Conserved water-mediated H-bonding dynamics of catalytic Asn 175 in plant thiol protease

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The role of invariant water molecules in the activity of plant cysteine protease is ubiquitous in nature. On analysing the 11 different Protein DataBank (PDB) structures of plant thiol proteases, the two invariant water molecules W1 and W2 (W220 and W222 in the template 1PPN structure) were observed to form H-bonds with the O_b atom of Asn 175. Extensive energy minimization and molecular dynamics simulation studies up to 2 ns on all the PDB and solvated structures clearly revealed the involvement of the H-bonding association of the two water molecules in fixing the orientation of the asparagine residue of the catalytic triad. From this study, it is suggested that H-bonding of the water molecule at the W1 invariant site better stabilizes the Asn residue at the active site of the catalytic triad.

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1. Introduction

Cysteine proteases are responsible for intracellular proteolysis of various types and an abnormal increase in their activity results in the degradation of muscle proteins (Komatsu *et al* 1986). Their activity is mainly controlled by the three residues, Cys 25, His 159 and Asn 175 of the catalytic triad (Polgár 1974). The papain molecule is folded to form two interacting domains, each comprising residues from both the N- and C-terminal sections of the polypeptide, which are separated by a cleft containing the active site. His 159, located at the β-sheet of the right domain, can polarize and interact with the SH group of Cys 25 at the N-terminus of the L1 α-helix of the left domain, forming a thiolate–imidazolium ion-pair which influences the substrate- or inhibitor-binding propensity of the protein (Polgár 1974). Asn 175 is found to stabilize the thiolate–imidazolium ion-pair (Vernet *et al* 1995) through the formation of an H-bond between its side chain amide oxygen atom and $N^{E2}H$ atom of the catalytic His 159. Since this bond is observed to be collinear to the covalent C_β–Cγ bond of His 159, the imidazole ring rotates about this bond without disrupting the hydrogen bond (Vernet *et al* 1995). Thus, the imidazole ring stays in an optimum position during the catalytic process.

In this study, we investigated the role of conserved water molecules around Asn 175 and their involvement in hydrogen-bonding interactions. It is proposed that the hydrogen bonds formed by oxygen atoms in water could influence the interaction of this Asn towards the catalytic triad. However, among the 13 conserved water molecules from the 11 different X-ray structures (with resolutions higher than 2.20 Å) of plant thiol proteases (table 1), only two are observed to form H-bonds with Asn 175 through the peptide backbone oxygen (O_b) atom. The average distances between these two conserved water molecules fall between 5.4 and 5.8 Å and they subtend an angle of ~120°–130°

Keywords. Conserved water in molecular recognition; MD simulation; plant cysteine protease

Abbreviations used: CHASA, conditional hydrophobic accessible surface area; IMD, interactive molecular dynamics; PDB, Protein DataBank; RMSD, root mean square deviation

with the O_b of Asn 175. The role of one of these two water molecules (W222, which occupies the hydrophilic site of 1PPN) has recently been described (O'Farrell and Joshua-Tor 2007). However, the other conserved water molecule (W220 in 1PPN) occupies a position that seems to hold the Cys 25 and Gln 19 residues (which are involved in the oxyanion intermediate) in the proper position through the H-bond with both the O_b atom of Asn 175 and N_b atom of Lys 17. Thus, determining the role of Asn 175 in the catalytic process, that is, ion-pair stabilization, is thought to be important.

2. Materials and methods

All the X-ray structures of plant thiol proteases were taken from the Protein Data Bank (PDB) (Berman *et al* 2000) and their important parameters are included in table 1. Only one molecule was observed to be present in all the crystals – 1PPN (Pickersgill *et al* 1992), 9PAP (Kamphuis *et al* 1984), 1PE6 (Yamamoto *et al* 1991), 1PPP (Kim *et al* 1992), 1KHP (Janowski *et al* 2004), 1KHQ (Janowski *et al* 2004), 1BP4 (LaLonde *et al* 1998), 1AEC (Varughese *et al* 1992), 2ACT (Baker and Dodson 1980), 1MEG (Katerelos *et al* 1996), 2CIO (Alphey and Hunter 2006). Two other mutant structures, 2DZZ (O'Farrell and Joshua-Tor 2007) and 2E00 (O'Farrell and Joshua-Tor 2007), of bleomycin hydrolases were also included for the molecular dynamics (MD)-simulation studies only.

