ABSTRACT: Angiotensin converting enzyme 2 (ACE2) plays a key role in renin–angiotensin system regulation and amino acid homeostasis. Human ACE2 acts as the receptor for severe acute respiratory syndrome coronavirus SARS-CoV and SARS-CoV-2. ACE2 is also widely expressed in epithelial cells of the lungs, heart, kidney, and pancreas. It is considered an important drug target for treating SARS-CoV-2 as well as pulmonary diseases, heart failure, hypertension, renal diseases, and diabetes. Despite the critical importance, the mechanism of ligand binding to the human ACE2 receptor remains unknown. Here, we have addressed this challenge through all-atom simulations using a novel ligand Gaussian accelerated molecular dynamics (LiGaMD) method. Microsecond time scale LiGaMD simulations have unprecedentedly captured multiple times of spontaneous binding and unbinding of a potent inhibitor MLN-4760 in the ACE2 receptor. With ligand far away in the unbound state, the ACE2 receptor samples distinct Open, Partially Open, Closed, and Fully Closed conformations. Upon ligand binding to the active site, conformational ensemble of the ACE2 receptor is biased toward the Closed state as observed in the X-ray experimental structure. The LiGaMD simulations thus suggest a conformational selection mechanism for ligand recognition by the highly flexible ACE2 receptor, which is expected to facilitate rational drug design targeting human ACE2 against coronaviruses and other related human diseases.
Figure 1. Root-mean-square deviations (RMSDs) of ten MLN-4760 inhibitor molecules relative to the bound X-ray conformation (PDB: 1R4L) are calculated from the 2000 ns (A) "Sim1" and (B) "Sim2" LiGaMD trajectories, in which the ligand RMSD reached a minimum of ∼0.99 Å. (C) Two views of the ligand binding pathway observed in "Sim2", for which the center ring of MLN-4760 is represented by lines and colored by simulation time in a blue−white−red (BWR) color scale. (D) Two views of the ligand dissociation pathway observed in "Sim2", for which the center ring of MLN-4760 is represented by lines and colored by simulation time in a blue−white−red (BWR) color scale.
tail on the intracellular side. The enzyme PD domain can be inhibited by compounds like MLN-4760, which bind to the protein active site and prevent substrate binding. MLN-4760 binding biases the receptor to adopt a “Closed” conformation, in which the protein active site formed by two subdomains of the PD is closed from the external environment (Figure S1A). Furthermore, the receptor undergoes conformational changes with hinge-bending movement of the dynamic N-terminal subdomain I relative to the stable subdomain II, e.g., ∼16° bending upon inhibitor binding. In the absence of ligand binding, the two subdomains move apart from each other, and the protein active site becomes exposed to solvent in an “Open” conformation. In complex with RBD of the SARS-CoV or SARS-CoV-2, the ACE2 receptor also adopts a “Partially Open” conformation, in which the subdomain I lies between the “Open” and “Closed” conformations (Figure S1A). Among over 20 experimental structures of the ACE2 receptor present in Protein Data Bank (PDB), most of them exhibit “Open” and “Partially Open” conformations, but only one structure has been identified in the “Closed” conformation (PDB: 1R4L).7 Despite tremendous efforts to determine these experimental structures,5-13 the dynamics and functional mechanism of the ACE2 receptor are still poorly understood.14

MLN-4760 is a highly selective and potent (IC50: 0.44 nM) small-molecule inhibitor of the ACE2 receptor.15 The inhibitor has two carboxylic groups contributing to −2 net charge of the molecule (Figure S1B). One of the negatively charged carboxylic groups interacts with the positively charged zinc ion, by which the ACE2 receptor functions as a metallopeptidase enzyme. Depending on ligand or viral RBD binding, the receptor adopts different conformations, but the pathways and mechanism of ligand binding in the ACE2 receptor remain unknown. In the context of SARS-CoV-2 and many other medical implications, it is important to understand the mechanism of ligand recognition by the ACE2 receptor in order to design effective drugs against the virus.

