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How Does Huperzine A Enter and Leave the Binding Gorge of Acetylcholinesterase? Steered Molecular Dynamics Simulations

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Abstract: The entering and leaving processes of Huperzine A (HupA) binding with the long active-site gorge of *Torpedo californica* acetylcholinesterase (TcAChE) have been investigated by using steered molecular dynamics simulations. The analysis of the force required along the pathway shows that it is easier for HupA to bind to the active site of AChE than to disassociate from it, which for the first time interprets at the atomic level the previous experimental result that unbinding process of HupA is much slower than its binding process to AChE. The direct hydrogen bonds, water bridges, and hydrophobic interactions were analyzed during two steered molecular dynamics (SMD) simulations. Break of the direct hydrogen bond needs a great pulling force. The steric hindrance of bottleneck might be the most important factor to produce the maximal rupture force for HupA to leave the binding site but it has a little effect on the binding process of HupA with AChE. Residue Asp72 forms a lot of water bridges with HupA leaving and entering the AChE binding gorge, acting as a clamp to take out HupA from or put HupA into the active site. The flip of the peptide bond between Gly117 and Gly118 has been detected during both the conventional MD and SMD simulations. The simulation results indicate that this flip phenomenon could be an intrinsic property of AChE and the Gly117–Gly118 peptide bond in both HupA bound and unbound AChE structures tends to adopt the native enzyme structure. At last, in a vacuum the rupture force is increased up to 1500 pN while in water solution the greatest rupture force is about 800 pN, which means water molecules in the binding gorge act as lubricant to facilitate HupA entering or leaving the binding gorge.

Introduction

Acetylcholinesterase (AChE, acetylcholine hydrolase, E. C. 3.1.1.7) is one of the most essential enzymes in the family of serine hydrolases, operating at a rate approaching the diffusional limit in substrate association and dissociation.^{1,2} By rapid hydrolysis of the transmitter acetylcholine (ACh), the enzyme effectively terminates the impulse transmission at cholinergic synapses.^{1,3} The three-dimensional structures of *Torpedo californica* AChE (TcAChE) and its complexes with various inhibitors^{4–7} revealed that there is a narrow active-site gorge

which is about 20 Å deep and consists of two separated ligand binding sites, acylation (or active) site and peripheral anionic site. The acylation site located at the bottom of the gorge contains residues involved in a catalytic triad (His440, Glu327, and Ser200) and Trp84. The peripheral anionic binding site is located at the mouth of the gorge. This segment includes a key negative charged residue, Asp72 (Asp74 in human AChE), located near a constriction at the boundary between the peripheral site and the acylation site. At the midway of the deep gorge, the aromatic side-chains of Phe330 and Tyr121 form a bottleneck with a size permitting a water molecule to permeate in the X-ray crystal structures.⁵ Because the cross-section of substrate and inhibitors, such as ACh and Huperzine A (HupA), is much larger than the size of bottleneck, large-amplitude fluctuations are necessary for substrate or inhibitors to enter or leave along this main gorge. Some molecular dynamics simulations have provided such fluctuations,^{8–10} and several crystal

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structures of inhibitor–AChE complex show that Phe330 is flexible.^{6,11} Beside the fluctuations of the bottleneck, the large arrays of buried water molecules in AChE, which is about twice per residue in other typical proteins,¹² might act as lubricant that permits or even facilitates large-scale fluctuations of the main active-site gorge to allow the inhibitors' dissociation or association.¹¹ However, the bottleneck together with the electrostatic field that favors to accelerate the penetration of the cationic ligands but to impede them to exit from the active-site¹³ arise a question whether there are alternative routes to facilitate traffic of ligand or solvent in to and out of the gorge. Several molecular dynamics studies have been performed to investigate the release of small molecule such as acetic acid and water molecule from acetylcholinesterase through the alternate pathways except the main gorge.^{10,14–18} One of these studies indicated that “back door” existed for the escape of water molecules may be fostered by a local conformational change near the active-site cavity involving residues Trp84, Val129, and Gly441.¹⁸

Because of the key role that AChE plays in the nervous system, AChE inhibitors are used in treatment of various disorders such as myasthenia gravis and glaucoma. Their use has been proposed as a possible therapeutic approach to allay the symptoms of Alzheimer's disease (AD) and for the recovery of neuromuscular block in surgery.^{1,4,19,20} Compared with other well-known AChE inhibitors such as physostigmine, galanthamine, tacrine, and donepezil, Huperzine A (HupA), a novel alkaloid isolated from the Chinese folk medicine *Huperzia serrata* (Qing Ceng Ta) that has been used in China for centuries to treat contusion, strain, swelling, schizophrenia, has attracted considerable interest because of its lower toxicity, higher selectivity and long time inhibition. What interests us most is the experiment result that HupA equilibrated with free enzyme AChE in several minutes while HupA-inhibited AChE complex dissociated at a much lower rate (several hours) to restore enzyme activity. This phenomenon is in contrast to the rapid on- and off-rates that characterize reversible inhibitors of AChE with similar potency and thereby it is assumed to have a longer residence time than other commonly anti-ChE drugs.^{21,22} The reason for this unusual binding and unbinding of HupA to AChE cannot be provided by experiment at present time. Another

noteworthy feature of HupA–TcAChE complex is the orientation of the peptide bond between Gly117 and Gly118, which distinctly points in the direction opposite to that observed in the native structure and other inhibitor complexes.⁵ This conformation change destroyed the oxyanion hole⁴ which stabilizes substrate's tetrahedral intermediate during hydrolysis reaction.

As mentioned above, despite the progresses in experimental and molecular simulation studies on AChE and its complexes, several questions are still open. How do the inhibitors or substrate enter and leave the active-site gorge of AChE? Why HupA equilibrates rapidly with free enzyme AChE while HupA-inhibited AChE dissociates at a much lower rate to restore enzyme activity? What causes the peptide bond between Gly117 and Gly118 to be flipped by 180° in HupA–TcAChE complex compared with the structures of native AChE and other inhibitor complexes? What role the buried water molecules play for HupA binding and unbinding? The current developed steered molecular dynamics (SMD) simulation method is a complementary approach in studying ligand-protein binding and unbinding,²³ and thereby can be used to reach the answers of above questions. Briefly, SMD is an extended MD simulation method mimicking the principle of the atomic force microscopy (AFM).^{24–26} SMD has been widely used to explore the binding and unbinding properties of biomolecules and their responses to external mechanical manipulations at the atomic level.²⁷ It has been successfully applied to identify several ligand binding pathways^{28–31} and to explain the elastic properties of some proteins.^{32–36} In SMD simulations, time-dependent external forces are applied to ligand to facilitate its binding and unbinding with a protein as shown in Figure 1. From the accelerated moving of the ligand, SMD simulation can reveal information about the enzyme's flexibility and its response to the association or dissociation of ligand. Analyses of interactions between the binding ligand and the protein (especially residues forming the binding pocket) and the relationship between the applied forces and the ligand position could yield important information about the structure–function relationships of the protein–ligand complex, the binding and unbinding pathway(s) and possible mechanisms of ligand recognition and inhibition.

