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Analysis of Protein Conformational Changes and Binding Mechanisms Using Enhanced Sampling Molecular Dynamics Simulations

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Abstract: Proteins rely on conformational fluctuations to achieve substrate recognition, signal transduction, and drug binding. Many functionally crucial intermediate states cannot be generated within the finite time of conventional molecular dynamics simulations. This paper selects 50 articles published in the past three years on metadynamics, umbrella sampling, Gaussian accelerated molecular dynamics, replication exchange, weighted ensemble, and adaptive sampling as research objects, comparing the effects of these methods in terms of conformational transitions, ligand binding pathways, free energy, and kinetic estimation. Applications such as hidden pockets, receptor activation, binding and dissociation intermediates, solvent rearrangement, and flexible protein recognition are reviewed, analyzing the influence of collective variable selection, reweighting, force fields, and experimental cross-validation on mechanistic conclusions. Existing research shows that multi-method coupling can improve the sampling effect of rare events, but the transition pathway, obtaining stable free energy curves, and reliable predicted rate constants belong to different levels of evidence; independent replication, experimental constraints, and uncertainty analysis should be used to interpret the results.

1 The Timescale Issue In Protein Dynamics Research

When proteins exchange between different conformational substates, ligands first bind to existing open states, or after the initial contact, they promote the recombination of loop regions and side chains. Docking provides candidate postures but cannot reflect the process of entering and leaving pockets. Although conventional molecular dynamics has atomic resolution, rare transitions often occur beyond the timescale accessible to a single trajectory. Zia et al. summarized the method of using binding and dissociation simulations to accelerate the process, and believed that the speed of path sampling and computational throughput should be divided according to the state [1]. Bemelmans et al. discussed the emergence of hidden pockets and pointed out that missing sites in unbound structures can be supplemented by transient open conformations [2].

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Enhanced sampling explores rare states by changing the frequency of accessing the energy barrier. The bias trajectory also needs to be processed before it can be used for equilibrium statistics. Li et al. extracted the reaction coordinates from energy relaxation and proposed a scheme for slow motions that are difficult to define in advance during protein conformational transitions [3]. Bose et al. tested the robustness of ligand binding transition states and suggested that the determination of transition states cannot be based on a seemingly reasonable trajectory alone [4]. The following sections discuss state discovery, free energy estimation and rate prediction: they require different sampling designs and evidence. The discussion mainly focuses on the identification of proteins and small molecules or flexible peptides, and also involves large-scale conformational transitions and the stability of multi-component interfaces.

2 From Trajectory Events To Combination Mechanisms

Seeing an open pocket or ligand exit in a trajectory only indicates that the simulation visited that path. To determine how common the open state is, the state probability needs to be reconstructed under target temperature and solvent conditions; to estimate the binding free energy, the contributions of the unbound state, standard state volume, and confinement potential must be accounted for; to calculate the residence time, the waiting time and path flux must be preserved or reliably recovered. Therefore, the same "binding mechanism" involves at least three levels: structural, thermodynamic, and kinetic. Protein conformation and ligand position are often coupled: the slow coordinates corresponding to the open pocket before binding, the intermediate state generated after contact, and the finally stabilized complex may be different. The entry and exit of water molecules and ions will also affect the effective energy barriers on these coordinates. The following section connects the methodological principles with specific protein systems, pointing out the phenomena that can be directly observed by the simulation and the required statistical tests at each inference point.

3 Scientific Questions Answered By Different Augmentation Sampling Algorithms

3.1 Energy Surface Exploration Driven by Collective Variables

Metadynamics accumulates bias along collective variables, and umbrella sampling constrains coordinates in adjacent windows and reconstructs the average potential; both require the selected coordinates to cover key slow motions. Shen et al.’s Adaptive CVgen, which uses reinforcement learning to adjust variables, tested sampling efficiency in folding and chemical reactions [5]. Ray and Rizzi combined OPES with its exploratory form to improve free energy convergence under suboptimal variables in trypsin–benzamidine binding and small protein folding systems [6]. Chatterjee et al. used interpretable surrogate models to generate variables so that acceleration directions could be traced back to the input descriptor [7]; Yang et al. augmented training data with physics-inspired interpolation [8], and Shao et al. filtered out fast modal interferences on variable learning [9]. Mapping the numerical changes of variables back to specific structures is equally important: Chatterjee et al. used explainable machine learning to connect collective variables with structural features of HSP90 conformational transitions [10]. These works improve the variable creation process, but cannot rule out checking for water exchanges and side-chain rearrangements that are not included.

