# SHORT COMMUNICATION

# Unconventional Interactions Between Water and Heterocyclic Nitrogens in Protein Structures

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***ABSTRACT*** **We report an unusual interaction in which a water molecule approaches the heterocyclic nitrogen of tryptophan and histidine along an axis that is roughly perpendicular to the aromatic plane of the side chain. The interaction is distinct from the well-known conventional aromatic hydrogen-bond, and it occurs at roughly the same frequency in protein structures. Calculations indicate that the water–indole interaction is favorable energetically, and we find several cases in which such contacts are conserved among structural orthologs. The indole–water interaction links side chains and peptide backbone in turn regions, connects the side chains in β-sheets, and bridges secondary elements from different domains. We suggest that the water–indole interaction can be indirectly responsible for the quenching of tryptophan fluorescence that is observed in the folding of homeodomains and, possibly, many other proteins. We also observe a similar interaction between water and the imidazole nitrogens of the histidine side chain. Taken together, these observations suggest that the unconventional water–indole and water–imidazole interactions provide a small but favorable contribution to protein structures. Proteins 2004;57:1–8.**


**Key words: protein structure; water–protein interactions; tryptophan fluorescence; molecular simulations; aromatic hydrogen bonds**

## INTRODUCTION

Through the hydrophobic effect and specific hydrogen-bonding patterns, water molecules contribute to the stability of proteins and their complexes. In addition to the hydrogen-bonding interactions with backbone amide and carbonyl groups and with the polarized atoms of side chains, water molecules may also donate hydrogen bonds to the aromatic rings of phenyalanine, tryptophan, and tyrosine residues, which have been known to act as hydrogen-bond acceptors (shown schematically on the right in Figure 1).[1,2] In fact, ab initio calculations of all possible interactions between a water molecule and an isolated indole in the gas phase indicate that the global minima correspond to hydrogen-bond donation to the aromatic ring and to the heterocyclic nitrogen.[3] Recently, another type of water–aromatic ring contact has been proposed where the lone pair of the water interacts with the purine π system in RNA.[4]

In the course of refining two, independent crystal structures of the DNA-binding domain of the Engrailed transcription factor at 2.1 Å resolution, we noted the persistent occurrence of a well-defined water molecule on the aromatic face of a conserved tryptophan.[5] However, the water molecule was not at the expected position for an aromatic hydrogen-bond but instead was positioned over the heterocyclic nitrogen along the vector approximately normal to the indole ring [Fig. 2(A)]. A similar water–indole interaction is found in other members of the homeodomain family. The apparent conservation suggests that the contact might be important structurally or functionally.

These observations encouraged us to explore further the indole–water interaction. We describe here energy calculations undertaken using the refined crystal structures of the Engrailed homeodomain. These calculations show that the indole–water interaction is energetically favorable, with contributions originating from both van der Waals and electrostatic effects. A comprehensive database search revealed the existence of similar interactions in other proteins. Strikingly, the interaction is often preserved among orthologous members of protein architectural families. The database search also revealed the prevalence of a similar interaction of a water molecule with the heterocy-

The Supplementary Materials referred to in this article can be found at http://www.interscience.wiley.com/jpages/0887-3585/suppmat/index.html

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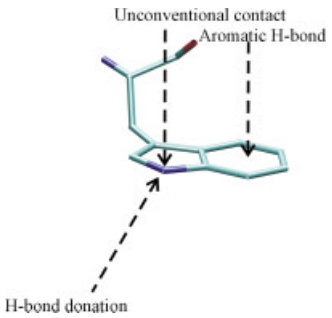


Fig. 1. A tryptophan and it potential points of polar interactions with the side chain. The unconventional contact is proposed here as a new water–indole association.

clic nitrogen of histidine. The salient results of this search are presented here, and these show how the interaction might play modest roles in stabilizing protein structures.

## MATERIALS AND METHODS

### Fluorescence Studies

Fluorescence was excited at 280 nm and emission spectra were scanned between 300 and 450 nm on a Perkin-Elmer LS 55 Luminescence Spectrometer. Buffer conditions were 50 mM sodium phosphate, 250 mM NaCl (pH 7.5) for native condition and 7.3 M Urea for denatured. The spectra were normalized to the maximum of the denatured state. Spectra were corrected for base-line, but not buffer profiles. The spectra in Figure 4 show a peak at 312 nm due to Raman scattering from water.

