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Hydration in drug design. 1. Multiple hydrogen-bonding features of water molecules in mediating protein–ligand interactions

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Summary

Water is known to play an important rôle in the recognition and stabilization of the interaction between a ligand and its site. This has important implications for drug design. Analyses of 19 high-resolution crystal structures of protein–ligand complexes reveal the multiple hydrogen-bonding feature of water molecules mediating protein–ligand interactions. Most of the water molecules (nearly 80%) involved in bridging the protein and the ligand can make three or more hydrogen bonds when distance and bond angles are used as criteria to define hydrogen-bonding interactions. Isotropic B-factors have been used to take into account the mobility of water molecules. The water molecules at binding sites bridge the protein and ligand, and interact with other water molecules to form a complex network of interconnecting hydrogen bonds. Some water molecules at the site do not directly bridge between the protein and the ligand, but may contribute indirectly to the stability of the complex by holding bridging water molecules in the right position through a network of hydrogen bonds. These water networks are probably crucial for the stability of the protein–ligand complex and are important for any site-directed drug design strategies.

Introduction

Over recent years, automated de novo methods for drug design have become increasingly developed [1,2]. Although most methods consider the design problem in vacuo [1,3], crystal structures of several protein–ligand complexes, determined to high resolution, reveal the presence of a number of ordered water molecules at the ligand binding sites. These molecules may play a significant rôle in mediating the protein–ligand interactions by bridging between the ligand and the protein [4–6]. However, there is not yet a convenient method for dynamic design in sites that enables solvent to move freely during each design step along the pathway of producing a novel structure. Various computational approaches have been adopted to remedy this deficiency. Two strategies are common: (i) if a crystal structure from the site is available and solvent molecules are present, the water molecules are retained in the same position they had the crystal; and (ii) the empty site can be scanned for positions of strong water binding, either by a grid-based method [7] or by rejection of weakly bound water molecules from a flood-filled site. An alternative approach would be to explore different structural factors that influence the binding of water molecules at the receptor site. In drug design we seek to answer the following questions: which water molecules are likely to be displaced during ligand binding, which of them have to be considered while designing a ligand, and do these water molecules have any structural significance? A detailed examination of protein–ligand complexes may reveal whether there are any common factors associated with water bridges that link the ligand to the protein. If these factors can be identified, it might be possible to incorporate them dynamically into a de novo drug design strategy, or to predict static hydration sites at the macromolecular receptor site. With this objective in mind, we have carried out a detailed analysis of high-resolution (< 2.0 Å) hydrated protein–ligand cocrystal complexes. Our aim has been to categorise water molecules in the complex in three ways.

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TABLE 1
PROTEIN–LIGAND COMPLEXES SELECTED FOR THIS STUDY

Protein–ligand complex	*R*-factor	Resolution (Å)	Number of subunits	Ref.
Dihydrofolate reductase with NADPH (3dfr)	0.15	1.7	1	10
Staphylococcal nuclease with 5'-deoxythymidine (1snc)	0.16	1.65	1	11
Lactate dehydrogenase with NADH and oxamate (9ldt)	0.23	2.0	4	12
H-*ras* p21 protein with guanosine-5'-triphosphate (5p21)	0.19	1.35	1	13
D-Xylose isomerase with D-xylose (8xia)	0.14	1.9	4	14
Holo-D-glyceraldehyde-3-phosphate dehydrogenase (1gd1)	0.17	1.8	4	15
Proteinase A with a tetrapeptide (5sga)	0.11	1.8	1	16
Chloramphenicol acetyl transferase with chloramphenicol	0.15	1.75	3	17
Ribonuclease T_1 with guanylic acid (1rnt)	0.19	1.9	1	18
Human neutrophil elastase (1hne)	0.16	1.84	1	19
FK506 binding protein (1fkf)	0.17	1.7	1	20
Citrate synthase coenzyme A (2cts)	0.16	2.0	2	21
D-Glucose binding protein with D-galactose (2gbp)	0.14	1.9	1	22
Ribonuclease A with acetyl deoxythymidine (8rsa)	0.16	1.8	2	23
L-Arabinose binding protein with D-galactose (5abp)	0.13	1.8	1	5
HIV protease with acetyl pepstatin (5hvp)	0.17	2.0	2	24
Acid proteinase with pepstatin-like renin inhibitor (4er2)	0.18	2.0	1	25
Thermolysin with a peptide (1tmn)	0.17	1.9	1	26
Aldose reductase with bound NADPH (1ads)	0.20	1.6	1	27

(1) Hydrogen-bonding interactions of all water molecules in the site have been analysed and assessed using strict hydrogen-bonding criteria of angles and distance. The mobility of the water molecules has been monitored by comparing their isotropic B-factors with those of neighbouring atoms. Hydrogen-bonding networks of water molecules have been determined.

(2) Surface shape properties of the local subsite, where the water molecules are found, appear on cursory examination to be partly responsible for determining water positions. The shape characteristics of these hydration subsites have been determined in terms of contact surface areas [8].

(3) The evolution of structure and function of proteins has largely been considered in terms of structural changes to the site by the deletion, addition or replacement of residues. Little attention has focussed on the conservation of water molecules in the site, unless they are functionally involved in the catalytic mechanism. In these studies we examine the conservation of water molecules, which seem to have a structural rôle in bridging the ligand to the site but are not involved in catalysis [9].

Materials and Methods

Data sample

In order to understand the rôle of water molecules in ligand binding to a receptor, water molecules at the binding sites of 19 different protein–ligand cocrystals have been analysed. The atomic coordinates of protein–ligand complexes, determined by X-ray crystallography, were obtained from the Brookhaven Protein Data Bank. Table 1 lists the complexes selected for this study. These crystal structures have been refined at a high resolution of 2.0 Å or better, to an *R*-factor of ≤ 0.23. All data sets contain the atomic coordinates for water oxygen atoms.

The complexes have been checked to see whether the ligand makes any intermolecular contacts with other subunits in the asymmetric unit. If an active site is situated between two subunits, or is composed of residues from different subunits, care has been taken to include all the site residues from different subunits. For example, the binary complex of chloramphenicol acetyl transferase with chloramphenicol (3cla) is a trimer with identical subunits. The active site of 3cla is located at the intersubunit interface and the site residues binding the substrate belong to two different subunits.

The present study concentrates on the hydrogen-bonding interactions of water molecules at the ligand-binding sites. Therefore all protein atoms at a distance of ≤ 5.0 Å from each ligand atom are considered in our analysis. The data sets were checked for multiple occupancies; if there were two occupancies for a given site point, the site point with a higher occupancy was selected.

Definitions and geometry

Water, with its tetrahedral coordination, acts as a potential donor as well as a potential acceptor and it can form multiple hydrogen bonds. Therefore, the hydrogen-bonding interactions of water molecules at the site with ligand atoms, protein-site atoms and other water molecules at the site are considered together in order to investigate the rôle of water molecules at binding sites in stabilizing the protein–ligand interactions.

A hydrogen bond is a strong electrostatic interaction between an electropositive and an electronegative atom.