Ligand Gaussian accelerated molecular dynamics (LiGaMD)16 is an enhanced sampling computational technique for efficient simulations of both dissociation and binding of ligand molecules. It is developed based on Gaussian accelerated molecular dynamics (GaMD), which works by adding a harmonic boost potential to smooth the biomolecular potential energy surface.17 GaMD greatly reduces energy barriers and accelerates biomolecular simulations by orders of magnitude.18 GaMD provides unconstrained enhanced sampling without the requirement of predefined collective variables or reaction coordinates. Moreover, because the boost potential exhibits a Gaussian distribution, biomolecular free energy profiles can be properly recovered through cumulant expansion to the second order.19 In LiGaMD,16 the ligand nonbonded interaction potential energy is selectively boosted to enable ligand dissociation. Another boost potential is applied to the remaining potential energy of the entire system in a dual-boost algorithm to facilitate ligand rebinding. LiGaMD has been demonstrated on host—guest and protein—ligand binding model systems. LiGaMD allows us to capture repetitive ligand binding and unbinding and thus characterize both ligand thermodynamics and kinetics simultaneously. The calculated ligand binding free energy and kinetic rate constants compared very well with experimental data.16

Here, we have applied all-atom LiGaMD simulations to investigate binding and unbinding of the MLN-4760 inhibitor and associated conformational changes of the ACE2 receptor. The MLN-4760 inhibitor-bound ACE2 receptor structure (PDB: 1R4L)7 was used to set up the simulation system (Figure S1C, see details in the Supporting Information). A total of 10 ligand molecules (one in the X-ray bound conformation and another nine placed randomly in the solvent) were included in the system. This resulted in ∼14 mM ligand concentration, being close to the ligand solubility of >5 mg/mL or ∼12 mM in aqueous solution. The simulation system was prepared as such since the simulation time needed to observe ligand binding would be inversely proportional to the ligand concentration in the solvent. During the LiGaMD equilibration, the bound ligand dissociated from the active site to the bulk solvent, accompanied by large conformational changes of the protein subdomain I (Figure S2). Upon ligand dissociation, subdomain I of the receptor changed from the “Closed” to “Open” conformation. After the equilibration simulation, ten independent 700–2000 ns LiGaMD production simulations (“Sim1”–“Sim10”) were further performed with randomized initial atomic velocities (Table S1).

Both binding and unbinding of the MLN-4760 inhibitor to the active site of the ACE2 receptor were observed in three of the ten LiGaMD simulations (“Sim1”, “Sim2”, and “Sim3” in Table S1), during which RMSD of the ligand relative to the 1R4L X-ray structure reached a minimum of ∼0.99 Å. During “Sim1” LiGaMD simulation, the MLN-4760 inhibitor bound to the active site of the ACE2 receptor during ∼500–1400 ns and then dissociated into the bulk solvent (Figure 1A). The inhibitor then bound to the receptor active site and dissociated quickly in two different events at ∼1500 and ∼1800 ns time, respectively. During “Sim2” LiGaMD simulation, the MLN-4760 inhibitor bound to the active site of the ACE2 receptor during ∼100–500 ns (Figure 1B) and then dissociated at ∼500 ns into the bulk solvent. The inhibitor then bound to the receptor active site and dissociated quickly at ∼700 ns. It bound to the receptor active site again at ∼1000 ns for ∼80 ns and dissociated at ∼1080 ns. Similarly, during “Sim3” LiGaMD simulation, the MLN-4760 inhibitor bound to the active site of the ACE2 receptor during ∼1780–1800 ns and then dissociated into bulk solvent (Figure S3A). Meanwhile, the receptor underwent large-scale conformational changes with fluctuations in the interdomain distance (Figure S4) and sampled different conformations including the “Open”, “Partially Open”, “Closed”, and “Fully Closed”, out of which three states (“Open”, “Partially Open”, and “Closed”) were consistent with the receptor experimental structures, and “Fully Closed” was a new low-energy conformational state discovered in the LiGaMD simulations. In the other seven LiGaMD production simulations (“Sim4” to “Sim10” in Table S1), no complete, stable ligand binding was observed (Figure S3). The three LiGaMD simulations that successfully captured ligand binding and dissociation (“Sim1” to “Sim3”) were used for further analysis of ligand binding pathways.

