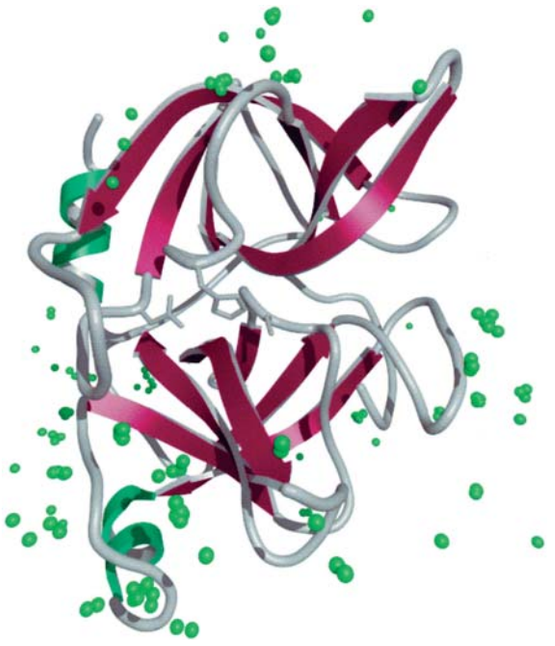

Fig. 23.4.4.6. Elastase structure represented as in Fig. 23.4.4.4. The crystallographic water molecules involved in crystal contacts in 11 superimposed elastase structures solved in a variety of solvents are shown in green.

isopropylanilide (Mattos *et al.*, 1994) or the trifluoroacetyl-Lys-Pro-*p*-trifluoromethylanilide (Mattos *et al.*, 1995) inhibitors in the structures of their complexes with elastase. These inhibitors span a large area of the active site, including an exosite not occupied by substrate analogue inhibitors (Mattos *et al.*, 1994, 1995). The water-binding sites in the active site are not very well conserved, with most sites represented in only two to four of the 11 structures. When all of the structures are superimposed, there is at least one water molecule in each of the subsites in the elastase active site. These water molecules are displaced either by inhibitors or by organic solvent molecules in the various structures. It is not surprising that in elastase, a protein with relatively broad substrate specificity, the active site in the uncomplexed native protein is populated by many displaceable surface water molecules. With the exception of a water molecule present in the oxyanion hole, these water molecules tend to make a single hydrogen bond with the protein. This hydrogen-bonding interaction is not generally conserved between different structures where a given site is occupied in multiple structures. The displacement of these water molecules upon ligand binding is entropically favourable, as they are released into bulk solvent, without too much enthalpic cost. This relatively small enthalpic cost can be compensated by the protein–ligand interactions.

Fig. 23.4.4.8 shows all of the 1661 water molecules colour-coded by the various classifications described above. Clearly, the entire surface of the protein is well hydrated. Notice how the yellow channel waters are often followed by a red buried water molecule. In addition, there is often no obvious spatial distinction between molecules categorized as crystal contacts (green) and those categorized as surface (blue).

23.4.4.2.2. *T4 lysozyme*

Over 150 mutants of T4 lysozyme have been studied to date, and, for the majority of these, the crystal structures are available. Although most of the mutant structures crystallize isomorphously to the wild type, many of them provide a view of the molecule in different crystal environments. This collection of structures leads to

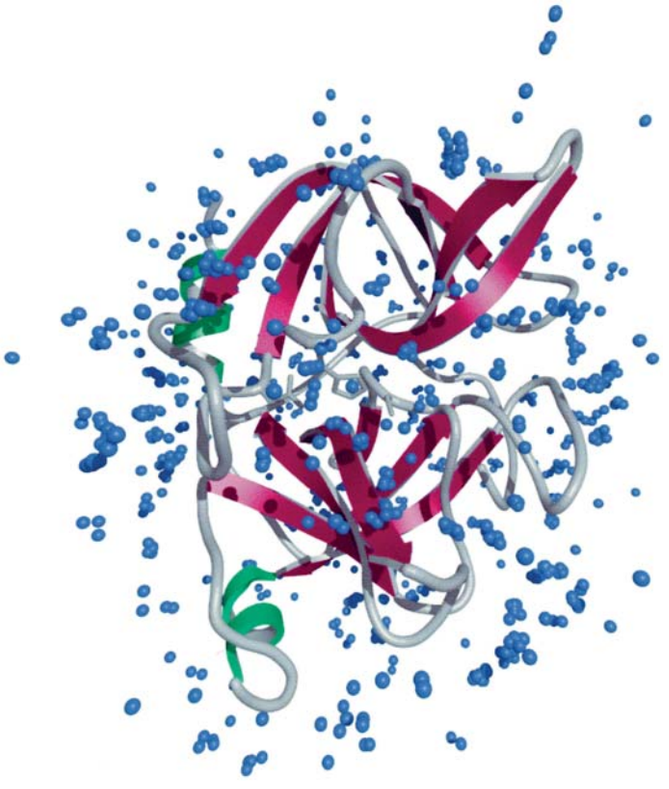

Fig. 23.4.4.7. Elastase structure represented as in Fig. 23.4.4.4. The surface crystallographic water molecules found in 11 superimposed elastase structures solved in a variety of solvents are shown in blue.

