Molecular dynamics analysis of conserved water mediated inter-domain recognition of His667-Trp669 in human ceruloplasmin

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Abstract:
The human ceruloplasmin (hCP) is the copper containing ferroxidase enzyme with multifunctional activities (NO-oxidase, NO2-synthase, oxidation of neurotransmitters including antioxidants). Therefore, it is of interest to probe the multi-domain hCP using molecular dynamics simulation. Results explain the role played by several conserved water centers in the intra and inter-domain recognition through H-bond interaction with the interacting residues. We observed seventeen conserved water centers in the inter-domain recognition. We show that five invariant water centers W13, W14, W18, W23 and W26 connect the Domain 5 to Domain 4 (D5···W···W4). We also show that five other water centers W19, W20, W27, W30 and W31 connects the Domain 5 to Domain 6 (D5···W···W6) that is unique in the simulated form. The W7 and W32 water centers are involved in the D1···W···W6 recognition. This is important for the water-mediated interaction of Glu1032 to the trinuclear copper cluster present at the interface between these domains. The involvement of W10 water center in the D3···W···D4 recognition through Gln552···W10···His667 H-bond interaction is critical in the complexation of CP with myeloperoxidase (Mpo). These observations provide insights to the molecular recognition of hCP with other biomolecules in the system.

Keywords: Conserved water molecules; MD-simulation; Ceruloplasmin.

Background:
Ceruloplasmin (hCP) is a copper containing ferroxidase enzyme. Beside the antioxidant properties of enzyme it also shows different activities e.g., oxidation of biogenic monoamines, NO-oxidase, NO2-synthase [1], glutathione-linked peroxidase [2] and able to prevent oxidative damage to protein, DNA and lipids [3] etc, however in most of the cases the reaction mechanisms are still unknown [4]. The X-ray structures have shown the presence of a trinuclear copper cluster and three mononuclear copper centers within the 3D-arrangement of six domains in the enzyme. Further MD-simulation studies have also indicated the role of conserved water molecules in the interaction with trinuclear and mononuclear
copper centers of CP [5,6]. Nevertheless the multifunctional enzyme need to have a well-defined and organized structure in order to maintain the functionality in physiological system, though some degree of flexibility of the domains and their recognition are required for catalytic activity or interaction with other macromolecular system. The structural and functional role of conserved water molecules and their endowment towards the intra and inter-domain recognition of proteins or metalloenzymes are well known [7,8]. However due to complexity in the structure of CP, the role of conserved water molecules in inter-domain recognition, hence their influence to the stability of overall structure or function is still not clear due to the limitations of resolution in the available crystal structures. Until now only one complex structure of CP with myeloperoxidase (the protein (Mpo) which is involved during inflammation) was solved at 4.69Å resolution by small angle X-ray diffraction method (PDB 1d 4EJX) [9]. Nevertheless the coupling between acidic and basic residues (of different domains) through conserved water molecules is also thought to be an important aspect for intra/inter-domain stabilization, moreover in few cases these water centers may also participate in the redox coupling or proton exchange reaction. MD-simulation studies have provided the key insights into the importance of conserved water molecules in the intra/inter-domain recognition and their influence to the stability of CP structure. Furthermore, investigation of conserved water mediated recognition between Glu552 to His667, alongwith the conformational dynamics of His667 and Trp669 have also been made because of their importance in the complexation of ceruloplasmin with macromolecule myeloperoxidase (Mpo).

Material and Methods:
The PDB structure Id: 2J5W of CP[10] having 2.8Å resolution was used for MD-simulation studies . In the asymmetric unit, few metal ions, small organic molecules and 341 number of water molecules were present along with the ceruloplasmin molecule. The numbering scheme for copper ions, amino acid residues, and water molecules were followed in according to 2J5W crystal structure.

