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Role of Molecular Dynamics and Related Methods in Drug Discovery

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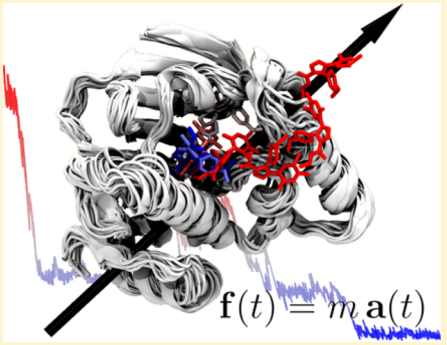
ABSTRACT: Molecular dynamics (MD) and related methods are close to becoming routine computational tools for drug discovery. Their main advantage is in explicitly treating structural flexibility and entropic effects. This allows a more accurate estimate of the thermodynamics and kinetics associated with drug−target recognition and binding, as better algorithms and hardware architectures increase their use. Here, we review the theoretical background of MD and enhanced sampling methods, focusing on free-energy perturbation, metadynamics, steered MD, and other methods most consistently used to study drug−target binding. We discuss unbiased MD simulations that nowadays allow the observation of unsupervised ligand−target binding, assessing how these approaches help optimizing target affinity and drug residence time toward improved drug efficacy. Further issues discussed include allosteric modulation and the role of water molecules in ligand binding and optimization. We conclude by calling for more prospective studies to attest to these methods' utility in discovering novel drug candidates.



1. INTRODUCTION

Computational drug discovery can accelerate the challenging process of designing and optimizing a new drug candidate.[1] The impact of computational structure-based drug design (SBDD) on drug discovery has intensified in the past decade because of the rapid development of faster architectures and better algorithms for high-level computations in a time-affordable manner.[2] Classical molecular dynamics (MD) simulations nowadays allow implementation of SBDD strategies that fully account for structural flexibility of the overall drug−target model system.[3,4] Indeed, it is now widely accepted that the two major drug-binding paradigms (induced-fit and conformational selection) have superseded Emil Fischer's rigid lock-and-key binding paradigm.[5−7] Receptor and ligand flexibility are crucial for correctly predicting drug binding and related thermodynamic and kinetic properties.[8,9] As a result, classical MD is no longer considered prohibitive for effective drug design. Instead, it is pushing the frontiers of computationally driven drug discovery in both academia and industry.[10]

Classical MD is a physical method for studying the interaction and motion of atoms and molecules according to Newton's physics. A force field is used to estimate the forces between interacting atoms and calculate the overall energy of the system. Then, during MD simulations, the integration of Newton's laws of motions generates successive configurations of the evolving system, providing trajectories that specify positions and velocities of the particles over time. From these MD trajectories, a variety of properties can be calculated, including free energy, kinetics measures, and other macroscopic quantities, which can be compared with experimental observables. The method was originally conceived within theoretical physics in the late 1950s but is now applied in chemical physics, materials science, modeling of biomolecules, and more recently, drug discovery.[11,12]

Pioneering studies by Karplus and McCammon[13] and by Warshel and Levitt[14] showed the crucial role of classical MD simulations in studying biological systems. They used MD simulations to obtain different conformations of proteins and nucleic acids, including early attempts to simulate spontaneously complex phenomena, such as protein folding.[13,14] In recent decades, researchers have increasingly recognized that MD can also overcome the major limitations of static structure-based drug design, such as those limitations that characterize routinely applied ligand docking calculations, which do not sample the major protein conformational rearrangements often observed during ligand binding.[15,16]

The issue of structural flexibility in SBDD was first addressed using advance molecular docking protocols, which permitted partial flexibility of the receptor when screening compound libraries.[17] In the past decade, for example, using multiple pre-existing conformations of a target has become widely accepted as a way to partially account for protein flexibility in SBDD.[18] One notable example is ensemble docking, where several independent protein conformations are targeted. The results

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are eventually merged together and averaged, leading to improved outcomes.[19] In addition to using experimental techniques to structurally characterize the receptor in ensemble docking,[20,21] MD simulations were an obvious way to obtain multiple conformations of macromolecular targets.[22,23] As early as 1994, Pang and Kozikowski extracted multiple conformations of the acetylcholinesterase enzyme from a 40 ps trajectory. They used these to successfully predict, through rigid docking, the bound pose of huperzine A.[24] Since then, numerous research groups have used snapshots extracted from MD trajectories to provide a discrete representation of the target plasticity.[25] The relaxed complex method developed by McCammon et al.[26] is a particularly notable example. In the past decade, MD protocols for ensemble docking have shown great potential in handling targets governed by significant structural flexibility. These include protein kinases[27] and G-protein-coupled receptors,[28] whose function is characterized by remarkable conformational plasticity.

Moving beyond protocols that use MD to incorporate target flexibility into standard docking calculations, it is now possible to run MD simulations for long enough to explore the free-energy landscape and kinetic profile associated with the overall drug-binding process (i.e., from the drug fully solvated in water to the drug−target bound state).[29] Today, a full dynamical description of the protein−ligand binding event can be obtained, with various degrees of accuracy. This is due to increasing computer power, the advent of graphical processor unit (GPU) architectures, and MD software that can efficiently run on these innovative hardware infrastructures. In pioneering studies by Buch et al. and Shan et al.,[30,31] for example, a ligand binding to a target protein was investigated by multiple replicas of microsecond-long MD simulations. More recently, Decherchi et al.[32] used a similar protocol in combination with machine learning algorithms to identify different routes to detect binding and to generate accurate free-energy profiles associated with these routes.

Although simulations lasting up to a few milliseconds (corresponding to 10^{12} time steps) are now possible,[33,34] several different trajectories are recommended to obtain adequate statistics and exhaustive sample of the conformational space. Thus, even when studying a single lead compound, the process is demanding. It is also common to find druglike molecules with long unbinding kinetics (in the order of several minutes).[35] Conventional MD methods, even if running on specialized hardware, still cannot describe such slow unbinding events. In fast-paced drug discovery programs, this is the major issue limiting the use of MD-based simulations for kinetic prediction.[10] However, the sampling issue has led to the development of many innovative algorithms that form the basis of "enhanced sampling methods". These speed up the description of *slow* processes, accelerating the rare events characterized by high-in-free-energy states.[36] Notable enhanced sampling methods (developed over the past 2 decades and now widely used) include free-energy perturbation,[37,38] umbrella sampling,[39] replica exchange,[40] metadynamics,[41] steered MD,[42,43] accelerated MD,[44] milestoning,[45] transition-path sampling,[46] and their many possible combinations. Researchers have recently demonstrated the power of these methods for studying protein−ligand binding and estimating the associated free energy and kinetics.[3,4] Recent studies have also shown how various implementations of MD can effectively engage targets[47] that are druggable but that do not appear to be very ligandable.[48] In these challenging cases, MD has aided the discovery of entirely new (usually allosteric) pockets.[49] These pockets are only transiently formed and are often too short-lived to be mapped on the protein surface by conventional experimental techniques.[50]

Given the ever-increasing role played by MD in the evolution of computationally driven drug discovery, we will here discuss a few theoretical concepts that form the basis of classical MD, focusing on those most related to drug discovery. We review some of the most recent and effective applications of plain MD for studying protein−ligand binding and unbinding and for retrieving major thermodynamic data such as binding free energy. We move on to examples where enhanced sampling methods combine with MD to characterize ligand−target conformational and energetic landscapes. Again, we focus on those methods most widely applied in drug discovery, such as free-energy perturbation, metadynamics, and steered MD.

