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# Increased throughput in methods for simulating protein ligand binding and unbinding

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**Abstract**

By incorporating full flexibility and enabling the quantification of crucial parameters such as binding free energies and residence times, methods for investigating protein-ligand binding and unbinding via molecular dynamics provide details on the involved mechanisms at the molecular level. While these advancements hold promise for impacting drug discovery, a notable drawback persists: their relatively time-consuming nature limits throughput. Herein, we survey recent implementations which, employing a blend of enhanced sampling techniques, a clever choice of collective variables, and often machine learning, strive to enhance the efficiency of new and previously reported methods without compromising accuracy. Particularly noteworthy is the validation of these methods that was often performed on systems mirroring real-world drug discovery scenarios.

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## Introduction

The study of ligand binding and unbinding processes provides thermodynamic and kinetic insights that can be used in drug discovery [1,2]. On the one hand, thanks to a low computational cost, standard docking tools can predict bound poses for entire libraries in a limited amount of time. On the other hand, these methods only provide a coarse estimation of binding energies and a limited understanding of the dynamic processes of protein − ligand interactions due to several well-known limitations: ligand conformational sampling is only performed within the boundaries of a predefined binding pocket, receptor flexibility is only partially explored, and scoring functions are based on very simplified heuristics [3−6]. Molecular dynamics (MD) simulations can be applied to obtain a more detailed exploration of the conformational space [7−11]. However, MD suffers, in turn, from a key drawback: an extensive sampling of high energy states would require exceedingly long trajectories, making the study of rare events impractical [12]. To overcome this issue, an assortment of enhanced sampling methods has been developed [13−16]. These methods accelerate the sampling of rare events by modifying potential energy functions or introducing biasing forces, in order to decrease barriers height, thus making efficient kinetic and thermodynamic quantitative predictions possible [12,17]. Enhanced sampling protocols have been thoroughly reviewed elsewhere [18−22]. In particular, case studies involving systems of pharmacological interest have been recently reported [23−25]. In general, the application of MD-based protocols to large datasets remains challenging due to longer calculation times and difficulties in obtaining a fully automated setup. The advantages and disadvantages of using these methods compared to docking are summarized in Figure 1.

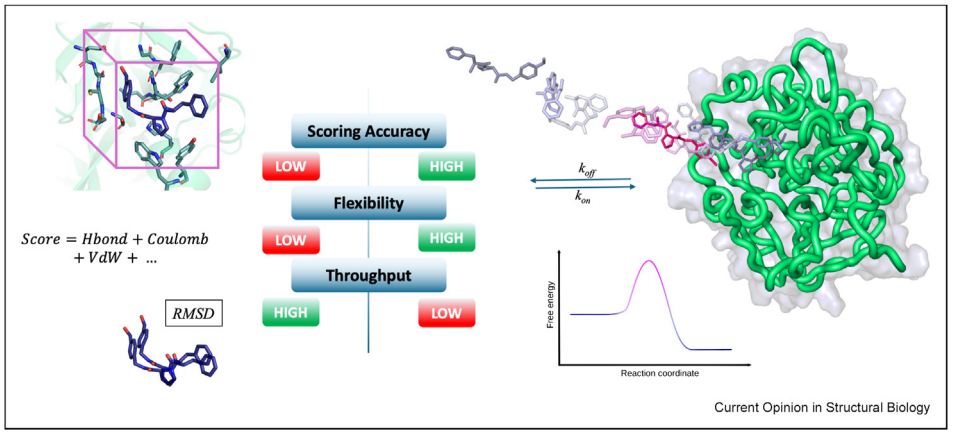
Here, we aim at providing an overview of the most recent updates concerning the development or the application of MD-based methods to study ligand binding and unbinding events.

