steigemann, 1975). Subsequently, 1 Å X-ray data were obtained from a different crystal form, and the new model was jointly refined with 1.8 Å neutron diffraction data (Wlodawer *et al.*, 1984). Minor differences in structure between the two crystal forms of BPTI were observed (Wlodawer *et al.*, 1984). The interesting contribution of the 1 Å model to the understanding of solvent structure resulted from the ability to refine occupancy at this resolution. A total of 63 water molecules were placed in the model, 20 of them within 1 Å of a water molecule found in the structure solved in the original crystal form. During refinement against the 1 Å data set, full occupancy was assigned to all protein atoms, and water occupancy was allowed to refine. Of the 63 water-molecule positions, 29 were found to be fully occupied. The remaining 34 had partial occupancies, with 0.4 being the minimum occupancy found. Given that there are very few contacts between protein molecules in the crystal (Wlodawer *et al.*, 1984), it is reasonable to assume that this observation is representative of water occupancies on protein surfaces in general. It is likely that well over half of the water positions found on protein surfaces are less than fully occupied, although there is no definitive proof that this is true.

#### 23.4.5.3. *Summary*

In general, small proteins serve as important models where results of X-ray crystallography, NMR and molecular-dynamics calculations can be easily compared and cross-validated, since larger proteins are more difficult to study by the latter two techniques. Small proteins are also more likely to form relatively ordered crystals, which are able to diffract X-rays to atomic resolution (of the order of 1 Å). With respect to understanding



references