### Classical Calculations

Calculations have been performed using three structures (pdb codes; 1ENH, 1P7I, 1P7J) of the Engrailed homeodomain. For the wild-type protein, calculations used subunit A and one of the symmetric subunits according to cell symmetry; for K52A mutant, subunits C and D; and for K52E, subunits B and D. Hydrogens were added to the coordinates and optimized in a standard way. In this procedure, heavy atoms were fixed to their crystallographic positions.

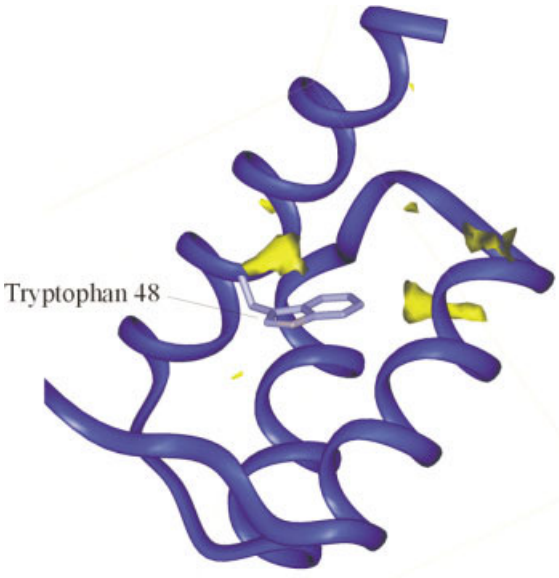


Fig. 3. Molecular interaction potentials contours in wild-type Engrailed homeodomain (1ENH). Regions with interaction energies (using a water probe) of −3 kcal/mol are shown in yellow.

All calculations were performed using the program cMIP,[6] which represent the interaction energy between a macromolecule and a probe molecule as the addition of an electrostatic and a van der Waals term. The electrostatic component was evaluated using the linear form of the Poisson-Boltzmann equation, with internal and external dielectric constants of 2 and 80. A low dielectric cavity was defined in all cases to contain both the protein and the water molecule under study. van der Waals interactions were determined using the standard Lennard-Jones formalism. AMBER force-field parameters were used to evaluate both electrostatic and van der Waals potentials.[7,8] The probe (in this case a water molecule) particle was placed in a regular grid centred in the position of the crystallographic water under study (wild type: water 424, K52A: water 98, K52E: water 80). The focusing procedure (with a final grid spacing of 0.5 Å) was used to improve the

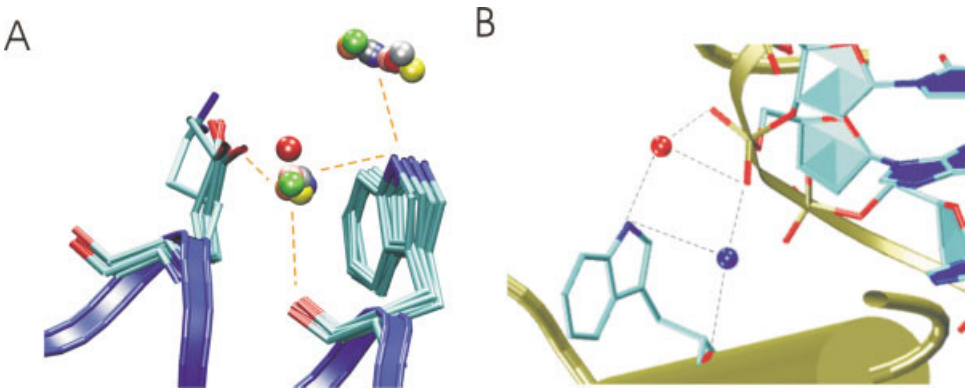


Fig. 2. The water–tryptophan interaction in the Engrailed homeodomain. (**A**) An overlay of the water in six copies from Engrailed homeodomain mutants (pdb codes 1P7I and 1P7J) and wild-type free (1ENH) and bound (3HDD). (**B**) The water in the protein–DNA complex (3HDD).

definition of the potential. In order to optimize the orientation of the water molecule in each grid position systematic rotational search in Euler space was performed.