This Perspective will also touch on the emerging and critical role of MD simulations and related methods in predicting the binding and unbinding kinetics of drugs, since these physicochemical observables play an increasingly central role in drug design and optimization. We close by outlining how MD simulations can help in understanding and using allosteric mechanisms for drug design and in examining the thermodynamic properties of the water molecules that solvate protein-binding sites, which may help in designing new and more potent lead compounds.

Overall, we provide an overview of the state of the art of classical MD in pharmaceutical research, highlighting key applications to drug design from recent years. We envisage a not-too-distant future wherein MD and related approaches are routinely used for the in silico screening of large libraries of small molecules, accelerating drug candidate identification and optimization. More generally, we anticipate the wider utilization of MD-based methods in pharmaceutical endeavors.

2. THEORETICAL BACKGROUND

2.1. Molecular Dynamics with Classical Potentials. The central idea behind MD simulations is to study the time-dependent behavior of microscopic systems. This is obtained by solving the second-order differential equations represented by Newton's second law:

$$\mathbf{f}_i(t) = m_i\mathbf{a}_i(t) = -\frac{\partial V(\mathbf{x}(t))}{\partial \mathbf{x}_i(t)} \quad (1)$$

where $\mathbf{f}_i(t)$ is the net force acting on the ith atom of the system at a given point in time t, $\mathbf{a}_i(t)$ is the corresponding acceleration, and m_i is the mass. In eq 1, the instantaneous configuration of the system is represented by the vector $\mathbf{x}(t)$, which describes the position of the N interacting atoms in the Cartesian space ($\mathbf{x} = \{x_1, y_1, z_1, x_2, y_2, z_2, ..., x_N, y_N, z_N\}$). Usually, in computational drug discovery, we adopt a classical mechanics description of the forces. Notably, this approximation holds for massive particles such as nuclei, while the electron motions must be averaged out. To achieve this, an empirical potential energy function is introduced ($V(\mathbf{x})$ in eq 1), and the model arising from this simplified representation is referred to as the force field (FF), or molecular mechanics (MM):

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$$V = \sum_{i}^{\text{bonds}} \frac{k_{l,i}}{2}(l_i - l_{0,i})^2 + \sum_{i}^{\text{angles}} \frac{k_{\alpha,i}}{2}(\alpha_i - \alpha_{0,i})^2 + \sum_{i}^{\text{torsions}} \left\{ \sum_{k}^{M} \frac{V_{ik}}{2}[1 + \cos(n_{ik}\cdot\theta_{ik} - \theta_{0,ik})] \right\} + \sum_{i,j}^{\text{pairs}} \varepsilon_{ij}\left[\left(\frac{r_{0,ij}}{r_{ij}}\right)^{12} - 2\left(\frac{r_{0,ij}}{r_{ij}}\right)^{6} \right] + \sum_{i,j}^{\text{pairs}} \frac{q_i q_j}{4\pi\varepsilon_0\varepsilon_r r_{ij}} \quad (2)$$

In eq 2, the first three terms represent intramolecular interactions of the atoms. They describe variations in potential energy as a function of bond stretching, bending, and torsions between atoms directly involved in bonding relationships. They are represented by summations over bond lengths (l), angles (α), and dihedral angles (θ), respectively. Bond stretching and bending contributions share the same functional form, as they are both described by harmonic potentials with reference values l_0 and α_0 and force constants k_l and k_α, respectively. However, because of their intrinsic periodicity, torsional terms are naturally defined by a cosine series of M terms for each dihedral angle. Thus, n_{ik} is a parameter describing the multiplicity for the kth term of the series, $\theta_{0,ik}$ is the corresponding phase angle, and V_{ik} is the energy barrier. Taken as a whole, this group of contributions is usually referred to as the "bonded" terms of the FF.

The fourth and fifth terms in eq 2 represent van der Waals and electrostatic interactions between atoms, respectively, and are denoted as "nonbonded" terms. These contributions act on every pair of atoms in the system that is not already covered by the bonded counterpart. The energy is expressed as an inverse power function of the distance between the considered atoms, r_{ij}. The van der Waals interactions are generally treated with a 12−6 Lennard-Jones potential, where ε_{ij} is a parameter defining the depth of the energy well, whereas $r_{0,ij}$ is the minimum energy distance that equals the sum of the van der Waals radii of the two interacting atoms. Finally, the electrostatic energy is described with the Coulomb potential, where q_i and q_j are the partial charges of a pair of atoms, ε_0 stands for the permittivity of free space, and ε_r is the relative permittivity (or dielectric constant), which takes a value of 1 in vacuum.

The major advantage of FFs is that they speed up calculations considerably, especially compared to quantum mechanics (QM). QM would be a more accurate theory for treating molecular-sized systems,[2] but it is intrinsically difficult to solve the Schrödinger equation for many interacting particles. This prevents QM from being applied practically to studying very large systems of several thousand atoms. Lately, however, accurate QM-based approaches have found increasing applicability in drug discovery.[51−53]

In this context, FFs have a long history of success, permitting extended MD simulations of large biomolecular systems, despite some intrinsic limitations (see below). FFs widely used nowadays for biomolecular simulations (condensed-phase FFs) include AMBER,[54] CHARMM,[55] and OPLS.[56] These have attained such a high standard of quality that the preference for one over the other is often dictated by practical considerations only, related to their implementation with the MD engine of choice. In addition to amino acids, parameters for nucleic acids, lipids, carbohydrates, and several ionic species have been included in the parent FFs in recent years by suitably extending the original parametrization. However, the structural variability of small molecules (i.e., ligands) has seriously challenged the condensed-phase FFs. To get around this, the user must often supply specific parametrizations. Much effort has been devoted to simplifying and automating this time-consuming and error-prone procedure, including the development of general force field sets for organic compounds (e.g., GAFF[57] for AMBER, and CGenFF[58] for CHARMM) and specific parametrization toolkits. Among the latter we mention the Antechamber program,[59] Paramfit,[60] and Hopkins and Roitberg's proposed procedure for AMBER,[61] while the general methodology GAAMP can be used to improve parametrizations relying on both GAFF and CGenFF libraries, with the added value of identifying, scanning, and optimizing all soft dihedral parameters of small molecules according to QM data.[62] Another promising general strategy is the systematic parametrization method provided by ForceBalance, which is able to derive parameters by combining theoretical and experimental data in a flexible way.[63] But QM-derived partial atomic charges and dihedral angle potentials should still be checked carefully when dealing with nonstandard small molecules.

