Finding Multiple Reaction Pathways of Ligand Unbinding

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Searching for reaction pathways of large systems presents a long-standing challenge in physics. We present a general molecular dynamics method to find multiple diverse reaction pathways of ligand unbinding through minimization of a loss function describing ligand–protein interactions. The method successfully overcomes large energy barriers using an adaptive bias potential, and constructs all possible heterogeneous reaction pathways along transient tunnels without initial guesses of intermediate or final states, requiring crystallographic information only. We examine the method on the T4 lysozyme L99A mutant which is often used as a model system to study ligand binding to proteins, and provide a previously unknown reaction pathway.

Molecular dynamics (MD) simulations provide sufficient temporal and spatial resolution to study physical processes. Unfortunately, MD fails to reach high energy barriers (≫ kT) that dictate mechanisms and kinetics of rare events. Transport in heterogeneous media, such as ligand unbinding, cannot be simulated directly, and even biased MD methods often fail to find possible reaction pathways along complex transient tunnels of proteins that form spontaneously during dynamics.\textsuperscript{1,2} Although many general purpose methods have been developed to sample rare events,\textsuperscript{3–5} finding multiple reaction pathways of ligand unbinding is especially difficult. Also, experimental methods used currently to quantify ligand binding, e.g., time-resolved crystallography and xenon binding focus primarily on gaseous species, providing indirect evidence for the migration of larger ligands, which makes most details of reaction pathways unresolved.

The main computational limitations that render the reconstruction of reaction pathways for ligand unbinding difficult stem from accounting for internal topological features of proteins (e.g., tunnels), which is related to the degree of coupling between protein dynamics and ligand conformational states. The structural flexibility of protein tunnels allows proteins to facilitate binding by adapting to binding partners along possibly multiple pathways to the binding site. This intrinsic dynamics poses a severe challenge to straightforward biased MD methods that have been used to sample reaction pathways in ligand unbinding.\textsuperscript{5–8} Typically, such methods either approximate reaction
pathways by linear Cartesian coordinates, or probe protein tunnels randomly.

An additional and ubiquitous obstacle in describing ligand unbinding is the overestimation of energy barriers and underestimation of exponentially dependent kinetic rates arising from inadequate initial guess of reaction pathways. As emphasized by Elber and Gibson, sampling should not overestimate preference to more direct and geometrically shorter reaction pathways. Producing and exploring multiple reaction pathways of a complex system remains a challenge.

In this Letter, we present a general biased MD method to find multiple diverse reaction pathways of ligand unbinding along transient protein tunnels. The method does not require an initial guess of intermediate or final states, which is a major challenge for existing methods. Its only prerequisite is the knowledge of the initial bound state, without requiring an initial reactive trajectory. The method also takes into account protein dynamics, which is important to observe transient tunnels. The method minimizes a loss function during MD simulations of a 3X set of ligand coordinates \( x \equiv (x_1, \ldots, x_{3X}) \) and a 3Y set of protein coordinates \( y \equiv (y_1, \ldots, y_{3Y}) \). To this aim, we propose the loss function must fulfill three important criteria, e.g., (i) describe physical interactions in a ligand–protein system, (ii) tend to infinity as the ligand moves too close to the protein, and (iii) decrease as the ligand unbinds from the protein; (ii) prohibits the method from sampling ligand configurations that clash with a protein, and (iii) provides a coarse estimate of how ligand conformations are buried within a protein tunnel.

**Minimization**—Because empty space in proteins and its intrinsic fluctuations constitute a key feature of tunnels, we use a coarse physical model for ligand–protein interactions, which accounts for steric interactions only. We motivated our decision by the simplicity of this approach. For \( i \)th pair of ligand–protein atoms, we define a partial loss function as \( \exp(-r_i)/r_i \), where the distance between the atoms is given by \( r_i = \lambda \|x_i - y_i\| \). The \( \lambda \) constant sets length scale in the loss function, and is equal to 1 when using ångströms, i.e., \( \lambda = 1\text{Å}^{-1} \). Hence, we used the loss function of the following form:

\[
s = \sum_{i=1}^{P} \frac{\exp(-r_i)}{r_i},
\]

where \( P \) is the number of ligand–protein atom pairs. The sum over all pairs meets the criteria of the loss function for ligand unbinding presented here. The aim of the proposed method is to efficiently sample the configurational space of the ligand–protein complex, and optimize Eq. (1) during MD simulations, so that the reconstructed reaction pathways of ligand unbinding minimize the loss function along multiple tunnels. In this method, many MD simulations are required to sample multiple reaction pathways.