### Quantum Mechanical Calculations

Density functional theory (DFT) was used to accurately characterize the intrinsic nature of water–indole interaction as well as to verify the accuracy of classical calculations. DFT calculations were carried out using the B3LYP function[9] and the 6-31G(d) basis set. In all the cases the interaction energies were by the basis set superposition error (BSSE) following Bois and Bernardi counterpoise method.[10]

We find that the magnitude of the gas-phase interaction between the indole and the water molecule obtained by Generalized Molecular Interaction Potential (gMIP)[11] calculations are very close to the values obtained at the B3LYP/6-31G(d) level, and also at the classical cMIP level. The 6-31G(d) basis set is used to describe atomic orbitals in all gMIP calculations.

### Database Surveys

The Macromolecular Structure Database[12] is a relational database for protein structure information and was used to search the protein structural database for examples of interaction of water with indole and imidazole. The database is organized in various data marts which are self-contained units of information about different properties of the protein structure data. The active site data mart is our first "derived information" data mart. This is generated by analyzing the environment around all the ligands found within the protein structures available in the database. These data include a catalog of all interactions between the ligand and protein molecule. A further extension to these data was to include information about interactions between water and protein residue (ARG, ASN, ASP, GLN, GLU, HIS, PRO, TRP, TYR) side chains. Two types of interactions were studied:

1. The interaction between the centre of the planar groups in the side chain and the water molecule (the pyrrole ring in the case of tryptophan)
2. The interaction between the water molecule and certain atoms in the side chain (e.g., heterocyclic N of TRP).

Once the data mart is populated, the data were searched using SQL queries to find instances of water molecules with restrictions on distance and angle just described. A detailed documentation and schema for the database is available at http://www.ebi.ac.uk/msd-srv/docs/dbdoc/.

The distance was chosen to select interactions that are at least within van der Waals contacts, and the conservative angle chosen was to restrict the cases to the near-normal orientation. We have used the same search limitations in the analysis of contacts to ring carbons, so that the results might be directly compared. In the supplementary material are graphs showing the distribution of distances between waters and indole/imidazole rings. The distance profiles show maxima at 3.0 Å and 2.7 Å to the plane center of indole and imidazole, respectively. The maxima for water/heterocyclic nitrogen are 3.6 Å for both imidazole and indole. Because the water–nitrogen interactions are longer than the water–plane center interactions, they are unlikely to be hydrogen-bonding contacts, as we discuss in the text. We also observe that the distribution of contacting waters as a function of solid angle increases monotonically in the range 0 to 5°, so we have not chosen an optimum for either the angle or the distance in the searches described here.

## RESULTS AND DISCUSSION

### A Conserved Tryptophan–Water Interaction in the Homeodomain Family

One of the most highly conserved residues in the homeodomain protein family is W48. Figure 2(A) shows the environs of W48 from several crystal structures of the *Drosophila* Engrailed homeodomain. Superimposed in the common reference frame of the indole ring are six independent copies from two stabilizing mutants and the free- and DNA-bound wild-type structures. In this overlay, a water molecule is seen to interact persistently with the heterocyclic nitrogen of W48 roughly along the normal to the indole plane. The water is orientated by the carbonyl group of the tryptophan, and in the K52E mutant, the water makes an additional hydrogen-bond to the E52 carboxylate. Also shown in Figure 2(A) is a second group of water molecules that are "in-plane" with the indole, and these form conventional hydrogen-bonds with the indole nitrogen. A similar "off-plane" water-tryptophan contact was found in several of the available homeodomain-DNA complexes, as represented by the Engrailed/DNA complex shown in Figure 2(B). In those complexes, one off-plane water and one in-plane water both interact with the same phosphate group of the DNA.

The off-plane waters in Figure 2(A) lie within 3.3 Å of the ring nitrogen and form a tight cluster, while the in-plane waters are closer to the nitrogen—about 2.8 Å, as expected for a conventional hydrogen bond—but these waters tend to be less tightly clustered than the off-plane group. Comparison of these two groups suggests that the off-plane interaction is a van der Waals contact that requires a more restrictive stereochemistry.