Although FFs can reduce the computational burden, eq 1 can still be analytically solved for only a few atoms. For biological macromolecule simulations, numerical methods must be used to split the integration of the equations of motion into discrete time intervals, called time-steps, δt. In doing so, forces are assumed to be constants during each integration step. However, as reported in eq 1, forces depend upon atomic positions that change over time. Thus, a small δt guarantees reliable forces over time. In practice, with appropriate workarounds, a time-step of 1 or 2 fs can be safely considered as a good compromise between calculation accuracy and efficiency. This is commonly achieved, for example, with the SHAKE and related algorithms.[64] Here, the equations of motions are solved while still satisfying the geometric constraint for the fastest degrees of freedom (i.e., the stretching and bending of bonds involving hydrogen atoms). Other strategies envision, for example, repartitioning the mass of heavy atoms onto the neighboring hydrogen atoms, allowing for a time-step increase of up to 4 fs.[65] As an example of an integrator, the velocity-Verlet[11,12] is a simple and widely used algorithm in MD codes. Within such an integrator, positions at time $(t + \delta t)$ are calculated by current positions, velocities ($\mathbf{v}(t)$), and accelerations according to

$$\mathbf{x}_i(t + \delta t) = \mathbf{x}_i(t) + \mathbf{v}_i(t)\,\delta t + \frac{1}{2}\mathbf{a}_i(t)\,\delta t^2 \quad (3)$$

Accelerations are in turn calculated by the forces acting on each atom, that is, by taking the first derivative of the potential energy with respect to positions with the opposite sign as shown in eq 1. Then, velocities are propagated as follows:

$$\mathbf{v}_i(t + \delta t) = \mathbf{v}_i(t) + \frac{1}{2}[\mathbf{a}_i(t) + \mathbf{a}_i(t + \delta t)]\,\delta t \quad (4)$$

As seen in eq 4, the second term on the right-hand side corresponds to the arithmetic mean of the accelerations taken at time t and $(t + \delta t)$. Moreover, to calculate accelerations at the next step, positions must be updated *before* advancing velocities. As a matter of fact, to be safely used in an MD simulation, any numerical integrator must satisfy some practical and theoretical requirements. They must follow the analytical trajectory as closely as possible and must preserve the physical properties of the equations of motion, with the total energy that is constant of motion, given by the sum of the potential and the kinetic energy ($K(\mathbf{p})$):

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$$H(\mathbf{x},\mathbf{p}) = V(\mathbf{x}) + K(\mathbf{p}) \quad (5)$$

where $H(\mathbf{x},\mathbf{p})$ is the classical Hamiltonian of the system that depends upon the coordinates and momenta of the particles and returns the total energy (E) of the system. From these considerations, it should be clear that as long as the integrator works properly, MD naturally follows the motion of a microscopic isolated system, where neither matter nor energy are exchanged with the surroundings. In other words, Newtonian dynamics sample a statistical ensemble of microstates characterized by a constant number of particles (N), volume (V), and energy (NVE = const, or microcanonical ensemble). Nonetheless, it is possible to better mimic actual macroscopic behavior by also controlling the system's temperature and pressure during simulation. Constant temperature is maintained through algorithms, called thermostats,[66] that allow fluctuations in kinetic energy as if the simulated system were immersed in a thermostatic bath (NVT = const, canonical ensemble). However, the analogy with a thermostatic bath should not be taken too literally, since heat flow is not simulated. Instead, the system temperature is forced to attain, on average, the desired macroscopic value through proper alterations of the equations of motion. Stochastic dynamics (SD) can also be used for this purpose.[66] Very similar considerations apply for the so-called barostat algorithms,[11,12] by which the pressure is controlled by opportunely scaling (isotropically or not) the system volume (NPT = const, isothermal−isobaric ensemble). In order to better describe bulk properties with finite size systems, periodic boundary conditions (PBC) are also commonly employed in MD. With the use of PBC, the model system is placed in a unit cell that is replicated in all directions to form an infinite lattice of image atoms. In this way, coordinates and velocities are stored and propagated for the unit cell only, although the evaluation of nonbonded terms must in principle be extended to every pair of atoms in the unit cell and periodic images.[11,12] For this reason, a spherical cutoff scheme with a radius of at least 10 Å can be used to contain the computational cost and, as such, it is employed in the calculation of the short-ranged van der Waals terms. Conversely, because of the intrinsically long-ranged nature of electrostatics interactions (decay as r^{-1}), the evaluation of the Coulomb energy for the entire periodic lattice is required. The calculation of full electrostatics is achieved through the Ewald sum methods, which treats differently the rapidly varying electrostatic interactions at short-range (in the real space) and the slowly decaying potential at large distances (in the reciprocal space). With the use of spherical cutoffs and an appropriate splitting parameter between short and long-range interactions, one can use the particle-mesh Ewald (PME) method[67] to greatly improve the efficiency of the computation. In this way, the point charges in reciprocal space are smeared on a regular grid, and the calculation is performed by taking advantage of fast Fourier transform.[68] The interested reader is encouraged to delve into the review articles and books that focus on PME.[69] By use of these algorithms, MD simulations nowadays can simulate the evolution of a protein in realistic conditions for hundreds of microseconds and more. This allows researchers to observe events such as a drug binding to its target and to estimate the kinetics and free energy associated with binding.

2.2. Major Limitations of MD with Classical Potentials. Here, we mention some key approximations of FF-based simulations. First, the atomic charge of each atom is fixed, disallowing any charge polarizability over time. To overcome this limitation, polarizable FFs have been developed, which can mimic, to a certain extent, the electronic redistribution in response to an external electric field (for recent reviews, see Masetti et al.[70] and Shi et al.[71]). This effect may play a non-negligible role in the energetics of certain protein−ligand complexes.[72] Polarizable FFs are very promising; however, they remain quite computationally demanding, with their use and parametrization being less user-friendly than that of their fixed-charge counterparts. Advances in polarizable FFs will certainly widen their use in the coming decades.

Another major limitation of FF-based simulations is that they cannot be used to study chemical reactivity, since chemical bonds cannot be broken or formed during MD. To address this, researchers can use methods that exploit a dual-resolution description. For example, QM/MM simulations[73] treat a very limited portion of the system with higher accuracy. This is typically the binding site of a protein where chemical reactions occur. The majority of the system (the remainder of the protein and solvent) is then treated at a less accurate level of theory. These methods have increasingly impacted drug discovery. One example is in the study of covalent inhibitors, which block the target by forming a chemical bond. During drug design, QM/MM calculations can be used to generate QM-based electrostatic potential maps of the receptor's binding site, to determine the protonation states of key residues of the binding pocket, and to dissect reaction mechanisms of enzymes that are drug discovery targets.[53,74,75] The ReaxFF[76] is one emerging approach to studying chemical reactivity using classical MD. This FF allows chemical reactions to be studied through a geometry-dependent parametrization of reactants and products. Unfortunately, this parametrization is not simple and has only covered limited classes of chemical species to date.