In the following, we present the results of SMD simulations for HupA entering and leaving TcAChE in water solution. To our knowledge these simulations are the first of studying HupA–AChE binding and unbinding processes kinetically. The purpose

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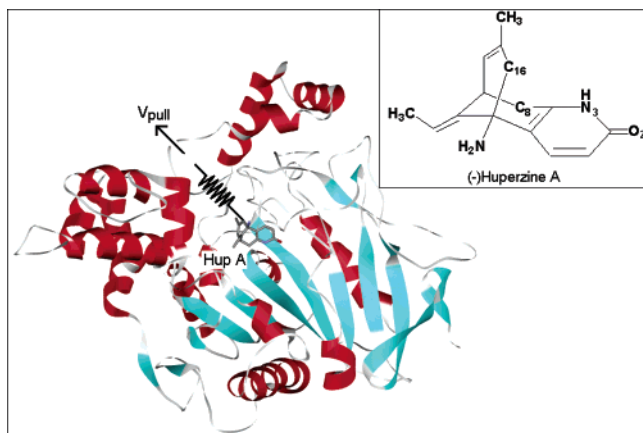


Figure 1. Ribbon schematic representations of solvated HupA–TcAChE model for the SMD simulations (water molecules not shown for clear view). During the SMD simulations, HupA is pulled away from or pushed into the binding gorge of AChE through a harmonic potential symbolized by an artificial spring connected to the atom C16 of HupA. This pulling potential moves with constant velocity V_{pull} (arrow) while the center of mass of AChE is kept fixed. The structure diagram of Huperzine A is shown at top right corner. This picture was rendered in the POV-Ray program.⁴¹

of the present work does not intend to obtain the exact forces required for HupA binding and unbinding, but to compare the forces acting on HupA concerning its binding and unbinding with AChE, which provides a valid interpretation for pronounced quick entrance but very slow dissociation of HupA to AChE. The detailed analyses for the structures and interactions in the SMD trajectories detected the important residues associated with HupA binding and unbinding, the flip of the peptide bond between Gly117 and Gly118, and the dynamic effect of the buried water molecules.

Models and Methods

The models used in the present study were built up based on the X-ray crystal structure of TcAChE–HupA complex at 2.5 Å resolution (PDB entry code 1VOT).⁵ The coordinates of the missing residues (Pro485, His486, Ser487, Gln488, and Glu489) and the missing atoms in many other residues were repaired according to the X-ray crystal structure of TcAChE–E2020 complex (PDB entry code 1EVE)³⁷ by using the molecular modeling software Sybyl 6.7 (Tripos Inc. St. Louis, MO). The repaired residues were subjected to energy minimization in Sybyl 6.7 using steepest descent method up to the gradient tolerance of 0.05 kcal/(mol·Å) to relieve possible steric clashes and overlaps of side chains with fixing the rest part of complex. The ionization states of some residues in TcAChE were determined by the method of Gilson et al.¹⁸ Residues His440, Glu443, and Asp392 were neutralized as pointed out by McCammon et al.⁸ All other ionizable residues were kept in their standard protonation states. The geometry of HupA was optimized at the HF/6-31G** level and then its partial atomic charges were determined by using the RESP fitting method³⁸ implemented in the NWChem 3.3.1 software package.³⁹ Topology file and other parameters except the charges for HupA were generated using the program XPLO2D.⁴⁰ The force field parameters of HupA are listed in Table S1 in the Supporting Information.

Before MD simulations, the TcAChE–HupA complex was solvated with 15,181 TIP3^{42,43} water molecules using the SOLVATE program.⁴⁴ The radius of the water sphere is 50 Å, which ensures the whole surface of TcAChE to be covered by a water layer with a thickness more than 12 Å, thereby the water layer above the mouth of the binding gorge is thick enough for pulling HupA out of TcAChE completely. The simulation system is totally composed of 51 362 atoms. Then water molecules, TcAChE and HupA were coupled separately to a temperature bath at 300 K using a coupling constant of 0.1 ps. A layer of 4 Å thick in the brim of the solvated system was subjected to SBOUND forces⁴⁵ to counterbalance surface tension and evaporation, and thus restrain the water molecules to a given volume. Afterward, a conventional MD simulation continued until to 5 ns and two SMD simulations were performed at a constant velocity of 0.02 Å ps^{−1} (the spring-constants is 2.8 Nm^{−1}) for the HupA unbinding from and binding toward TcAChE.

The SMD simulation procedure of Grubmüller et al.^{28,34,36,46} was adopted. In the SMD simulations, the main gorge is set as the pulling pathway of HupA binding and unbinding. Although previous simulations suggested there are alternate pathways except the main gorge, the sizes of the alternate pathways are narrow only for water molecule or acetic acid release.^{14,15,18} Accordingly, the main gorge is the mostly correct pathway for HupA entering and leaving. A harmonic potential was assigned to the carbon atom, C16 (shown in the top right corner of Figure 1), to pull HupA out of the long binding gorge along the previously defined axis that extends from atom Ile444-C^δ to the center of mass of atomic group of Glu73-C^α, Asn280-C^β, Asp285-C^γ, and Leu333-O.⁴⁷ At the beginning of pulling HupA back to the active site along the same axis, HupA exchanges positions with several water molecules above the binding gorge along the axis. After equilibrated for about 200 ps the whole system was stable, and this stabilized system was taken as the starting point for further SMD simulation. During this SMD simulation, the harmonic potential was assigned to the carbon atom C8 (also shown in the top right corner of Figure 1).

All the simulations were performed using the parallel version of MD program EGO⁴⁸ with the CHARMM19 force field.⁴⁹ Fast multiple-time-step structure-adapted multipole method (FAMUSAMM) developed by Eichinger et al.⁵⁰ was applied to rapidly evaluate the electrostatic interactions, whereas the lengths of bonds involving hydrogen atoms were held fixed with the SHAKE algorithm.⁵¹ During the simulations, the integration step was set up as 1 fs and structures were saved every 1000 steps (1 ps). LIGPLOT,⁵² a program for automatically plotting protein–ligand interactions, was used to analyze the hydrogen bonds, water bridges and hydrophobic interactions between TcAChE and HupA in the SMD simulation trajectories. Water bridge is defined as that there is only one linking water molecule between the ligand and residues.