During the “Sim2” LiGaMD trajectory, starting from the bulk solvent, one of the MLN-4760 inhibitor molecules first attached to the interface between the receptor 3α H4 and α5 helices within ∼100 ns, moved up into the space between the two protein subdomains and entered the active site of the ACE2 receptor between ∼100 and 160 ns (Figures 1B and 1C). The ligand bound at the active site of the receptor during ∼100–500 ns. At ∼500 ns, the ligand dissociated from the active site to bulk solvent (Figure 1D). The dissociation pathway was observed to be different from that of binding. Ligand dissociated from the opening between the receptor α2
and α4 helices as the subdomain I transitioned from the "Closed" to "Open" conformation (Figure 1D). In contrast, the ligand bound through the space just above the βII H4 and α5 helices (Figure 1C). However, during multiple binding events in "Sim1" and "Sim3", the inhibitor bound and dissociated from the active site through the space between the receptor α2 and α4 helices. The inhibitor dissociated through the same pathway in the LiGaMD equilibration as well (Figure S2A).

This showed that the inhibitor can take either of these pathways (between the receptor α2 and α4 helices or through the interface between the receptor βII H4 and α5 helices) for binding to the active site and just one pathway (between the receptor α2 and α4 helices) for dissociation. During the ligand binding and dissociation in "Sim1"–"Sim3" LiGaMD trajectories, subdomain I of the receptor sampled different conformations. However, such conformational changes were also observed in other seven simulations ("Sim4"–"Sim10") regardless of ligand binding/dissociation.

A 2D potential of mean force (PMF) free energy profile was calculated with the ligand root-mean-square deviation (RMSD) relative to X-ray conformation and the interdomain distance by combining the ten independent LiGaMD production trajectories (Figure 2A, Figures S4 and S5). The protein interdomain distance was calculated between the Ca atoms of residues Glu56 and Ser128, which were located at the tip of the α2 and α4 helices, respectively. Nine low-energy conformational states of the receptor were identified from the PMF profile, including the "Bound (B)" and "Intermediate-1 (I-1)" states. Particularly, the system adopted the "Bound" state with ligand RMSD < 5 Å, the "Unbound" state with ligand RMSD > 35 Å, and intermediate states with 5–35 Å ligand RMSD relative to the 1R4L X-ray structure. The PMF free energy profiles were calculated through reweighting of the LiGaMD simulations with trajectories of all ten ligands considered. The PMF minima indeed highlight the lowest energy states of ligand binding to the ACE2 receptor.

In the "Bound" state, the ligand was bound at the protein active site, and the protein interdomain distance was ~12–14 Å. The ligand exhibited a minimum RMSD of 0.99 Å compared with the X-ray structure (Figures 2A and 2B). The system sampled four different intermediate states during ligand binding to the active site through the space between the receptor α4 helices or through the interface between the receptor βII H4 and α5 helices.
binding, i.e., “Intermediate-1 (I-1)”, “Intermediate-2 (I-2)”, “Intermediate-3 (I-3)”, and “Intermediate-4 (I-4)”. In the “I-1” state, the ligand RMSD was $\sim$9.8 Å and the interdomain distance was $\sim$18−22 Å (Figures 2A and 2C). The ligand was located near the active site, making interactions with residues of the $\alpha 5$ helix, $\alpha 11$ helix, $\alpha 14$ helix, $\alpha 18$ helix, and $3_{10}H4$ helix in the two protein subdomains. In the “I-2” state, the ligand RMSD was $\sim$25.6 Å, and the interdomain distance was $\sim$5−7 Å (Figures 2A and 2C). The ligand interacted with the $\alpha 17$ helix, $\alpha 18$ helix, and $\alpha 19$ helix of the subdomain II in the receptor. In the “I-3” state, the ligand RMSD was $\sim$30.4 Å and the interdomain distance was $\sim$13−20 Å (Figures 2A and 2C). The ligand interacted with the $\alpha 2$ helix of subdomain I and the $\alpha 4$ helix of subdomain II in the receptor. In the “I-4” state, the ligand RMSD was $\sim$31.7 Å and the interdomain distance was $\sim$25−26 Å (Figures 2A and 2C). The ligand interacted with the $\alpha 8$ helix, $\alpha 14$ helix, and $3_{10}H4$ in the protein subdomain II (Figure 2C). The system sampled four “Unbound-1 (U-1)”, “Unbound-2 (U-2)”, “Unbound-3 (U-3)”, and “Unbound-4 (U-4)” states, where the ligand RMSD was $\sim$80 Å and the interdomain distances were $\sim$5−7, 10−12, $\sim$20−21, and $\sim$25 Å, respectively. In these states, the ligand was found far away from the receptor in the bulk solvent, and the receptor could change among the “Fully Closed”, “Closed”, “Partially Open”, and “Open” conformations (Figures 2A and 2D).