Inspection of the hydrogen-bonding patterns in the homeodomain–DNA complexes show the off-plane water is forming conventional hydrogen bonds with two obligate acceptors (a peptide carbonyl and the non-esterified phosphate oxygen). This can be seen in Figure 2(B), where the perspective is along the pyrimidal axis of the water. The location of the obligate acceptors fixes the positions of the water protons and, by inference, one of the water's lone pairs must be oriented toward the indole nitrogen's proton, but at a glancing angle of roughly 45°; the other lone pair is pointing away from the indole in the direction of the ring. From this, we deduce that the interaction is not a conventional hydrogen bond.

### Energy Calculations of the Water–Indole Interaction

To explore all the potential interactions of water with the protein, a classical molecular interaction potential (cMIP)[6] was calculated using a water probe and the X-ray coordinates in which the experimentally observed water molecules had been removed. This calculation reveals an energetically favorable pocket above the nitrogen group of W48, in agreement with the observations from crystallography (Fig. 3), and suggests that this pocket is among the most hydrophilic places in the protein. Calculations using other probes, such as $Na^+$, show less favorable values, indicating that the binding site is specific for water (data not shown).

To further characterize the interaction between the protein and off-plane water, we undertook classical energy calculations with the refined crystal structures of the K52A, K52E, and wild-type Engrailed homeodomains using the experimentally observed water positions. Energy values were estimated for the complete interaction with the protein (top lines for each protein entry in Table 1). These show that the interaction is stabilizing and that it combines both favorable electrostatic and dispersion contributions. Most of the energy can be attributed to interactions with W48 for the K52A and wild-type proteins (second lines for each protein in Table I). But for the K52E mutant, the additional hydrogen bond between the water and glutamate dominates the interaction energetically. We separated the interaction for the individual components: the carbonyl group, the entire backbone, and the entire indole side chain (third to fifth lines of each entry in Table I). This decomposition analysis show that the total Trp/water interaction energy is almost equally due to the interaction of the indole and the carbonyl groups. The indole interaction combines favorable electrostatic and van der Waals terms, while the carbonyl interaction is mostly electrostatic (Table I).

A simplified representation of the W48/water interaction was generated from an isolated methyl-indole and water pair with the same geometry as found in the protein structures. Energy calculations were undertaken of this simplified system in the gas phase (last line for each protein in Table I). The interaction energy remains favorable, even in the absence of the protein and solvent dielectric effects. Finally, single-point quantum mechanical calculations yield a stabilization energy of around −2.0 kcal/mol, in good agreement with the classical calculations. When the position and orientation of water was fully optimized in the gas phase, the energy of the water/indole pair decreases by only 0.8 kcal/mol, and the displacement of water from the position determined in the crystal was negligible, which suggests that the water occupies a local energy minimum (See Figure S1 in the supplementary material). Thus, the calculations support the conclusion that the interaction is favorable.

**TABLE I. Interaction Energies Between Optimized Water Molecules and the Protein[†]**

| | vdW | Electrostatic | Total |
|---|---|---|---|
| Wild type | | | |
| Protein–water | −2.87 | −2.41 | −5.28 |
| W48 | −1.00 | −3.42 | −4.42 |
| Backbone | −0.22 | −2.08 | −2.30 |
| (CO) | −0.08 | −2.06 | −2.14 |
| Side chain | −0.77 | −1.34 | −2.11 |
| Indole–Water (Gas) | −0.79 | −2.27 | −3.05 |
| K52A | | | |
| Protein–water | −2.20 | −3.25 | −5.45 |
| W48 | 0.12 | −3.25 | −3.13 |
| Backbone | 0.21 | −2.32 | −2.11 |
| (CO) | 0.38 | −2.22 | −1.84 |
| Side chain | −0.09 | −0.92 | −1.01 |
| Indole–water (Gas) | 0.03 | −2.11 | −2.08 |
| K52E | | | |
| Protein–Water | −1.84 | −9.00 | −10.84 |
| W48 | 0.60 | −3.96 | −3.36 |
| Backbone | 1.32 | −3.25 | −1.93 |
| (CO) | 1.50 | −3.13 | −1.63 |
| Side chain | −0.72 | −0.71 | −1.43 |
| E52 | 0.36 | −5.12 | −4.76 |
| Backbone | −0.22 | 0.63 | 0.41 |
| Side chain | 0.58 | −5.73 | −5.15 |
| Indole–water (Gas) | −0.68 | −1.34 | −2.02 |

[†]Columns indicate the van der Waals (vdW) and electrostatic components. Energy values are given in kcal/mol. The interaction energy for each residue has been decomposed into individual component energies (CO refers to the carbonyl group within the backbone). Only residues with interaction energies better than −1 kcal/mol are indicated. Values marked indole–water (gas) correspond to the interaction between a water and a methyl indole molecule in the corresponding positions in the gas phase.