2.1 *Identification of conserved structural water molecules*

The conserved water molecules of plant thiol proteases in their X-ray structures (table 1) were identified by a standard least-square fitting algorithm using the Swiss PDB Viewer program (Guex *et al* 2001). The X-ray structure of 1PPN was taken as the template and the reference structures were superimposed on the backbone atoms of the template structure and the root mean square deviation (RMSD) values were within ~0.3–0.7 Å. After superimposing the concerned individual structures on the template (1PPN), the conserved water molecule occupation sites were compared and located between the two respective structures included in table 2. Water molecules that were found to be within 1.5 Å (Mustata and Briggs 2004) in the PDB structures were taken as conserved.

2.2 *Water mobility*

The mobility of the crystal water molecules was calculated by using the respective B-factors (Mustata and Briggs 2004).

$$\text{Mobility } W_i = \frac{\text{BWi/B-aver}}{\text{OWi-O-aver}}$$

where BWi represents the temperature factor for the particular crystal water molecule, and B-aver denotes the average temperature factor for all the water molecules present in the respective X-ray structures. Occupancy of OWi indicates occupancy for *i*th water molecule (usually 1) and O-aver is the average occupancy of all water molecules present in the individual structure.

2.3 *Energy minimization of PDB structures*

All the PDB structures of plant thiol proteases (including water molecules) were taken for energy minimization.

Table 1. Surveyed crystal structures of some plant thiol proteases and bleomycin hydrolase

PDB code (Year deposited)	Resolution [Å]	R-factor	Mutation (if any)	No. of water molecules	Solvent other than water	Reference
1PPN* (1991)	1.60	0.16	No	226	1	Pickersgill *et al* 1992
9PAP (1986)	1.65	0.161	No	195	29	Kamphuis *et al* 1984
1PE6 (1991)	2.10	0.159	No	181	14	Yamamoto *et al* 1991
1PPP (1993)	1.90	0.194	No	205	1	Kim *et al* 1992
1KHP (2001)	2.00	0.185	No	53	–	Janowski *et al* 2004
1KHQ (2001)	1.60	0.149	No	105	-	Janowski *et al* 2004
1BP4 (1998)	2.20	0.190	No	88	-	LaLonde *et al* 1998
1AEC (1992)	1.86	0.145	No	268	-	Varughese *et al* 1992
2ACT (1979)	1.70	0.171	No	272	-	Baker and Dodson 1980
1MEG (1996)	2.00	0.193	D158E	94	-	Katerelos *et al* 1996
2CIO (2006)	1.50	0.179	No	161	-	Alphey and Hunter 2006
2DZZ (2006)	2.15	0.190	N392V	258	-	O'Farrell and Joshua-Tor 2007
2E00 (2006)	2.00	0.212	N392L	259	-	O'Farrell and Joshua-Tor 2007

Table 2. Invariant water molecules in the different X-ray crystal structures of plant thiol protease

S. no.	1PPN	9PAP	1PE6	1PPP	1KHP	1KHQ	1BP4	1AEC	2ACT	1MEG	2CIO
1	215	4	238	273	302	312	415	3	3	247	24
2	216	1	237	221	301	304	413	7	7	248	11
3	217	7	241	289	325	303	450	9	9	223	130
4	218	3	242	224	319	307	421	2	2	225	38
5	220	8	240	222	303	302	411	14	14	224	131
6	221	13	264	404	345	301	449	26	26	221	129
7	222	2	263	246	323	305	453	8	8	236	134
8	232	10	244	226	321	323	423	5	5	251	39
9	237	5	251	363	336	317	414	17	17	258	108
10	252	19	250	351	311	341	436	15	15	281	101
11	254	27	262	395	331	316	457	16	16	240	146
12	259	12	254	240	313	315	438	29	29	234	109
13	279	16	228	223	346	308	451	6	6	220	132

*1PPN is taken as the template structure.

*All the equivalent water molecules of the different structures were placed horizontally retaining their original PDB numbering scheme (as present in the individual PDB structure).

This was performed by a GROMOS 96 force field (Van Gunsteren and Mark 1992) (500 steps of the steepest descent followed by 1000 cycles of conjugate gradient) implemented in the Swiss PDB Viewer program with a 10 Å cut-off distance (Pisabarro *et al* 1994) for non-bonded interactions and a distance-dependent dielectric constant. During minimization, all residues and water molecules were allowed to move freely.