TABLE 2
WATER MOLECULES AT THE BINDING SITES INTERACTING WITH LIGAND AND PROTEIN[a]

Protein–ligand complex	Water molecule	Total no. of H-bonds[b]	No. of H-bonds			Protein–ligand complex	Water molecule	Total no. of H-bonds[b]	No. of H-bonds		
			with ligand	with protein	with water				with ligand	with protein	with water
Dihydrofolate reductase	**208**	**5**	**1**	**3**	**1**	Holo-D-glyceraldehyde-3-	390	2	2	0	0
with NADPH (3dfr)	276	3	1	0	2	phosphate dehydrogenase	416	3	1	0	2
	279	**2**	1	**1**	**0**	(1gd1)	430	2	1	0	1
	301	**5**	**1**	**1**	**3**		478	2	1	0	1
	302	4	**1**	0	3	Proteinase A with a	**241**	**2**	**1**	**1**	**0**
	318	**3**	**1**	**1**	**1**	tetrapeptide (5sga)	**346**	**4**	**2**	**1**	**1**
	326	3	2	0	1		**356**	**2**	**1**	**1**	**0**
	373	2	1	0	1	Chloramphenicol acetyl	**252**	**3**	**1**	**1**	**1**
	401	**2**	**1**	**1**	**0**	transferase with chlor-	**308**	**5**	**1**	**1**	**3**
	439	**2**	**1**	**1**	**0**	amphenicol (3cla)	313	3	1	0	2
Staphylococcal nuclease	**157**	**5**	**1**	**1**	**3**		360	2	2	0	0
with 5'-deoxythymidine	170	3	2	0	1		403	2	1	0	1
(1snc)	**171**	**5**	**1**	**3**	**1**	Ribonuclease T_1 with	148	2	2	0	0
	173	**4**	**1**	**2**	**1**	guanylic acid (1rnt)	**158**	**3**	**2**	**1**	**0**
	187	**5**	**1**	**2**	**2**		**186**	**4**	**1**	**1**	**2**
	213	**2**	**1**	**1**	**0**	Human neutrophil elastase	**19**	**2**	**1**	**1**	**0**
	217	**3**	**1**	**1**	**1**	(1hne)	**108**	**3**	**1**	**2**	**0**
Lactate dehydrogenase with	**1**	**4**	**1**	**2**	**1**	FK506 binding protein	13	2	2	0	0
NADH and oxamate	10	2	1	0	1	(1fkf)	33	2	1	0	1
(9ldt)	**21**	**4**	**1**	**3**	**0**		**43**	**3**	**1**	**1**	**1**
	28	**3**	**1**	**1**	**1**		**69**	**2**	**1**	**1**	**0**
	41	**2**	**1**	**1**	**0**	Citrate synthase	**41**	**3**	**1**	**2**	**0**
H-*ras* p21 protein with	**170**	**2**	**1**	**1**	**0**	coenzyme A (2cts)	**49**	**3**	**1**	**2**	**0**
guanosine-5'-triphos-	**172**	**5**	**3**	**2**	**0**	D-Glucose binding protein	**313**	**5**	**2**	**1**	**2**
phate (5p21)	**173**	**6**	**2**	**4**	**0**	with D-galactose (2gbp)					
	175	**4**	**1**	**2**	**1**	Ribonuclease A with acetyl	**2199**	**4**	**1**	**2**	**1**
	187	**3**	**1**	**1**	**1**	deoxythymidine (8rsa)					
	281	3	1	0	2	L-Arabinose binding protein	**309**	**4**	**2**	**2**	**0**
	282	2	1	0	1	with D-galactose (5abp)					
D-Xylose isomerase	**17**	**3**	**1**	**2**	**0**	HIV protease with acetyl	**308**	**3**	**1**	**2**	**0**
with D-xylose (8xia)	**192**	**5**	**1**	**3**	**1**	pepstatin (5hvp)					
	212	**4**	**1**	**1**	**2**	Acid proteinase with	**166**	**3**	**2**	**1**	**0**
Holo-D-glyceraldehyde-3-	353	3	2	0	1	pepstatin-like renin	175	2	1	0	1
phosphate dehydro-	**354**	**3**	**1**	**1**	**1**	inhibitor (4er2)					
genase (1gd1)	**356**	**4**	**1**	**3**	**0**	Thermolysin with a peptide	**362**	**3**	**2**	**1**	**0**
	366	2	1	0	1	(1tmn)					
	367	**4**	**1**	**2**	**1**	Aldose reductase with	**484**	**7**	**4**	**1**	**2**
	373	2	1	0	1	bound NADPH (1ads)					
	388	**4**	**1**	**3**	**0**						

[a] Water molecules in bold are those bridging the ligand and the protein.

[b] In some cases, the ligand and protein atoms interacting with water can act as an acceptor as well as a donor with respect to a water molecule. In such situations, only one interaction is taken into account while calculating the number of possible hydrogen bonds associated with a given water molecule. See Tables 3–5.

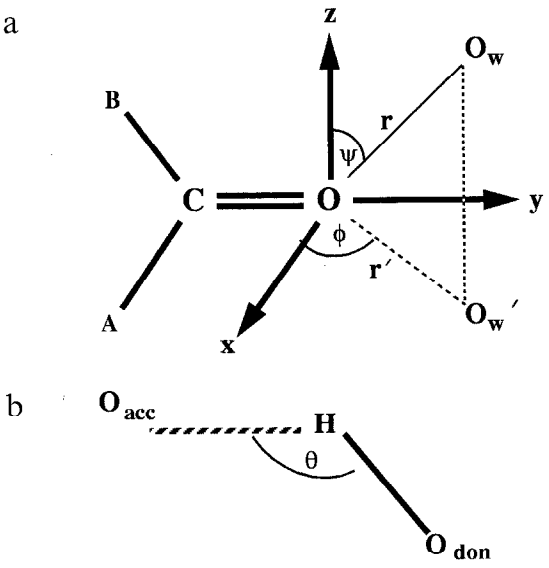

Fig. 1. (a) Acceptor geometry described by the spherical polar coordinates r, ϕ, and ψ. The z-axis is normal to the plane containing the side-chain atoms C, A, B and the acceptor atom O. O_w is the position of the water oxygen at the distance r, and r' is the projection of the radius vector r in the x-y plane. (b) Donor geometry described by the angle θ.

Thus, there is no definite or unique definition of a hydrogen bond for crystallographic usage. In general, in crystallographic studies only the distance between heavy atoms is taken as the criterion to define a hydrogen bond. In this study, we rely on both the distance and angle criteria to be satisfied to define a hydrogen-bonding interaction. A water molecule is considered to be 'bound' only if it satisfies the criteria for both the distance and angles for a hydrogen-bonding interaction with any other hydrogen-bonding atom.

The interactions of the type X-H···O_W and X···H-O_W, where X is a ligand, a site atom or a water oxygen, are studied here. The interactions of water oxygen with the polar atoms oxygen, nitrogen and sulphur are considered. These polar atoms can act as both donors and acceptors, depending on their hybridization state. As the ligand–water interactions are of principal interest, in addition to commonly occurring donor and acceptor groups in proteins, many other hydrogen-bonding groups are included in this study. Acceptor groups included are carbonyls (C=O), amines (NH_2), nitriles (C≡N), nitro groups (NO_2), ether oxygens (-O-), phosphates (PO_4^{2-}) and nitrogens in five- and six-membered rings. The geometry of the acceptor group and the position of a bridging water molecule with respect to an acceptor group are described by the spherical polar coordinates r, ϕ and ψ, as shown in Fig. 1a. The donor groups considered are rotatable (OH and SH) or rigid (NH). The donor geometry is given in Fig. 1b, where the donor angle is described by the angle θ.