Because chemical reactivity is not allowed in conventional FF-based simulations, specific protonation and tautomeric states must be preassigned to all system residues (amino acids, nucleic acids, ligands, etc.). This is often referred to as the system's topology, which is specified by the user during the setup phase and maintained throughout the simulation. Usually, assuming a neutral solution, acidic amino acids such as aspartate and glutamate can be safely modeled in their deprotonated state (negatively charged), while basic residues like lysine and arginine are modeled in their protonated state (positively charged). However, cysteine and histidine, by virtue of their pK_a (around 8−9 and 6−7, respectively), sample a population of states at physiological pH rather than being a single dominant form. For histidine, the issue is further complicated by the fact that two tautomeric forms (N^{ε} and N^{δ} protonated) are at equilibrium in the neutral state. In this regard, the so-called constant-pH simulations allow dynamical changes in topology during dynamics, mimicking more realistic conditions for biological model systems. Interestingly, the AMBER and CHARMM engines have recently started to support explicit solvent−constant-pH implementations to be used in conjunction with their own native FF,[77,78] while an in-house version of GROMACS is available to run this kind of simulation.[79] However, constant-pH simulations are generally much more expensive than ordinary setups, and a significant increase in computational power is probably required before they achieve widespread use.

In summary, the inherent approximations of FFs unavoidably introduce a systematic error into simulations. This error can always be reduced using a better (yet more computationally

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demanding) description of the model system. In addition, MD simulations are always affected by another approximation, related to the limited sampling allowed (far from being exhaustive). This creates a random error, which can be decreased by extending the length of the simulations. In particular, just as experiments must be replicated to assess the reliability of results, it is becoming computationally affordable to adopt this good practice when running MD simulations too. Indeed, the easiest and most general way to evaluate the statistical uncertainty is to repeat independent runs with different (uncorrelated) initial conditions and/or random seeds (e.g., different velocity distributions). More specific strategies can also be used in the context of single runs.[80] However, certain types of calculations (especially free-energy estimates, see below) are often so time-consuming that, until recently, they excluded repeated simulations. Continued increases in computational power will lead to better statistics, thus improving the reliability of MD-derived findings. With these considerations in mind, we describe here how classical MD simulations can be used to make informed choices in drug discovery programs.

2.3. MD-Derived Observables for Drug Discovery. Many observables can be obtained from MD runs. The principles of statistical mechanics allow quantitative estimates of important thermodynamic observables to be computed from MD trajectories. These include internal energy, pressure, and heat capacity.[11,12] However, the key thermodynamic quantity in drug discovery is the protein−ligand binding free energy. The noncovalent association between a protein P and a ligand L in solution, to form the complex PL,

$$\mathrm{P} + \mathrm{L} \rightleftarrows \mathrm{PL} \tag{6}$$

is described with the equilibrium (or association) constant K_a, which is defined as

$$K_a = \frac{[\mathrm{PL}]_{eq}}{[\mathrm{P}]_{eq}[\mathrm{L}]_{eq}} \tag{7}$$

where the square brackets indicate a concentration, usually expressed in molar units. The reciprocal of eq 7 is simply the equilibrium constant for the opposite reaction, namely, the dissociation constant ($K_d = 1/K_a$). Because K_d corresponds to the ligand concentration for which an equal probability of bound and unbound protein is achieved, this is used more than K_a in pharmaceutical research. Moreover, for enzyme inhibitor assays, K_d is usually replaced by the inhibition constant K_i, which has the very same chemical interpretation, so long as competitive inhibition is considered. The link between the experimentally accessible equilibrium constants and thermodynamics is provided by the standard free-energy change for binding at constant temperature and pressure:

$$\Delta G_b^\circ = -k_B T \ln(K_a C^\circ) = k_B T \ln\left(\frac{K_d}{C^\circ}\right) \tag{8}$$

where C° is a constant defining the standard concentration, which is 1 M by convention (1 molecule in the volume of 1663 Å^3). Importantly, multiplying the association constant by C°, or equivalently dividing the dissociation constant by the same amount, makes the argument of the logarithm in eq 8 a dimensionless number, as it should be. Since the standard binding free energy (sometimes also referred to as "absolute" binding free energy) depends on the equilibrium constant and the reference value of C° (see eq 8), this conventional concentration must be properly considered to make meaningful comparisons between computational free-energy estimates and absolute experimental data (see below).[81,82]

As mentioned previously, the calculation of free-energy differences is not simple. Together with entropy-related quantities, free energy is the thermodynamic observable whose estimation suffers the most from sampling limitations. A rigorous formulation of the problem would require statistical mechanics arguments that are outside the scope of this Perspective. Therefore, we limit ourselves here to recalling that the probability of visiting microstates in the canonical ensemble is proportional to the Boltzmann factor:

$$p(\mathbf{x}) \propto e^{-V(\mathbf{x})/(k_B T)} \tag{9}$$

Because of the exponential relationship, high-in-energy configurations are much less frequently visited than low-energy states, and barriers larger than a few $k_B T$ units can severely hamper the efficient exploration of space, which is required to achieve reliable (or converging) free-energy estimates. For this reason, transitions between states underlying significant free-energy barriers can be viewed as *rare events* relative to the time scales accessible through MD simulations. From a statistical mechanics standpoint, eq 8 can be reformulated as[83]

$$\Delta G_b^\circ = -k_B T \ln\left(\frac{p_{bound}(\mathbf{x})}{p_{unbound}(\mathbf{x})}\right) + k_B T \ln\left(\frac{C^{box}}{C^\circ}\right) \tag{10}$$

The first term in eq 10 represents the computational free-energy difference expressed as a probability ratio. Here, $p_{bound}(\mathbf{x})$ and $p_{unbound}(\mathbf{x})$ are the probabilities of observing the protein in the bound and unbound states, respectively, provided that a clear distinction between them can be achieved by defining an appropriate reaction coordinate (or collective variable, see below). However, the second term in eq 10 is a correction that must be introduced to retrieve the standard binding free energy. This term takes into account both the reference concentration of the standard state (C°) and the concentration of the interacting partners in the simulation box (C^{box}). This correction is only relevant for absolute comparisons with experimental data, as it cancels out when computing relative free-energy differences ($\Delta\Delta G_b$). However, reliable binding free energies through the direct use of eq 10 require extremely long (and possibly repeated) simulations. In principle, many binding and unbinding events must be observed to assess a statistically meaningful probability ratio between states. To date, this approach has been successful for weakly interacting solutes (solvent mapping).[84,85] If stronger binding is considered (approximately larger than 3−4 kcal/mol), unbiased MD is typically unable to recover transition rates, and methods based on enhanced sampling procedures can be applied.