## Recently reported molecular dynamics-based methods for studying ligand binding and unbinding

### CF-SMD

Constant force steered molecular dynamics (CF-SMD) is a protocol to evaluate dissociation rates for protein-ligand complexes [26,27]. In multiple runs, a constant force is applied along a collective variable (CV) until a dissociation event is observed. In each iteration, a

**Figure 1**




Schematic representation of the main advantages and disadvantages of using standard ligand docking (on the left) versus enhanced sampling methods for studying ligand binding and unbinding (on the right).

distinct value of the constant force is applied. The dissociation time devoid of applied bias is estimable through extrapolation from a function that optimally interpolates dissociation time against applied force, employing Bell's and DHS models [28,29]. Utilizing both models, the method returns a prediction of the dissociation time within a range bounded by lower (Bell) and upper (DHS) limits. While predicting absolute values for the kinetic constants remains a challenging task, the method is very effective for ranking relative dissociation times among different systems [26].

## MMVT-SEEKR2

In SEEKR2, kinetic and thermodynamic constants are estimated by studying target-ligand binding and unbinding events through Markovian milestoning with Voronoi tessellation (MMVT) [30]. This approach requires defining a CV, such as the distance between the center of mass (COM) of the ligand and the COM of the binding site residues. Voronoi cells are established as concentric spheres expanding along the CV. Initial structures within each cell are generated by gradually extracting the ligand from the binding site using steered molecular dynamics (MD) while applying a harmonic restraint until reaching the outermost cell. Subsequently, MMVT simulations are conducted within each cell until convergence is achieved, with trajectories confined within the cell boundaries through reflective boundary conditions. Notably, no external bias is introduced during this phase. Thanks to the concurrent use of multiple partitions, SEEKR integrates both MD and Brownian dynamics (BD) approaches, exploiting MD when explicit solvent and full molecular flexibility are required, while resorting to BD when molecules can be treated as semi-rigid objects in implicit solvent. Ultimately, MMVT returns the ligand residence time while generating a free energy profile for the unbinding event. The protocol was efficiently applied to predict and rank the residence times of a series of JAK2 and JAK3 inhibitors [31].

## MLTSA

In 2022, Badaoui et al. described an enhanced sampling protocol that characterizes the free-energy profile of ligand unbinding [32]. The method utilizes an iterative strategy to identify key molecular features relevant to the unbinding process. In this way, an aprioristic approach to the CV definition is not required. CVs are dynamically determined and adapted as the ligand advances along the unbinding trajectory. First, a standard MD simulation is carried out to establish an initial set of CVs based on interatomic distances and to determine the required bias. These CVs and the bias are then iteratively updated as the distance between the ligand and the protein increases. When all ligand-protein contacts are lost, the process halts. Subsequently, the finite-temperature string method is utilized to generate a converged free energy profile and to estimate a reliable transition state ensemble [33]. A machine learning (ML)-based transition state analysis is hence applied to identify relevant molecular descriptors, which can be used in rational molecular design. In detail, downhill unbiased simulations are initiated near the identified transition state and classified as "*in*" or "*out*", depending on the final state (bound or unbound), that is achieved. From these trajectories, a set of CVs is selected to train a ML model. Two ML approaches were used for validation: a multilayer perceptron and a gradient boosting decision tree. Both techniques could efficiently predict the trajectory outcome and identify key features that drive the ligand through the binding or unbinding event. The protocol was successfully applied to study a kinase and a G-Protein-Coupled Receptor (GPCR) [32,34].