2.4 *Solvation*

All the water molecules and inhibitors present in the X-ray structures were removed and all the PDB structures were then solvated by using the conditional hydrophobic accessible surface area (CHASA) program (Fleming *et al* 2005).

2.5 *MD simulation*

The 22 PDB- and CHASA-solvated structures were initially energy-minimized (100 cycles to eliminate initial contacts which would destabilize the integrator) using the CHARMM force field (Brooks *et al* 1983). After energy minimization, all the aquated structures (crystal and CHASA water molecules) were simulated using auto-interactive molecular dynamics (IMD) connected between the visualization program Visual Molecular Dynamics v. 1.8.5 (Humphrey *et al* 1996) and the MD program Nanoscale Molecular Dynamics v. 2.6 (Nelson *et al* 1996; Phillips *et al* 2005). The simulation was run on a workstation Xeon® 5160 hardware system of 3 GHz and 2 Gb RAM. MD simulations were performed for every structure by selecting a 10 Å zone around the Asn 175 residue of the catalytic triad for the IMD molten zone and fixing an 8 Å zone around the IMD-molten zone for the IMD-fixed zone. The MD of all the structures were followed for 2 ns at 300 K temperature and a 2 fs time-step by means of Langevin dynamics using the CHARMM force field, which relaxed the water molecules and propagated them towards the structural equilibrium position. The fluctuations in potential energy, kinetic energy and total energy were monitored. Plots of the potential energy and kinetic energy against time at constant temperature revealed that the equilibrium was closed when the average potential energy and kinetic energy were constant, and the simulations were adequately converged within 2 ns. Different snapshots were taken at 0.5 ns intervals during the 2 ns simulation, which clearly revealed similar types of water-mediated H-bond distances between different residues of the protein structures. Moreover, the two other mutant X-ray structures (2DZZ and 2E00) of bleomycin hydrolase were also simulated for up to 2ns.

3. Results and discussion

On detailed analysis of the X-ray structures, 13 water molecules were found to be structurally conserved in all the eleven plant thiol proteases (table 2). The two conserved water molecules W220 and W222 were seen to interact with Asn 175 of the catalytic triad in the template 1PPN structure and the equivalent conserved water molecules in the other

structures are included in table 2. Here, W1 and W2 are assumed to be equivalent to the W220 and W222 water molecules of the 1PPN template structure and corresponding water molecules in the other structures.

The water molecules W1 and W2 form H-bonds with the backbone oxygen (O_b) atom of Asn 175. However, the W2 to O_b distance was observed to be slightly more than the W1 to O_b distance in most of the structures. The temperature factors (B-factors) and mobilities of the two water molecules (W1 and W2) are given in table 3. The comparable average B-factors and the subsequent mobility of these two conserved water molecules in most of the structures with their H-bonding affinities towards Asn 175 indicate the structural importance of these conserved water molecules.

The asparagine residue of the catalytic triad has been found to form two more H-bonds, one with the backbone nitrogen (N_b) atom of Asn to the backbone oxygen (O_b) atom of Gly 185 at an average distance of 3.2 Å, and the other with the side chain oxygen atom of Asn with the backbone nitrogen (N_b) atom of the Ser 176 residue at an average distance of 2.90 Å .

In all the X-ray structures, the W1 water molecule was observed to be present within H-bonding distance from both the O_b atom of the catalytic Asn 175 residue and backbone nitrogen (N_b) atom from Lys 17 (Arg 17 in 1MEG) (figure 1) with an O_b to W1 to N_b angle ~120° (table 3). Interestingly, another water molecule was also observed to form an H-bond with the W1 water molecule in all the PDB structures. The appearance of a water molecule at the W1 site has also been indicated and supported in the auto-solvated (CHASA) X-ray structures and energy-minimized structures (table 4), although no other water molecule was observed to occupy the W2 water molecule position in the solvated PDB structures.