It should be emphasized that the pulling velocity (V_{pull}) is an important parameter in the SMD simulations. The simulations at higher pulling velocity may lead to remarkable nonequilibrium effects, which may introduce obvious errors to the simulation results.²³ The low-

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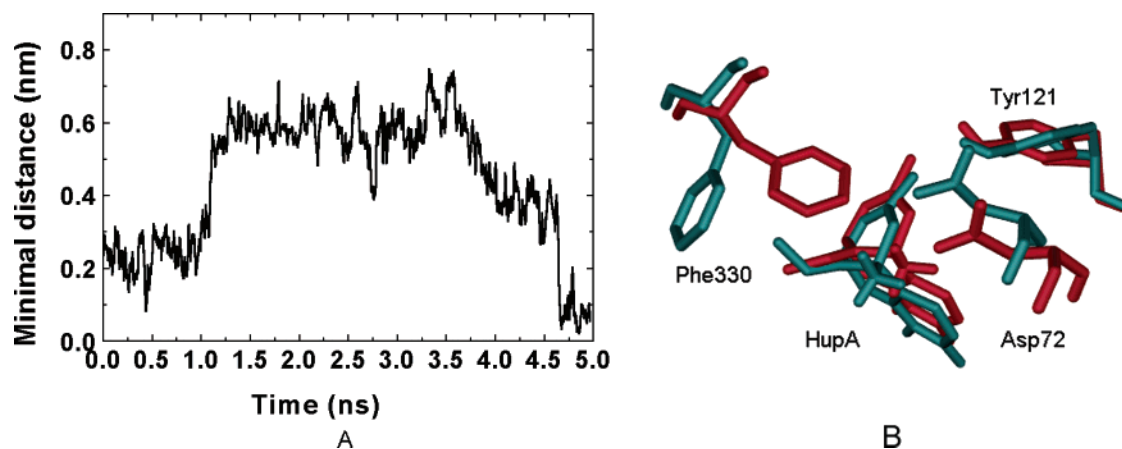


Figure 2. (A) Time dependence of the minimal van der Waal distance between Tyr121 and Phe330, which forms the bottleneck in the deep gorge. (B) The superposition of the crystal structure (red) and the snapshot structure of 1.2 ns (cyan) for the bottleneck. This picture was rendered in the POV-Ray program.⁴¹

velocity SMD simulations that carried out on a millisecond time scale can overcome these disadvantages and reproduce actual atomic force microscopy experiments; however, the corresponding computational cost will be very expensive. To find an appropriate simulation velocity, several SMD simulations should be carried out at different pulling velocities.²⁸ The pulling velocity of $0.02 \text{ \AA} \cdot \text{ps}^{-1}$ in this study was selected based on others' publications^{28,34,36,46} and our own experience,^{53,54} which produced reasonable results for several ligand–protein systems. To validate whether this pulling velocity is suitable for simulating HupA–TcAChE binding, additional test SMD simulations were performed at other two different pulling velocities, 0.1 and $0.01 \text{ \AA} \cdot \text{ps}^{-1}$. Additionally, these two test SMD simulations were performed from different starting points. The simulation results are listed in Figures 1S and 2S in the Supporting Information. For the SMD simulations under the three pulling velocities (0.1, 0.02 and $0.01 \text{ \AA} \cdot \text{ps}^{-1}$), the distributions of the direct hydrogen bonds, water bridges and hydrophobic interaction pairs between HupA and TcAChE along with the active-site gorge are similar. Most importantly, the global shapes of the force profiles under the three pulling velocities for the unbinding simulations of HupA–TcAChE complex are similar (Figures 1S and 2S, and Figure 3 below). Therefore it can be concluded that the unbinding process of HupA–TcAChE complex may not be significantly affected by the pulling velocity. Moreover, the aim of this study is to address the dynamical processes of the HupA–TcAChE binding and unbinding rather than reproduce the accurate binding force or binding free energy. Therefore, we only use the SMD simulation results at velocity of $0.02 \text{ \AA} \cdot \text{ps}^{-1}$ to discuss the binding and unbinding processes of the HupA–TcAChE complex.

Results

Fluctuation of Bottleneck. The AChE crystal structures revealed that a bottleneck, which mainly composed of Tyr121 and Phe330, locates at the middle of the binding gorge.⁴ The ligand cannot enter or leave the binding gorge of AChE unless the bottleneck opened. The minimum van der Waals distance between Tyr121 and Phe330 (Y–F distance) was monitored during the 5 ns conventional MD simulation. Figure 2A displays the fluctuations of the Y–F distance of the MD simulation. The fluctuation feature of the bottleneck is similar to the result of a 10 ns molecular dynamics simulation by Tai et al.^{9,10} Obviously, the bottleneck began to open at about 1.1 ns, the aromatic side

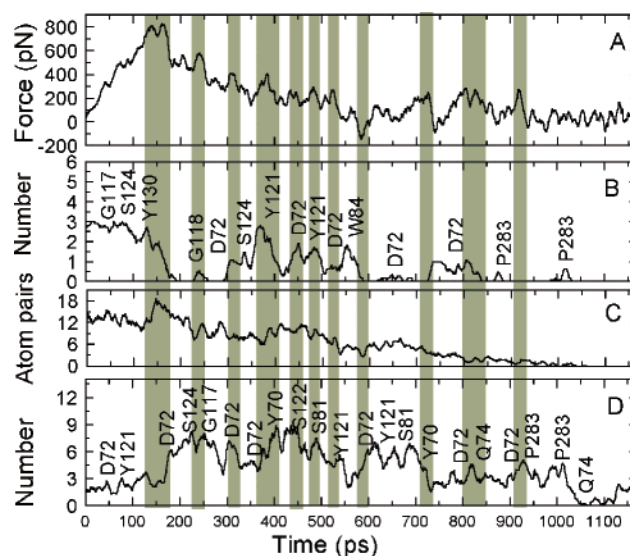


Figure 3. Rupture force (A), numbers for direct hydrogen bonds (B), atom pairs of hydrophobic interactions (C), as well as water bridges between HupA and TcAChE (D) vs time in the process of HupA leaving the TcAChE binding gorge. The main residues of AChE that contact with HupA are highlighted. All curves are obtained by 10 ps averaged.

chain of Phe330 began to turn aside from the axis of the binding gorge, and at about 1.2 ns the bottleneck opened up completely as shown in Figure 2B. The open state holds about 3.0 ns, and at about 4.0 ns the bottleneck is closed up again. This open time scale is enough for a ligand to enter or leave the active site gorge in the next SMD simulations. The MD simulation result indicates that the aromatic side chain of Phe330 is more flexible than that of Tyr121, which corroborates the previous suggestion based on the X-ray crystallographic determination.⁶ The flexibility of Phe330 side chain was verified by our X-ray crystal structure determination of Huperzine B–TcAChE complex,⁵ in which Phe330 was refined in two alternative conformations: one conformation which is similar to that seen in complexes with (\pm)HupA, was refined with 65% occupancy, and another conformation with only 35% occupancy. This result can also explain why HupA–HAcHE (human AChE) complex is approximately 200-fold more stable than its Tyr337Ala mutation (Y337 of human AChE is equal to F330 of TcAChE).²²

The root-mean-square deviation (rmsd) values of the C_{α} atoms and all atoms of TcAChE from its crystal structure as a function