2.4. Enhanced Sampling in MD. To overcome limitations inherent to Boltzmann sampling, several theoretical methodologies have been developed. These are generally referred to as "enhanced sampling methods", since they attempt to escape Boltzmann statistics while retaining the correct distribution of states in the given statistical ensemble.[36] A large group of these methodologies exploit the fact that the free energy is a state function; thus, differences in free energy do not depend upon the path from state A to B. This means that we can transform state A into state B without worrying about the physical transition (i.e., the binding/unbinding path). From a practical standpoint, this is achieved by introducing a hybrid (or mixed)

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Hamiltonian, which is a function of the potential energy of states A and B through the coupling parameter λ:

$$V(\lambda) = (1 - \lambda)V_A + \lambda V_B \quad (11)$$

In eq 11, the general case of linear interpolation between the two potential energy functions is shown. This is not always the best possible solution, and more sophisticated and effective mixing can be used (nonlinear interpolation schemes, the use of soft-core potentials, and the separated coupling of electrostatics and van der Waals terms). For relative free-energy differences, this can be thought of as performing a smooth and progressive transformation of a ligand A into a ligand B, in both the protein-bound and unbound states (bulk solvent). Then, the free-energy changes for the two transformations are calculated with appropriate theoretical estimators and practical precautions (stratification, forward and backward sampling, etc.), and the relative contributions are finally combined through a suitable thermodynamic cycle to recover the $\Delta\Delta G_b$ (Figure 1).

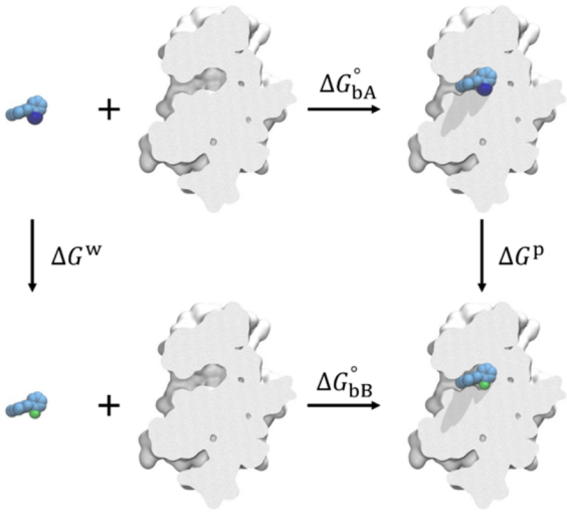

Figure 1. Thermodynamic cycle used to calculate relative binding free energies ($\Delta\Delta G_b = \Delta G^\circ_{bA} - \Delta G^\circ_{bB}$) between congeneric ligands. The horizontal legs correspond to the physical binding process, whereas vertical legs indicate the unphysical transformation of ligand *A* (blue) into ligand *B* (green) performed in bulk solvent (left) and in the protein binding site (right). FEP is used to compute the free energy associated with the vertical legs of the cycle, and the relative binding free energy is calculated as the difference between the free energy required to transform the ligands in the binding site (ΔG^p) and in solution (ΔG^w).

Because of the unphysical path used for the free-energy calculation, this class of enhanced sampling methods is sometimes referred to as "alchemical transformations", although in drug design they are mostly known as "free-energy perturbation" (FEP) methods.[38] FEP is commonly used to calculate the free-energy contribution associated with the vertical legs of the cycle in Figure 1. But, in principle, other approaches can be used, such as thermodynamic integration (TI). In fact, these methods can be grouped into a more general class of hybrid Hamiltonian techniques.

Standard binding free energy can also be recovered using complex thermodynamic cycles and more challenging simulations (Figure 2). For example, during MD simulations, the ligand is reversibly changed into a fictitious noninteracting particle. In other words, it is decoupled from the environment

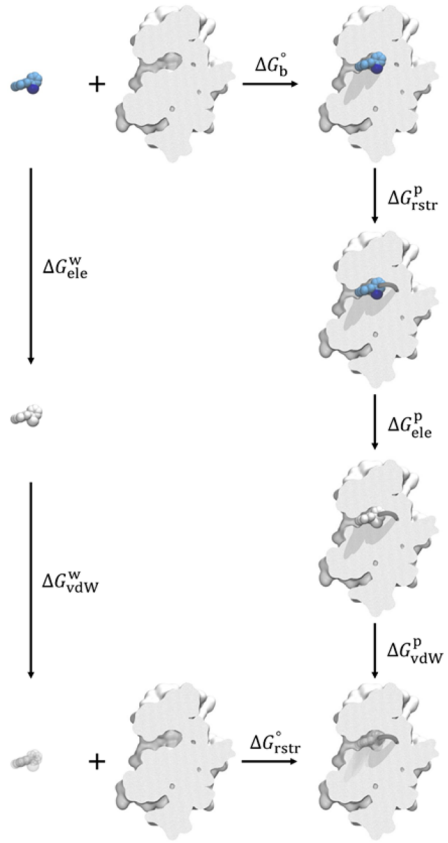

Figure 2. Typical thermodynamic cycle used to compute absolute binding free energy differences (double decoupling method, DDM). In the simplest implementation, the cycle involves the introduction of rototranslational restraints to the bound ligand in order to avoid the "wandering ligand" problem. The restraints are depicted as a ring fastening the ligand to the protein, and their associated free-energy penalty is ΔG^p_{rstr}. Eventually, conformational restraints might also be envisioned (not shown in the picture for simplicity). Then the ligand is reversibly decoupled from the protein (or the solvent), usually, in two steps: first, electrostatics is turned off (ΔG^p_{ele} or ΔG^w_{ele}), then the van der Waals contributions are switched off (ΔG^p_{vdW} or ΔG^w_{vdW}). The "uncharged" state is represented by the ligand colored in white, whereas the transparent ligand depicts the fully decoupled state. The free-energy penalties associated with confining the decoupled ligand in the binding-site volume are calculated analytically (horizontal leg at the bottom of the cycle), and the standard state correction (ΔG°_{rstr}) is usually added to this contribution.

(the protein and the solvent). For this reason, this theoretical framework is called the double decoupling method (DDM).[81] The method was first introduced by Jorgensen in 1988 as the double annihilation method (DAM)[86] and later formalized by Gilson et al.[81] and others.[87−89] A critical aspect of the procedure is that to avoid the "ligand wandering" problem associated with highly decoupled states, suitable restraining potentials must be introduced to keep the ligand in place in the binding site during the whole transformation (see Figure 2). These restraints must be applied in such a way as to allow an exact recovery for the free-energy penalty due to their introduction. Moreover, during ligand decoupling, a cavity is created in the binding site that must be filled by an appropriate number of water molecules. However, this process can be slow enough to challenge the effectiveness of the whole procedure. To address this, Helms and Wade introduced a complex hybrid Hamiltonian scheme to replace the ligand with a certain

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amount of water molecules.[90] With a more general purpose, Woods et al.[91] proposed the water-swap reaction coordinate, where an equivalent volume of bulk water molecules replaces the ligand in the binding pocket. This method is better suited to describing both buried and superficial binding cavities.

Enhanced sampling methods based on unphysical pathways can be effectively used to compute free-energy differences, providing valuable insight into the binding thermodynamics. However, these approaches do not reveal kinetic aspects of the drug-binding process that are often of interest. Accounting for the binding kinetics requires methods that use physical pathways for free-energy calculations, through which free-energy barriers and possible intermediates along the (un)-binding path can be determined.