Besides the biological or structural importance of the W2 water molecule, the involvement of the W1 water molecule in the Cys 25–His 159 ion-pair stabilization through interaction with Asn 175 was also seen in all the

Table 3. H-bonding distances (Å), angles (in degree) and the different parameters of the two invariant water molecules (W220 and W222 of 1PPN and their equivalent water molecular sites [W1 and W2] in the other plant thiol protease PDB structures)

	W1		W2		
PDB of protein	Water molecular PDB-id (distance from Asn 175 O_b*atom, Lys 17 N_b**/ Angle O_b – W1 – N_b) [B-factor of W1 in Å^2] Mobility	H-bonding association with other water molecules and the distances / (Å)	Water molecular PDB-id (distance from Asn 175 O_b atom) [B-factor of W2 in Å^2] Mobility	Distance between the two water molecules W1 and W2	Angle (°) subtended at Asn 175 O_b* by W1 and W2 W1–O_b–W2
1PPN	W220 (2.80/2.83/117.25) [9.95] 0.2628	W 219 (2.68)	W222 (3.22) [7.26] 0.1917	5.41	127.64
9PAP	W8 (2.80/2.70/118.15) [10.09] 0.2466	W28 (2.34)	W2 (3.48) [7.19] 0.1757	5.61	120.01
1PE6	W240 (2.93/2.85/116.09) [10.45] 0.2709	W239 (2.74)	W263 (3.63) [5.95] 0.1542	5.79	123.66
1PPP	W222 (3.06/2.99/107.71) [5.20] 0.1800	W 288 (2.45)	W246 (3.41) [4.01] 0.1388	5.83	128.53
1KHP	W303 (2.67/2.83/124.13) [22.14] 0.6065	W330 (2.56)	W323 (3.54) [25.39] 0.6955	5.50	123.94
1KHQ	W302 (2.78/2.85/118.67) [16.61] 0.4668	W393 (2.77)	W305 (3.38) [15.8] 0.4440	5.51	126.74
1BP4	W411 (2.87/2.79/113.89) [6.90] 0.2470	W468 (2.62)	W453 (3.37) [14.43] 0.5167	5.60	127.58
*1AEC	W14 (2.72/2.90/120.32) [5.61] 0.1621	W 19 (2.80)	W8 (3.52) [5.59] 0.1615	5.58	126.26
*2ACT	W14 (2.82/2.85/117.59) [10.63] 0.2949	W 19 (2.76)	W8 (3.52) [9.39] 0.2605	5.61	127.82
*1MEG	W224 (2.91/2.68/117.68) [5.76] 0.2784	W 222 (2.93)	W236 (3.13) [6.35] 0.3070	5.38	125.77
2CIO	W131 (2.72/2.78/115.70) [10.20] 0.2968	W51 (2.67)	W134 (3.28) [9.04] 0.2631	5.45	130.43

*Lys 17 and Asn 182 – for 1AEC, 2ACT. **Arg 17 and Asn 179 – for 1MEG.