In this study, we have used permissive cutoff criteria [28–30] for distance and angles to define hydrogen bonds. In all three types of interaction, i.e., ligand–water (L-H···O_W and L···H-O_W), water–protein (P-H···O_W and P···H-O_W) and water–water interactions (O_W-H···O_W and O_W···H-O_W), the heavy atoms with a distance between 2.5 and 3.5 Å were considered to satisfy the distance criterion for hydrogen bonding. A donor angle θ greater than 130° was chosen to describe a donor interaction. The limits for acceptor angles ϕ and ψ are chosen as $0° \leq \phi \leq 90°$ and $40° \leq \psi \leq 90°$, respectively.

The donor angles were calculated by adding the hydrogen atoms in idealized positions to both ligand and protein atoms. In the case of rotatable donor groups like hydroxyl, the angle ROW made by water oxygen, hydroxyl oxygen and the non-hydrogen atom bonded to hydroxyl oxygen was considered in deciding the possible donor interaction. In the case of sp^3 hybridized oxygens, it is not possible to associate the acceptor geometry exactly with Fig. 1a. In such cases, only the angle ϕ was taken into consideration. Only for water–water interactions it was not possible to distinguish between acceptor and donor interactions of water molecules, since the atomic coordinates are available only for heavy atoms. Therefore, the angle LOW or WOP, made by either a ligand or a protein atom and two mutually interacting water oxygens, was considered in deciding water–water interactions (as it was not possible to add hydrogen atoms to water molecules).

In a few cases, some of the water molecules interact with ions bound at the ligand binding sites. Interactions of water molecules with the ions are more complex and need a separate study. Therefore, in this study we concentrate only on the interactions of water molecules with the protein and the ligand.

Temperature factors and water mobility

Mobility of water molecules at the binding site has been taken into account in deciding the hydrogen-bonding interactions of water molecules with ligand and protein site atoms. The isotropic B-factors are used to calculate the mean displacement $\overline{U}$ of a water molecule, where the temperature factor B is given by $B = 8\pi^2 \overline{U}^2$. An imaginary sphere is constructed around each water molecule with a radius equal to a mean displacement $\overline{U}$ of the water about its mean position. The hydrogen-bonding angles that a given water molecule can make with any possible acceptor or donor atom are calculated as it fluctuates about its mean position. If polar coordinates r', ϕ' and ψ' describe the position of any point P' in the sphere, a 3D grid is generated within the sphere, with two radial intervals and 12 angular intervals of 30° each, for ϕ and ψ. The hydrogen-bonding geometry is calculated at each of these grid points in the sphere. If the water molecule makes an appropriate angle(s) with respect to either an acceptor or a donor at any point in the sphere, the water molecule is considered to make a hydrogen bond with the donor or the acceptor in question.

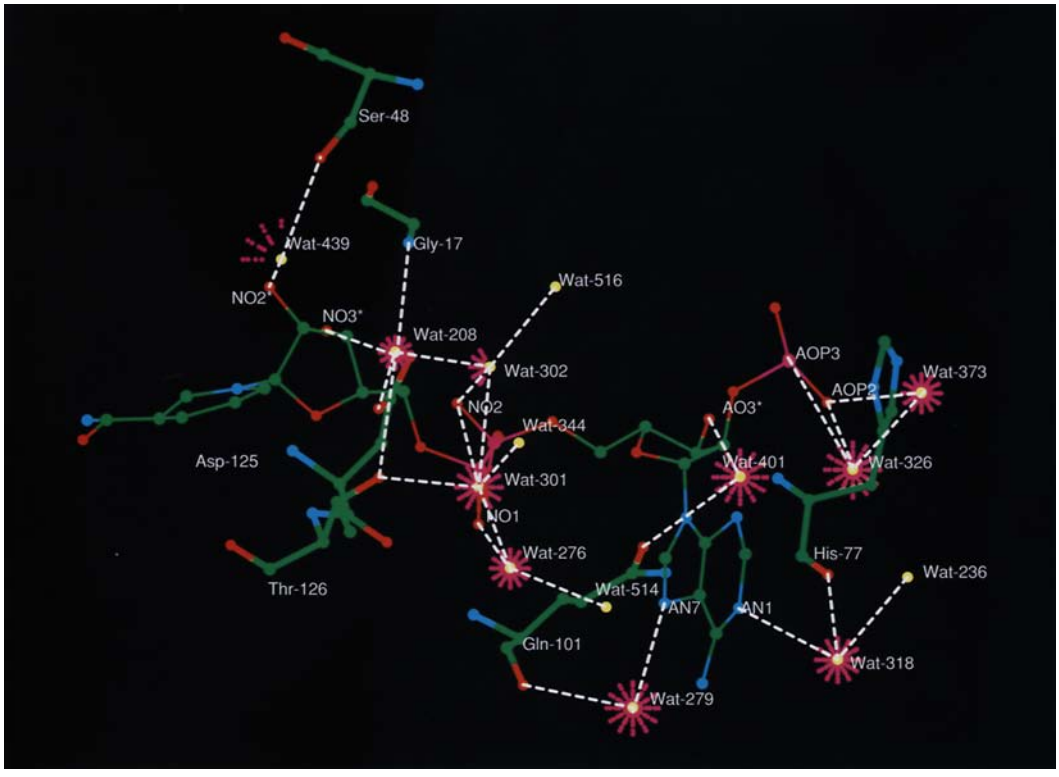

Fig. 2. Hydrogen-bond network of water molecules at the binding site of dihydrofolate reductase (3dfr) involving NADPH and protein-site residues. The protein residues are shown in bold. Water molecules bound to the ligand are represented by yellow circles associated with star-like features. Dots in magenta (within the sphere) are the positions of water where it assumes the correct geometry to either donate or accept a hydrogen bond as it fluctuates about its mean position. Note the multiple hydrogen bonds associated with the bridging water molecules. Phosphate oxygen AOP3 is masked by the phosphorus atom. Colour code: red, oxygen atoms; blue, nitrogen atoms; green, carbon atoms.

