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Discover binding pathways using the sliding binding-box docking approach: application to binding pathways of oseltamivir to avian influenza H5N1 neuraminidase

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Abstract Drug binding and unbinding are transient processes which are hardly observed by experiment and difficult to analyze by computational techniques. In this paper, we employed a cost-effective method called "pathway docking" in which molecular docking was used to screen ligand-receptor binding free energy surface to reveal possible paths of ligand approaching protein binding pocket. A case study was applied on oseltamivir, the key drug against influenza a virus. The equilibrium pathways identified by this method are found to be similar to those identified in prior studies using highly expensive computational approaches.

Keywords Influenza · H5N1 · Neuraminidase · Docking · Oseltamivir · Binding pathway

Introduction

Structured-based computer-aided drug design has undoubtedly made significant impacts to the drug development process [1–12]. Major research focus in this area has been on predicting the end-point of the ligand binding process, namely the lowest-energy binding pose of a ligand and its corresponding binding energy. Toward this end, various docking methods were developed and continually improved to perform virtual screening of compound libraries for lead optimization. How a ligand makes its way to this end-point state has been increasingly recognized to be of equal importance. In particular, knowledge of the binding pathway such as different channels leading to the same binding pose, transition states and metastable minima along these channels provides insight to the binding mechanism, its kinetics and thus helps to understand drug responses [13].

The most popular way to study drug kinetics by far is molecular simulation. This intuitive approach encounters a number of challenges in discovering ligand binding pathways, the two most significant of which are (1) specifying the starting point and (2) overcoming the barriers along the binding pathway. On the first challenge, due to the funnel-like shape of the space available to ligand toward binding site, number of degrees of freedom greatly increases when stepping away from the binding site, requiring more exhaustive sampling to effectively explore this space. The second challenge arises from the fact that ligands may need to surpass energy barriers along the binding pathway, making it even more tricky to conventional molecular dynamics method. This could explain why until now, there have been very few efforts using conventional MD to study the binding pathway [14–16].

To address the starting point problem, Chang et al. [17] employed Brownian dynamics method to simulate a large number of association trajectories with different starting points to explore possible binding pathways. To make it computationally feasible, coarse-grained models for both the protein and ligand were developed. The only drawback of this approach is the expertise required and the time consumed for developing such coarse-grained models.

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To speed up the process of going over a barrier and at the same time reduce computational demand in conventional MD method, one approach is to introduce an external force to help ligands surmounting these barriers more easily as in the steered molecular dynamics (SMD) method [18, 19]. However, when applying this approach to study the ligand unbinding process, the problem of choosing direction and amplitude of the external force would lead to bias in the observed kinetics. To reduce such bias, targeted molecular dynamics (TMD) method proposed by Schlitter et al. [20, 21] modifies the force field by implementing a time-dependent constraint on structural deviation that could drive a system to the target structure. During a TMD simulation, the system is guided towards the final target structure by means of steering forces. A less prejudiced method is biased molecular dynamics (BMD) [22] in which the system would only feel the external biasing potential whenever it moves away from the target [23, 24]. These three methods are widely used to study transitional processes of biomolecules, sharing the common idea of introducing an external perturbation to the system to drive it from a predefined initial state to the target state, but differing in how the perturbation is applied [25]. A different approach is to combine directional guiding, Monte–Carlo search and minimization as proposed by Straber et al. [20, 21]. In this procedure, ligands are translated to the binding pocket on a virtual guiding line. At each step, rotation over all pre-defined rotatable bonds of the ligands and of target protein is carried out and the structure of the whole complex is consequently energy-minimized to obtain a new generation. With a similar scheme, Ram et al. [15] combine energy minimization and MD simulation to look for the binding pathway. Ligand is moved incrementally from the pocket to the surface. At each of intermediate states, energy minimization and MD simulation are carried out. All of the above methods suffer the same difficulty in specifying the initial unbound state and the 'guiding' line or force without any preconceived notion about the actual pathway from the final structure.