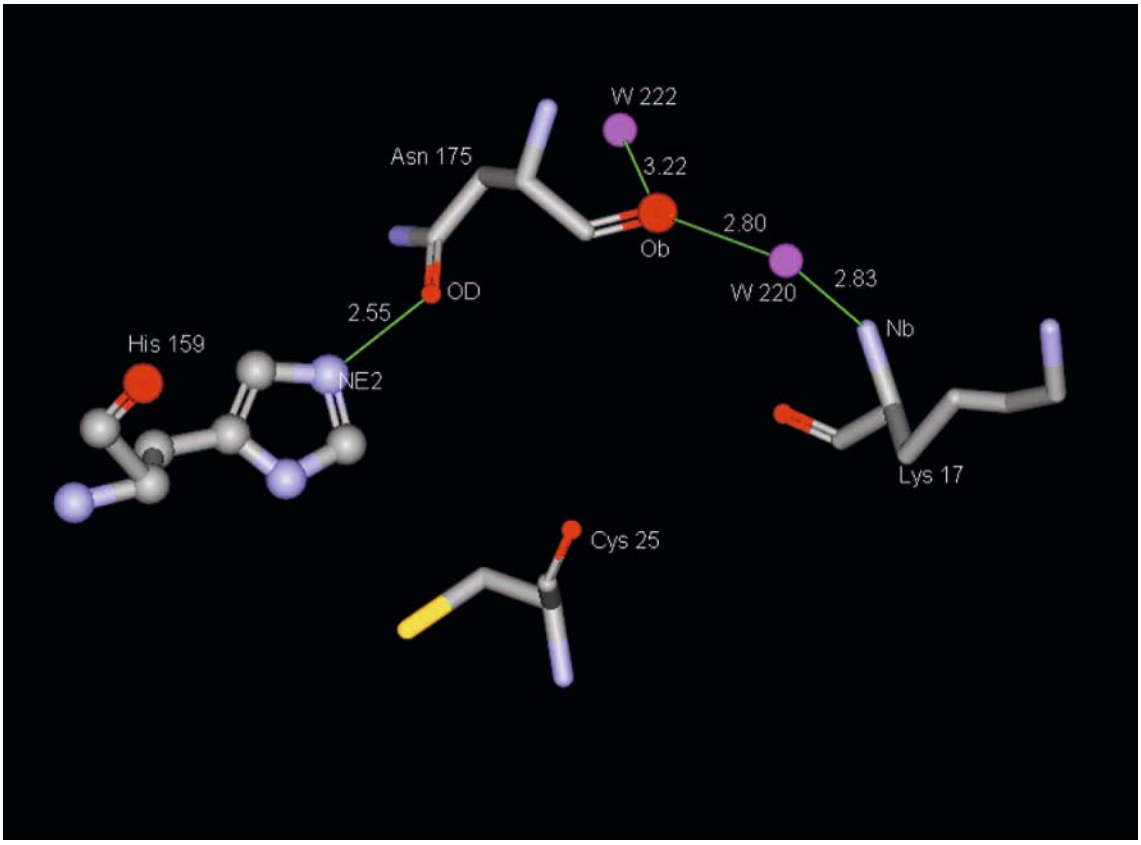

Figure 1. The H-bonding network of W1, W2 and asparagine residue of the catalytic triad.

Table 4. H-bonding parameters of the invariant W1 water molecule with the Asn 175 and Lys 17 residues in the different energy minimized (E-Min), PDB and auto-solvated structures

Protein (PDB water molecule) (positionally equivalent water molecule in the EM PDB and auto-solvated structures)	Distance of W1 (Å) from the Asn 175 O_b/Asn 182 O_b*/Asn 179 O_b** in the EM PDB and auto-solvated structures	Distance of W1 (Å) from the Lys 17 N_b/Arg 17 N_b** in the EM PDB and auto-solvated structures	Angle O_b–W1–N_b (°) in both the EM PDB and auto- solvated structures
1PPN (W220)/[W321]	2.96/2.50	2.90/2.95	130.63/123.73
9PAP (W8)/[W314]	3.20/2.51	2.95/2.95	140.01/119.49
1PE6 (W240)/[W316]	3.33/2.93	2.90/2.95	132.14/113.02
1PPP (W222)/[W321]	3.73/2.92	6.58/2.95	60.73/112.54
1KHP (W303)/[W322]	3.59/2.82	3.15/2.95	128.26/114.86
1KHQ (W302)/[W317]	3.24/2.71	2.72/2.95	120.18/117.15
1BP4 (W411)/[W323]	3.40/2.75	2.88/2.95	116.76/112.57
1AEC (W 14)/[W336]	3.44/2.60	2.72/2.95	129.23/123.09
2ACT (W 14)/[W335]	2.73/2.56	2.72/2.95	127.96/122.92
1MEG (W224)/[W331]	3.65/2.49	2.83/2.95	127.87/123.02
2CIO (W131)/[W320]	3.05/2.48	2.82/2.95	118.81/117.72

*Lys 17 and Asn 182 $^-$ for 1AEC, 2ACT **Arg 17 and Asn 179 $^-$ for 1MEG.

*Lys 17 and Asn 175 $^-$ for other PDB structures.

energy-minimized structures. After energy minimization, the W2 water molecule shifts from 2.80 Å to an average distance of 5.95 Å from the Asn 175 O_b atom, whereas the W1 water molecule almost retains its original previous