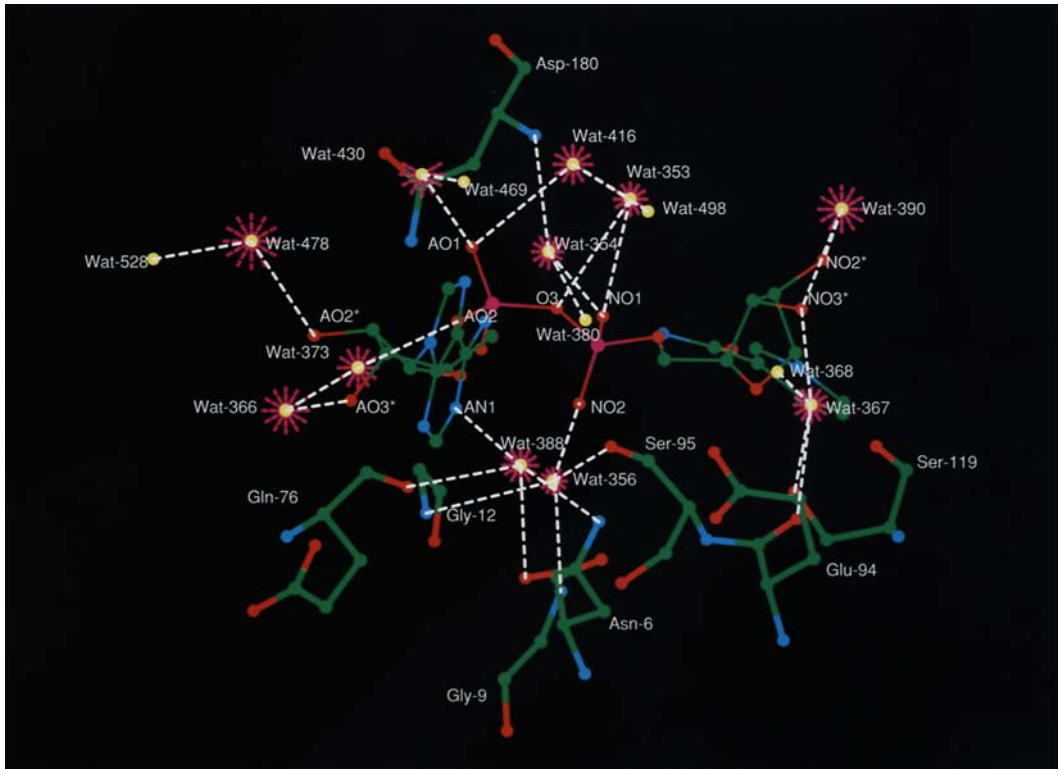

Fig. 3. Hydrogen-bond network of water molecules at the binding site of H-*ras* p21 (5p21), involving NAD and protein-site residues. Other features are described in the legend of Fig. 1.

A program was written to extract all water molecules at the site as well as their possible interactions with ligand and protein atoms and other water molecules at the site. The ligand data with added hydrogens were given as input to the main program. In the first stage, the program selects all the water molecules that are at the hydrogen-bonding distance from each acceptor and donor atom of the ligand. Then the program chooses those water molecules that are in an appropriate geometry, either to accept a hydrogen bond from or to donate a hydrogen bond to the ligand. In calculating the hydrogen-bonding geometry, the program takes into account the thermal fluctuations of these water molecules as described earlier. In the next step, protein-site atoms (polar atoms) are selected that are at a hydrogen-bonding distance to these ligand-bound water molecules (hydrogen bonded to the ligand atoms). Then the program chooses all protein site atoms that satisfy the angle criteria to make hydrogen bonds with these bridging water molecules. Once again the thermal fluctuations of the bridging water molecules are considered in calculating the hydrogen-bonding geometry of water–protein interactions. Finally, the interactions of these bridging water molecules with other water molecules in the site, which are at a hydrogen-bonding distance, are checked to see whether they are in an appropriate geometry to interact with the bridging water molecules.

Results

This study focusses on water molecules at the binding sites of protein–ligand complexes and especially on the water molecules that form a bridge between the potential hydrogen-bonding groups of the ligand and the site. The protein–ligand complexes chosen for the study are listed in Table 1. Details of the hydrogen-bonding interactions of water molecules at the binding sites of 19 selected complexes are presented in Table 2. As expected, the number of water molecules at the binding site of any protein–ligand complex depends on the number of hydrophilic groups associated with the ligand. There are six bridging water molecules at the binding site of dihydrofolate reductase (3dfr) and staphylococcal nuclease (1snc). The H-*ras* p21 (5p21) has five water molecules bridging the ligand with the site, while lactate dehydrogenase (9ldt) and glyceraldehyde dehydrogenase (1gd1) each have four such water molecules. In total, 7 of the 19 protein–ligand complexes have more than three water molecules mediating the protein–ligand interactions. The other 12 complexes have either one or two bridging water molecules at the site. Some of the protein and ligand hydrogen-bonding atoms can act as acceptors as well as donors with respect to a given water molecule. In such cases, only one interaction is considered for calculating the number of hydrogen bonds associated with a water molecule.

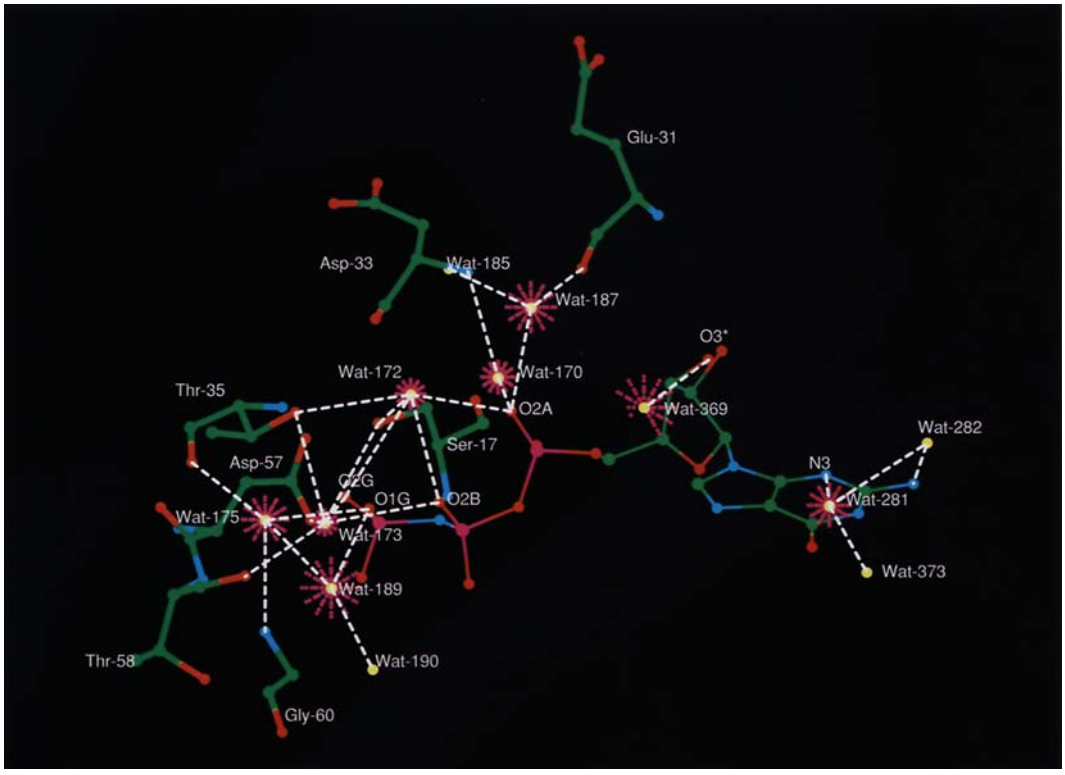

Fig. 4. Hydrogen-bond network of water molecules at the binding site of holo-D-glyceraldehyde-3-phosphate dehydrogenase, involving NAD and protein-site residues. Other features are described in the legend of Fig. 2.