### LiGaMD2

Gaussian accelerated molecular dynamics (GaMD) evolved from accelerated MD (aMD) [35–38]. The original idea behind aMD was to enable extensive conformational sampling required for describing free energy surfaces of complex systems within a feasible timeframe using standard hardware [39]. A harmonic boost is applied to the potential energy of the system to enhance the conformational sampling. The boost is only applied when the potential energy is beneath a certain threshold and its amplitude is continuously updated according to the difference between the threshold and the potential energy at each timestep during the simulation. In this way, the boosted energy surface results smoothened while maintaining the original shape. In GaMD, the boost follows a Gaussian distribution, which, reducing statistical noise, allows a more accurate recovery of the original free energy landscape by cumulant expansion to the second order [39]. Derived from GaMD, the Ligand Gaussian accelerated molecular dynamics (LiGaMD) implementation is specifically tailored for exploring protein-ligand interactions [40]. In LiGaMD, users have the option to specify whether to apply the boost solely on the potential energy of the nonbonded interactions of the ligand or on both the potential energy of the nonbonded interactions of the ligand and the total potential energy of the system [40]. In the recently reported LiGaMD2, the possibility to selectively boost both the ligand and the binding site residues was introduced. This approach enhances performance, particularly for systems featuring buried ligands and closed binding pockets, and it has been successfully validated on the T4 lysozyme [41].

### BiKi Hydra

BiKi Hydra is a tool originally developed to characterize the energetic profile and persistency of water molecules in binding pockets [42]. Since hydration is critically important in determining the thermodynamics and kinetics of protein-ligand (un)binding, building upon BiKi Hydra's framework, a CV based on dehydration (dehydration bias) was developed [43]. By tuning the local hydration of binding partners, this bias helps overcoming the dehydration barrier, thus increasing the probability of observing successful native binding events in a short amount of simulated time. The technique was efficiently applied to accelerate the binding of an inhibitor against Src kinase from an unbound, solvated state. In detail, multiple short-length simulations were performed. Unbiased simulations were used to establish a baseline, and predictably, no binding event was observed. When the dehydration bias was only applied to the ligand, this would systematically get in proximity of the binding pocket, but the actual bound conformation could never be recovered. When the bias was applied to both the ligand and the binding site, the crystallographic pose could be recovered in at least one run. The binding event was in line with that observed thanks to microsecond-long unbiased MD simulations, as reported by Shan et al. [44]. Eventually, another attempt was made, initially biasing the ligand and forcing partial dehydration in the binding pocket only at a later stage (delayed bias). In this case, true binding events increased, but together with false positives, suggesting a propensity of the delayed bias to generate artifacts. The advantage of this technique is that the dehydration bias is mild: it selectively desolvates targeted regions while minimally disrupting the water network. Upon reaching a predetermined dehydration threshold, the bias is automatically deactivated. In this way, the system reverts to an unbiased state when, after crossing the dehydration energetic barrier, the ligand reaches the binding site.

### Targeted MD for the estimation of drug-target residence time

Ziada et al. recently presented a protocol based on reverse targeted MD ($TMD^{-1}$) that ranks compounds based on their residence time (RT) [45]. $TMD^{-1}$ entails the application of a harmonic constraint to the Root Mean Square Deviation (RMSD) between the current position of the ligand and a reference position. The system evolves towards a target RMSD value, which is set at a predefined higher value, while the reference position is gradually modified until the ligand leaves the binding site. The RT is closely related to the difference between the energy of the unbinding transition-state and that of the bound state. However, the total energy added by the bias includes both: i) the work exerted in pushing the ligand *uphill* toward the peaks of the energy barriers, and ii) the bias employed during descents toward metastable states. Here, a new function was developed that only encompasses the former, while ignoring the latter. The obtained RTscore is assumed to be linearly related to the natural logarithm of the experimental RT. Using this approach, the results showed that it was possible to discriminate among short RT ligands ($RT < 1.5$ min), medium RT ligands ($1.5\ \text{min} \leq RT \leq 1$ h), and long RT ligands ($RT > 1$ h). The main advantage of the RTscore is that it includes all the energy barriers encountered during the dissociation process, making it possible to evaluate complex multistep kinetic processes. The other advantage is the low computational cost that it requires. In fact, each simulation ran for only 1.5 ns [46].