### Fluorescence Studies of Engrailed Homeodomain Folding

It has been noted that the folding of homeodomains is associated with quenching of fluorescence of W48, but it is not clear what causes this effect. It has been reported earlier that the quenching is not due to interaction with a neighboring terminal amino group or aromatic hydrogen bonding.[5] We wondered if this effect might be attributed to the water/indole interaction described here. For instance, the water could orientate the tryptophan for electronic transfer to the peptide backbone.[13]

The fluorescence emission spectra are shown in Figure 4 for the Engrailed homeodomain wild-type protein and several of its mutants (K52A, K52E, K52L) in the folded and denatured states. We observe a 5-fold decrease in fluorescence intensity with folding for the wild type, which is in agreement with earlier reports,[14,15] and the mutants have a comparable extent of quenching. Interestingly, we find that the K52L mutant shows only a 2-fold decrease in fluorescence intensity with folding (Fig. 4). This mutant has the same folding free energy as the wild-type protein in the buffer conditions of the experiment[5] so the change in quenching is not attributed to stability. Although we were unsuccessful in our attempts to obtain the crystal struc-

**TABLE II. Relative Occurrence of Contacts Between Water and Heterocyclic Atoms and Ring Centers in the Protein Structural Database[†]**

| Amino acid | Contacts to heterocyclic nitrogen | Contacts to ring center[a] | Average number of contacts per ring carbon |
|---|---|---|---|
| Tryptophan | 150 | 155 | 78 |
| Phenylalanine | — | 170 | 55 |
| Tyrosine | — | 281 | 61 |
| Histidine | 225[b] | 276 | 180 |

[†]The numbers have been scaled in proportion to the frequencies relative to tryptophan. Distances are between 2.51 and 3.49 Å and within 5° of the normal to specified atom or the ring center. Only structures of 2.5 Å resolution or better were used in this analysis.
[a]For tryptophan, contact to center of the indole 5-membered ring.
[b]Average number of contacts for the two heterocyclic nitrogens.

ture of this mutant to determine the presence or absence of the water molecule, our modelling studies indicate that the side chain would most likely displace the orientation of the tryptophan and partially occlude the pocket for the water. We also observe red-shift of the emissions with folding of the wild type and K52A and K52E mutants, but a smaller shift for the less quenched K52L mutant. These observations are consistent with environmental differences of the tryptophans and suggest a possible role of the water molecule in quenching the tryptophan fluorescence.

### Database Searches

Using a relational database query system,[12] we examined the protein database for other examples of the indole–water interaction. Over a hundred protein structures from the databank were found where a water molecule is within 3.5 Å of the heterocyclic nitrogen and directed along the normal to the plane (within a solid angle of 5°).

We examined how frequently a water molecule is found in the off-plane orientation over the imidazole and indole nitrogens. The interaction is rare, representing only 0.13% of the tryptophans and 0.19% of the histidines in the structural database (with the conditions that the water is within 5° of the normal and the resolution of the structure is 2.5 Å or better). However, these values are similar to the corresponding fraction of tyrosines and phenylalanines that make the known water–aromatic hydrogen bond. The relative occurrence of water contacts to different atoms of the aromatic residues is summarized in Table II. Both the conventional aromatic hydrogen bonds and the unconventional water–indole contacts are more frequent than the equivalent water–ring carbon interaction, which suggest they are not simply random molecular contacts. The imidazole nitrogen/water interaction is comparatively less enriched over center-contacts, but this could be due to common modelling errors in choosing between the two possible orientations of the histidine ring in electron-density maps. The unusual water/indole and water/imidazole interactions were not noted in an earlier database analysis of water–side-chain interactions.[16] An analysis of these earlier data indicate the existence of the water/ring contacts, but at a lower frequency compared with conventional hydrogen bonding with the nitrogen, in agreement with our analysis.