TABLE 3
DETAILS OF HYDROGEN-BONDING INTERACTIONS INVOLVING NADPH, WATER MOLECULES AND PROTEIN ATOMS AT THE BINDING SITE OF THE TERNARY COMPLEX OF DIHYDROFOLATE REDUCTASE BOUND TO NADPH AND METHOTREXATE

Ligand atom	Type of interaction[a]	H-bond distance (Å)	Bridging water molecule	B-factor of water molecule (Å^2)	Protein atom	Type of interaction[a]	H-bond distance (Å)	Water molecule	H-bond distance (Å)
NO3*	Acc	2.73	208	13.40	N Gly^{17}	Don	2.85	302	3.01
			208		OD1 Asp^{125}	Acc	2.73		
			208		OG1 Thr^{126}	Daa	3.26		
NO1	Acc	2.65	276	18.70				301	2.91
			276					514	2.79
AN7	Acc		279	36.00	O Gln^{101}	Acc	2.90		
NO2	Acc	3.03	301	27.50	OG1 Thr^{126}	Daa	2.81	344	3.01
			301					276	2.91
			301					302	3.23
NO2	Acc	2.66	302	20.00				208	3.01
			302					516	2.71
			302					301	3.23
AN1	Acc	2.95	318	25.40	O His^{77}	Acc	3.06	236	2.85
AOP2	Acc	2.99	326	39.30				373	3.05
AOP3	Acc	3.35	326						
AOP2	Acc	2.60	373	19.30				326	3.05
AO3*	Acc	2.94	401	37.50	OE1 Gln^{101}	Acc	3.10		
NO2*	Acc	2.98	439	78.70	OG Ser^{48}	Daa	2.85		

[a] Acc is a ligand or a protein atom acting as an acceptor; Don is a ligand or a protein atom acting as a donor; and Daa is a ligand or a protein atom acting as both acceptor and/or donor.

The striking feature of water molecules bridging the ligand and the site is the possibility that they have multiple hydrogen bonds associated with them, resulting from interactions with ligand and site atoms and other water molecules. Of the 48 bridging water molecules found in 19 complexes, 15 can have three hydrogen bonds, 12 can make four hydrogen bonds and nine are able to form five hydrogen bonds. One of the water molecules at the binding site of H-*ras* p21 (5p21) has as many as six possible hydrogen bonds, while seven hydrogen bonds are possible with the only water molecule involved in bridging NADPH and the site in aldose reductase (1ads). Thus, in total, 38 of the 48 bridging water molecules listed in Table 2 can have three or more hydrogen bonds and the 10 remaining water molecules have two hydrogen bonds.

The data presented in Table 2 illustrate the importance of the bridging water molecules, which may stabilize the protein–ligand interactions (especially in complexes like 3dfr, 1gd1, 9ldt, 1snc and 5p21). These five complexes have at least four water molecules at the site, mediating the protein–ligand interactions with multiple hydrogen bonds (predominantly four or five hydrogen bonds). Although many of the complexes chosen for the study have only one or two water molecules in their binding sites, it is interesting to note that most of these water molecules have at least three hydrogen bonds. For example, those at the binding site of galactose binding protein (2gbp) and L-arabinose binding protein (5abp) have four and five hydrogen bonds, respectively. These bridging water molecules, together with their hydrogen-bonding partners, generally satisfy tetrahedral or near-tetrahedral geometry.

Further analysis of water molecules at the binding sites of individual protein–ligand complexes reveals many more interesting features. To illustrate their importance in binding the ligand with the site residues, we discuss in detail the interactions of each water molecule at the binding sites of 3dfr, 5p21 and 1gd1.

Figures 2, 3 and 4 illustrate the water-mediated interactions of ligand and protein residues in 3dfr, 5p21 and 1gd1, respectively. Stars in magenta depict the water molecules bound to the ligand, most of which are bridging the ligand and the site residues. These stars describe a sphere in which the water molecules fluctuate about their mean position at the centre of the sphere, as described earlier. Other water molecules at the binding site, which interact only with the bridging water molecules and not with either ligand or protein, are shown as yellow circles. These water molecules are not directly involved in bridging interactions, but presumably contribute to the stability of the network of water molecules, linking the ligand and the protein residues at the binding site.

Water molecules at the binding site of NADPH in dihydrofolate reductase

Figure 2 shows water molecules at the binding site of dihydrofolate reductase bound to NADPH. A total of 10 water molecules are bound to the ligand, six of which are involved in bridging the ligand and the site residues; they are listed in Table 2. The details of these interactions are given in Table 3. The water molecules Wat^{373} and Wat^{326} are bound to each other and also make contact with the oxygens of AMN (adenine mononucleotide) ribose 2'-

phosphate; they do not directly interact with the protein. Wat^{302} is bound to Wat^{208}, which mediates the interactions of NMN (nicotinamide mononucleotide) ribose oxygen and the residues Asp^{125}, Thr^{126} and Gly^{17}. Wat^{302} is also bound to Wat^{301} which bridges the residue Thr^{126} with NMN ribose 5'-phosphate. Furthermore, Wat^{276}, bound to NMN ribose 5'-phosphate, indirectly interacts with the site residue Thr^{126} through Wat^{301}. In addition, Wat^{276} and Wat^{302} are held in position by Wat^{514} and Wat^{516}, respectively; the latter water molecules may possibly make further contacts with other protein residues. Table 2 gives the details of interactions of six water molecules involved directly in bridging the ligand and the site residues. Wat^{208} has the highest number of hydrogen bonds (five) and the lowest temperature factor (13.4 $Å^2$) of all, indicating a greater stability. The water molecules Wat^{279} and Wat^{439} have two hydrogen bonds each. Wat^{279} bridges the main-chain oxygen of Gln^{101} with the N7 of adenine. Wat^{401} mediates the interaction of Gln^{101} at OE1 with NMN ribose 5'-phosphate. Wat^{439} has a high temperature factor, yet is involved in bridging the hydroxyl oxygen of NMN ribose and OG1 of Ser^{48}. Wat^{318} interacts with Wat^{236} and bridges N1 of adenine with the main-chain oxygen of His^{77}. Six water molecules at the binding site of NADPH in dihydrofolate reductase directly mediate the interaction of NADPH and the site residues, and two water molecules (Wat^{276} and Wat^{302}) indirectly mediate through other bridged water molecules. It is interesting to note that there are other water molecules at the binding site, not directly involved in bridging the ligand and the site residues, but possibly contributing to stabilization of the protein–ligand interactions by forming an interconnecting network of hydrogen bonds with the bridging water molecules.

Water molecules at the binding site of the H-ras p21–GTP complex

The complex of H-*ras* p21 protein with guanosine-5'-triphosphate (GTP) has eight water molecules hydrogen bonded to the ligand (Fig. 3). The details of these interactions are given in Table 4. Of the eight water molecules at the site, five are involved in bridging the phosphate group of GTP with the protein residues. Wat^{172} and Wat^{173} have very low temperature factors (11.63 $Å^2$ and 8.22 $Å^2$, respectively) compared to those of other water molecules at the site, and can make five and six hydrogen bonds, respectively. Wat^{172} bridges the oxygens O2A, O2B and O2G of the phosphate group with residues Ser^{17} and Thr^{35}. The residues Ser^{17}, Thr^{35} and Asp^{57} interact with phosphate oxygens O2B and O2G of GTP. Wat^{175} bridges O1G of GTP with residues Thr^{35} and Gly^{60}. Wat^{175} makes its fourth hydrogen bond with Wat^{189}, which in turn interacts with O1G and Wat^{190}. Wat^{187} mediates the interaction of O2A of GTP and Glu^{31} and simultaneously interacts with Wat^{185}, while Wat^{170} bridges Asp^{33} with O2A of the phosphate. Wat^{281} is bound to N3 and is held in position by Wat^{282} and Wat^{378}. Wat^{282} bridges N1 of the guanine base with Wat^{281}.