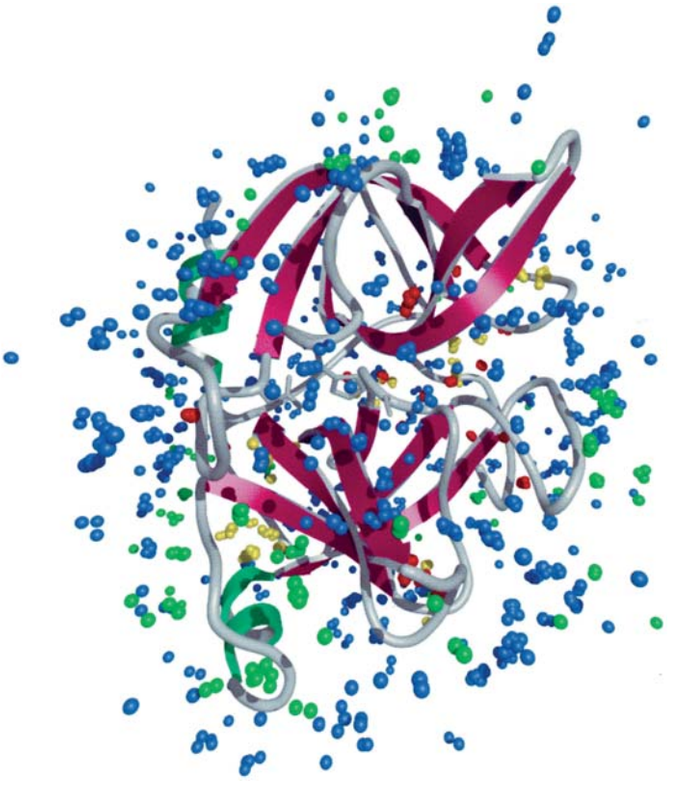

Fig. 23.4.4.8. Elastase structure represented as in Fig. 23.4.4.4. The 1661 water molecules found in 11 superimposed elastase structures of elastase are colour-coded as in Figs. 23.4.4.4–23.4.4.7.

the comparative analysis of the solvent positions in ten different crystal forms of T4 lysozyme, providing a clear picture of the effect of crystal contacts on the hydration sphere of a protein viewed by X-ray crystallography (Zhang & Matthews, 1994). The resolution and degree of refinement of the structures involved varied significantly, from 2.6 to 1.7 Å resolution, and the number of water molecules included per protein molecule ranged from 38 to 160. Nevertheless, this study revealed important features. A striking observation is that 95% of the solvent-exposed residues on T4 lysozyme were involved in at least one crystal contact in one or another of the crystal forms studied, showing that any part of the protein surface can be involved in crystal contacts. A corollary to this finding is that any of the surface water molecules can be displaced or involved in bridging protein–protein contacts in the crystal.

Of the 1675 individual water molecules observed in the 18 independently refined T4 lysozyme molecules included (Fig. 23.4.4.9), the ones that were within a sphere of radius 1.2 Å were considered to occupy the same site on the protein. As in the case of elastase described above, all of the water molecules observed upon superposition of the 18 T4 lysozyme structures represent a large portion of the first hydration shell. This reinforces the concept that multiple structures of a protein of interest provide a more complete picture of the protein hydration than possible with a single structure. There are four buried water sites that are occupied in at least 15 out of the 18 structures and are independent of crystal contacts. Two of these buried sites are at the hinge-bending region between the two helical domains and appear to play a functional role in the opening and closing of the active site (Weaver & Matthews, 1987). The other two play a structural role at the protein core. Other than the four buried water molecules, the most conserved water sites appear at the active-site cleft between the two domains and at the N-termini