A representative selection of proteins with this water–indole interaction is shown in Figure 5(A). In the majority of cases, the water is also supported through contacts with a backbone carbonyl and at least one additional group. Often, the indole-contacting water had a lower crystallographic temperature factor compared with neighboring waters, which suggests that it is comparatively well localized. As far as we can see, the interaction does not occur in the absence of adjacent hydrogen bonding interactions. We suggest that the interactions require a special peptide/side chain geometry, and it is unlikely to persist in solution for the unfolded state.

Specific examples of the water/indole and water/imidazole interactions are shown in Figure 5. For these representative structures, the water–nitrogen distances range from 3.0 to 3.4 Å, which indicates that the atoms are in van der Waals contact. This is in accord with the observations from the energy calculations for the homeodomain structure, which indicate that van der Waals contacts are a favorable component of the water–indole interaction.

In the Gelsolin protein, shown in Figure 5(A), the off-plane water links a β-strand to two α-helices and a loop through other water molecules. Also in this figure are shown a water in contact with W108 of lysozyme; this interaction links a section of loop to a short turn between helices. Also shown is a water–indole interaction that occurs in a turn and at an oligomeric interface in alcohol dehydrogenase. In catalase, neighboring helices are linked through a water molecule that pairs with a tryptophan. Finally, the water in hyaluronate lyase links side chains from alternating β-strands within a sheet. These examples (and others not shown) demonstrate that the water–indole interaction consolidates networks of conventionally hydrogen-bonding groups to help join different parts of the protein.

We also examined the database for examples of unconventional interactions of waters with histidine side-chain nitrogens and again found over a hundred examples. As can be seen in the example shown Figure 5(B), the histidine–water interaction may connect backbone with side chain and links neighboring structural elements—just as seen for the tryptophan–water interaction. The Nϵ appears to be preferred over the Nδ in these water–imidazole interactions.

In most of the above examples, the proteins are members of families for which several orthologous structures are available. We find in these cases that the indole–water interactions are maintained generally in identical positions among diverse family members. For instance, the sequence alignment of the lysase family indicates that the interacting tryptophan is conserved, as well as the tyrosine that forms a hydrogen-bonding network with the off-plane water [Fig. 6(A)]. An overlay of multiple orthologous structures in the reference frame of the tryptophan shows the conserved position of the contacting water molecule and the principal tyrosine [Fig. 6(B)].

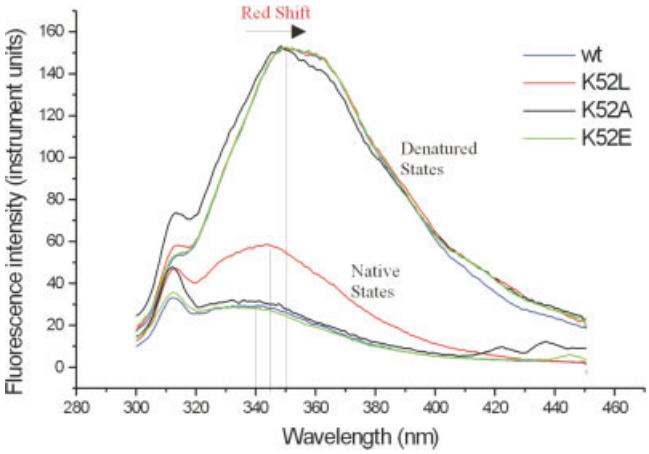


Fig. 4. Fluorescence characterization of Engrailed homeodomains. The denatured state is in 7.3 M Urea, the spectra were normalized to the maximum of the denatured state.

## CONCLUSION

In our studies presented here, we have identified nonconventional indole–water and imidazole–water interactions in protein structures. The results of database searches and calculations indicate that these interactions are energetically and structurally favorable.