In this study, we proposed a rather simple method for discovering binding pathways. The method utilizes molecular docking in a novel way in order to flag possible points along ligand binding pathways and is called the sliding grid-box docking approach. Protein–ligand docking, as mentioned earlier, has been normally used to find the lowest-energy binding pose of a ligand in the active site of a target protein. Typical docking methods explore the conformational space of a ligand within a specified 3D grid-box containing the active site. Here the idea is to change the size and the location of the grid-box to search for low-energy configurations in different protein regions enclosed by the grid-box. Thereby a series of docking calculations with grid-box sliding from the opening surface of the protein to the buried active site will establish continuous paths of discrete low-energy poses. Such paths are approximations to the steepest descent paths on the free energy landscapes. This approach enables prediction of binding pathways while avoiding initial biases of specifying the starting point and the direction which the ligand follows.

To illustrate this method, we apply it to study binding pathways of oseltamivir to avian influenza H5N1 neuraminidase. Oseltamivir, also known as tamiflu, is an inhibitor of H5N1 neuraminidase and has been a key drug to fight bird flu as well as the 2009 pandemic H1N1 flu. The drug eventually became ineffective due to the appearance of single-point mutations which are specifically challenging since they confer drug resistance without being close to the drug binding site. A number of computational studies have been done to investigate the interaction of oseltamivir with H5N1 neuraminidase [26–29], yet only a few focused on the binding pathway of this drug. Our recent study suggested that disruption in the pathway of oseltamivir to the binding pocket would be the reason of observed drug resistance from a single-point mutations His274Tyr or Asn294Ser [30]. Although the study employed the SMD approach and thus suffered from limitations mentioned above, it illustrated the significance of ligand binding pathway in the drug development process. Thus, by using the binding pathway of oseltamivir to H5N1 neuraminidase as an illustrating example of the sliding grid-box docking approach proposed here, the results can also help to confirm our previous suggestion of the mechanism of drug resistance in this system.

Computational details

Sliding grid-box docking approach

The central idea of the sliding grid-box docking approach is using the grid-box to restrict the docking algorithm to search for low-energy ligand conformations in narrow slices of the binding channel. Docking calculations on a series of overlapping grid-boxes will result in overlapping low-energy binding poses of ligand in the space covered by these boxes. This practice is similar to umbrella sampling in simulations of potential of mean force. Cluster analysis on these continuously overlapped ligand poses would lead to the discovery of possible binding pathways.

For each grid-box, docking calculation is performed to generate low-energy binding poses inside the box. Thus for boxes that do not contain the binding site, these low-energy binding poses may be far from the end-point, yet would be among the favorable binding poses of the drug on its way

to the binding site. In this work, we used the Autodock Vina program [31] which implements an iterated local search with global optimization method using an empirical scoring function. Thus, convergence of finding a binding pose as well as the applicability of the present method is governed by the limitations of the Autodock Vina program. Thus this method is applicable to finding binding pathway for all types of binding sites from surface binding positions to interior binding sites. It is limit only by the accuracy of the docking scoring function of the docking procedure. For AutoDock Vina, the scoring function is known to work better for binding sites that are buried rather than those exposed to solvent.

To facilitate both the computation and analysis, the target protein is oriented so that the direction from its main binding pocket to the opening exposed to solvent, i.e. the binding channel, is along the z-axis. Each grid-box is defined to cover a slice of the protein with a minimal depth or height (z-dimension length) that is just slightly larger than the full length of ligand and sufficient for the docking algorithm to explore the full conformational space of the ligand inside the box and the base xy plane is sufficiently large to cover the entire protein area. Since in AutoDock, the ligand is fully flexible, the z-dimension should be slightly larger than the maximum length of the ligand when it is fully stretched. A series of overlapping grid-boxes sliding along the z-direction starting from sufficiently high above the surface of the protein (5 Å in this case) to its binding site were created. The initial slide grid-box should cover some fraction of the protein surface to facilitate cluster analysis and ensure the docking scoring function would provide meaningful results. Each box is overlapped by 75 % with neighbor ones. As illustrated in Fig. 1, this procedure resulted in 6 grid-boxes of 48 × 48 × 10.5 Å in size for binding of oseltamivier to avian influenza H5N1 neuraminidase.

Points along a possible binding pathway were then revealed by clustering analysis. Binding poses in all overlapping grid-boxes were clustered using the k-means algorithm [32] on four dimensions, namely x, y, z coordinates of the ligand center of mass and the binding energy weighted by a factor of 0.7. The use of binding energy as part of the clustering analysis helps to discriminate binding poses that are close in space thus have close centers of mass but have different orientations. All outlier points that are sparsely distributed or unable to reach the binding pocket in a continuous path were removed prior to the analysis. This step resulted in seven clusters, each of which consists of points that are close enough in space and not too much different in the binding energy (Fig. 2).