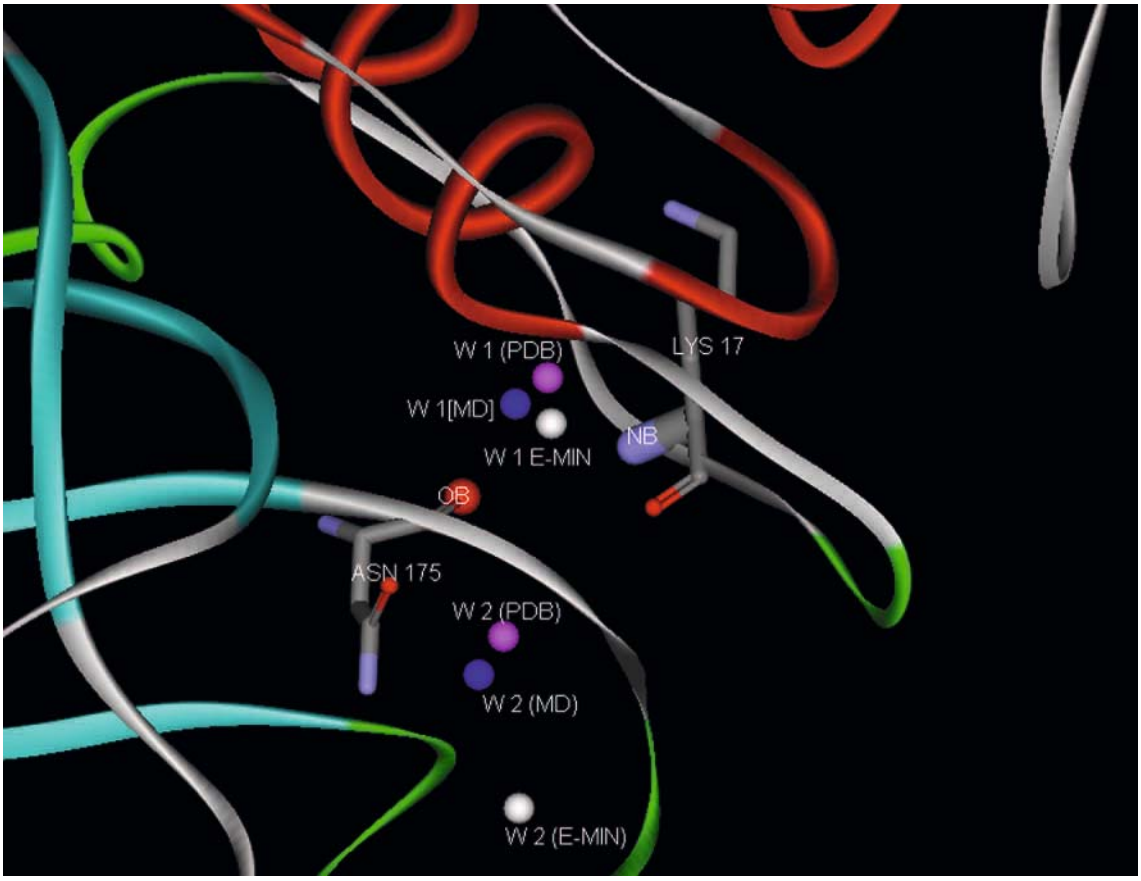

Figure 2. The relative positions of the W1 and W2 sites in PDB, E-Min (energy-minimized) and MD (molecular dynamics)-simulated structure

position. However, if the two water molecules W1 and W2 are removed from the structures and all the other water molecules are retained, energy minimization reveals the appearance of a new water molecule (W219 in 1PPN) at the W1 site near Asn 175. This W219 water molecule (which is not H-bonded to any residue of the protein) is at a distance of ~2.68 Å from the W1 invariant site. However, no water molecules are observed within 3.5 Å of the conserved molecular position of W2. Again, following energy minimization, retaining all water molecules except W1 reveals the appearance of another water molecule at the W1 invariant site, which indicates the importance of the hydrophilic W1 water molecule. Thus, the invariant position of the W1 water molecule may be indispensable in the architecture of the papain fold or protease activity.

The MD simulation studies of three CHASA-solvated PDB structures (1KHP, 2ACT and 1AEC) clearly revealed the presence of the W1 water molecule at a conserved hydrophilic centre. However, another water molecule was observed to occupy the position during the 2 ns simulation of the 1PE6, 1PPP, 1BP4 and 2CIO structures. During the simulation, the W1 water molecule was observed to form H-bonds with both the O_b of Asn 175 and N_b atom of Lys 17 at average distances of ~2.67–3.3 Å and ~2.97–3.23 Å, respectively, in the CHASA-solvated and X-ray structures (table 5) (figure 2). Some discrepancies in the H-bond distances were observed in the 9PAP and 1MEG structures. It should be noted that the W2 water molecule site was not found to be occupied during MD simulation in all CHASA-solvated structures, implying that H-bonding of the W1 water molecular site is more pertinent when compared with the W2 conserved centre. During the simulation of up to 2 ns, the consecutive fluctuation of W1 to O_b (Asn 175) H-bond lengths was from 2.81 to 3.45 Å, and W2 to O_b (Asn 175) was 2.64 to 3.37 Å (at successive 0.5 ns intervals). The RMSD calculation of the backbone atoms of individual conformation (relative to the minimized crystal structures) at different time intervals showed the involvement of the W1 and W2 water molecules in fixing the orientation of the Asn residue of the catalytic triad. The involvement of the two water molecules was further studied by MD simulation of the two mutant (X-ray) structures, 2DZZ and 2E00. On analysis of the MD simulation of 2DZZ