Finally, we note that the water–indole interaction might explain some of the changes in tryptophan fluorescence that accompanies the folding of the homeodomain and most other proteins. A study with model peptides show that tryptophan fluorescence quenching may arise from electronic interaction with the peptide backbone.[13] This study found that fluorescence lifetimes of tryptophan drastically vary for each of its discrete rotameric states, which suggests that the quenching effect would occur only for restrained and defined conformations. We propose that the water's role in fluorescence quenching is indirect; for example, it may help define the relationship between the

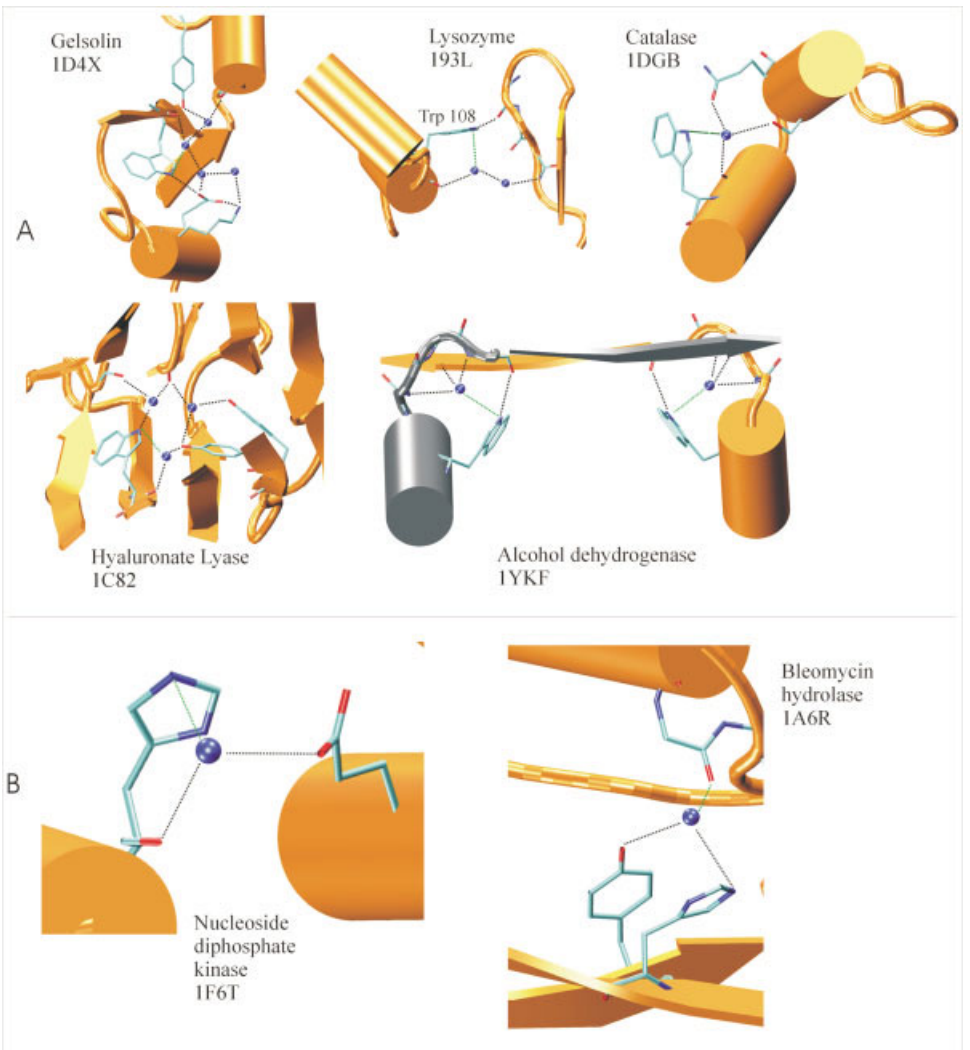


Fig. 5. (**A**) Representative examples of tryptophan–water interactions in the protein database. The structural sub-elements that are linked by waters are shown. The dotted lines represent conventional hydrogen bonds (black) and unconventional water–indole interactions (green). For each structure the entry code is given for the protein structural database. (**B**) Examples of off-plane histidine–water interactions.