The main advantage of this approach is its simplicity and being highly cost-effective since it employs docking as principal calculations and does not require any modification

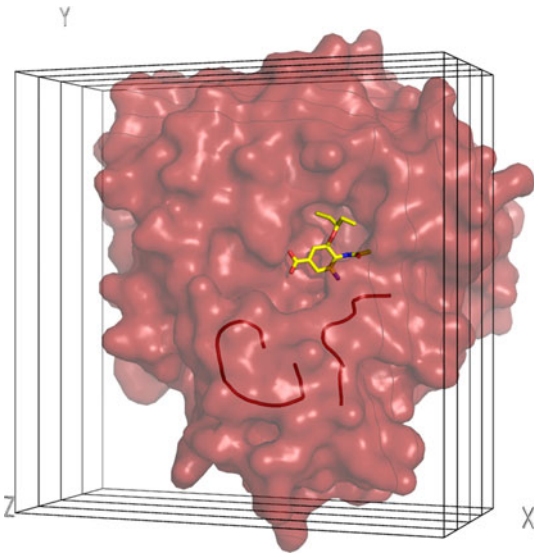

Fig. 1 Pathway docking methodology. Grid-box is defined to cover a large area on protein surface and moved along *z* axis toward the active site (indicated by the bound oseltamivir molecule). Neuraminidase structure is re-oriented to maximize the opening area cut by XY-*clipping plane*. 430-loop and 150-loop are displayed in cartoon

of the docking program. The entire calculation can be done in one submitting script that repeats the docking calculation over the number of grid-boxes. The drawback, however, is the uncertainty in the accuracy of binding energies generally and especially those that are far from the binding site. The reason is that docking scoring functions, including that from Autodock Vina, are generally fine-tuned to experimental binding complexes which are end-point. Accordingly, force field-based scoring functions are expected to yield more physically reasonable results. To avoid unphysical results, we observed the distribution of binding energies along the binding pathways. As discussed in the result section below, the binding energies slowly decreased from the entrance toward the binding pocket, indicating that Autodock Vina's scoring function is acceptable for regions outside of the binding pocket in this case. Furthermore, pathways identified by this approach have a limited resolution, leaving large regions in between the flagged points undetermined, due to the use of the k-mean cluster analysis and the requirement of the grid-box to be sufficiently large to fully contain the ligand. However, lacking of point in region between two clusters along a binding path could also indicate the possibility of it being a transition state.

Receptor structure

Protein flexibility can be accounted for by using the ensemble-based docking approach, which is increasingly common in virtual screening [33–35]. This approach allows including of large protein motions such as loop flapping that is important for ligand binding pathways

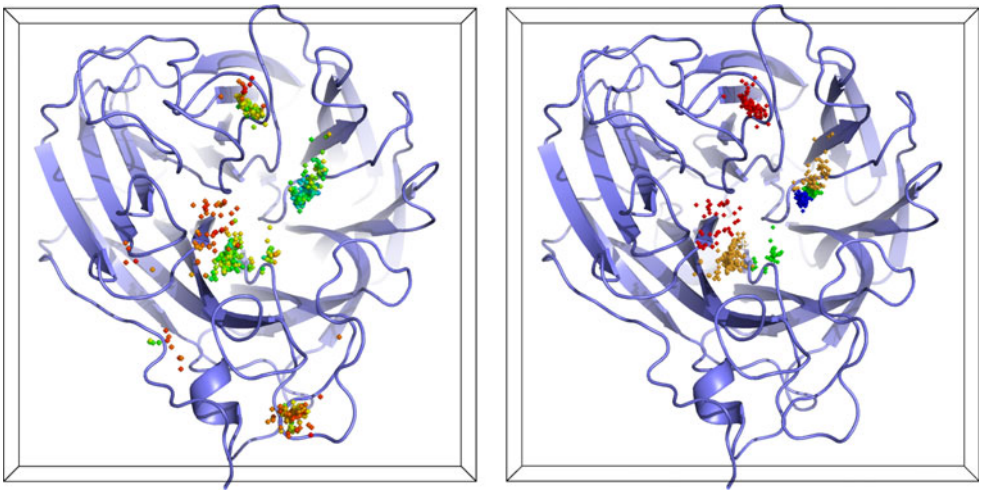

Fig. 2 Pathway identification based on k-means clustering of ligand centers of mass. Centroid are colored by binding energy *(left)* with *red* binding least favorable binding energy poses and *blue* being most favorable binding energy poses and by cluster *(right)*