Structure preparation:
A) (i) O₂-bound hCP (from 2J5W PDB-structure):
The N-acetyl-D-glucosamine (NAG) molecules, an oxygen atom near to Cu3049, glycerol molecules, Ca²⁺ ion and one extra Cu²⁺ ion (which were incorporated in the crystal during crystallization) were removed from the 2J5W-PDB structure. The missing residues at the sequences 476-482 (Tyr-Asn-Pro-Gln-Ser-Arg-Ser), 885-889 (Tyr-Leu-Lys-Val-Phe) and 1042-1046 (Asp-Thr-Lys-Ser-Gly) were successively added to protein structure. The six integral copper atoms of trinuclear cluster and T1 mononuclear centers alongwith the O₂ molecule was kept fixed at their respective crystallographic positions. Successive energy minimization of the structure was followed by steepest descent (1000 steps) and conjugate gradient (2000 steps) methods. Superimposing it on 2J5W crystal structure checked the final structure of protein, stereochemical arrangements of the residues were verified using Ramachandran plot.

(ii). O₂–bound hCP (from 4ENZ PDB-structure) [9]
Superimposition between the 2J5W and 4ENZ crystal structures has shown the RMSD value of 0.34Å. The 4ENZ crystal structure (having 2.6 Å resolution) of CP contains only 82 number of water molecules, so we have also compared the water molecular position in both the PDB structures. The N-acetyl-D-glucosamine (NAG) molecules, glycerol molecules (and the other ions which were incorporated in the crystal during crystallization) were removed from the 4ENZ-PDB structure. The missing residues at the different sequences were successively added to protein structure. All the copper atoms of trinuclear cluster and T1 mononuclear centers and O₂ molecule were kept fixed at the respective crystallographic positions. Successive energy minimization of the structure was done by steepest descent (1000 steps) and conjugate gradient (2000 steps) methods. Superimposing it on 4ENZ crystal structure checked the final structure of protein, and stereochemical arrangements of the residues were verified using Ramachandran plot.

B) Apo structure of CP:
The six copper atoms and O₂–molecule were removed from the final energy minimized modelled structure of CP (built from the 2J5W-PDB structure). Then energy minimization was followed for removing the steric clashes, abnormal bond lengths and angles. Finally, the stereochemical arrangement of all the residues was checked again by Ramachandran plot. The standard deviation of the protein backbone between the simulated structures of 2J5W and Apo form of hCP was 0.08Å.Identification of conserved water molecules. The 3DSS server [11] and Swiss PDB viewer program [12] were used to find out the conserved water molecules among the MD simulated and X-ray structures. The 2J5W PDB structure [13] was taken as reference and the MD-simulated structures at different time of simulation were successively superimposed on it. The cut-off distance between the pairs of superposed water molecules was taken to be 1.8 Å and only those were considered which have at least one hydrogen bond with the protein residue [14,15]. When the water molecule is found at a particular position (or within 1.8 Å) in the X-ray structures of a macromolecule or has high residual frequency (~98-100%) at that site during simulation then it considered as static conserved water molecule (site), on the contrary when that water site in the X-ray structure was occupied...
by different water molecules at different time of simulation then that hydrophilic site is defined as dynamic conserved water center.