Low-energy intermediate conformational states “I-1”, “I-2”, “I-3”, and “I-4” of the MLN-4760 inhibitor binding to the human ACE2 receptor identified from the LiGaMD simulation free energy profiles are shown in Figures 3A, 3B, 3C, and 3D, respectively. Polar and charged groups present in different parts of the receptor made favorable interactions with the charged carboxylic groups and the polar chloride and nitrogen...
These interactions played important role in recognition and binding of the MLN-4760 inhibitor to the receptor. In the intermediate “I-1” state, the receptor adopted a “Partially Open” conformation with ~18–22 Å interdomain distance. The ligand molecule was located near the receptor active site with ~9.8 Å RMSD relative to the X-ray structure and formed interactions with residues from both subdomains of ACE2. One of the ligand carboxylate groups formed ionic interaction with the positively charged protein residue Arg273 (Figure 3A). Another carboxylate group formed an ionic interaction with the Asn149 positively charged nitrogen group. Similarly, the ligand’s central ring formed a π−π interaction with the aromatic protein residue Phe274. The ligand chloride group formed polar interactions with protein residues Thr371, S409, and Thr445. In the intermediate “I-2” state, the receptor adopted a “Fully Closed” conformation, and the ligand formed polar and hydrophobic interactions with the residues of receptor subdomain II. One of the chloride atoms formed polar interactions with residue Glu552. One of the ligand’s negatively charged carboxylate groups formed ionic interactions with the protein positively charged groups of residue Arg559 and Asn572. The ligand also formed hydrophobic interactions with protein residue Leu568.

In the intermediate “I-3” state, the receptor adopted the “Partially Open” conformation, and the ligand interacted with the α2 helix of subdomain I and the α4 helix of subdomain II in the receptor (Figure 3C). One of the ligand chloride atoms formed polar interactions with protein residues Ser70 and

Figure 4. RMSDs of (A) subdomain I and (B) subdomain II of the ACE2 receptor relative to the closed X-ray conformation (PDB:1R4L) are calculated from three independent LiGaMD production simulations. (C) 2D potential of mean force (PMF) of the subdomain I RMSD and interdomain distance calculated by combining the ten LiGaMD simulations. Four low-energy conformational states of the receptor are identified in the PMF profile, including the “Fully Closed”, “Closed”, “Partially Open”, and “Open”. “Closed”, “Partially Open”, and “Open” low-energy conformational states are similar to the 6LZG, 6ACK, and 1R4L PDB structures, respectively. (D) Low-energy conformations of the ACE2 receptor with subdomain I found in the “Open” (red), “Partially Open” (blue) “Closed” (green), and “Fully Closed” (brown) states in the LiGaMD simulations. Subdomain II is stable and colored in white.

atoms in the ligand molecule. These interactions played important role in recognition and binding of the MLN-4760 inhibitor to the receptor. In the intermediate “I-1” state, the receptor adopted a “Partially Open” conformation with ~18–22 Å interdomain distance. The ligand molecule was located near the receptor active site with ~9.8 Å RMSD relative to the X-ray structure and formed interactions with residues from both subdomains of ACE2. One of the ligand carboxylate groups formed ionic interaction with the positively charged protein residue Arg273 (Figure 3A). Another carboxylate group formed an ionic interaction with the Asn149 positively charged nitrogen group. Similarly, the ligand’s central ring formed a π−π interaction with the aromatic protein residue Phe274. The ligand chloride group formed polar interactions with protein residues Thr371, S409, and Thr445. In the intermediate “I-2” state, the receptor adopted a “Fully Closed” conformation, and the ligand formed polar and hydrophobic interactions with the residues of receptor subdomain II. One of the chloride atoms formed polar interactions with residue Glu552. One of the ligand’s negatively charged carboxylate groups formed ionic interactions with the protein positively charged groups of residue Arg559 and Asn572. The ligand also formed hydrophobic interactions with protein residue Leu568.