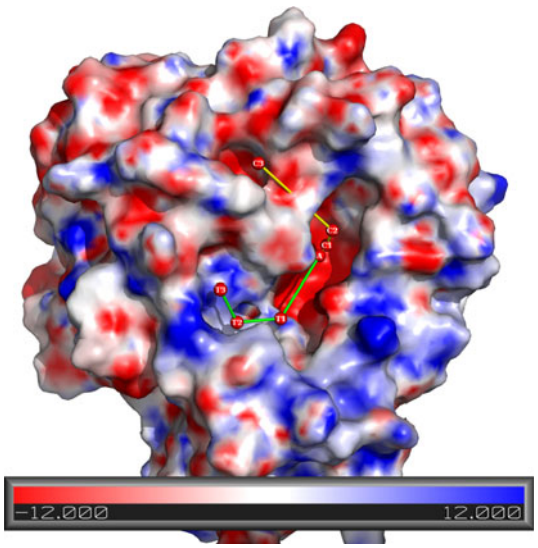

Fig. 3 Two possible binding pathways of oseltamivir approaching neuraminidase active site (*labeled A*). The climbing pathway is represented by C3–C2–C1–A, and the tunneling pathway by T3–T2–T1–A

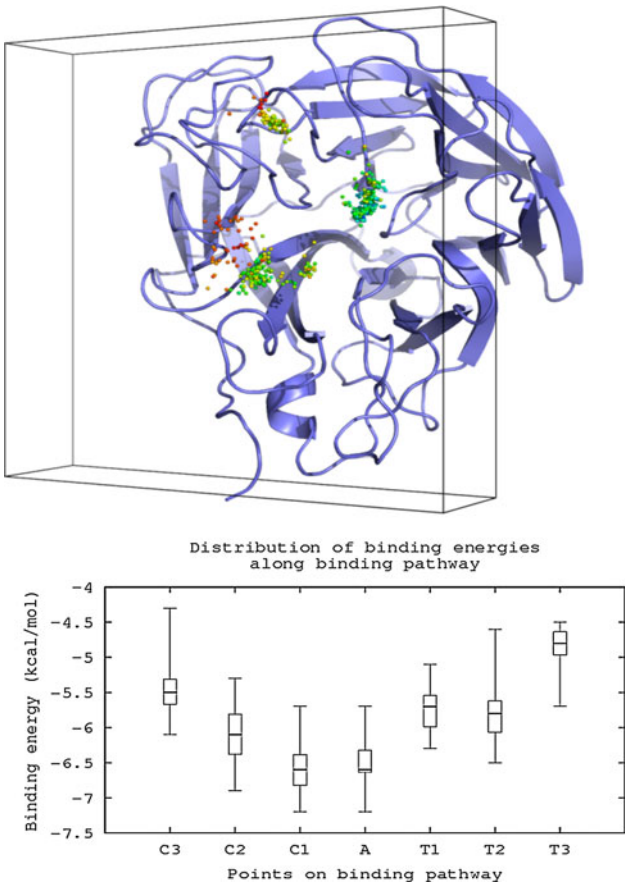

Fig. 4 Distribution of binding energies of docked conformations. *Upper:* Docked conformations represented by ligand centers of mass, colored by binding energies, with highest energy in *red*, lowest in *blue*. *Lower:* Statistical distribution of binding energies at each point of along the binding paths

whereas flexible docking programs can only account for limited flexibility of certain side chains in the binding site, which is important only for the end-point binding pose and energy.