**Molecular dynamics (MD) simulation:**

Molecular dynamics simulation of both the O2-bound CP structures (2J5W and 4ENZ) and apo-form of ceruloplasmin structure were performed by NAMD v.2.6 [16,17] with CHARMM36 force field [18–20]. For O2-bound CP structure, the charges for copper atoms (Cu3046: 0.7937, Cu3047: 1.4304, Cu3048:1.4957, Cu3049: 1.4158, Cu3051: 0.7108, Cu3052:1.0425) and oxygen molecule (O1:-0.5084, O2:-0.5309) were obtained from our previous studies and they were successively added to the respective copper and oxygen atoms of O2 molecule [5] in both the 2J5W and 4ENZ structures. Then both the apo and O2-bound CP structures were converted to Protein Structure File (PSF) by Automatic PSF Generation Plug-in within VMD program v. 1.9.2 [21]. All the crystal water molecules, 341 in 2J5W and 82 in 4ENZ were added to the respective structures. In apo structure water molecules of the 2J5W structure were added accordingly. Then all these water molecules of the structures were converted to TIP3P water model [22]. Then adding appropriate number of sodium and chloride ions neutralized each system. Subsequent energy minimizations of the structures were performed by conjugate gradient method. The process was conducted in two successive stages; initial energy minimization was performed for 1000 steps by fixing the backbone atoms, followed by a final minimization for 2000 steps were carried out for all atoms of the system to remove residual steric clashes. Then each energy minimized structure was simulated separately at 310 K temperature and 1atm pressure by Langevin dynamics [23] using periodic boundary condition. The Particle Mesh Ewald method was applied for full-electrostatics and the Nose–Hoover Langevin piston method used to control the pressure and dynamical properties of the barostat. Then for each structure (apo-form and the two O2-bound form of CP) water dynamics was performed for 2 ns by fixing the protein residues and allowing the water molecules to move freely. Then all-atom molecular dynamics simulations for 50ns were carried out separately for both the apo and O2-bound human ceruloplasmin (2J5W modeled) structures. Moreover, 50ns MD-simulation of 4ENZ-modeled structure was also done. Atomic coordinates were recorded at every 2 ps for analysis. For each simulated structure, root mean square deviation (RMSD) of MD structures were calculated (by taking the X-ray structure as reference molecule) using RMSD trajectory tool in VMD (Figure 1).

**Results and discussion:**

The structure of ceruloplasmin is mainly build up with the six domains (D1-D6) having sequences: 1-192 (D1), 193-340 (D2), 347-553(D3), 554-703 (D4), 704-884(D5) and 891-1040 (D6) [24]. Several water molecules are observed to involve in the intra and inter-domain recognition in both the X-ray and MD-simulated structures of CP through H-bond interaction with the residues. Almost thirty-four number of water molecules are found to be conserved in both the X-ray and MD-simulated structures of 2J5W PDB-structure. Among these, seventeen number of water centers are played role in the stabilization of intra-domain residues whereas the other seventeen centers are involve to inter-domain recognition which have given in Table 1. The occupation frequencies (O.F.) of those water sites are also included in that table. The conserved water centers that are involved in the inter-domain recognition have shown in (Figure 2). The interaction of conserved water centers with the different residues in the X-ray and MD-simulated structures of 2J5W are given in (Table 2).
Table 1: The occupation of different water molecules at the conserved hydrophilic sites during MD-simulation of O₂-bound ceruloplasmin structure.