For a schematic depiction of the method, see Fig. 1. The method proceeds as follows: (i) it finds a minimum of the
FIG. 1. Sampling of ligand unbinding pathways using the presented biased MD method. As an example, the unbinding of benzene from T4 lysozyme L99A along a reaction pathway is shown. The unbinding is initiated from the bound state (X-ray binding site) of the T4L–benzene complex, and ends once the ligand reaches solvent. Cross-sections through X-ray structure of T4L shows no apparent tunnels for benzene to leave the protein, which means that the protein must undergo structural changes to open possible exits. A reaction pathway characterizing atomistically the unbinding along the transient exit tunnel is identified locally during the MD simulations. To determine \((k+1)\)th intermediate, the positions of benzene are sampled in the neighborhood (constrained by the sampling radius) of \(k\)th intermediate. Then, from the sampled ligand conformations the optimal direction of biasing is calculated by selecting the ligand conformation which is represented by the lowest loss function score.

loss function in the neighborhood of the current position of the ligand, and, (ii) the position of the ligand is biased in the direction of the localized minimum of the loss function. The minimization is repeated during the MD simulation every \(\Delta t\), and the biasing is performed until a new solution in the neighborhood of the current position is calculated, which will be described further.

The minimization of the loss function is performed using simulated annealing.

To this end, the method checks if a randomly chosen neighboring position of the ligand \(x'\) is preferred in terms of the loss function. The neighbor is selected as a next solution according to the Metropolis–Hastings algorithm with the Boltzmann factors (for simplicity, we omit protein coordinates \(y\) in notation):

\[
p = \begin{cases} 
\exp(-\beta(s(x') - s(x))) & \text{if } s(x') > s(x), \\
1 & \text{otherwise},
\end{cases}
\]  

(2)
FIG. 2. Reaction pathways of benzene unbinding from T4L. Only the T4L C-terminal domain is depicted, but the complete protein was used in all simulations. The crystallographic bound conformation of benzene is shown. Benzene conformations sampled during the MD simulations are biased by an adaptive bias potential to find multiple exits of T4L via the reaction pathways. The reaction pathways are named pwa–e, which corresponds to the T4L tunnels indicated by helices, i.e., D/F/G tells that the unbinding pathway is located near the D, F, and G helices.

where $\beta = 1/T(t)$ is a parameter introduced to decrease the probability of acceptance of a worse solution as the minimization procedure proceeds. $T(t)$ is reduced according to $T(t) = kT(t - 1)$, where $t$ is the time during each optimization phase, and $k = 0.95$ to promote convergence to an optimum. The minimization procedure is reiterated to find an optimal solution.

This minimization procedure, however, clearly needs constraints to optimize the loss function locally, since intermediate states are searched for sequentially, and a global minimization of the loss function would identify only the final state of ligand unbinding. A naive approach is to sample ligand conformations constrained to a sphere with a constant radius positioned at the center of mass of the ligand, but this requires an estimate of the radius, which is clearly system-dependent and should changed as protein dynamics is simulated. To alleviate this issue, we take the sampling radius equal to the minimal distance between the ligand–protein atom pairs, e.g., $r_s = \min_i r_i$. By doing so, the method dynamically adjusts the conformational space available for the sampling.
FIG. 3. Loss function along an example trajectory of benzene unbinding from T4L. Two sudden jumps in the loss function indicate conformational transitions of the benzene–T4L complex during unbinding. The first transition at about $t = 1.85$ ns is the change from the bound conformation to an intermediate, during which benzene samples the exit along pwa via D/F/G. Next, at about $t = 4.6$ ns the complex switches its state to unbound.