3.2 Changing the potential energy distribution and parallel sampling

GaMD uses modified potential energy surfaces to cross energy barriers, and then reweights them

after biasing. Wang and Miao's LiGaMD3 adds three boost terms to the energy terms associated with dissociation, rebinding, and conformational changes in the system. In MDM2, Nutlin 3, and flexible peptide systems, they simulated the phenomenon of repeated binding and compared the kinetic and free energy results with available experimental data [11]. Hasse et al. combined many GaMD parameters with replication exchange and used the exchange between copies to expand the conformational search [12]. The solute temperature regulation scheme proposed by Stratmann et al. is mainly to improve the sampling efficiency of protein conformation and binding events [13]. If comparing the chemical states of different ligands, Lai et al. used a combination of multi-site λ kinetics and umbrella sampling to establish the corresponding free energy surfaces [14]. Karrenbrock et al. used OneOPES to estimate the absolute binding free energy [15]. These outputs should give constraints, standard state correction, reweighting, and statistical error, respectively. The recurrence of binding events does not necessarily guarantee the accuracy of the rate estimation.

3.3 Path Sampling that Preserves Dynamic Information

Weighted ensemble replication, trajectories merging and statistical weights can increase the number of samples for low-probability paths. Bose et al. pointed out that when using weighted ensemble data to build Markov state models, if the model lag time is greater than the trajectory resampling interval, then merging trajectories will cause errors; they proposed a correction algorithm and verified it on models and biomolecular systems [16]. Leung et al. put the progress coordinates of NTL9 folding unsupervised learning on trajectory assignment, making trajectory assignment more consistent with the folding process [17]. Chong et al. summarized the state division, software and rate application of weighted ensembles, giving flux estimation a methodological background [18]. Another adaptive sampling is to select the next starting point from the existing trajectory; Sarkar et al. found in their study on ligand transport of buried active sites that how prior knowledge affects seed selection is affected by the initial distribution, that is, the initial distribution itself will affect the channel search [19].

Table 1. Applications and limitations of major methods in protein binding problems.

Method	Priority questions	Key checks
Metadynamics and OPES	Path and relative free energy basin	Slow variable coverage, bias correction, repeated crossing
Umbrella sampling	Average potential force on specified coordinates	Window overlap, path selection, standard state correction
GaMD and Solute Temperature Regulation	Unknown conformational substates and coupled motions	Copy swapping, reweighting, potential energy model
Weighted ensembles and adaptive sampling	Rare paths and potential rates	State definition, independent and repeated, steady state and weighted variance

Actual selection should be based on a reverse design driven by the expected output. To determine the existence of hidden open states, one can first use multiple copies, solute temperature regulation, or GaMD to increase conformational coverage, and then use unbiased short trajectories to test its stability. If comparing the binding free energies of two ligands, it is essential to ensure that the important protein conformations and hydration states sampled from both systems are consistent before considering path bias or alchemical transformations. If the goal is to estimate the dissociation rate, it is best to preserve trajectories that can recover time information, or strictly

satisfy the assumption of a reduced rate from biased kinetics. Simply replacing "finding a dissociation path" with dwell time often ignores factors such as path probability, state boundaries, and waiting time. For macromolecular systems, it is also necessary to calculate the number of solvent atoms, multiple copies, and the actual computational power consumed by round-trip events, rather than simply comparing the length of a single trajectory. The method-specific uses and validation checks are summarized in Table 1.

4 How Conformational Changes Participate In Molecular Recognition

4.1 Concealed Pocket Opening and Design Selection

Hidden pockets are often closed in unbound structures. Vithani et al. linked enhanced sampling with binding capacity assessment to expand candidate hidden sites; whether a geometrically open pocket can stably bind a ligand also depends on hydration and the interaction environment [20]. In KRAS G12D, the same team explored hidden pockets along collective motions associated with the normal modes, demonstrating a method for finding temporarily open substates in unbound conformations [21]. Routh et al. studied mutation-guided holo-like conformational recovery, suggesting that amino acid changes can alter conformational groups compatible with ligands [22]. These studies point to a sequence problem: open states may exist before ligand arrival or continue to promote pocket expansion after ligand contact. When judging the relative contributions of conformational selection and induced fit, one should not only look at the probability of unbound states, the order of approach, the order of contact formation, and the subsequent rearrangement process, but should consider all aspects. In particular, pocket volume, hydrophobicity, hydrogen bond donors and acceptors, water entry, etc., should be examined, and transient geometric cavities should not be regarded as drug-forming sites.