Enhanced sampling methods, which rely on physical pathways, can reconstruct the free energy of the investigated event as a function of a few reaction coordinates, usually called collective variables (CVs), able to account for relevant degrees of freedom of protein−ligand binding and unbinding. The projection of the free energy along the relevant degrees of freedom is called the potential of mean force (PMF). In principle, it can be used to extract both thermodynamic and kinetic information (see Figure 3).[92] In fact, we can distinguish

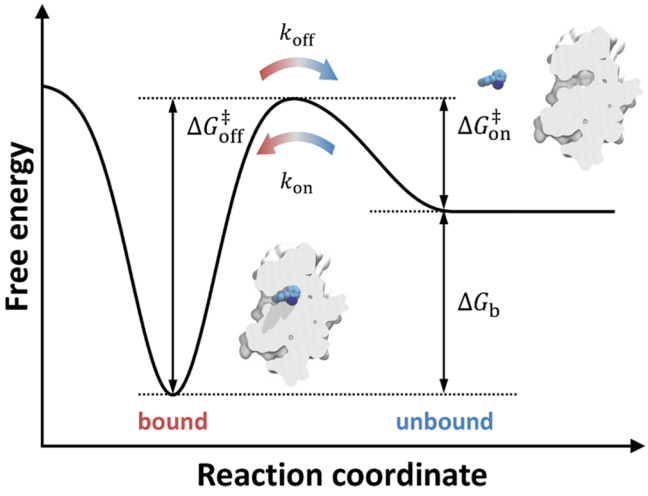

Figure 3. Simplified free-energy profile for the (un)binding process. The drug−receptor complex is shown as a deep free-energy minimum on the left (bound state), and the dissociated complex is represented as a higher-in-energy minimum on the right (unbound state). The thermodynamics of binding is quantified (in first approximation) by the free-energy difference between these minima (ΔG_b), and the kinetics of (un)binding is determined by the dissociation and association rate constants, k_{off} and k_{on}. These quantities are related to the free-energy differences between minima and the transition state, $\Delta G^{\ddagger}_{off}$ and $\Delta G^{\ddagger}_{on}$, respectively.

between those methods that explicitly use CVs during sampling and those that do not. The first method class allows the exploration of rare events by biasing the MD simulations along the chosen CVs. This can be achieved in several ways, such as acting on the forces or introducing external potentials that may or may not change over time (leading to equilibrium or nonequilibrium approaches, respectively). Thus, CV-based methods require an a priori definition of the reaction coordinate to sample. In most cases, it is not simple to define a proper CV (or an ensemble thereof). Much chemical intuition and/or trial simulation is required to achieve a satisfying description.[36] Umbrella sampling (US)[39] is one notable equilibrium-CV-based enhanced sampling method, while steered MD[42,43] and metadynamics[41] are certainly the most popular nonequilibrium ones. The second method class, which does not explicitly use CV, acts by heating all the degrees of freedom of the system (or a fraction of them) at once. Here, we further underline that temperature must be understood figuratively, since the same enhancement in sampling can be obtained by actually heating the system or by opportunely scaling the Hamiltonian. This is analogous to what takes place for the enhanced sampling methods based on unphysical pathways. Temperature replica exchange MD (T-REMD, also known as "parallel tempering")[40] and Hamiltonian replica exchange MD (H-REMD, also known as "solute tempering")[93] are examples of these methodologies. The recently proposed scaled MD[94] can also be grouped in this class of techniques. These methods are devoid of any preconceived notion of the rare event and CVs. For this reason, however, they are also less specific and often less efficient than CV-based methods. Interestingly, because of their complementarity, CV-based and replica exchange methods are sometimes coupled to fully exploit the potential of both approaches (e.g., US or metadynamics combined with T/H-REMD).[36] Moreover, replica exchange methods can also be coupled to enhanced sampling techniques based on unphysical pathways, such as FEP.[93,95,96]

3. COMBINING MOLECULAR DYNAMICS WITH LIGAND DOCKING AND VIRTUAL SCREENING

Ligand docking has been successfully used for drug discovery in recent years.[19] It attempts to predict the three-dimensional structure and binding free energy of the complex formed by a receptor, usually a protein, and a small ligand. When applied iteratively to a library of small molecules, each member of the library is docked into the receptor, assigned a predicted binding energy, and ranked accordingly. This computational approach is called "structure-based virtual screening".[97] The topmost ranking compounds are then prioritized for further in silico studies or experimental testing.

Despite its widespread and successful use,[98] docking suffers from one main drawback: the ability to handle proteins' intrinsic flexibility in docking is either absent or limited.[15,99] This downside has greatly limited this technique's prospective applicability. To address this, researchers have implemented several strategies, including soft docking,[100] rotamer libraries,[101] and local optimization of side chains.[102] However, these relatively simple heuristics do not allow extended rearrangements of the protein structure, including significant conformational changes at the backbone level.[103]

3.1. MD for Generating Ensembles of Receptor Conformations. One practical alternative to on-the-fly modeling of receptor plasticity is the use of pre-existing multiple receptor conformations (MRC) of the binding pocket. This is facilitated by the steadily increasing number of X-ray and NMR protein structures.[17,104] Different experimental structures of the same receptor, if available, are used to populate the conformational ensemble used in MRC docking.[105] However, the number of targets whose three-dimensional structure has been experimentally solved is limited, considering the estimated extent of the druggable genome.[106] Moreover, the amount of conformational space explored by experimental structures is usually quite limited and biased toward a few known ligand−receptor complexes. While several

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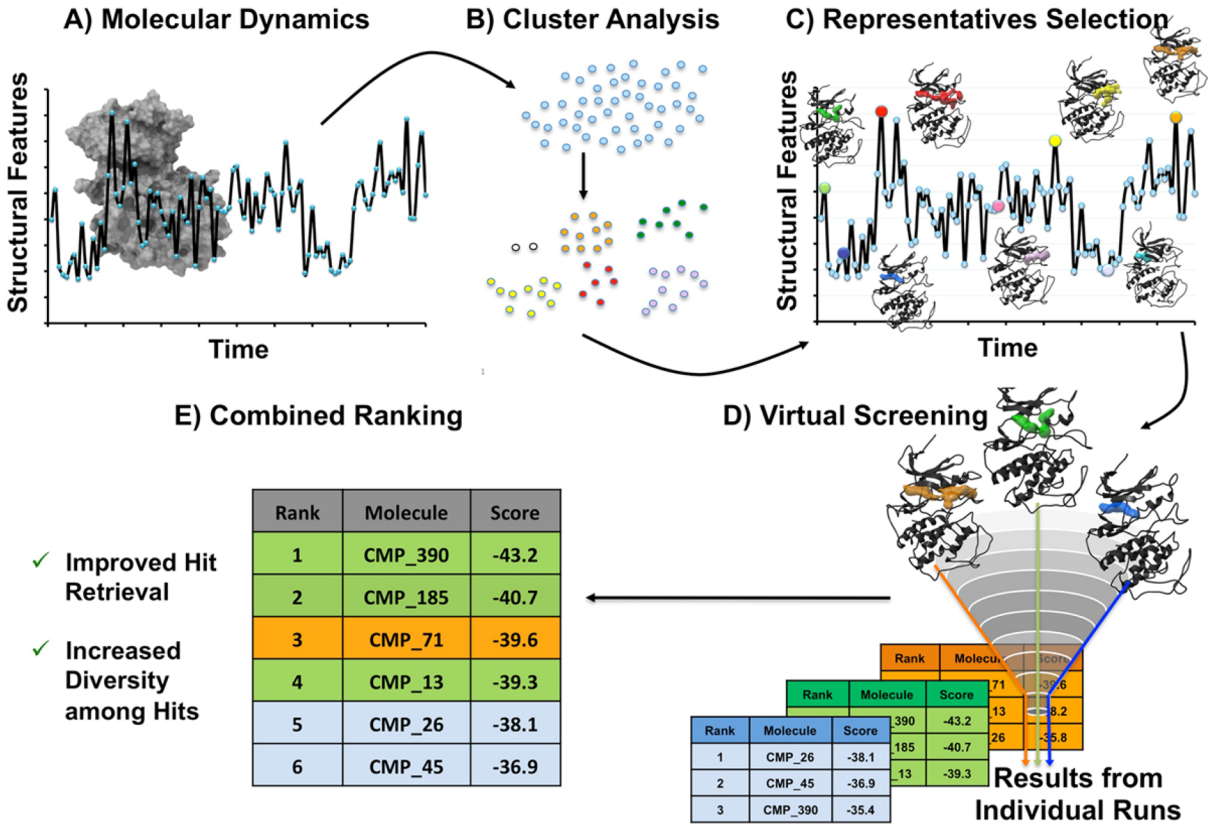