Water molecules Wat^{185}, Wat^{190} and Wat^{378} do not directly mediate the GTP interaction with the site residues, but may hold the bridging water molecules in position. Thus, these water molecules may contribute to the stabilization of the hydrogen-bonding network involving the bridging water molecules. Wat^{185}, Wat^{190} and Wat^{378} could possibly be hydrogen bonded to other protein atoms.

In addition to the protein–GTP interactions, which are mediated by five water molecules at the site, it is interesting to note the complex network of hydrogen bonds

TABLE 4
DETAILS OF HYDROGEN-BONDING INTERACTIONS INVOLVING GTP, WATER MOLECULES AND PROTEIN ATOMS AT THE BINDING SITE OF H-*ras* PROTEIN BOUND TO GUANOSINE-5'-TRIPHOSPHATE

Ligand atom	Type of interaction[a]	H-bond distance (Å)	Bridging water molecule	B-factor of water molecule ($Å^2$)	Protein atom	Type of interaction[a]	H-bond distance (Å)	Water molecule	H-bond distance (Å)
O2A	Acc	2.86	170	13.63	N Asp^{33}	Don	2.91		
O2B	Acc	3.13	172	11.33	OG Ser^{17}	Daa	3.12		
O2G	Acc	3.37	172		OG1 Thr^{35}	Daa	3.20		
O2A	Acc	2.71	172						
O2G	Acc	3.06	173	8.22	OG Ser^{17}	Daa	3.13		
O2B	Acc	3.19	173		OG1 Thr^{35}	Daa	3.18		
			173		OD1 Asp^{57}	Acc	2.66		
			173		O Thr^{58}	Acc	2.61		
O1G	Acc	2.85	175	27.47	O Thr^{35}	Acc	2.96	189	2.85
			175		N Gly^{60}	Don	3.49		
O2A	Acc	2.93	187	32.42	O Glu^{31}	Acc	2.94	185	2.79
O1G	Acc	3.12	189	46.80				190	3.20
			189					175	2.85
N3	Acc	2.93	281	23.13				282	3.06
			281					378	2.84
N2	Don		282	53.25				281	3.06

[a] Acc is a ligand or a protein atom acting as an acceptor; Don is a ligand or a protein atom acting as a donor; and Daa is a ligand or a protein atom acting as both acceptor and/or donor.

TABLE 5
DETAILS OF HYDROGEN-BONDING INTERACTIONS INVOLVING NAD, WATER MOLECULES AND PROTEIN ATOMS AT THE BINDING SITE OF HOLO-D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

Ligand atom	Type of interaction[a]	H-bond distance (Å)	Bridging water molecule	B-factor of water molecule (Å^2)	Protein atom	Type of interaction[a]	H-bond distance (Å)	Water molecules	H-bond distance (Å)
O3	Acc	3.48	353	11.30				416	2.76
NO1	Acc	2.92	353						
NO1	Acc	2.91	354	11.76	N Asn^{180}	Don	2.80	380	2.90
NO2	Acc	2.75	356	7.14	N Gly^{9}	Don	3.12		
			356		N Gly^{12}	Don	2.92		
			356		O Ser^{95}	Acc	2.85		
AO3*	Acc	2.88	366	22.35				373	2.95
			366						
NO3*	Acc	3.02	367	12.29	O Glu^{94}	Acc	2.83	368	2.93
			367		OG Ser^{119}	Daa	3.12		
AO2	Acc	2.67	373	17.26				366	2.95
AN1	Acc	2.82	388	12.95	OD1 Asn^{6}	Acc	3.43		
			388		ND2 Asn^{6}	Don	3.06		
			388		O Glu^{76}	Acc	2.98		
NO3*	Acc	2.66	390	23.10					
NO2*	Acc	3.35	390						
AO1	Acc	3.10	416	15.61				353	2.76
			416					498	2.67
AO1	Acc	2.92	430	20.23				469	2.76
AO2*	Acc	2.69	478	32.47				528	2.85

[a] Acc is a ligand or a protein atom acting as an acceptor; Don is a ligand or a protein atom acting as a donor; and Daa is a ligand or a protein atom acting as both acceptor and/or donor.

formed by these water molecules, interconnecting the phosphate oxygens and the site residues with other water molecules through multiple hydrogen bonds. The temperature factors associated with the bridging water molecules are low and comparable to those of the protein atoms to which they are bound. We discuss this aspect in detail below. Thus, these bridging water molecules act like any other protein-site atom, mediating the interaction between the protein residues and the ligand, and may contribute to the stability of the complex.

Water molecules at the binding site of holo-glyceraldehyde-3-phosphate dehydrogenase

The asymmetric unit of holo-glyceraldehyde-3-phosphate dehydrogenase contains a tetramer. For our analysis we have considered only one subunit (O), as the contacts and geometry of the NAD molecule bound to the coenzyme are similar in all four subunits [33].

Multiple hydrogen-bonding interactions of water molecules involved in binding the ligand NAD to the coenzyme are shown in Fig. 4. The details of these interactions are given in Table 5. Of the 11 water molecules bound to the ligand, four take part in bridging the ligand and the protein atoms. Wat^{354} binds pyrophosphate oxygen NO1 with Asp^{180} and interacts with Wat^{380}. The residues Gly^{9}, Gly^{12} and Ser^{95} interact with NO2 of the pyrophosphate through Wat^{356}. NO3* of NMN ribose is bridged to the residues Glu^{94} and Ser^{119} by Wat^{367}. Furthermore, Wat^{367} interacts with Wat^{368} and Wat^{388} mediates the interaction of residues Asn^{6} and Gln^{76} with AN1 of the adenine ring, making four hydrogen bonds.

An additional feature that can be seen from Fig. 5 is the intramolecular interaction of the NAD molecule, mediated through one or two water molecules. Wat^{353} directly bridges the oxygens O3 and NO1. Wat^{416} bridges between AO1 of AMN phoshate and Wat^{353}. Wat^{363} and Wat^{366} form a chain between AMN ribose and AO2 of AMN phosphate. Oxygens of NMN ribose appear to be held in position by Wat^{390}. Water molecules Wat^{430} and Wat^{478} interact with AO1 and AO2* of the ribose, respectively, and may stabilize the other hydrogen-bonded networks formed by other bridging water molecules. It is evident from Fig. 4 that these water molecules at the binding site of glyceraldehyde-3-phosphate dehydrogenase also form a complex network of interconnected hydrogen bonds, stabilizing the NAD interaction with the site residues.

A general observation from different complexes is the preferential hydration of phosphate groups where these are present. The detailed analysis of water molecules at the binding sites of three protein–ligand complexes (3dfr, 5p21 and 1gd1) discussed above shows that the majority of the water bridges involve the phosphate oxygens compared to other ligand atoms.

The data presented in Table 2 with respect to bridging water molecules may be slightly different from those reported in the original papers on structure determination, because of the more stringent criteria of both angles

and distance we use to define hydrogen bonding. For example, in the case of L-arabinose binding protein bound to galactose, two water molecules at the binding site (Wat^{309} and Wat^{310}) are referred to as mediating protein–ligand interactions. According to our analysis, we find Wat^{309} satisfying both the angles and distance criteria for the hydrogen-bonding interactions, whereas Wat^{310} satisfies only the distance criterion.