In the intermediate “I-3” state, the receptor adopted the “Partially Open” conformation, and the ligand interacted with the α2 helix of subdomain I and the α4 helix of subdomain II in the receptor (Figure 3C). One of the ligand chloride atoms formed polar interactions with protein residues Ser70 and
Asn117, while the other chloride atom formed polar interactions with protein residue Ser113. One of the ligand’s negatively charged carboxylate groups formed ionic interactions with the protein positively charged groups of residues Lys114 and Asn64, while the other carboxylate group formed ionic interactions with positively charged nitrogen of Gln60. The ligand central ring’s nitrogen atom formed ionic interactions with negatively charged oxygen group of protein residue Thr118 (Figure 3C). In the intermediate “I−4” state, one of the ligand chloride atoms formed polar interactions with protein residues Ser280 of subdomain II (Figure 3D). One of the ligand charged carboxylate groups formed polar interactions with positively charged protein residue Lys247. One of the nitrogen atoms in the ligand’s central ring formed ionic interactions with negatively charged oxygen of Pro284 and Gln287 in the receptor. In addition to free energy profiles calculated by combining all ten LiGaMD simulations (“Sim1” to “Sim10”), we calculated PMF profiles for each of the ten independent LiGaMD production simulations as shown in Figure S5. These free energy profiles showed clear differences, in terms of positions and free energy values of the PMF minima and energy barrier heights, suggesting that the LiGaMD simulations were still not converged.

During the LiGaMD simulations, Zn$^{2+}$ was stabilized by ionic interactions with the Glu375 and Glu402 residues near the active site of human ACE2 receptor. The distances between the positively charged zinc and the C$_{6}$ atoms of Glu375 and Glu402 were maintained at ∼2−3 Å. This was observed consistently in all the ten LiGaMD independent simulations (Figure S6).

In the LiGaMD simulations, while the protein subdomain II was stable maintaining the 1R4L X-ray conformation with ∼2−4 Å RMSD (Figure 4B), subdomain I in the human ACE2 receptor exhibited high flexibility and underwent large conformational changes with ∼3−10 Å RMSD compared with the X-ray conformation (Figure 4A). We calculated 2D PMF profiles regarding the interdomain distance and subdomain I RMSD relative to the 1R4L X-ray conformation. Four low-energy conformational states were identified in the PMF profile, including the “Open”, “Partially Open”, “Closed”, and “Fully Closed” (Figure 4C).

In the “Closed” conformation, subdomain I moved near subdomain II closing the active site. The receptor interdomain distance was ∼14−15 Å, and the RMSD of subdomain I was ∼5 Å compared with the 1R4L X-ray structure (Figures 4C and 4D). In the “Fully Closed” conformation, the subdomain I can further move toward the subdomain II with interdomain distance ∼12−13 Å. The subdomain I RMSD was ∼4.5 Å. In the “Partially Open” conformation, the receptor interdomain distance increased to ∼17−18 Å and the RMSD of subdomain I RMSD was ∼6 Å compared with the 1R4L X-ray structure (Figures 4C and 4D). Finally, the receptor interdomain distance could increase further to ∼19−20 Å, and the subdomain I RMSD relative to the 1R4L X-ray structure increased to ∼7 Å in the “Open” conformation. Notably, conformations of the ACE2 receptor in the “Partially Open” and “Open” low-energy states were closely similar to the experimental 6ACK cryo-EM and 6LZG X-ray structures, respectively (Figures 4C and 4D). Therefore, the different low-energy states of ACE2 receptor revealed from our LiGaMD simulations highlighted the receptor conformational plasticity during its function for ligand binding and interactions with other proteins (e.g., the coronavirus spike protein).