### dcTMD-based Workflows

Dissipation–corrected targeted molecular dynamics (dcTMD) exerts an external pulling force along a CV by using a moving distance constrain. The method accounts for deterministic and stochastic dynamics to model realistic representations of biological systems [47]. In the work of Wolf et al. Jarzynski's equality was used to compute from dcTMD simulations the free energy profile and the friction field, which were, in turn, utilized in a numerical integration of the Langevin equation. Thereby, temperature-boosted Langevin simulations were run to unveil coarse–grained dynamics

of biological systems and reveal pathway−dependent kinetics of rare events in reasonable computational times. The Hsp90−inhibitor and trypsin−benzamidine protein−ligand complexes were successfully modeled by using this method [48]. Using another approach based on dcTMD, a 2D model (multi-CV) was employed to assess the binding/unbinding of the trypsin−benzamidine complex [49]. The pulling force was applied along one CV, while the system could evolve independently along the second CV. This strategy was adopted to capture a more detailed and comprehensive representation of the system under investigation. However, Jarzynski's approximation only holds if the work exerted on a system follows a Gaussian distribution. As the system evolves across multiple dimensions along different reaction coordinates, the aggregate work distribution might deviate from normality. A theoretical framework was devised to split the total reaction flux into multiple pathways. Path−specific rates were weighted based on their probability, and then combined into global binding/unbinding rates, demonstrating the relative contribution of each rate to the global reaction rate. Recently, to automatize the process of detecting reaction pathways, an algorithm based on an unsupervised machine learning method for clustering trajectories was developed. The approach allows the identification of pathways of transitions between metastable states in MD simulation, as well as the characterization of CVs or other underlying factors liable for protein-ligand binding and unbinding phenomena. The technique was applied to the streptavidin−biotin and a $A_{2a}$ adenosine receptor − ligand complexes [50].

### TRAM

A recent study reported a Transition−based Reweighting Analysis Method (TRAM) alongside Markov state models to investigate ligand dissociation processes from the protein kinase PYK2 [51]. The approach is computationally efficient as biased and unbiased simulations are blended. τ−Random acceleration molecular dynamics (τ−RAMD) was used in combination with umbrella sampling for the detection of dissociation pathways. TRAM provided computed dissociation rates within an order of magnitude from experimental data. Moreover, coarse−graining microstates obtained from Markov state models into a small number of macrostates afforded an easy interpretation of the ligand dissociation mechanism at the molecular level.

## Conclusions

The MD-based methods reported in recent years for studying ligand binding and unbinding share some common features: i) largely exploiting enhanced sampling, these methods have been developed with efficiency in mind, aimed at returning results in a timeframe compatible with fast-paced drug discovery projects; ii) along the same line, the validation of the proposed methods usually goes beyond simple toy models and has actually been performed on pharmaceutically-relevant systems, including flexible enzymes and membrane proteins; iii) like many others, this field is being deeply impacted by the widespread adoption of machine learning. Supervised and unsupervised approaches can both be found in mixed workflows with ML. One limitation of current techniques is related to possible unrealistic states of the system generated by methods that work at high temperatures and/or bias the system's energy. In principle, this could limit the accuracy of path predictions and, thus, the confidence in the gathered insights for rational drug design. Reasonably, it will soon be possible to simulate fully flexible binding and unbinding events, concurrently extracting thermodynamic and kinetic observables, in large screening campaigns, approaching the throughput of standard ligand docking methods. In due time, these methods will provide actionable insights for the design of new and, possibly, better compounds and will standardly be employed in drug discovery workflows.

## CRediT authorship contribution

**Syeda Rehana Zia:** Investigation, Writing - Review & Editing; **Adriana Coricello:** Investigation, Writing - Review & Editing; **Giovanni Bottegoni:** Conceptualization, Supervision, Funding Acquisition, Writing - Review & Editing.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Syeda Rehana Zia and Adriana Coricello declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Giovanni Bottegoni owns shares of BiKi Technologies srl, a company based in Genova (Italy) that commercializes software for computational medicinal chemistry.

## Data availability

No data was used for the research described in the article.

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