Table 5. Molecular dynamics – simulation studies of the different plant thiol protease structures

Protein	Water present before MD at W1 position in the auto-solvated PDB structures with the distances (Asn 175 O_b atom / Lys 17 N_b) in (Å) and angle (O_b–W1–N_b) in degrees	Water present after MD at W1 position in the auto-solvated PDB structures with the distances (Asn 175 O_b atom/ Lys 17 N_b) in (Å) and angle (O_b–W1–N_b) in degrees	W 1 position in the MD-simulated X-ray structures (distances of W1 to Asn 175 O_b/Lys 17 N_b and angle O_b –W 1–N_b)
1PPN	W 321 (2.50 / 2.95) / 123.73	W 297 (2.67 /3.21) / 129.60	3.45 / 2.88 / 105.12
9PAP	W 314 (2.51 / 2.95) / 119.49	W 314 (6.26 /4.73) / 76.04	2.65 / 3.21 / 119.74
1PE6	W 316 (2.93 / 2.95) / 113.02	W 293 (2.80 / 3.23) / 154.61	2.80 / 2.77 / 159.34
1PPP	W 321 (2.92 / 2.95) / 112.54	W 299 (2.82 / 3.6) / 132.38	3.10 / 3.88 / 87.21
1KHP	W 322 (2.82 / 2.95) / 114.86	W 322 (3.14 / 2.89) / 140.60	2.60 / 2.76 / 122.20
1KHQ	W 317 (2.71 / 2.95) / 117.15	W 343 (2.77 / 6.9) / 61.07	2.94 / 3.02 / 124.57
1BP4	W 323 (2.75 / 2.95) / 112.57	W 296 (2.64 / 2.97) / 161.96	2.70 / 3.33 / 118.74
1AEC	W 336 (2.60 / 2.95) / 123.09	W 336 (3.30 / 3.09) / 126.82	3.01 / 2.97 / 159.83
2ACT	W 335 (2.56 / 2.95) / 122.92	W 335 (2.71 / 2.97) / 126.30	2.84 / 3.23 / 130.34
1MEG	W 331 (2.49 / 2.95) / 123.02	W 357 (3.32 / 6.16) / 63.93	3.14 / 2.85 / 116.00
2CIO	W 320 (2.48 / 2.95) / 117.72	W 299 (2.89 / 3.0) /121.24	2.65 / 2.79 / 172.84

(where Asn 392 is mutated by Valine), no water molecule was found to occupy the W1 and W2 invariant sites, and the SG atom of the catalytic Cys 73 residue shifted by around 0.9 Å. But in 2E00 (where Asn 392 is mutated by Leucine), the two water molecules were found to occupy the W1 and W2 invariant sites and the SG atom of the catalytic Cys 73 shifts by 0.3 Å only. With the fluctuation of the H-bond distances between the water molecules and backbone oxygen atom, the side-chain orientation of the catalytic asparagine may possibly change due to the change in the H-bonding pattern of Asn during simulation. Hence, the W1 and W2 water molecules may be thought to play a role in fixing the orientation of the side chain of catalytic Asn and thus controlling the H-bond distances between its side chain amide oxygen atom and the $N^{E2}H$ atom of the imidazole ring of catalytic His 159.

4. Conclusions

These results suggest that both the water molecules (W220 and W222 of 1PPN) at the W1 and W2 invariant sites are responsible for orientating the asparagine residue of the catalytic triad, which is important for the formation of the H-bond between its side chain amide oxygen atom and the $N^{E2}H$ atom of the imidazole ring of catalytic His 159. From this study, it is suggested that H-bonding of the invariant site of the W1 water molecule is more important in stabilizing the asparagine residue at the active site of the catalytic triad.

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