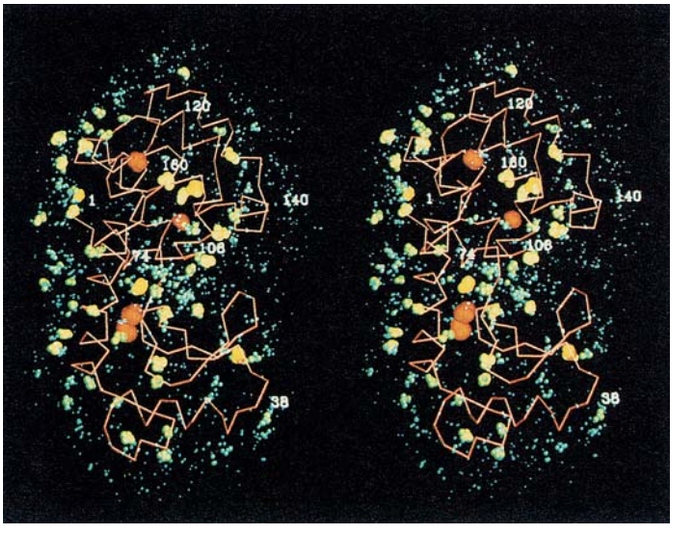

Fig. 23.4.4.9. Distribution of solvent-binding sites in 18 mutant T4 lysozymes from ten refined crystal structures. The lysozyme structures were compared to identify common sites of hydration. A total of 1675 solvent molecules were included in the comparison. Each solvent molecule is represented by a coloured sphere. The size of the sphere is proportional to the number of lysozyme structures in which solvent was observed at the same site (*i.e.* within 1.2 Å). In addition, the colour of the solvent changes from blue for the least-conserved sites to red for the most-conserved ones [*e.g.* the red spheres indicate that solvent is observed with high frequency (15–17 times) at the four internal sites]. The numbers indicate representative residue positions along the backbone of the lysozyme molecule. Reprinted with the permission of Cambridge University Press from Zhang & Matthews (1994). Copyright (1994) The Protein Society.

of α-helices. As is the case in the previous works reviewed above, the 20 most conserved water sites appear in well conserved protein environments and generally have low temperature factors. Buried or highly conserved water molecules also tend to make at least three hydrogen bonds with protein atoms or other water molecules. The less-conserved water sites appear more randomly on the protein surface and are strongly influenced by the particular crystal environment in which the structure was solved.

23.4.4.2.3. *Ribonuclease T1*

A group of four crystal structures of ribonuclease T1 in complex with guanosine, guanosine-2′-phosphate, guanylyl-2′,5′-guanosine and vanadate were used for an analysis of conserved water positions that contribute to the structural stabilization of the protein (Malin *et al.*, 1991). The four structures were obtained from isomorphous crystals and ranged in resolution from 1.7 to 1.9 Å. Conserved water molecules were considered to be those found within a sphere of 1 Å from each other in all four structures. All other water molecules were excluded from the analysis. 30 water molecules were found to be conserved. Of these, ten were observed near crystal contacts, although only one appears to be dictated by the crystal contact itself, making a single hydrogen bond with each of the symmetry-related protein molecules. Ten other water molecules form a channel that brings together an α-helix and a hairpin-like loop structure and then go on to wrap around the calcium ion, providing half of its coordination sphere. The first five of these water molecules are completely buried, holding together the two secondary-structure elements, which would otherwise collapse (Malin *et al.*, 1991). Two water molecules are found to stabilize the N and C termini, which are brought together by a disulfide bond. The remaining eight conserved water molecules hold together various elements of secondary structure or are located in the active site.

An interesting extension to this study included four additional structures: the E58A mutant in complex with guanosine-2′-monophosphate, the H92A mutant crystallized under two different conditions and wild-type RNase T1 in complex with guanosine-3′,5′-biphosphate. Two of these crystal forms were not isomorphous with the native protein crystals or with each other. Thus a total of eight structures solved in three different space groups were analysed (Pletinckx *et al.*, 1994). Although the effect of crystal packing on the three-dimensional structure of the protein is minimal, there are some significant differences in the solvent structure. In particular, there is no evidence of the calcium-binding site and its coordinating water structure in any crystal forms other than the canonical wild type. Instead, the E58A mutant has a sodium-binding site at a different position, along with three previously unobserved water molecules. It is clear that the presence of the metal ions is fortuitous and linked to the crystallization conditions.

There are 25 water molecules structurally conserved throughout the different packing arrangements studied. Ten of these are single sites, there are three clusters of two water molecules and a larger cluster originally described by Malin *et al.* (1991) to hold together the core of the protein. As was observed for the study on T4 lysozyme (Zhang & Matthews, 1994), the strictly conserved water-binding sites present in crystal structures solved across different space groups are involved in bridging protein secondary-structure elements and seem to be crucial for the integrity of the protein structure.

23.4.4.2.4. *Ribonuclease A*

Ribonuclease A is not homologous to ribonuclease T1 in either sequence or structure, but both have evolved to catalyse the same reaction with specificity for different substrates (compare Figs. 23.4.4.10 and 23.4.4.11). Ribonuclease A cleaves RNA after pyrimidines, while ribonuclease T1 cleaves specifically after

references