| S.No. of conserved water sites | Id No. of Crystal water molecules (PDB Id 2J5W) | Id No. of water molecules which are occupied in the conserved hydrophilic water sites during MD-simulation (of 2J5W PDB structure) at different time (ns) | Occupation frequency (%) / type of conserved water centers | Conserved water centers played role in the intra-/inter-domain (D) recognition during MD-simulation | Conserved hydrophilic positions occupied by water molecules in 4ENZ PDB structure |
|--------------------------------|-----------------------------------------------|-------------------------------------------------------------------------------------------------|----------------------------------------------------------|-----------------------------------------------------------------|----------------------------------------------------------------------------------|
| 10                             | 20                                            | 30                                                 | 40                                                      | 50                                                             | 100/ dynamic                                                              | W1225                                                                 |
| W1                             | W2005                                         | W2003                                             | W2003                                                   | W2065                                                         | W2065                                                                | Intra (D1)                                                               |
| W2                             | W2040                                         | W2040                                             | W2040                                                   | W2040                                                         | W2040                                                                | Intra (D1)                                                               |
| W3                             | W2043                                         | W2043                                             | W2048                                                   | W2048                                                         | W2076                                                                | Intra (D1)                                                               |
| W4                             | W2054                                         | W2054                                             | W2054                                                   | W2054                                                         | W2054                                                                | Intra (D1)                                                               |
| W5                             | W2066                                         | W2066                                             | W2066                                                   | W2066                                                         | W2066                                                                | Intra (D1)                                                               |
| W6                             | W2080                                         | W2037                                             | W2260                                                   | NA                                                            | W2162                                                                | Inter                                                                    |
| W7                             | W2090                                         | W2331                                             | W2331                                                   | W2331                                                         | W2331                                                                | Inter                                                                    |
| W8                             | W2106                                         | W2106                                             | W2106                                                   | W2106                                                         | W2106                                                                | Inter                                                                    |
| W9                             | W2115                                         | W2115                                             | W2115                                                   | W2115                                                         | W2115                                                                | Inter                                                                    |
| W10                            | W2126                                         | W2126                                             | W2126                                                   | W2126                                                         | W2126                                                                | Inter                                                                    |
| W11                            | W2148                                         | N2                                                 | W2148                                                   | W2148                                                         | 95/ dynamic                                                           | Inter                                                                    |
| W12                            | W2152                                         | W2152                                             | W2152                                                   | W2152                                                         | W2152                                                                | Inter                                                                    |
| W13                            | W2154                                         | W2154                                             | W2154                                                   | W2154                                                         | W2154                                                                | Inter                                                                    |
| W14                            | W2156                                         | W2156                                             | W2156                                                   | W2156                                                         | W2156                                                                | Inter                                                                    |
| W15                            | W2160                                         | W2257                                             | W2257                                                   | W2257                                                         | 100/ static                                                           | Inter                                                                    |
| W16                            | W2167                                         | W2318                                             | W2318                                                   | W2079                                                         | W2079                                                                | Inter                                                                    |
| W17                            | W2168                                         | NA                                                 | W2075                                                   | W2261                                                         | W2317                                                                | 95/ dynamic                                                           |
| W18                            | W2179                                         | W2178                                             | W2249                                                   | W2157                                                         | 100/ dynamic                                                           | Inter                                                                    |
| W19                            | W2197                                         | W2197                                             | W2197                                                   | W2197                                                         | 100/ dynamic                                                           | Inter                                                                    |
| W20                            | W2199                                         | W2199                                             | NA                                                      | W2241                                                         | 97/ dynamic                                                           | Inter                                                                    |
| W21                            | W2221                                         | W2221                                             | W2277                                                   | W2221                                                         | 100/ dynamic                                                           | Inter                                                                    |
| W22                            | W2244                                         | W2244                                             | W2244                                                   | NA                                                            | W2266                                                                | 95/ dynamic                                                           |
| W23                            | W2256                                         | W2191                                             | W2160                                                   | W2226                                                         | W2111                                                                | 100/ dynamic                                                           |
| W24                            | W2270                                         | W2270                                             | W2270                                                   | W2270                                                         | 100/ static                                                           | Inter                                                                    |
| W25                            | W2271                                         | W2181                                             | W2181                                                   | W2181                                                         | 100/ static                                                           | Inter                                                                    |
| W26                            | W2273                                         | W2256                                             | W2236                                                   | W2320                                                         | 100/ dynamic                                                           | Inter                                                                    |
| W27                            | W2279                                         | W2279                                             | W2279                                                   | W2279                                                         | 100/ dynamic                                                           | Inter                                                                    |
| W28                            | W2286                                         | W2286                                             | W2286                                                   | W2286                                                         | 100/ static                                                           | Inter                                                                    |
| W29                            | W2300                                         | W2300                                             | W2300                                                   | W2300                                                         | 100/ static                                                           | Inter                                                                    |
| W30                            | W2302                                         | W2302                                             | NA                                                      | W2302                                                         | 97/ static                                                            | Inter                                                                    |
| W31                            | W2303                                         | W2231                                             | W2231                                                   | W2231                                                         | 100/ static                                                           | Inter                                                                    |
| W32                            | W2311                                         | W2033                                             | W2033                                                   | W2033                                                         | 100/ static                                                           | Inter                                                                    |
| W33                            | W2316                                         | W2316                                             | W2316                                                   | W2316                                                         | 100/ static                                                           | Inter                                                                    |
| W34                            | W2321                                         | W2322                                             | W2322                                                   | W2189                                                         | 100/ dynamic                                                           | Inter                                                                    |

Table 2: Hydrogen bonding interaction of the residues with the different conserved hydrophilic water sites during the simulation of O₂-bound ceruloplasmin and also in the X-ray structures (PDB Id 2J5W).