Since its discovery in 2000, the ACE2 receptor has been recognized as a critical protease enzyme with multiple physiological roles in the renin–angiotensin system, amino acid transport, gut microbiome ecology, and innate immunity. The ACE2 receptor has also been identified as the functional receptor for SARS-CoV and SARS-CoV-2. The COVID-19 pandemic caused by SARS-CoV-2 has been recognized as a serious global health threat as it has no proper treatment and continues to spread across the world. With the infection cases rising daily, it is critical to develop therapeutics against SARS-CoV-2. Here, we have applied all-atom simulations using a novel LiGaMD method to investigate the mechanism of ligand binding to the human ACE2 receptor.

Through LiGaMD enhanced sampling simulations, we have, for the first time, successfully captured both binding and dissociation of a ligand in the human ACE2 receptor. During the simulations, the receptor could sample distinct conformational states, revealing remarkable conformational plasticity of the receptor. When the ligand binds to the active site of the receptor in the Bound state with the ligand RMSD < 5 Å, the interdomain distance was confined to be small at ∼12−14 Å, being closed. This suggested that the ligand binding biased the receptor conformational ensemble to the Closed state, suggesting a conformational selection mechanism rather than induced fit. Furthermore, the MLN-4760 ligand has two carboxylate groups contributing to net −2 charge of the molecule. Hence, ligands repelled each other with no significant ligand–ligand interactions observed in the simulations. This finding suggested that electrostatic interactions played an important role in the recognition and binding/dissociation of the MLN-4760 inhibitor to the ACE2 receptor, being consistent with previous findings of “electrostatic steering” in recognition of charged ligands by proteins.

Despite our encouraging simulation findings, it is important to note that we sampled ligand dissociation and binding events in 3 out of 10 LiGaMD simulations. The free energy profiles calculated for each of the ten individual simulations (Figure S5) and all ten simulations combined (Figures 4A and 4C) showed clear differences, in terms of the positions and free energy values of the PMF minima and energy barrier heights. This suggested that the LiGaMD simulations were still not converged, and the calculated free energy profiles were not accurate for direct comparison with experimental ligand binding free energy. It remained challenging to sample enough events of repetitive ligand binding and unbinding along with the large protein conformational changes. More sufficient sampling would be needed in order to obtain converged simulations and calculate accurate ligand binding free energies and kinetic rates. This can be potentially achieved through additional and longer simulations as well as further method developments combining LiGaMD with other enhanced sampling algorithms such as replica exchange and Markov state models.

In this context, the MLN-4760 inhibitor binds to the human ACE2 receptor with high affinity (IC$_{50}$: 0.44 nM). It is extremely difficult to simulate the ligand dissociation and binding with long-time scale conventional MD (cMD) and even the enhanced sampling methods. A recent study showed that the MLN-4760 ligand could dissociate from the ACE2 receptor upon binding of the viral RBD. This study was not able to characterize ligand binding to the ACE2 receptor. In comparison, our LiGaMD simulations could...
capture both ligand binding and dissociation in the human ACE2 receptor.

We further highlighted the dynamic nature of the ACE2 receptor in terms of the large-scale movement of subdomain I upon ligand binding. Furthermore, because the human ACE2 receptor shows conformational selection for ligand binding as revealed from the LiGaMD simulations, virtual screening using ensemble docking24–26 with receptor structural ensembles generated from the LiGaMD simulations will be a promising approach to designing potent drug molecules of the ACE2 receptor.

In summary, we have successfully simulated both ligand binding and dissociation in the human ACE2 receptor using the novel LiGaMD enhanced sampling method. During the LiGaMD simulations, the receptor could sample distinct Fully Closed, Closed, Partially Open, and Open conformational states, being consistent with previous experimental structures. Ligand binding could bias the receptor conformational ensemble toward the Closed state, suggesting a conformational selection mechanism. Therefore, the LiGaMD simulations have allowed us to understand the mechanism of ligand recognition by the ACE2 receptor, which is expected to facilitate rational drug design targeting ACE2 for the therapeutic treatments of COVID-19 and other related human diseases.

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**Author Contributions**

Y.M., A.B., and S.P. conceived the study; A.B. performed the simulations; A.B. and S.P. analyzed the data; and A.B., S.P., and Y.M. wrote the manuscript.

**Notes**

The authors declare no competing financial interest.

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