4.2 Movement of Local Ring Regions and Large-Scale Structural Domains

Odstrcil et al. used enhanced sampling to investigate the conformational changes and molecular mechanisms of human NTHL1, linking local motion with functional explanations [23]. Juraschko et al. found that potential energy models can lead to deviations in the description of intermediate states by comparing the conformational dynamics of bacterial ribotransporters under different force fields [24]. Activation of membrane receptors is accompanied by changes in transmembrane helices, intracellular coupling sites, and pocket water networks. Aureli et al. created variables for β1-adrenergic receptors that included microswitches, sodium ion pockets, and important water molecules, and used OneOPES to compare the changes in the activation free energy surface under ligand-free and adrenaline-bound states, thus deriving the stepwise activation process [25]. Roessner et al. combined metadynamics with Markov state models and used state networks to analyze the conformational transitions of G protein-coupled receptors [26]. Mosa et al. compared the conformational switching of the aryl hydrocarbon receptor under agonist and antagonist conditions [27]. In the kinase system, Wang et al. used metadynamics plus umbrella sampling to study the selectivity of CDK inhibitors[28], and Ghosh et al. linked LRRK2 mutations with free energy landscape rearrangement and changes in inhibitor affinity[29]. Therefore, it is not enough to use only root mean square deviation to determine "stability"; the distribution of gating residues, salt bridges, ions, water networks, and ligand contacts in each state should be compared.

4.3 From Combination Posture to Free Energy and Dwell Time

The equilibrium state probability is determined by comparing free energy. The dwell time is the

time required to leave the bound state. The two cannot be substituted for each other. Liao et al. improved the calculation of alchemical free energy for systems with binding accompanied by significant protein conformational changes, thus solving the sampling problem caused by insufficient conversion between different structures [30]. Lee et al. used information bottleneck variables with state prediction capabilities to learn and then estimated the dwell time of ligands by enhancing sampling [31]. Votapka et al. combined multi-scale milestones with meta-dynamics to explore the dissociation dynamics between tyrosine kinase receptors and ligands [32]. Summa et al. calculated the absolute free energy, enthalpy and entropy of drug binding and found that the decomposition of equilibrium binding strength requires reliable energy and conformational sampling [33]. Rate prediction must clearly define the bound state boundary and the time recovery assumption, and cannot be simply replaced by a path leaving the pocket.

Pocket water can alter the burial of polar groups, hydrogen bond networks, and dissociation barriers. Deng et al. used nonequilibrium grand canonical Monte Carlo and polarization force fields to improve the sampling efficiency of protein cavity hydration states [34]. Wagle et al. used state separation to solve the problem of water retention in relative binding free energy calculations [35]. Jiang et al. accelerated the kinetics of the solvent layer and studied the collective functional response of receptors in alchemical calculations [36]. It can be seen that without considering water exchange, judging the relationship between biased ligands and proteins solely based on distance will ignore some important slow processes. For various binding postures appearing on the trajectory, Zhang et al. used kinetics to determine that there are several different binding states in the T4 lysozyme L99A system [37]. Ray et al. used a data-driven approach to classify different dissociation pathways [38]. State boundaries and exit branches will affect the “mechanism” description and will also affect flux and rate estimation.

5 Mechanistic Evidence Presented In Cases From The Past Three Years

Sahil et al. used long-scale simulation, adaptive path search and state model analysis to study the recognition process of geldanamycin for HSP90 and identified three states along the binding pathway: unbound, long-lived intermediate, and bound; the L4 ring region has differences between the intermediate state and the final state, which can explain the multi-step binding phenomenon observed in the experiment [39]. In larger assembly systems, Lynch et al. used weighted ensembles to study the conformation of the hepatitis B virus capsid and the mechanism of action of the regulators, and found that the conjugates would change the state distribution on the assembly path [40]. Palit et al. combined weighted ensembles with electron paramagnetic resonance distance to explore the closed-open transition of lysine, arginine and ornithine binding proteins, and used site-directed mutagenesis to confirm the inferred interactions between important residues [41]. Atik et al. used weighted ensembles to evaluate the stability of molecular glues and shifted the focus from binary ligand binding to multi-component interfaces [42].