Conserved hydrophilic (water) sites: Residues interacts with the conserved water sites in the X-ray and MD-simulated structure during 50ns* | Interdomain (D) recognition by conserved water center (D=W–D) | X-ray MD-structure

| Interact in the X-ray structure | Interact only during MD-simulation | X-ray MD-structure |
|--------------------------------|-----------------------------------|--------------------|
| W1 Gly173(0B), Phe73(0B), Tyr53(NB) | Lys23 (NZ), Glu22(OE1), Phe248(OB) | D1=W–D2 |
| W2 Gly173(0B), Phe73(0B), Tyr53(NB) | D1=W–D2 |
| W3 Leu186(0B) | D1=W–D2 |
| W4 Gly207(OE1), Lys50(NZ), Lys49(OB), Ser210(OC) | Glu207(OE1), Lys50(NZ), Lys49(OB), Ser210(OC) | D1=W–D2 |
| W5 Ser242(OC), Asn244(NB), Glu265(NB) | Ser242(OC), Asn244(NB), Glu265(NB) | D2=W–D6 |
| W6 Leu302(0B), Val996(0B), Ser994(OC) | Leu302(0B), Val996(0B), Ser994(OC) | D2=W–D6 |
| W7 Gly242(0E1), Arg242(0H2) | Gly242(0E1), Gly242(0E1), Arg242(0H2) | D3=W–D10 |
| W8 Trp900(NE1), Tyr908(0E1), Asn467(0B), Ile456(0B) | Trp900(NE1), Tyr908(0E1), Asn467(0B), Ile456(0B) | D3=W–D10 |
| W9 Gly552(0E1), Cys512(0NB), Val514(0NB) | His657(0N2, Cys552(0E1), Cys512(0NB), Val514(0NB) | D3=W–D10 |
| W10 Ser603(OC), Asn605(NB), Phe626(0B), Gly606(NB) | Ser603(OC), Asn605(NB), Phe626(0B), Gly606(NB) | D2=W–D12 |
| W11 Gly320(0E1), Asn321(0NB), Ala330(0NB) | Gly320(0E1), Asn321(0NB), Ala330(0NB) | D2=W–D12 |
**Residue to water distances which are within 1.90Å to 3.50Å (during simulation) are included in this table. **Conserved water molecules/centers which are involved in the inter-domain recognition through the interaction of acidic (Aspartic, Glutamic) and basic (Lysine, Arginine) residues

*Figure 2:* The conserved water molecules involved in the inter-domain recognition of ceruloplasmin. The domain 1 is shown in red, domain 2 in yellow, domain 3 in green, domain 4 in blue, domain 5 in cyan and domain 6 is shown in magenta colour. Red and grey spheres show the water molecules and copper atoms.
Conserved water molecules in intra-domain recognition:

Among the seventeen conserved water molecules (Tables 1 and 2), three water centers (W1, W2, and W3) are observed to interact with the residues of D1, W5 interacts with D2, two water centers (W8 and W9) to D3, three water centers (W11, W15 and W17) recognize D4, four water centers (W21, W22, W24, and W25) are interacting with D5 and the other four water centers (W28, W29, W33, and W34) have interact with D6 domain. During simulation of CP the static or dynamic character of the conserved water centers have been mentioned in (Table 1). Fifteen water centers (W1, W2, W3, W5, W8, W9, W11, W15, W21, W24, W25, W28, W29, W33, and W34) are observed to have ~100% occupation frequency (O.F.) and the rest other (W17 and W22) have ~95%. Stabilization of the intra-domain residues through conserved water (W1, W3, W8, W26) mediated salt-bridge interaction (acidic—water—basic) have also been observed in the simulated structures of 2J5W (Table S2), however some of them were not found in its crystal structure. Superimposition of 4ENZ crystal structure on the 2J5W crystal has also revealed the presence of eight invariant water molecules at the W2, W9, W21, W24, W25, W28, W29 and W33 sites (Table 1). The interactions of water molecules with the residues are almost found to be same in the crystal and simulated structures of 2J5W. Moreover, simulation studies of apo-ceruloplasmin structure however, some of them were not found in its crystal structure. However in 2J5W—X-ray—simulated structure, at least seventeen conserve water molecules in the MD simulated structures of Apo form of CP (Table S1) and they were also observed to be static in the 2J5W crystal and simulated structures with 100% O.F. Compiling all these results it may be presumed that at least the four invariant water centers W2 (W2040), W5 (W2066), W24 (W2270) and W29 (W2300) may play role in the structural stabilization of the respective D1, D2, D5 and D6 domains.