In this study, target protein conformations were taken from a 40-ns MD simulation of neuraminidase N1 in complex with oseltamivir using the Gromacs program with AMBER99SB force field. The complex structure (PDB ID:2HU4) was solvated by TIP3P waters, neutralized with Na^+ ion, minimized, gradually heated to 300 K and then equilibrated for 40 ns with a time step of 1 fs, having list of nonbonded pair within 1 nm updated every 10 fs. A cutoff of 1.4 and 1.0 nm was imposed for evaluation of vdW and electrostatic interactions, respectively. Long-range electrostatic interactions are treated with particular-mesh Ewald summation. The simulation is an extension of that performed by Nguyen et al. for the wild-type system [36]. Trajectories of the last 20 ns were extracted to generate representative ensembles for docking. RMSD structural

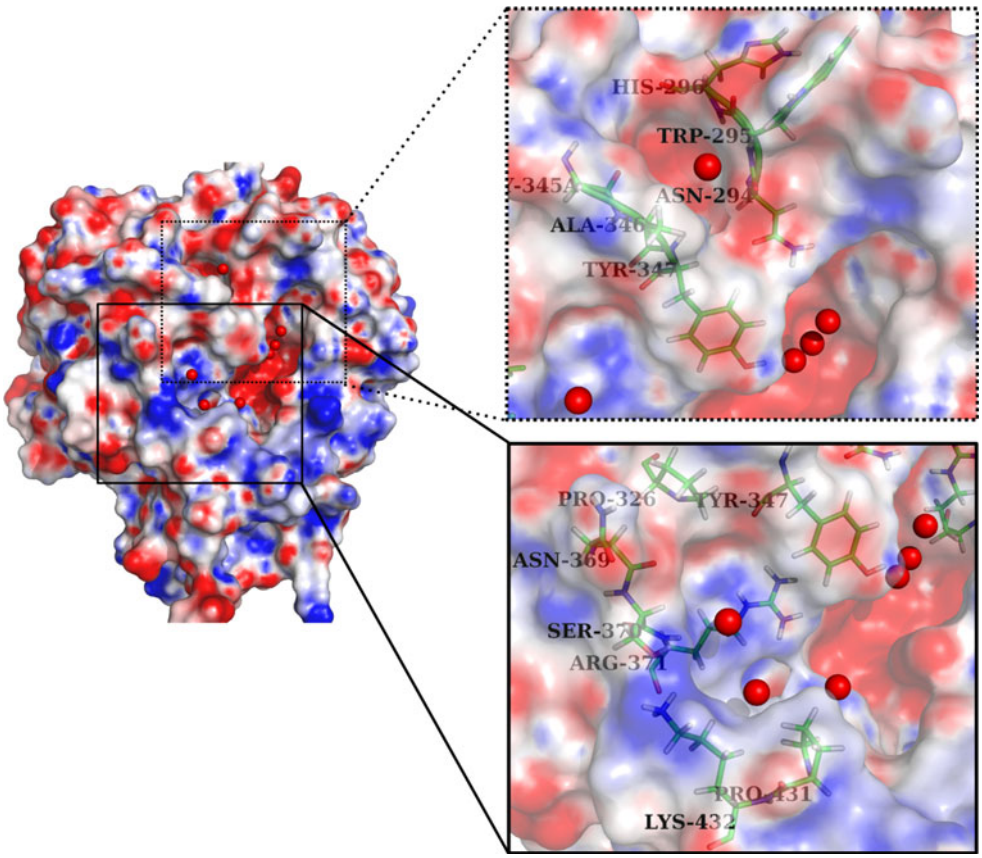

Fig. 5 Important interactions in the early stage of binding along the climbing pathway *(upper frame)* and along the tunneling pathway *(lower frame)*

analysis shows large motions of the 150-loop (Asn146–Arg152) and the 430-loop (Arg430–Thr439) consistent with previous MD simulation [37]. Clustering analysis was performed using the g cluster program provided with the Gromacs package, at 0.9 Å cutoff resulting in 127 clusters. All 127 clusters were used as receptor conformations in docking calculations for each binding-box.

Results and discussion

Binding pathway of oseltamivir to influenza neuraminidase

Docking results over 127 representative receptor structures proposed two lowest-energy pathways approaching *sialic acid binding site* in influenza N1 *neuraminidase* (Fig. 3). The first pathway, referred to as the climbing path, is indicated in Fig. 3 as the line C3–C2–C1–A and the second pathway, referred to as the tunneling path, as the line T3–T2–T1–A. Distribution of docked conformations (represented by center of mass) and averaged binding energies of point clusters along these two pathways are plotted in Fig. 4. These two pathways are relatively similar to the previous MD simulation studies [30].