Oh et al. proposed interpretable discriminative variables to track the changes in the state of human retinol-binding protein 2 caused by different ligands [43]. Disordered proteins cannot be described by a pocket for their recognition process. Zhu et al. performed enhanced sampling and machine learning analysis on the disordered N-terminal domain Tau-5 region of the androgen receptor, found nine metastable states, and found aromatic stacking and structured water-mediated hydrogen bonds in the EPI-002 binding model. The study also used binding and cell experiments to verify the screened candidate molecules [44]. Gu et al. used hierarchical AF2RAVE to explore multiple conformations of S100 calcium-binding protein, thereby carrying out virtual screening work [45]. Aoki et al. added various protein structures predicted by artificial intelligence to the free energy calculation of enhanced sampling [46]. Elangovan et al. used data-driven enhanced sampling

to discover mechanistic pathways [47]. It can increase the coverage of initial structures and pathways, but for states that do not appear, it is necessary to extrapolate with the help of other starting points or experimental constraints.

The above cases are mutually corroborating evidence. Trajectory clustering can obtain candidate states, and only the results of multiple independent round trips and stable reweighting indicate that the state is relatively stable; if the calculated affinity, structural distance, key residue mutations and binding experiments are consistent with each other, then the identification model is more convincing. In the study of HSP90, there is an intermediate state, which is confirmed by independent funnel element dynamics, and the open-close transition is limited by EPR and mutation. For disordered proteins, conformational groups and proportions should be given, and membrane receptor simulations should also clearly explain the relationship between membrane, ions and receptors. Table 2 compares representative systems with the mechanism claims they support and the remaining checks.

Table 2. Representative Cases and the Levels of Conclusions They Can Support

System	Research Focus	Supportable mechanism conclusions	Factors that still need to be verified
KRAS concealed pocket	Open substates and candidate associativity	There are potential target conformations	Pocket ratio and actual measurement
Receptors and kinases	Spiral or toroidal rearrangement	Ligand alteration conformational balance	Different force fields and receptor states
HSP90 and disordered proteins	Multiple postures or instantaneous combinations	Recognition is not a single static pose	Experimental positioning and water network
Capsule and molecular glue	Large-scale or multi-component interactions	Different paths exist between states.	System size and rate error

6 Reliability Of Results And Unresolved Issues

Kang et al. compared four enhanced sampling strategies in terms of ligand binding, protein conformational changes, kinase activation, membrane permeation, and ion transport, and concluded that the methods performed differently in different systems, and even if the commonly used self-consistent convergence index met the requirements, the free energy profile might still deviate from the independent reference [48]. Therefore, the smooth curve is only the result of the calculation and needs to be checked for independent starting points, repeated trajectories, forward and reverse conversions, reweighted stability, and consistency between different methods or different experiments. Umbrella sampling should have window overlap, replication exchange should be checked for round trips, and weighted ensemble should be checked for weight distribution and flux changes over time. The free energy can also be affected by changes in force field, protonation, water model, ions, and membrane composition. The rate results should give the number of events, state boundaries, and assumptions about time recovery. Experimental mutations can cause changes in folding stability and binding interfaces, so changes in activity cannot be directly correlated to a specific contact.

Zhu et al. summarized the algorithms and applications of machine learning-assisted augmented sampling, and also showed that the connection between variable learning, generative models and physical sampling is becoming the focus of research [49]. Sheen et al. used structural motifs to guide transition path sampling, giving another constraint to finding paths in complex landscapes

[50]. If the training set does not contain important intermediate states, then machine learning variables will turn the missing into a sampling blind spot. Therefore, it is necessary to compare the states obtained from different starting points and descriptors, and to disclose the training data, the history of biases and the reweighting process. In addition to the number of transitions found per unit of computing power, subsequent benchmarks should also compare state probabilities, free energy, path branches and experimental rates. For new systems, hypotheses should be proposed first from the structure, and then statistical convergence and experimental constraints should be used to verify thermodynamic and kinetic inferences in order to turn observable trajectory events into credible combination mechanisms.

7 Conclusion

Enhanced sampling expands the scope of protein research from single, stable structures to exchangeable conformations and binding pathways. Algorithm selection should first determine whether the objective is to find states, estimate free energy, or predict rates, and then configure the algorithm based on the corresponding variables, reweighting, and validation. For hidden pockets, membrane receptors, and disordered proteins, combining various sampling methods with experimental information is most likely to yield reliable mechanistic explanations, rather than accepting a trajectory as conclusive evidence simply because it has not been independently validated.

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