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of time for the 5 ns MD simulation are shown in Figure S3 in the Supporting Information, suggesting the whole structure showed nonequilibrium drift. Similarly, the 10 ns MD simulation of AChE by Tai et al. also indicated that this system would not be well equilibrated within 5 ns simulation.⁹ However, two local equilibrium regions were found in the 5 ns MD trajectory, viz. 1 to 1.3 ns and 1.75 to 2.25 ns. According to the bottleneck fluctuation situation, the 1285 ps snapshot situated at the first local equilibrium region in the MD trajectory was selected for the next SMD simulation of HupA unbinding. Because the large rmsd of the whole structure of *TcAChE* was originated from the large fluctuations of the C-terminus, which is far away from the active-site gorge, it would not necessarily invalidate the SMD simulations and the results.^{36,53}

SMD Simulations. Leaving of HupA from AChE. The first SMD simulation was performed to investigate the unbinding process of HupA from *TcAChE*. To map the molecular mechanism of HupA leaving the gorge at the atomic level, the rupture forces were calculated during the SMD simulation. Figure 3A shows the rupture force profile in the leaving trajectory of HupA. The largest peak is located at about 150 ps, which points out that the largest rupture force is about 820 pN; at about 580 ps there is a deepest valley, indicating that the smallest rupture force acts on HupA. To elucidate the fluctuation of the rupture forces along with the HupA moving trajectory, the interactions between HupA and *TcAChE* in the process of HupA leaving the gorge were analyzed. The direct hydrogen bonds (DHB), water bridges (WB) and hydrophobic interactions (HI) between HupA and *TcAChE* were considered. The interaction features were analyzed by using program LIGPLOT,⁵² and are shown in Figure 3B–D.

While moving along the binding gorge, interaction between HupA and *TcAChE* changes from one kind to another, such as old hydrogen bonds (HBs) break and the formation of new HBs occurs continuously. Figure 3B illustrates the variation of the direct HBs (DHBs) formed between HupA and *TcAChE*. The number of DHBs is always less than 3. From 0 to 190 ps, HupA forms DHBs mainly with Gly117, Ser124 and Tyr130; from 200 to 300 ps only very few DHBs exist between the ligand and the enzyme; when HupA moves toward to the peripheral anionic site at about 350 ps, HupA forms new DHBs with Asp72, Tyr121, Ser122, and Trp279, Trp84; this DHB rich time holds up to 590 ps; then DHB vacuum appears again in the time range from 600 to 720 ps; at about 800 ps, HupA arrives at the mouth of the gorge, it forms DHB again with Asp72; after 1050 ps no DHB forms between HupA and *TcAChE*, HupA has left the gorge at this time. Most DHBs are formed between N3 and O2 (Figure 1) of HupA and residues of *TcAChE*.

Figure 3C presents the change for hydrophobic interaction (HI) pairs between HupA and *TcAChE* during HupA moving through the gorge. In general, the HI pairs are decreasing along with HupA leaving away the active binding site, except a peak appears at about 150 ps, which corresponds to the highest rupture force peak (Figure 3A). Around the highest peak in the rupture force profile (Figure 3A), from 100 to 250 ps, the aromatic residues such as Trp84, Phe330, Phe331, and Tyr121 contribute about 80% to the HI pairs. In the whole process of HupA leaving, the aromatic residues contribute about 60% to the hydrophobic interactions. This hydrophobic contribution may

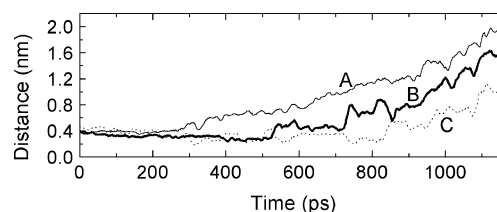


Figure 4. Time dependence of the minimal distance from Phe330 (A), Tyr121 (B) and Asp72 (C) to HupA, displayed as 10 ps averaged.

assign to the fact that the long active site gorge is lined with fourteen aromatic residues.⁴

Both X-ray crystal structures of inhibitor-AChE complexes^{4–7} and theoretical analyses^{11,17} have demonstrated the importance of water molecules in the ligand-AChE binding, i.e., water molecules bridge ligands with AChE. Therefore, more attention was paid to the water bridges (WBs) between HupA and AChE during the SMD simulation. The number fluctuation of WBs between HupA and the residues of *TcAChE* through a water molecule is displayed in Figure 3D. The number of WBs fluctuates when HupA goes through the active site gorge. From 0 to 160 ps about 3 WBs form between HupA and Asp72 or Tyr121; after HupA crosses the bottleneck, at about 200 ps, the number of WBs increases up to 6, then fluctuates between 3 and 9 until 1050 ps. During this period, WBs form with and break from Asp72, Ser124, Gly117, Tyr70, Ser122, Ser81, Tyr121, Gln74, and Pro283, respectively. Figure 3D shows that Asp72 is an important residue for WBs, it often forms WBs while HupA moving in the gorge.

As mentioned above that, at about 150 ps, the DHBs between HupA and *TcAChE* begin to break, of particular interesting that it is the time for the HI pairs and WB number increasing, demonstrating that the energy loss due to DHB breaking can be compensated partially by hydrophobic interaction and WBs. Nevertheless, the highest force peak at this time indicates that DHBs breaking is a major source for rupture force increasing. Although the SMD simulations were carried out after the bottleneck opened, steric hindrance of the bottleneck may still affect HupA dissociation. Accordingly, we monitored the minimal distances of HupA to Tyr121, Phe330, and Asp72 in the SMD simulation. The results are presented in Figure 4, which reveals that these three residues are very close to HupA from 0 to 250 ps, the three minimal distances fluctuate around 4 Å. Figure 4 also indicates that the minimal distances of HupA to Tyr121 and Asp72 are shorter than that of HupA to Phe330. This is in agreement with the result of the 5 ns conventional MD simulation, i.e., after 1 ns MD simulation, Phe330 has made way for HupA, whereas Tyr121 and Asp72 are still on the road. Another reason is that HupA forms DHBs and WBs with Tyr121 and Asp72, making it more close to these two residues.