Figure 4. Fundamental steps in a virtual screening workflow combining docking and MD simulations. (A) An MD trajectory is used to explore the receptor conformational space. (B) From the trajectory, several snapshots are extracted and redundancy is eliminated by means of cluster analysis. (C) From each cluster, a representative structure (e.g., medoid) is selected. (D) Virtual ligand screening is independently carried out at each representative conformation. (E) Activity predictions returned by independent runs are combined together in a global ranking.

computational techniques, including homology modeling[107] and normal modes analysis,[108] can complement experimental structures for the MRC ensemble, MD is an obvious way to generate receptor conformations.[23,29]

Figure 4 reports a general framework for combining molecular dynamics and MRC virtual ligand screening. While others have applied a similar workflow,[28,109] the relaxed complex method developed by McCammon's group is one of the most well-known examples of this strategy for drug design.[110] First, MD simulations of the selected target are used to generate a diverse set of receptor conformations, which also characterize the structural flexibility of most relevant regions of the receptor for ligand binding. Enhanced sampling methods can be used, allowing a most efficient exploration of the conformational space.[111] For example, in their studies on PARP-1, Antolin et al. used temperature replica exchange MD.[112] Then, at regular intervals, multiple snapshots are extracted from the trajectories (Figure 4B). Each snapshot represents a conformational variant of the binding pocket. In principle, every snapshot could be used for MRC virtual screening.

Notably, a selection of the MD-generated snapshots is normally needed to avoid redundant structures. This procedure has demonstrated that some conformers perform better than others in enriching active compounds.[113] Nevertheless, if no a priori knowledge indicates the single best performing conformation, some general guidelines exist for building a representative ensemble of targeted structures.[114] From a survey of the literature, the most important concept is probably that a combination of two or three conformational variants of the target usually performs better than random single-conformation rigid docking. A limited conformational ensemble can improve both the final enrichment and the chemical diversity of the resulting hits. In fact, if the set of target conformations for MRC is too large and diverse, this often generates an overwhelming noise that deteriorates the virtual screening performance.[115] For these reasons, a limited (yet hopefully significant) number of conformations is advisible for efficient MRC virtual screening. This can be selected by means of cluster analysis, as one example.[116] Ideally, the selected snapshots should capture the entire structural diversity of the target, sampled along the trajectory, with the minimum number of significant conformers (Figure 4C). For instance, a nonredundant set of conformations could be obtained using rmsd-based hierarchical-agglomerative cluster analysis protocols and clusterization methods based on QR-factorization.[117] In a comparative study, Nichols et al.[116] demonstrated how MD-generated receptor variants can match and possibly outperform crystal structures in retrospective virtual screening experiments. Each variant is used in an independent run to generate an individual set of results (Figure 4D). These separate rankings are eventually joined together (Figure 4E).[118,119] Importantly, the relaxed complex method was successfully used to prospectively identify several modulators of relevant pharmaceutical targets, as reviewed in detail by Ivetac and McCammon.[25]

3.2. MD for Postprocessing of Docked Protein–Ligand Complexes. Because sampling and scoring algorithms

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for docking suffer from several limitations,[99] MD can be used as a postprocessing tool to validate and/or refine docking solutions. Here, the underlying assumption is that a bad docking pose will generate an unstable MD trajectory, during which the ligand could even leave the binding site. Conversely, a meaningful docking pose will display stable and specific interactions with the target, showing a low rmsd over time, with respect to the starting conformation. Furthermore, MD could provide evidence of so-called "induced-fit effects", in which the binding site adapts to the starting pose of the ligand, strengthening the interactions already captured by docking and establishing additional interactions during the MD runs. Finally, MD in explicit solvent can suggest the role of structural water molecules within the binding site, which is important for correctly predicting ligand binding.[120]

Our work in 2004 is one example of how docking and MD simulations can be fruitfully coupled. Docking and cluster analysis allowed us to identify several binding modes of propidium at the peripheral anionic site of the human acetylcholinesterase enzyme.[121] We investigated these initial poses using MD and eventually identified two alternative binding modes. Both binding modes were compatible with the electron density map, with one resembling the crystallographic pose and the other being flipped by 180°. This study showed that even a few nanoseconds of MD simulations could discriminate good docking poses from bad, when surface solvent-exposed binding sites were considered. In another study, Kacker et al.[122] used a combination of QM calculations, docking, and MD to define the protonation state of BACE-1's catalytic machinery in complex with different ligands. MD was crucial in discriminating stable hydrogen bonds established between ligands and the catalytic dyad from more transient ones and in assessing the role of bridging water molecules. Complementing docking and MD, Rastelli et al.[123] devised an automatic pipeline, in which they rescored docking-generated conformations using MM-PB/GBSA free-energy calculations. With an acceptable increase in calculation time, they improved hit enrichment in retrospective virtual-screening experiments. Focusing on GPCR, particularly the human A_{2A} adenosine receptor, Sabbadin et al.[124] proposed a sequential approach to studying stability in an explicit lipid−water environment of GPCR−ligand complexes generated by docking. Trajectories were analyzed in terms of individual electrostatic and hydrophobic contributions to the binding energy of each residue contacting the ligand. The bioactive conformation was expected to display a persistent interaction network along the trajectory. On the other hand, it is worth mentioning the recent study of Lauro et al.,[125] which represents a counterexample where MD, employed to perform linear interaction energy calculations, while efficient, could not significantly improve ligand-ranking accuracy with respect to results generated by high quality docking scoring functions. For several other applications of MD as a ligand-docking postprocessing tool, the reader may refer to the review article by Alonso and colleagues.[22]

4. UNBIASED MD FOR INVESTIGATING DRUG BINDING

During unbiased MD simulations, the model system evolves freely over time, without any (biasing) force acting on it. Thus, this seems the simplest way to simulate and observe the spontaneous evolution of a drug binding from the bulk of the solvent into the biological target. That is, one can run simulations for long enough to let the drug diffuse into the solvation waters until it finds its way into the target binding pocket, leading to the final bimolecular complex. However, for slow binding events (i.e., weak binders), very long simulations may be required, which can easily exceed today's affordable simulation time (up to hundreds of microseconds and milliseconds).