**Table 1.** Conserved water molecules in the MD simulated structures of Apo form of CP.

| Water positions | 2J5W X-ray Structure | MD-simulated structures at different time (ns) |
|-----------------|-----------------------|---------------------------------------------|
| WA1             | W2022                 | W2022 W2022 W2022 W2022 W2022 W2022 W2022 W2022 |
| WA2             | W2036                 | W2036 W2078 W2080 W2068 W2168 |
| WA3             | W2040                 | W2040 W2040 W2040 W2040 W2040 |
| WA4             | W2054                 | W2054 W2054 W2054 W2095 W2095 |
| WA5             | W2066                 | W2066 W2066 W2066 W2066 W2066 |
| WA6             | W2080                 | W2080 W2020 W2317 W2168 W2080 |
| WA7             | W2085                 | W2085 W2085 W2085 W2085 W2085 |
| WA8             | W2102                 | NA W2102 W2102 W2102 W2102 |
| WA9             | W2103                 | W2106 W2091 W2119 NA W2054 |
| WA10            | W2116                 | W2116 W2116 W2116 W2116 W2116 |
| WA11            | W2127                 | W2127 W2127 W2127 W2127 W2127 |
| WA12            | W2154                 | W2157 W2183 W2183 W2183 |
| WA13            | W2159                 | W2160 W2134 W2274 W2134 W2134 |
| WA14            | W2184                 | NA W2184 W2205 W2065 W2065 |
| WA15            | W2221                 | W2221 W2229 W2229 W2229 W2229 |

Conserved water molecules in inter-domain recognition:

In the 2J5W—simulated structure, at least seventeen conserve water molecules (Table 1) are observed to involve in the inter-domain recognition through H-bond interaction of the residues of different domains (Table 2). Among these conserve water centers, ten are found to be static having ~100% O.F. and other seven sites are dynamic in nature though their residential frequencies are observed to be high. Inter-domain (D) recognition of the residues by H-bond interactions through the conserved water centers: D1—W4—D2, D2—W6—D6, D4—W14—D5, D5—W19—D6, D5—W20—D6, D5—W27—D6, D5—W30—D6 and D1—W32—D6 are observed in both the X-ray and simulated structures of 2J5W (Table 2). However, stabilization of inter-domains by H-bonding interaction through conserved water centers like D1—W7—D6, D3—W10—D4, D2—W12—D4, D4—W13—D5, D4—W16—D6, D4—W18—D5, D4—W23—D5, D4—W26—D5 and D5—W31—D6 have been observed only in the simulated structures. Nevertheless the recognition between the domain 1 and domain 6 through two conserved water W7 and W32 center is thought to be important because the trinuclear copper cluster is situated at the interface between these two domains. The W7 water molecule seems to connect the Glu1032 to that copper cluster (Glu1032—W7—Cu-cluster) which may be important for water mediated electron transfer process in CP[5,25]. The W10 water center has stabilized the His667 rotamer, which might be important for complexation of CP with Mpo. Nonetheless in the simulated structure of 2J5W several acidic and basic residues of the helix and loops (present at the surface of different domains in protein) have stabilized by conserved water mediated salt-bridge (Table S2) interaction: D1—W4—D2 (Glu207 (OE2)—W4—Lys50(NZ)), D4—W14—D5(Asp671(OD1)—W14—Arg845(NH2)), D4—W23—D5(Asp652(NH2)—W23—Glu844(OE1)), D4—W26—D5 (Arg652(NH1)—W26—Glu844(OE2)), D5—W31—D6(Glu784 (OE2) —W31—Arg945(NH1)) though few of them are observed in its crystal structure. However in D5—W27—D6 and D1—W4—D2 inter-domain recognition in the 4ENZ—PDB structure are made through water mediated ionic interaction Lys761(NZ)—W1209—Glu906(OE2) and Lys50(NZ)—W1214—Glu207(OE1).
In the simulated structure of 2J5W, beside these water mediated hydrogen bonding between the acidic and basic residues, some of those residues have also been stabilized by direct fork–fork type of salt-bridge between Glu844 and Arg652, where the OE1::NH2 and OE2::NH1 distances were varied from 2.7-3.1 and 2.74-3.2 Å. The Glu844 (OE1) residue also forms a water (W26) mediated salt-bridge with Arg882 (NH1). Similar type of fork-fork geometry has also been found in the salt-bridge between Glu784 and Arg 945, where the OE1::NH2 and OE2::NH1 distances were ranging from 2.7-3.2 and 2.63-3.23Å respectively. Moreover, fork-stick type of geometry has been observed in the salt-bridge Glu207(OE1)::Lys50(NZ) and Asp671(OE2)::Arg 845(NH1) where the distances were ranging from 2.5 to 3.0 and 2.51 to 2.8 Å respectively and these interactions were also observed in the 2J5W X-ray structure.

Role of water molecule in conformational dynamics of His667 and Trp669

MD-simulation studies have also revealed the importance of a conserved or pseudo conserved water center (W10) in the recognition of D3::W10::D4 domains. The influence of that water molecule in the conformational dynamics of His667 and Trp669 residues has also been observed. In the 2J5W and 4ENZ PDB-structures these two residues are observed to stabilize by stacking interaction (with distance ~4.1 Å), where the respective torsion angles χ1 (NB-CA-CB-CG) and χ2 (CA-CB-CG-CD2) of Trp669 in the structures are -62.78 and 84.31 in 2J5W, and -63.53 and 87.73 in 4ENZ. In stacking condition the χ1(NB-CA-CB-CG) and χ2(CA-CB-CG-ND1) values of His667 are -152.29 and 138.31 in 2J5W, ~145.97 and 136.80 in 4ENZ structure. However, the existence of another rotamer of His667 (χ1=63.04 and χ2=167.79) has also been indicated in the 4ENZ structure, which thus stabilized, by His667::W1202::Gln552 H-bond interaction. In both the crystal structures, Gln552 of domain 3 is found to stabilize by a water molecule of W10 site (W2126 in 2J5W and W1202 in 4ENZ structures) through H-bonds (~2.99Å), which were given in Tables 1 and 2. Nevertheless, such stabilization mechanism of His667 rotamer has also been observed in the simulated structure of CP though there were some variations in the torsion angles.

During simulation of 4ENZ structure, His667 shows two preferred conformations I and II, where the χ1 and χ2 values are ~176° and 50° (for I), and ~75° and -80° (for II). The rotamer I of His667 exists from 0 to 6.2 and 19.5 to 37.8ns, whereas the II-rotamer is exists from 6.25 to 19.45 and 37.83 to 50ns. The variation of torsion angles of that residue with time has shown in Figure 3. From the initial stage, the conformation I of His667 is stabilized by stacking interaction with Trp669 upto 6.2ns. However, after adopting conformaion II at ~6.25ns the residue is stabilized by water mediated inter-domain D3::W10::D4 interaction through His667(NE2)::W10::Gln552(OE1) H-bonds. After 19.5ns, His667 is again revert back to conformation I, and the water molecule (W1202) is observed to migrate from that conserved site (W10) at ~20ns. The occupation frequency of W10 water center is observed to be ~40%. Again the imidazole residue seems to adopt conformation II at ~37.83 which exists upto 50ns. However in the entire simulation period, the Trp669 stays almost at its position (conformation I), where the χ1 and χ2 values are ~ -65° and 104° which are shown in Figure 3.