The structural snapshots of HupA on the way through the gorge may give a clearer picture of interactions. Figure 5A shows the structural state of HupA arriving at the bottleneck. The corresponding position in the rupture force profile is at 150 ps, with the highest force on the way leaving the gorge. At this position, HupA forms a DHB with Gly117, two WBs with Asp72, a WB with Trp84, and several HI pairs with Trp84, Tyr121, and Phe330. Therefore, these strong interactions have to be overcome if HupA moves on ahead. Additionally, the hindrance from Tyr121 prevents HupA from going through the bottleneck, the bridge methyl group of HupA points to the

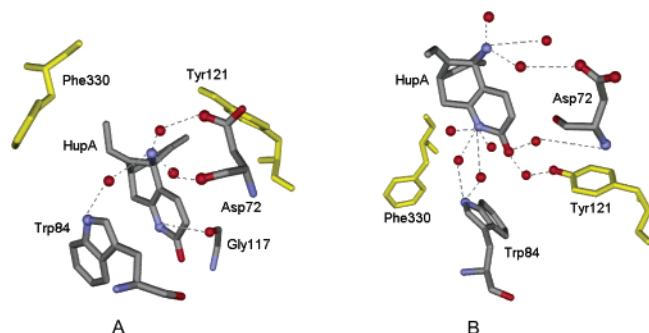


Figure 5. Snapshots for HupA to cross over the bottleneck (A) and to overcome the bottleneck (B) in the process of HupA leaving the gorge. The bottleneck is composed by the residues colored in yellow. The red balls represent the water molecules. The hydrogen bonds are shown as dashed lines. This picture was rendered in the POV-Ray program.⁴¹

aromatic side chain of Tyr121. All of these cause HupA receiving the highest rupture force at this position. Figure 5B is a snapshot when HupA crosses over the bottleneck at about 580 ps in the SMD simulation. From Figure 3, it can be seen that, at this position, DHBs disappear, and less HI pairs exist. This is why the rupture force at this point is the lowest one (Figure 3A).

Considering three kinds of interactions, steric hindrance and the fluctuation of the rupture force profile in parallel, the relevant important residues were found (displayed in Figure 3). Gly117, Ser124, and Tyr130 make the most DHB before HupA goes across the bottleneck. Asp72 is the most active residue and plays a key role (discussed below). The steric hindrance of bottleneck that may include residue Asp72 in our simulation might be the dominating factor to result in the maximal and minimal force along this process.

Entrance of HupA into the Binding Gorge. After the simulation of HupA–TcAChE unbinding finished, we simulated the binding process of HupA with TcAChE. Because HupA spins frequently in the bulk water layer, first HupA was pushed back to the mouth of the active-site gorge quickly with a 200 ps simulation, and the pulling velocity for HupA was set as $0.1 \text{ \AA} \cdot \text{ps}^{-1}$. Then the velocity was switched to $0.02 \text{ \AA} \cdot \text{ps}^{-1}$ during driving HupA back to its binding site. In the following only the simulation results at velocity of $0.02 \text{ \AA} \cdot \text{ps}^{-1}$ are presented.

The pushing force profile of HupA entering the binding gorge is displayed in Figure 6A. Compared with the rupture force profile (Figure 3A), there is no sharp peak in the pushing force profile. The largest pushing force is about 360 pN, which is much smaller than the largest rupture force, 820 pN. Similar analyses were carried out to assess the behaviors of DHBs, HI pairs and WBs during HupA moving back to the active site, the results are shown in Figure 6B–D. The force peak groups are shaded in Figure 6. Breaking the DHBs formed by HupA with Asp72, Tyr121, and Tyr70 and the WBs with Tyr70, Ser122, and Asp72 results in the first force peak group. Breaking WBs of HupA with Gln69, Tyr70, Ser124, Ser81, and Arg298 produces the second force peak group. It is particularly interesting that there are more valleys in the pushing force profile. Of them what arise our interesting most are the two lowest valleys, at about 185 and 640 ps in Figure 6A. Snapshot structure corresponding to 185 ps shown in Figure 7A indicates that four residues, Gln74, Asp72 and Tyr70 and Tyr334, contact with HupA through WBs; whereas Gln74, Asp72 and Tyr70 form two hydrogen bonds with HupA through water molecules.

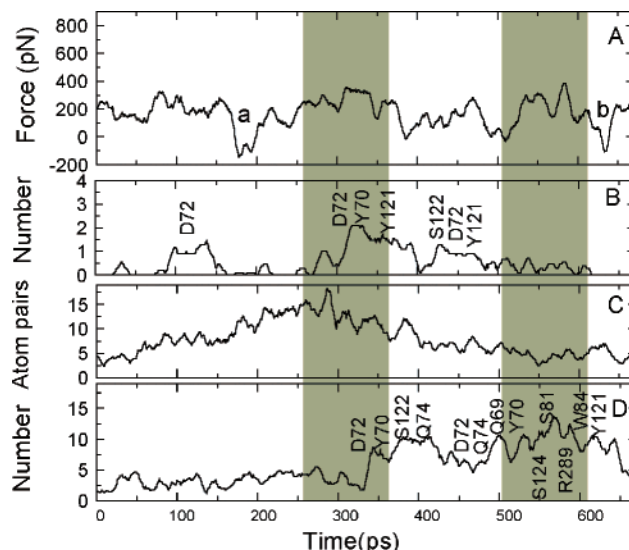


Figure 6. Rupture force (A), numbers for direct hydrogen bonds (B), atom pairs of hydrophobic interactions (C), as well as water bridges between HupA and TcAChE (D) vs time in the process of HupA entering the TcAChE binding gorge. The main residues of AChE that contact with HupA are highlighted. All curves are obtained by 10 ps averaged.

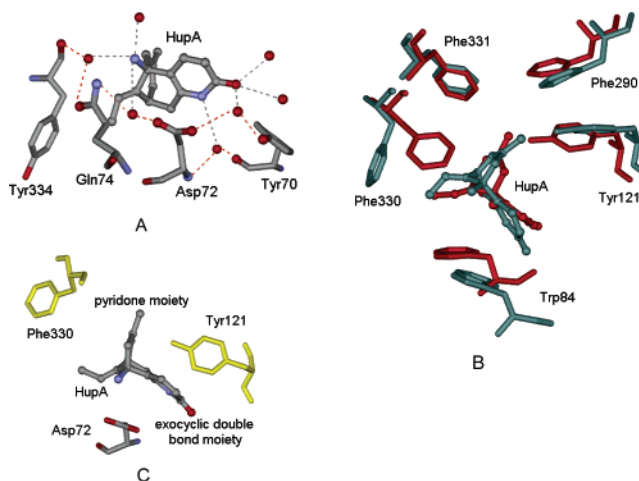


Figure 7. Three snapshots for HupA entering the binding gorge. (A) The complicated interactions of HupA with the residues Tyr334, Gln74, Asp72, and Tyr70 at 185 ps. Red dashed lines depict the water bridges; gray dashed lines display the hydrogen bonds between water molecules and HupA. (B) The superposition of 640 ps snapshot (cyan) with the crystal structure (red). At this time the rmsd of HupA from the crystal structure is 2.8 \AA . (C) The platform (viewed from the top of the deep gorge) of HupA entering the bottleneck (yellow color) at 370 ps. The aromatic side chain of Tyr121 just wedges perfectly between the pyridone moiety and exocyclic double bond moiety of HupA, and the side chain of Phe330 gives way to HupA entering. This picture was rendered in the POV-Ray program.⁴¹

Moreover, these residues link together through water molecules. Thus HupA, Gln74, Asp72, Tyr70, and Tyr334 as well as some water molecules form a hydrogen bond (H-bond) network. It indicates that the H-bond network forms a trap for capturing HupA (Figure 7A). Overcoming the binding forces of this H-bond network, HupA may arrive at its active site smoothly. The rmsd from the X-ray crystal structure of HupA (data not shown) was examined to determine whether and when HupA could recover its position and orientation in the X-ray structure of HupA–TcAChE complex. The superposition of the snapshot structure at 640 ps with the crystal structure of HupA–TcAChE complex, as shown in Figure 7B, and the rmsd of HupA

demonstrate that at this time HupA is the closest one to its original position and orientation in the X-ray structure.