Adaptive biasing—Once the optimal ligand conformation $x' = \min_{x} s$ is calculated, the position of the ligand is biased in the direction of $x'$ along transient protein tunnels. This stage is performed by biasing the position of the ligand using an adaptive harmonic potential:

$$V(x) = \frac{k}{2} \left( v \Delta t - (x - x'_{i-1}) \cdot \frac{x'_{i} - x}{||x'_{i} - x||} \right)^2,$$

(3)

where $x'_{i}$ is $i$th optimal solution, $v$ is the biasing rate, $\Delta t$ is the interval at which the loss function is minimized, and $k$ is the force constant. The bias potential from Eq. 3 is a generalization of the harmonic biasing potential introduced by Heymann and Grubmüller to curvilinear reaction pathways. Several methods introduced recently used a constant bias to sample complex reaction pathways. In contrast to these approaches, the bias shown by Eq. 3 is adaptive, and dependent on the optimal reaction pathways calculated by minimizing Eq. 1.

Unbinding benzene from T4 lysozyme L99A—We illustrate the method on T4 lysozyme L99A (T4L) with bound benzene, which is considered as a model system to study ligand unbinding from proteins. In this example, 300 10-ns trajectories were run to reconstruct the reaction pathways of benzene unbinding from the protein. We used the biasing rate $v = 0.02$ Å/ps with the force constant in the stiff-spring regime $k = 7.2$ kcal/(molÅ). The optimal position of the ligand was recalculated by minimizing the loss function every $\Delta t = 200$ ps. We found that for lower biasing rates the method is unable to find the reaction pathways in the desired span of 10 ns for a single simulation. This is, however, only a technical nuisance that can be overcome by sampling longer MD trajectories. The method was implemented in a development version of the plumed-2.4.2 code and will be available soon. For details concerning the model of T4L with bound benzene and the MD simulations, see Supporting Material.
FIG. 4. Bias potentials from all the simulations that took a specific pathway projected along the reconstructed reaction pathways. Here, we used the loss function $s$ to project the bias potential to depict in what stage of unbinding the bias is higher. The high value of the loss function indicates that the ligand is bound (b) to the T4L matrix (in the X-ray structure the loss function reaches about 4.5), whereas the low value an unbound (u) state (at end of MD simulations the loss function decreases to 0). As can be seen, the characterization of the reaction pathways is heterogeneous, showing different mechanisms of benzene unbinding, and indicates different barriers for the reaction pathways close to one another, for instance, pwa and pwe near the D helix.

We directly compared our results with reaction pathways found in previous studies. The method identified five reaction pathways for benzene exit from the binding cavity buried in T4L. These reaction pathways correspond to five tunnels of T4L, named pwa—D/F/G (tunnel through helices D, F, and G), pwb—C/D, pwc—F/G/H, pwd—H/J, and pwe—D/G (Fig. 2). The reconstructed reaction pathways pwa–d are mostly in agreement with a recent study by Nunes-Alves et al. in which the reaction pathways of benzene unbinding were sampled using temperature-accelerated MD simulations. Other studies also reported pwa and pwc. To our knowledge, ligand unbinding via pwe is first identified here.

Apart from the work of Nunes-Alves et al., other studies found only one reaction pathway, probably because of the employed biased MD methods. Biased MD methods employed by Wang et al. and Miao et al. may limit the search in configuration space to a most optimal solution. Wang et al. used metadynamics to bias a reaction pathway identified initially by self-penalty walk which agrees with the observation that such methods strongly rely on the initial guess of a pathway. Thus, it may not be possible to identify all possible heterogeneous reaction pathways that exist in the form of transient sparse tunnels in the studied ligand–protein complex. Interestingly, the reaction pathways identified here agree mostly with exit tunnels retrieved by Nunes-Alves et al. where MD simulations with elevated temperature were used to overcome large energy barriers along reaction pathways and increase the probability
of the rare event. In both temperature-accelerated MD and the method presented in this article there is no need for initial guess of trajectories, which clearly improves sampling of diverse pathways.