Structural significance of water molecules at the binding sites

In order to understand the structural significance of water molecules at the binding sites, a comparative analysis has been made of the isotropic B-factors (temperature factors) of water molecules at the binding site and those of random surface water molecules bound to a single protein atom. Of the 19 protein–ligand complexes, seven complexes with more than three bridging water molecules in their binding sites have been chosen for this analysis; they are listed in Table 6. Hereafter, we refer to the binding-site water molecules as 'bound water molecules' and to the surface water molecules bound to one protein atom as 'surface water molecules'.

In Fig. 5, the apparent mean displacement $\overline{U}$ of each water molecule is plotted against the mean displacement $\overline{U}$ of the protein atoms to which it is bound, for both bound water molecules and surface water molecules. If the bound water molecules were to have any structural significance, the thermal displacements of bound water molecules would be expected to be relatively small compared to those of surface water molecules. In addition, the data corresponding to the bound water molecules are expected to be more linear compared to those of surface water molecules. The data in Fig. 5a are scattered for bound water molecules as well as for surface water molecules. As the data were taken from seven different data sets, individually refined to different *R*-factors, Fig. 5 does not show the expected features to indicate any structural significance of the bound water molecules. The scatter masks the individual contributions. The data in Fig. 5b, taken from H-*ras* p21, show the expected features to indicate the structural importance of bound water molecules at the site.

A 'matched-pair analysis' [31] of both bound water molecules and surface water molecules with their associated protein atoms has been made to demonstrate a 'general trend' observed in all seven complexes. Matched-pair analysis is the Student's *t*-test for paired data, where the given data are analysed to understand whether an 'observed feature' is significant through all the data. For all seven protein–ligand complexes listed in Table 6, the data are tested individually for both surface water molecules and bound water molecules. Probability values of less than 0.05 indicate a significant difference in the paired variables. From the significance probability values presented in Table 6, it can be seen that the differences in the mean displacements, $\overline{U}_{sw} - \overline{U}_{swp}$ (where $\overline{U}_{swp}$ is the mean thermal displacement of protein atoms bound to surface water molecules), are significant in the case of surface water molecules. In contrast, there is no significant difference in the mean displacements of bound water molecules ($\overline{U}_{bw}$) and those of the associated protein atoms ($\overline{U}_{bwp}$). This is true for all the complexes, except lactate dehydrogenase. The results suggest that the B-factors of the bridging water molecules at the binding sites are close to those of the protein atoms to which they are bound. Therefore, water molecules at the binding sites may be structurally significant. Table 6 confirms the structural significance of

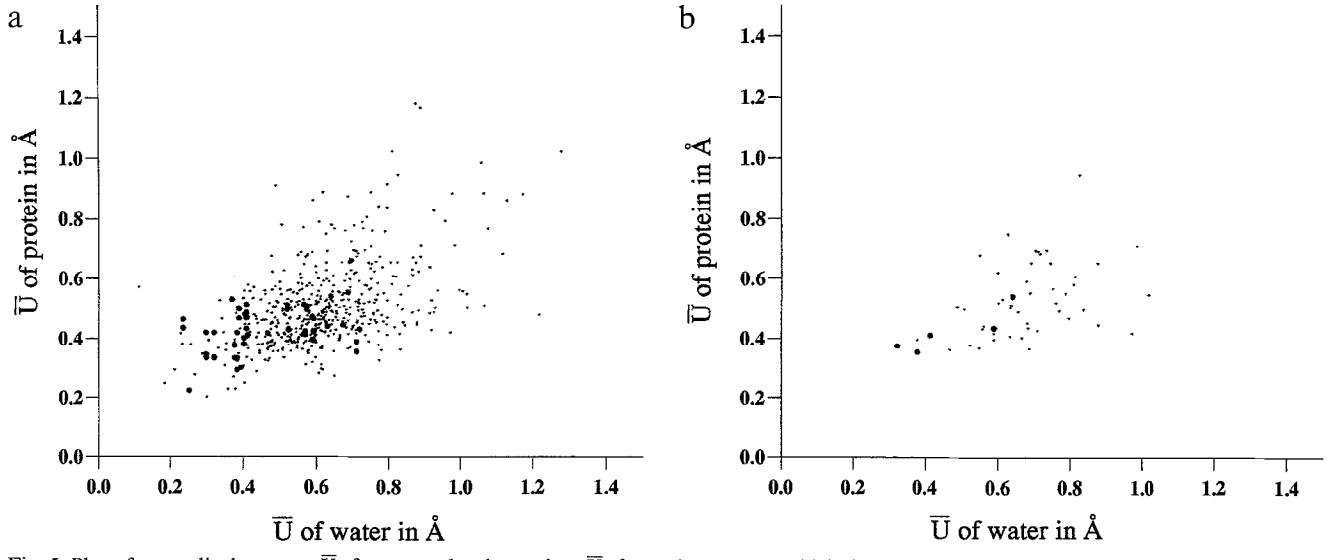

Fig. 5. Plot of mean displacement $\overline{U}$ of water molecules against $\overline{U}$ of protein atoms to which they are bound. Dots represent the data corresponding to surface water molecules and filled circles show the data corresponding to bound water molecules. Data for (a) were taken from seven different protein–ligand complexes listed in Table 6; data for (b) were taken from H-*ras* p21.

TABLE 6
SIGNIFICANCE PROBABILITIES FOR SURFACE WATER MOLECULES AND BOUND WATER MOLECULES AT THE SITE[a]

Protein–ligand complex	Surface water molecules			Bound water molecules			$\langle\overline{U}_{sw}\rangle$[d] (Å)	$\langle\overline{U}_{bw}\rangle$[e] (Å)	$\langle\overline{U}_{prot}\rangle$[f] (Å)
	No.[b]	$\langle(\overline{U}_{sw}-\overline{U}_{swp})\rangle$[c] (Å)	Probability	No.[b]	$\langle(\overline{U}_{bw}-\overline{U}_{bwp})\rangle$[c] (Å)	Probability			
Dihydrofolate reductase with NADPH	115	0.187	0.331×10^{-11}	8	0.104	0.151	0.723	0.586	0.572
Staphylococcal nuclease with 5'-deoxythymidine	33	0.111	0.276×10^{-2}	10	0.082	0.615	0.622	0.529	0.563
Lactate dehydrogenase with NADH and oxamate	50	0.095	0.28	7	–0.049	0.360	0.509	0.396	0.492
H-*ras* p21 protein with guanosine-5'-triphosphate	48	0.170	0.424×10^{-6}	10	0.067	0.380	0.684	0.469	0.531
D-Xylose isomerase with D-xylose	98	0.157	0.148×10^{-14}	6	0.110	0.023	0.634	0.593	0.488
Holo-D-glyceraldehyde-3-phosphate dehydrogenase	77	0.160	0.328×10^{-7}	9	0.057	1.000	0.567	0.371	0.442
Proteinase A with a tetrapeptide	81	0.112	0.992×10^{-5}	3	0.066	0.089	0.521	0.493	0.456

[a] Water molecules bound to a single protein atom are defined as surface water molecules; water molecules bridging the ligand and site residues are defined as bound water molecules.
[b] Number of water molecules.
[c] Mean of the difference in mean displacement of water molecules and the protein atoms to which they are bound.
[d] Mean of mean displacements of surface water molecules.
[e] Mean of mean displacements of bound water molecules.
[f] Mean of mean displacements of protein atoms.

these bound water molecules, where the average values of mean displacements, $\overline{U}_{sw}$ for surface water molecules, $\overline{U}_{bw}$ for bound water molecules and $\overline{U}_{bwp}$ for protein atoms associated with the bound water molecules, are listed for the same set of seven protein–ligand complexes.