Discussion

Distinction between the Processes of HupA Binding and Unbinding. In this study, we have obtained the processes of HupA leaving and entering the active site of *TcAChE* with the SMD simulations, and thus we can gain some insights into the binding and unbinding properties of HupA–AChE interaction. Many distinct differences exist between these two processes, binding and unbinding, of HupA interacting with *TcAChE*. The rupture force of pulling HupA out of the binding site is much larger than the force pushing HupA back to the active site. For HupA entering process the largest pushing force is less than 400 pN, whereas the largest rupture force is more than 800 pN for the leaving process (Figures 3A and 6A). The difference of force profiles implies that it is easier for HupA to bind to the active site rather than to leave it. This is in good agreement with the experimental evidence that the association of HupA with *TcAChE* is much quicker than its dissociation.^{21,22} The bottleneck plays an important role for the binding and unbinding of HupA. Structurally, the bottleneck has less steric hindrance for HupA approaching to the active site than for HupA leaving the gorge when it is in the open state. For HupA entering, the aromatic side chain of Tyr121 just wedges perfectly between the pyridone moiety and exocyclic double bond moiety of HupA (Figure 7C), which avoids the steric crash with HupA in the leaving pathway. The X-ray structure and MD simulations demonstrate that the peripheral site of AChE has enough space for HupA to adjust its orientation to pass across the bottleneck, which is beneficial to HupA entering the active site; the active site however has no such advantage in space for HupA to adopt an appropriate orientation to leave it. This could be the reason that the bottleneck is favorable to HupA entering. Therefore, the SMD simulations have given a valid explanation at the atomic level to the experiment result that HupA equilibrates rapidly with either free enzyme or enzyme–substrate complex and HupA-inhibited AChE dissociates at a much lower rate to restore the enzyme activity.²¹

Role of Asp72. Residue Asp72 of *TcAChE* located at the boundary between the peripheral site and the acylation site (active site) is a key residue with which the ligands interact. Previous studies have observed that Asp72 contributed very little to a general electrostatic effect to the cationic ligands, whereas mutation of Asp72 reduced affinity to charged ligands by measuring the equilibrium dissociation constant.^{55,56} However, these conclusions obtained by the experiments of binding kinetics did not provide the atomic details for the role Asp72 plays when HupA enters or leaves the active-site gorge.

As a negatively charged residue at the mouth of the deep gorge, Asp72 may generate electrostatic field affecting the cationic substrates or inhibitors, and water molecules around it can, however, screen most charges of the ion residues and ligands. The SMD simulations demonstrate that Asp72 does not often directly contact with HupA, but forms a lot of WBs throughout HupA leaving and entering the AChE binding gorge (Figures 3D and 6D). Figure 8 is a typical snapshot structures

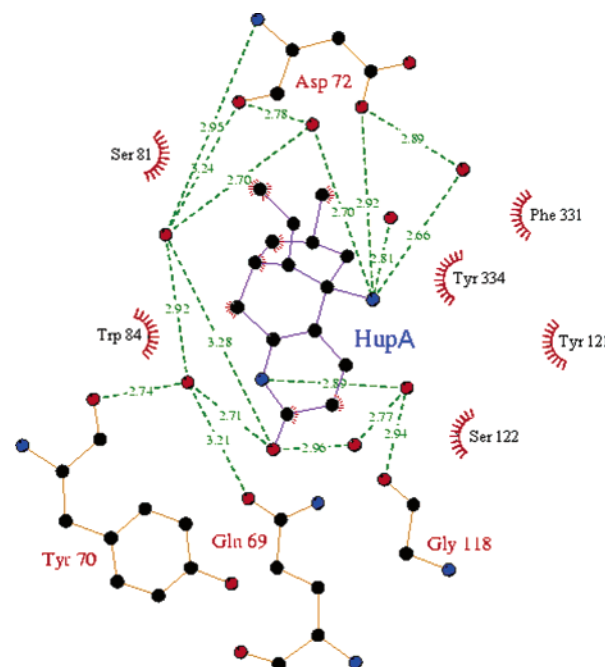


Figure 8. Schematic representation of hydrogen bond and hydrophobic interactions between HupA and *TcAChE* at different stages of rupture 350 ps in the process of pulling HupA out of the active site gorge. Dashed lines represent hydrogen bonds and spiked residues form hydrophobic interactions with HupA. This picture was made with the program LIGPLOT.⁵²

at 350 ps in the unbinding SMD trajectory, which shows that the main DHB (salt bridge) and WB interactions between Asp72 and HupA. Five WBs and one salt bridge between Asp72 and HupA form the H-bond network. With this H-bond network, Asp72 acts as a clamp to take out HupA from or put HupA into the active site. Because of the importance of Asp72 in the binding and unbinding of HupA with AChE, the mutation of this residue into other residue resulted in decreasing of the ligand–AChE binding affinity⁵⁵ is not surprising. Our SMD simulations gave a dynamic insight into the role of Asp72 at the atomic level.

Flip of the Peptide Bond between Gly117 and Gly118. The oxyanion hole formed with the backbone NH groups of Gly118, Gly119, and Ser201 plays a critical role in stabilizing the structures of the transition states and lowers the activation barriers of AChE-catalyzed ACh hydrolysis.⁴ The most striking observation seen in the structures of AChE complex with HupA analogues is the flip of the peptide bond between Gly117 and Gly118.⁵ As a consequence, the position of the main chain nitrogen of Gly118 in the oxyanion hole is occupied by the carbonyl of Gly117. This renders the oxyanion hole less accessible to substrate and less capable of stabilizing its tetrahedral intermediate for nucleophilic attack by Ser200. Furthermore, the flipped conformation is stabilized by Gly117O which forms H-bonds with Gly119N and Ala201N.⁵ Compared with the native structure of AChE and the structures of other inhibitor complexes, this peptide bond flip has been observed only in AChE complexes of HupA analogues.^{4–6} Previous dynamics simulation on the native *TcAChE* structure observed the peptide bond flip in the first 100 ps of a 500-ps equilibrium simulation.⁵⁷ To deeply understand this peptide bond flip, the