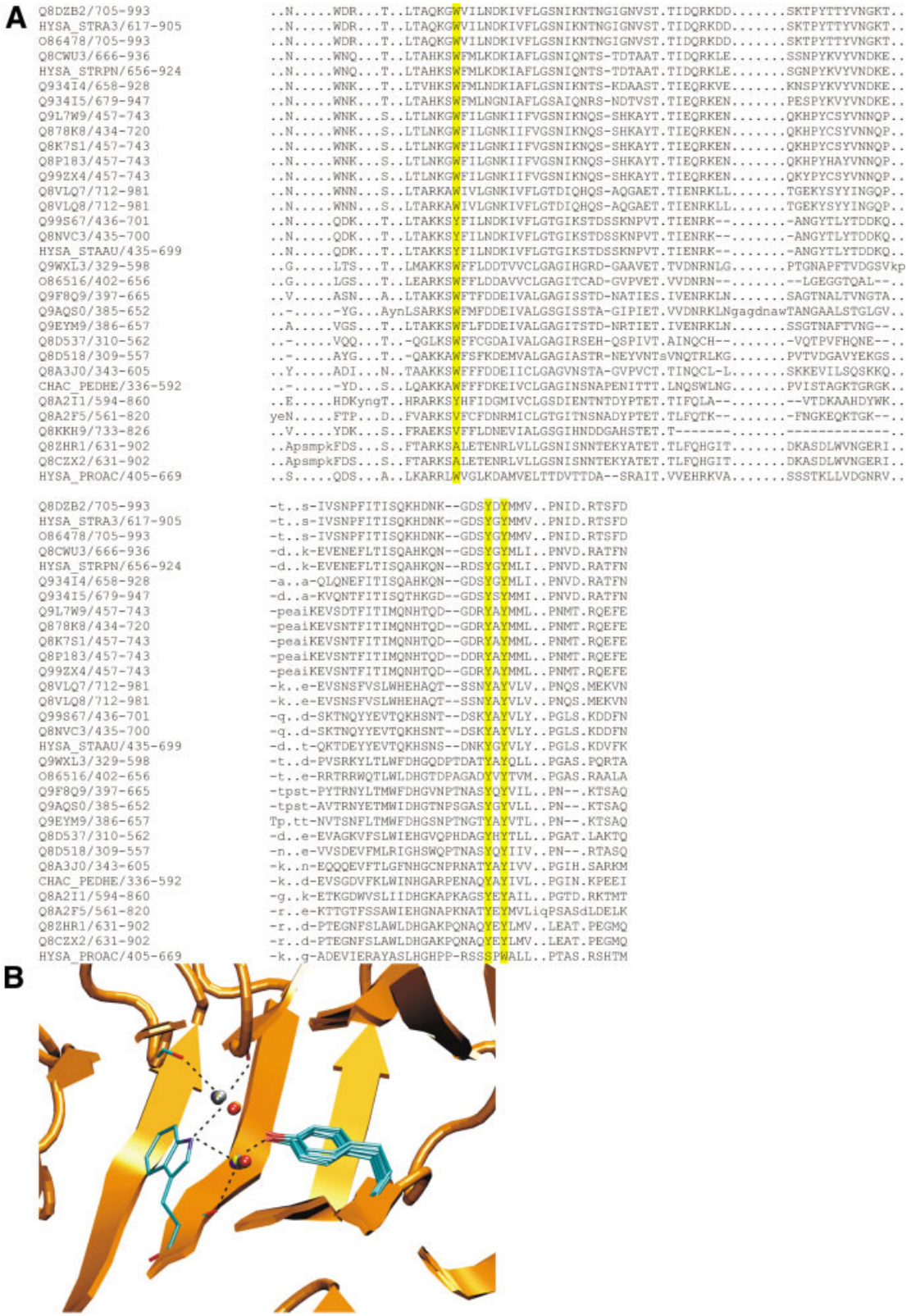


Fig. 6. Conservation of indole-water interaction in the hyaluronate family. (**A**) Alignment for the tryptophan and two tyrosines in the super β-barrel domain of hyaluronate lyase (32 members across the bacterial phylogeny from Pfam). The tryptophan and tyrosines involved in the interaction are highlighted in yellow. (**B**) An overlay of multiple orthologous structures in the reference frame of the tryptophan, showing the conserved orientation of the contacting water molecule and the principal tyrosine.

tryptophan and the peptide for electronic interaction. Additionally, the hydrogen bond to the peptide carbonyl might shift the electronic transition in the peptide bond to favor the electronic interaction between the indole and the peptide. The interaction is likely to exist only for defined geometries of the peptide backbone and tryptophan side chain, which create a special pocket for the water; this would not persist in the unfolded state and hence the fluorescence quenching of the tryptophan would be observed only with the transition to the folded structure.

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