In the climbing path, inhibitor molecule was loosely held on the enzyme surface in a small cavity between the two loops 294–296 (Asn–Trp–His) and 345–347 (Gly–Ala–Tyr) before moving toward the active site (Fig. 5). This small cavity may not be a prevailing feature on the enzyme surface, as it is not found in the crystalized structure. However, it can provide a harbor that favors landing of freely diffusing ligands on protein surface. Our results do not provide a clear picture on how oseltamivir moves from C3 to C2. It is possible that the ligand climbs over a barrier as indicated by the lack of low-binding energy poses between C3 and C2 along with movements of Asn294 and Tyr347 side chains and then slide to C2. Moving from C2 through C1 to A is favorable as indicated by a significant decrease in binding energies (Fig. 4) and is consistent with the strong electrostatic interaction along the negatively charged funnel as suggested by Le et al. [30]. Note that Asn294 is at the entrance of the barrier region for going from C3 to C2 (see Fig. 5), this implies Asn294 may behave as a gate, specifically provide more favorable hydrogen bonds to help the ligand overcoming the barrier. Thus mutation of this residue would affect ligand binding process along this pathway and thus lead to oseltamivir resistance. Our observation about the role of Asn294 supports the speculation in prior study [30] that mutation

Asn294Ser, locating at the negatively charged pathway, prevents oseltamivir from entering the sialic acid binding site.

In the tunneling path, oseltamivir was found to be in close contact with Ser170 which is among the minimum pattern of secondary sialic acid binding site [38, 39] and with Pro431 and Lys432 which are residues of the flexible 430-loop. Interaction analysis also showed that Arg371 has a high frequency of hydrogen bonds with oseltamivir carboxylate due to the guanidinium group on its side chain, while Pro431 and Lys432 contribute to the binding mainly via side chain hydrophobic contacts. These interactions indicate that this path may be similar to the second path suggested by Cheng et al. [33] and then by Le et al. [30] which emphasizes the movement of 430-loop during ligand binding. However, a closer look at distribution of binding energies in this area raises a question if it is possible for ligand to reach T1 from T2. Although T1 is closer to the active site, average binding energy of T1 group is not significantly different from, and even less favorable than, that of T2 group (Fig. 4). This distribution of binding energies shows a low possibility of ligand moving from T2 to T1, which is also in good agreement with conclusion of Le et al. [30] that the binding path from this direction is less favorable than the climbing path.

The present results reveal a number of interesting facts regarding the slide binding-box approach. It can predict stable intermediates along binding pathways. Such intermediates are important for constructing the correct binding mechanism and thus would be crucial for understanding drug binding/unbinding kinetics. Since the methodology can only predict the low-energy binding poses within the space of a given grid-box and the constraint on the width of the box discussed in the computational details section, the method cannot precisely identify potential barriers but can however suggest the possibility for its existence by the lack of data points in a certain region along a 'projected' line of data points as evidence of a binding pathway. The results though help to localize region in a large protein multi-dimensional space to search for potential barrier by a constraint MD method. Such problem has been a great challenge in bio-simulations.

Conclusion

We presented a simple and cost-effective computational approach, the sliding grid-box docking method for finding binding pathway. This method does not suffer the difficulty of specifying the starting point and of going over the barrier as in previous simulation methodologies. Pathways identified by this method represent an approximation to the minimum free energy paths and can be used to define the collective variables in accurate simulations of free energy profiles [30].

The simplicity and cost-effectiveness as well as limitations of the method suggest that it should be used as the necessary first step in studying ligand binding pathway, namely discovering all possible binding pathways. Subsequently, equilibrium MD simulations can be performed at each point along the binding pathway to provide more details about interactions involved in the pathway or simulation of free energy profiles along such path.

Despite its simplicity, application of the sliding grid-box docking method to binding of oseltamivir to H5N1 neuraminidase has revealed more important details about the roles of residues Asn294 and Tyr347 in the climbing path (negatively charged pathway as in Ref. [30]) as well as the roles of residues Pro431 and Lys432 in the tunneling path (the secondary path via 430-cavity as in Ref. [30]) which were found but not fully understood. The result obtained also provides explanation on why mutation Asn294Ser locates about 14 Å away from the sialic acid binding site but still induces significant drug resistance. More importantly, the agreement of the present results with previous studies using different simulation techniques validates the applicability of the sliding grid-box docking method. Further applications to different biological systems will help to identify the applicability as well as limitations of the presented method. Such studies are currently being done and will be published in forthcoming papers.

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