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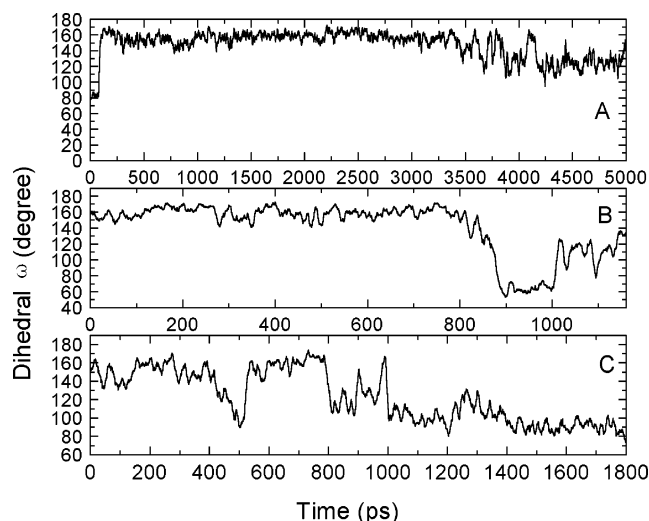


Figure 9. Fluctuations of the dihedral angle (C–N–C–C, ω) of the peptide bond between Gly117 and Gly118 of the equilibration MD simulation (A), unbinding (B) and binding (C) SMD simulations.

fluctuations of the dihedral angle of Gly117–Gly118 peptide bond (C_{α} –N–C– C_{α} , ω) were monitored during the unbinding and binding SMD simulations. The results are presented in Figure 9.

In the structure of native *TcAChE* ω is 143.8° , whereas in the structure of HupA–*TcAChE* complex it is 82.6° . The structure of Gly117–Gly118 peptide bond in the HupA–*TcAChE* complex ($\omega \approx 82.6^\circ$) lasted only about 100 ps during the 5 ns conventional simulation; then it flipped to $\omega \approx 150^\circ$, this value of ω has less fluctuation in the whole MD simulation. The snapshot structures show that after 100 ps equilibrium HupA moves far away from the Gly117–Gly118 peptide bond (Figure 10A). Meanwhile, Gly117 forms H-bonds and WBs with HupA, which induce the peptide bond to flip back to about 150° . In addition, the distance between the carbonyl oxygen atom of Gly117 and the carbonyl oxygen atom of HupA has been enlarged to about 3.95 Å after 100 ps simulation, therefore the carbonyl–carbonyl repulsion does not exist. This indicates that the Gly117–Gly118 peptide bond of AChE mostly tends to adopt the unbound structure even in the HupA complex.

For the SMD simulation of HupA unbinding, the Gly117–Gly118 peptide bond does not fluctuate dramatically before 900 ps (Figure 9B), it adopts structures of $\omega \approx 150^\circ$. The peptide bond at 215 ps shown in Figure 10B (purple) is a typical orientation in this period, which is more close to the native structure of *TcAChE* (gray in Figure 10B). At about 900 ps, when HupA has almost leaven the binding gorge, the peptide bond flips back to a state (yellow in Figure 10B) which resembles the crystal structure of HupA–*TcAChE* complex (green in Figure 10B). The peptide bond keeps flipping after HupA totally left the binding gorge because ω increases to 120° at about 1050 ps (Figure 9B). This is in agreement with the simulation of Tara et al.⁵⁷ Accordingly, we can conclude that the flip of the Gly117–Gly118 peptide bond should be an intrinsic property of AChE, the crystal structure of the native *TcAChE* and inhibitor complexes records just one state of its.

For the SMD simulation of HupA entering the active site and its following equilibration process, ω fluctuates dramatically. The ω decreases from 150° to 90° for the first 500 ps simulation, afterward ω increases to 150° again at about 650 ps and this

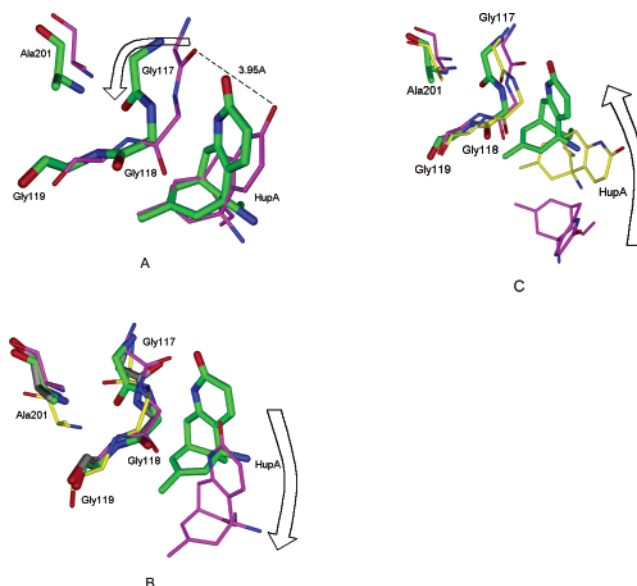


Figure 10. Flip of the peptide bond between Gly117 and Gly118 in MD and SMD simulations. (A) The superposition of the crystal structure of *TcAChE*–HupA complex (green) with snapshot (purple) at 500 ps in 5 ns conventional simulation. The flip of the peptide bond is shown by arrow. (B) The superposition of the crystal structure of *TcAChE*–HupA complex (green) with the native *TcAChE* (gray) and two snapshots at 215 ps (purple) and 945 ps (yellow) in the HupA unbinding SMD simulation. HupA in the snapshot at 945 ps is not shown because it is far away from the active site. Arrow labels the pathway of HupA leaving the gorge. (C) The superposition of the crystal structure of *TcAChE*–HupA complex (green) with the snapshot at 415 ps (purple) of the HupA binding SMD simulation and the snapshot of 1115 ps (yellow) of the equilibration simulation after HupA enters the binding site. The pathway of the binding process is also shown by arrow. This picture was rendered in the POV-Ray program.⁴¹

value kept unchanged before 800 ps; then ω decreased gradually to about 80° (Figure 9C). The superposition of the crystal structure of HupA–*TcAChE* with two snapshot structures isolated from the SMD trajectory (Figure 10C) indicates that peptide bond flip may not be induced by a carbonyl–carbonyl repulsion between HupA and Gly117, which is different from that shown in the X-ray crystal structure,⁵ for when HupA is far away from the binding site (purple in Figure 10C) the orientation of the peptide bond will flip to the structure of HupA–*TcAChE* complex. Therefore, the fluctuations of ω in the binding process also suggest that the flip of the Gly117–Gly118 peptide bond is an intrinsic property of AChE.