TABLE I. Reaction pathways of benzene unbinding from T4L. Quantities describing the reaction pathways were calculated from an ensemble of trajectories for the identified exits. These quantities include the number of trajectories (out of 300) that proceed through each pathway, the mean of the distribution of unbinding times that it takes for benzene to unbind in the biased simulations, and its standard deviation, and the average radius of each identified tunnel $r_s$ and its standard deviation. Errors were estimated by a bootstrapping procedure (see Supplementary Material).

| pathway tunnel | no. trajectories | unbinding time [ns] | $r_s$ [Å] |
|----------------|------------------|--------------------|-----------|
| pwa D/F/G      | 65               | 3.37 ± 0.01        | 2.37 ± 0.01|
| pwb C/D        | 82               | 2.61 ± 0.07        | 2.31 ± 0.01|
| pwc F/G/H      | 34               | 2.68 ± 0.09        | 2.41 ± 0.02|
| pwd H/J        | 27               | 2.29 ± 0.08        | 2.36 ± 0.03|
| pwe D/G        | 92               | 2.45 ± 0.11        | 2.34 ± 0.01|

The detailed characteristics of the reaction pathways for benzene unbinding from T4L are shown in Tab. I. We found an additional reaction pathway that, to the best of our knowledge, was not identified previously. This pathway corresponds to benzene unbinding along the T4L tunnel between helices D and G. We integrated the bias potential along each pathway projecting it on the loss function as (Fig. 4) describing ligand unbinding using

$$V(s) = \int dx \delta(s - s(x))V(x).$$

This way we were able to identify bottlenecks in tunnels indicated by high values of the bias potential or the sparsity of conformational space available for sampling. Despite roughly the same level of the bias along each reaction pathway (Fig. 4), it is clear that the pathways employ different mechanisms of unbinding. This is underlined by the bias barriers along pwd and pwe, and a rather smooth decrease of the bias along pwa, pwb, and pwc. Moreover, it is perhaps possible to explain the bias barriers by inspecting the average radius of each tunnel. For instance, pwd and pwe have $r_s$ at about 2.36 Å and 2.34 Å, respectively, and the highest barriers among pathways. This is an indication that the pathways are heterogeneous, and their specific atomistic mechanism of unbinding would not be obvious by calculating averages of the full ensemble of the unbinding trajectories, without decomposition into classes first.

It should be noted that the method lends itself to use as an optimal initial guess of reaction pathways in other biasing MD methods to estimate thermodynamic and kinetic quantities, i.e., metadynamics or variationally enhanced sampling. We point out that computing reaction pathways for the T4L–benzene complex is not needed when
calculating the mean-first-passage times of binding and unbinding as shown by Wang\textsuperscript{27} however, it is important in estimating how the mechanisms of binding varies between the calculated reaction pathways, including free energies and conformational changes. Recently, it was shown that some protein–ligand systems can exhibit pathway hopping\textsuperscript{28,29} and the method presented here can be used to quantify this process.

We note that the reaction pathways of ligand unbinding sampled using the method presented here diverge to diverse suboptimal basins. This is the feature that enables sampling multiple heterogeneous reaction pathways and allows to overcome the problem of the intrinsic dynamics of protein tunnels. This is due to the used sampling which is constrained by the protein structure to provide a local minimum. Also, the probability of selecting a new solution given by the Metropolis-Hastings algorithm is important for the heterogeneity of the reaction pathways. The method searches for an optimal ligand conformation locally to extend the current reaction pathway step by step. This way, the method is able to sample multiple possible unbinding pathways, which for a rare event as with ligand unbinding is necessary to explore configurational space of tunnels exhaustively.

Overall, we think the method should be applicable to proteins in which prominent structural motions on a larger scale are important for ligand unbinding (e.g., trypsin\textsuperscript{30}). We expect it to be possible with a careful selection of the biasing parameters, for instance, by lowering the biasing rate to observe unbinding events on a time scale of large conformational changes. Or simply by enhancing protein fluctuations by increasing temperature. This aspect of the method will be studied soon.

In conclusion, we have presented a general method for finding reaction pathways of ligand unbinding, starting only from available crystallographic information. The method does not need any prerequisite guesses of intermediate or final states. The introduced approach uses an adaptive bias to drive the ligand to unbind from the fluctuating protein, in the direction effectively calculated by minimizing a simple loss function. The methods adapts to transient tunnels of proteins by estimating the configurational space from which it samples plausible ligand conformations. We provided a rigorous method to find all possible reaction pathways, which can be used as a initial reference trajectory to reconstruct thermodynamic and kinetic data. Overall, our results from studies of ligand unbinding from T4L suggests that the method presented here can improve the reconstruction of heterogeneous reaction pathways along transient tunnels, and serve as an optimal choice for other biasing methods, limiting overestimation of free energy barriers.
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