During simulation of 2J5W structure, His667 is found to stabilize by Trp669 through π...π interaction upto 0.5ns, however after that period histidine adopts conformation II which exists upto ~50ns (where the χ1 and χ2 values are ~ -75° and -80°) and it is stabilized by Gln552 bound water molecule W2126 through His667(NE)::W2126::Gln552(OE) H-bond interaction, where the
His667(NE)···W2126 and Gln552···W2126 distances were varied from 2.86 to 3.11 and 2.65 to 2.98Å. Actually W2126 water molecule has occupied the conserved water site W10 with ~100% O.F. During simulation of hCP, Trp669 shows different conformations, initially from 0 - 9.98ns, it stays almost at its initial position with conformation I (where the torsion angles $\chi_1$ and $\chi_2$ are ~ -68° and 98°). But after ~10ns the indole ring is parallel displaced from its initial position and adopts conformation II ($\chi_1$ and $\chi_2$ angles are ~ 68° and -100°) which exists up to ~17.5ns, where the residue was stabilized by H-bond interaction with water molecules (W···Trp669(NE)). The conformation II (of Trp669) has reappeared at ~21.9ns and exist up to ~40ns. After that period, tryptophan adopts conformation III (where the $\chi_1$ and $\chi_2$ values are ~ 25° and -100°), where the indole ring lies almost perpendicular to previous conformation-I thus it stabilized by Trp669 (π)···water (W2175) interaction. The variations of torsion angles for Trp669 and His667 with time are shown in Figure 3. In the two simulated structures variation of occupation frequency of Gln552 bound water molecule (W10) may arise due to lower number of water molecules in the asymmetric unit of 4ENZ crystal compared to 2J5W structure. In ceruloplasmin, several conserved water molecules are playing role in the inter-domain recognition and structural stabilization. The W7 water center is playing role in the interaction of Glu1032 to trinuclear copper cluster. It is interesting to observe the role of conserved water molecule in the dynamics of His667 and Trp669 residues which may be important for the interaction of CP with the macromolecule myeloperoxidase (Mpo). Possibly, nature of these conserved water centers and their interaction with the intra and inter-domain residues are also thought to be important for keeping the proper structural flexibility of that multifunctional enzyme and the recognition of hCP with other biomolecules.

**Conclusion:**
Molecular dynamics analysis of the O2-bound ceruloplasmin structure show 34 conserved water sites. We observed that 17 centers are directly interacting and stabilizing the intra-domain residues through H-bonds. However, 17 other water centers are involved in the inter-domain recognition and are connected with the inter-domain residues through conserved water mediated H-bonds. The four invariant water molecules at the W2, W5, W24 and W29 sites are involved in the structural stabilization of ceruloplasmin. We report 10 conserved water centers involved in the inter-domain stabilization of domain 5 (D5). The 5 water centers W13, W14, W18, W23 and W26 are connected with domain 4 (D5···W···D4). Moreover, the 5 other water centers W19, W20, W27, W30 and W31 are involved in D5···W···D6 recognition. The W7 and W32 water centers connect the D1-domain to D6-domain through H-bonds. These water-mediated interactions (Glu1032···W7···Cu-cluster) are important to the electron transfer process of hCP as the trinuclear copper cluster is situated at the interface between domain 1 and 6 as described elsewhere [5]. The water molecule at the W10 center participates in the D3···W10···D4 recognition by Gln552···W10···His667 H-bond interaction, which stabilizes the complexation of CP with Myeloperoxidase (Mpo). This is interesting. The conserved water mediated interaction of the residues and their involvement to inter-domain stabilization have implication in the recognition biology of CP with other biomolecules.
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Conflict of Interest:
Author declares that he has no conflict of interest.

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