Role of Water Molecules. Functions of water molecules in proteins have attracted many biologists.¹² AChE and its complexes with inhibitors contain a large number of buried water molecules, which is twice the number per residue as it in typical proteins.¹¹ Koellner et al.¹¹ analyzed structural aspects of the buried water molecules in *TcAChE* and of the water molecules within its active-site gorge by comparing five crystal structures of AChE complexes. They suggested that the large arrays of buried water molecules act as lubricant that permits or even facilitates large-scale fluctuations of the loops forming the active-site gorge and water molecules in main gorge are more energetically activated compared to bulk water.¹¹ More recently, Henchman et al. used new methods to analyze the properties of water molecules in a 10 ns simulation of mouse AChE, which examined the population of gorge waters and the mobility of water in the middle and entrance to the gorge.^{17,58} Our attention was attracted by the dynamic effect of water

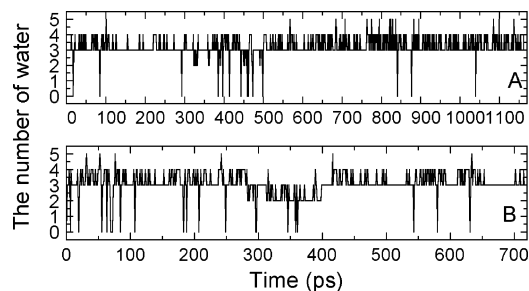


Figure 11. Number of water molecules forming hydrogen bonds with the ammonium group of HupA vs time for the HupA unbinding (A) and binding (B) processes.

molecules on HupA binding and unbinding. Figure 11 shows the average water molecules around the ammonium group of HupA during the SMD simulations. The ammonium group of HupA plays an important role in the HupA-AChE binding. X-ray crystal structure indicated that the ammonium group interacts with several residues of AChE through a H-bond network composed of a series of water bridges.⁵ Another hypothesis suggested that the cation- π interaction of cationic substrates or inhibitors with the aromatic residues lined the gorge wall is a driving force for their binding and unbinding.^{4–6} Our SMD simulations show that about three water molecules always form hydrogen bonds with the ammonium either in binding or unbinding process of HupA (Figure 11). MP2/6–31** calculation resulted that the cation- π interaction between the ammonium and the aromatic residues could be reduced rapidly as the increase of water molecules forming hydrogen bonds with the ammonium cation.⁵⁹ This demonstrates that cation- π interaction may not be a main driving force for HupA binding and unbinding. But what role do the water molecules play for HupA binding and unbinding?

As discussed above, water molecules in the binding gorge form WBs between HupA and AChE, and also attempt to form the H-bond networks between residues of AChE and HupA (Figures 3, 5, and 6). Formation and break of the WBs and H-bond networks facilitate HupA to move along the binding gorge. Therefore, the water molecules act as lubricant to help HupA enter or leave the binding gorge. To validate this hypothesis, a SMD simulation of HupA leaving the *TcAChE* gorge without water molecules was performed. The rupture force increased dramatically, the largest rupture force is even up to 1500 pN (Figure 12). Accordingly, the buried water molecules in the binding gorge would reduce the rupture force by about 700 pN. (Figure 3A and 12).

Structure analyses along with the SMD trajectories can also reveal the importance of water molecules for HupA binding and unbinding. For example, at about 185 ps when HupA goes back to the active site, HupA forms several WBs and a perfect H-bond network with AChE (Figure 7A), these interactions

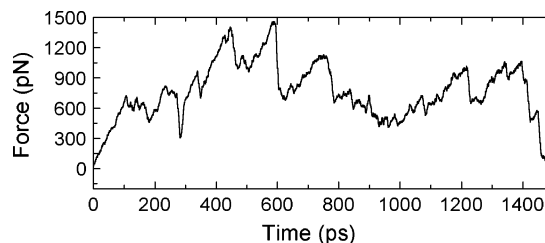


Figure 12. Force required for pulling HupA out from the binding gorge of *TcAChE* in a vacuum at a constant velocity of 0.02 \AA ps^{-1} , shown as 10 ps averaged.

stabilize the structure of the enzyme and also of the HupA-AChE complex, forming a trap for capturing HupA. Overcoming the bottleneck barrier, similar WBs and H-bond network may stabilize the structure of complex and help HupA go back the active site. As discussed in section “The Role of Asp72” and shown in Figure 8, WBs and H-network formed by the buried water molecules and some important residues (such as Asp72) act as clamps to take out HupA from or put HupA into the active site.

Conclusions

The present SMD simulations have provided a new insight into the binding and unbinding of HupA with *TcAChE* at the atomic level. In the nanosecond simulations, HupA was pulled out from and then pushed back into the long binding gorge of AChE, and the unbinding and binding forces were obtained. The peaks and troughs in the force profiles of the SMD trajectories can be assigned to the forming and breaking of specific direct hydrogen bonds, hydrophobic contacts, water bridges and H-bond networks. In particular, our SMD simulations of HupA-*TcAChE* binding and unbinding allow for the following conclusions:

1. Distinct difference exists in binding and unbinding processes of HupA interacting with *TcAChE*. The largest pushing force for HupA entering the binding gorge (360 pN) is less than the largest rupture force for HupA leaving the binding gorge (820 pN). In addition, the bottleneck is favorable to HupA entering. This is in good agreement with the experimental evidence that the binding process of HupA is much quicker than its dissociation.

2. Asp72 forms a lot of WBs throughout HupA leaving and entering the AChE binding gorge, acting as a clamp to take out HupA from or put HupA into the active site.

3. The flip of the peptide bond between Gly117 and Gly118 has been detected in both the conventional MD and SMD simulations. The simulation results indicate that this flip phenomenon may be an intrinsic property of AChE and the Gly117–Gly118 peptide bond in both HupA bound and unbound AChE structures tends to adopt the native enzyme structure, $\omega \approx 150^\circ$.

4. The buried water molecules in the binding gorge act as lubricant, facilitating HupA entering or leaving the binding gorge. They also form a water-bridge net with several residues to capture HupA into the main gorge.

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(59) The cation- π interaction between benzene and methylammonium were calculated as water molecules was introduced into the complex one by one at the MP2/6–31** level by program NWChem. The cation- π interaction between benzene and methylammonium without water molecule is -18.46 kcal/mol (the cation- π interaction between benzene and tetramethylammonium is -11.08 kcal/mol at the same level). The cation- π interaction reduces to -15.43 kcal/mol with one water molecule introduced into the complex, -12.87 kcal/mol with two water molecules and -36.75 kcal/mol with three water molecules. Therefore, the cation- π interaction will be reduced sharply as water molecule is introduced into the complex. The detailed result will be published elsewhere.

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Supporting Information Available: The rupture force, numbers for direct hydrogen bonds, atom pairs of hydrophobic interactions, and water bridges between HupA and *TcAChE* versus time in the process of HupA leaving the *TcAChE* binding

gorge at pulling velocities of 0.1 and 0.01 Å·ps⁻¹ are available in Figures S1 and S2, respectively. Time dependence of the rmsd from the crystal structure of HupA-*TcAChE* complex for the C_α atoms (A) and all atoms (B) of *TcAChE* for the 5 ns conventional MD simulation, shown as 10 ps averaged, are shown in Figure S3. The topology and force field parameter files of HupA produced by the XPLO2D program are listed in Table S1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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