B-factors and the number of H-bonds of bridging water molecules at the binding site

Careful scrutiny of Figs. 2–4 shows that the sizes of the star-like features are different for different water molecules at the binding site. It is interesting to note that the B-factors of the water molecules in general are inversely proportional to the number of hydrogen bonds that a water molecule can make (observe the B-factors of water molecules in Tables 3–5), indicating a decreased mobility of the water molecule. It can be seen from Figs. 2–4 that the B-factors of water molecules making multiple hydrogen bonds (four to six) are considerably smaller than those of water molecules making two hydrogen bonds. This further emphasises the structural importance of these water molecules at the binding site, which is confirmed using matched-pair analysis by comparing the mean displacement $\overline{U}$ of water molecules making one hydrogen bond with that of water molecules making multiple hydrogen bonds. The significance probabilities obtained for the water molecules making four and five hydrogen bonds are 0.0017 and 0.0131, respectively. These probability values indicate a significant difference in the B-factors of water molecules making multiple hydrogen bonds as compared to those making a single hydrogen bond. The mean displacements $\overline{U}$ of water molecules making two hydrogen bonds are not significantly different from those of water molecules making one hydrogen bond.

Discussion

In drug design, current de novo structure generation methods do not take into account the presence of water molecules at the binding sites in generating a candidate ligand structure, the reason being the lack of knowledge about possible water mediation in ligand binding. In this study, an attempt has been made to understand the rôle of water molecules in binding the ligand to its receptor. This study, based on 19 different high-quality protein–ligand complexes, suggests that water molecules at the binding sites can play a significant rôle by forming multiple hydrogen bonds, bridging the ligand with the protein-site atoms and possibly stabilizing the protein–ligand interactions. By comparing the B-factors of the bridging water molecules with their associated protein atoms through the use of a statistical test, it has been shown that the water molecules involved in mediating the protein–ligand interactions are as structurally important as the other polar protein-site atoms. Another interesting finding is the network of hydrogen bonds formed by the water molecules at the site, interconnecting the ligand with the protein and with themselves. These water molecules may possibly contribute significantly to the stabilization of protein–ligand interactions.

Bolin et al. [10] showed the importance of the water molecules at the binding site of the ternary complex of dihydrofolate reductase bound to NADPH and methotrexate (3dfr). Their crystallographic study demonstrated

that the contacts of the adenine ring and AMN ribose are essentially hydrophobic; the hydrogen bonds involving the adenine ring and the AMN ribose are made by the fixed water molecules. Our analysis (using a strict definition of hydrogen bonding) identifies all these bridging interactions, except the interaction between the AMN ribose and Arg^{44} through Wat^{401}. The program does not recognize this interaction, as the donor angle between water and donor group NH_2 of Arg^{44} is small (107°) and lies outside the donor angle limit defined in the program.

Crystallographic studies of L-arabinose-binding protein (ABP) with L-arabinose, D-fucose and D-galactose show the importance of water molecules at the binding site with their specificity and affinity in substrate binding [5]. The water molecules equivalent to those involved in substrate binding in ABP are absent in galactose-binding protein (GBP). The inability of GBP to bind L-arabinose and D-fucose is thought to be due to the absence of these equivalent water molecules in GBP. The importance of active-site water molecules is indicated in the crystallographic study of the complex of staphylococcal nuclease with deoxythymidine 3',5'-biphosphate [11], where N3 and O4 of the nucleotide base make no specific contacts with the enzyme but make hydrogen bonds with a string of water molecules. Skarzynsky et al. [15] have drawn attention to the presence of a large number of water molecules in the active-site pocket and around the NAD molecule. Crystallographic studies on the HIV proteases reveal the presence of a conserved water molecule at the active site, bridging the amide nitrogens of Ile^{50} and Ile^{150} and the ligand [24]. Our analysis confirms all these water-mediated interactions of ligand and protein site atoms. Some of them may not be listed in Table 2, as this study requires both the angle and distance criteria to be satisfied to recognise a hydrogen-bonding interaction. Recent crystallographic studies have revealed that a conserved water and its associated hydrogen-bond network at the active site is central to the structural transition between the oxidation states of cytochrome *c* [32]. The transition state of chloramphenicol acetyl transferase bound to chloramphenicol is suggested to be stabilized by a bound water molecule at the active site, through its multiple hydrogen bonds [18]. The protein–DNA interaction in *trp* repressor protein has been found to be stabilized only by water-mediated interactions, where there are no direct hydrogen bonds or nonpolar contacts between the DNA bases and the protein [33,34]. Four water molecules at the active site of human dihydrofolate reductase have been observed to have strong NOEs [6]. Two of the four long-lived water molecules mediate the interactions of methotrexate and the enzyme. The remaining two water molecules bridge between cofactor NADPH and the enzyme.

Our analysis finds many more hydrogen-bonding atoms at a hydrogen-bonding distance from a bridging water, apart from those presented in Table 2. These atoms fail to satisfy angle criteria for hydrogen bonding. This picture may be more meaningful in a dynamic situation, where the ligand approaches the site and binds. The water molecules at the site presumably make and break hydrogen bonds with the protein and ligand atoms as the complex is formed.

The water molecules are small compared to large polar residues and can easily occupy small potential voids and holes in the enclosed binding sites. In addition to the chemical nature of the binding-site surface, the physical shape may also influence the water binding. More precisely, the curved surface forming the small indentations on the site surface may be helpful in holding the water molecules more firmly. The influence of local surface shape of the binding-site surface on the water molecules bound in voids and deep grooves at the protein–ligand interface is presented in the second paper in this series [8]. Water molecules bound in grooves and cavities have been studied in great detail [35–37] and the buried water molecules have often been observed to be structurally conserved [38–43]. In the third paper, we examine the homologous structures of protein–ligand complexes to see if the water molecules at the ligand binding sites are conserved during evolution [9]. Structural conservation of water molecules at the binding sites emphasizes their importance in ligand binding.

To summarize the results of this study, most of the water molecules (nearly 80%) involved in bridging interactions can make three or more hydrogen bonds that bind ligand and protein atoms together with other water molecules in the site. There are water molecules at the binding sites that are not directly involved in bridging the ligand with the protein. These may contribute to the stabilization of the complex by interacting with other bridging water molecules, holding them in the right position. The water molecules at the binding sites form a complex network of hydrogen bonds, interconnecting the ligand with the protein-site residues through multiple hydrogen bonds. This suggests that water molecules at the binding sites of protein–ligand complexes may play a significant rôle in stabilizing the protein–ligand interactions. It is the size, the geometry, the polarity and the orientational flexibility of the water molecules that gives them structural and functional importance. The results of this study will be useful in predicting hydration sites at ligand-binding sites and may be applicable in automated methods for de novo drug design.

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