Until recently, the simulation of drug-binding events was far beyond the reach of atomistic MD simulations. Great progress has been made since the very first simulations of 9 ps in vacuo of the small bovine pancreatic trypsin inhibitor protein, reported in 1977's landmark study by McCammon, Gelin, and Karplus.[13] Specialized supercomputers designed for MD simulations, such as the Anton machine created by the DE Shaw lab, can run millisecond-long continuous single-trajectory MD simulations of small globular proteins.[29,33] Even longer aggregated sampling times have been reported using Folding@Home from the Pande lab.[126] While these highly efficient computers and MD systems are available to just a few researchers, the growing distribution of graphics processor units (GPUs) is increasing the MD community's ability to run long simulations at an affordable cost. Nowadays, researchers can quite routinely generate tens of microseconds of simulated time for medium-size models (50−100K atoms).[4] Only 10 years ago, it was hard to envisage such a rapid increase in MD time scales. Remarkably, this has mostly been achieved because of technological progress rather than the development of novel theories.

For drug discovery, typical model systems only include the solvated target and ligand(s), which do not usually exceed a few hundred thousand atoms in size. In the past few years, unbiased MD simulations of these models have been used quite extensively to describe the process by which drugs bind to targets in several systems. Hundreds of ∼100 ns runs of unbiased MD were used to identify possible pathways for the spontaneous binding of benzamidine to the trypsin.[30] Tens of microsecond-long simulations were run to detect the spontaneous binding of G-protein-coupled receptor (GPCR) agonists and antagonists, as well as protein kinase inhibitors.[31,127−129] More recently, Decherchi et al. ran extensive unbiased MD simulations (about 1 μs each) to investigate the tight binding event of a transition state analog (DADMe-immucilin H) into the pharmaceutical target, purine nucleoside phosphorylase.[32] Despite observing spontaneous binding through different routes, the authors used the pathways obtained by MD simulations to determine the free-energy profiles associated with the diverse binding mechanisms. By combining MD with machine learning and enhanced sampling methods (see below), they could observe the binding and estimate the associated kinetics and thermodynamics. Overall, these unbiased MD simulations have validated the computational methodology, reproducing the known crystallographic pose of the drug quite well (with an rmsd that was usually within 2 Å). In addition, the free-energy difference between the bound and unbound states can be calculated in many formally equivalent ways and compared to the experimental K_i, when available. Kinetic quantities, such as k_{on} and k_{off}, are further important observables that can be retrieved from these long MD simulations. They must also reproduce the experimental data to further validate the simulated drug-binding process (see below for further details on binding kinetics). In this way, the accuracy of the computational approach to simulating the drug-binding process is endorsed and, to some extent, benchmarked.

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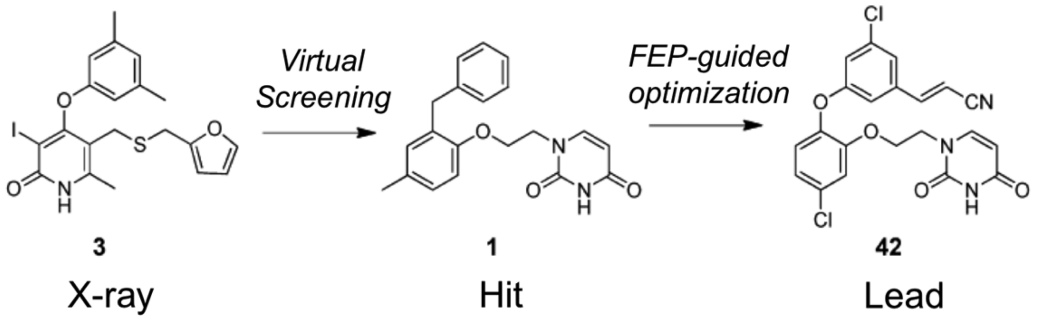

Figure 5. From the crystal structure of compound R221239 (compound **3**), the modest anti-HIV activity of the initial docking hit (compound **1**), 5 μM potency toward WT HIV-1 reverse transcriptase, was efficiently evolved into highly potent catechol diethers such as compound **42**, a 55 pM non-nucleoside inhibitor of HIV-1 reverse transcriptase (NNRTIs) discovered with the aid of the computational analyses guided by FEP results.[143]

Only then can MD trajectories be trusted, analyzed, and interpreted in a meaningful way. Doing so reveals a wealth of potentially useful insights into how the drug can reach the final bound conformation, highlighting key transient interactions that drive drug binding. For example, these simulations can reveal mesostates and high-energy intermediates formed along the path to the final binding into the cavity. It is easy to raise concerns about the real existence of these metastable conformational states. However, these concerns can be addressed by providing rational hypotheses and corroborating data retrieved from the MD simulations. This can explain, for example, the experimental drug−target affinity, which the MD simulations must reproduce well.[32]

Indeed, these first successful applications of unbiased MD to reconstructing spontaneous drug-binding processes, and the related thermodynamics constants, are a great achievement. Their success demonstrates the potential of unbiased MD to predict (and not just reproduce) plausible binding poses of new compounds in the not-too-distant future. Simulations can reveal key drug−target interactions that must be formed and/or disrupted to achieve tight drug binding. This can suggest hot spots (on both the ligand and target) that could in principle be modified to modulate their activity. It can also help prioritize compounds in medicinal chemistry campaigns.[130,131] As mentioned above, these unbiased trajectories can also provide quite realistic initial configurations and guess paths for subsequent simulations.[132] These can be used to improve sampling along that initial path and to estimate the kinetics and thermodynamics of drug−target binding.

5. MD COUPLED TO ENHANCED SAMPLING METHODS FOR INVESTIGATING LIGAND BINDING

While unbiased MD is a straightforward but costly way of simulating drug-binding processes, enhanced sampling methods are often coupled to MD to accelerate this process and to retrieve useful thermodynamic and kinetic data.[92] Indeed, enhanced sampling methods (mostly steered MD and metadynamics) were used for the first simulations of drug-binding events, with the ligand moving into and/or out of the binding pocket.[43,133,134] Researchers can now rank compounds according to the relative free energy of binding, calculated using the enhanced sampling scheme. Of the enhanced sampling techniques developed in recent years, we focus here on FEP,[37,38] umbrella sampling,[39] steered MD,[42,43] and metadynamics.[41] These methods have appealed most broadly to the drug discovery community, with demonstrated potential for impacting structure-based drug design. Other existing methods will be discussed in the final paragraph.

5.1. Free-Energy Perturbation (FEP). FEP theory and calculations relate the free energy of an initial reference state to the final target state of a system, using Monte Carlo (MC) or atomistic MD as a sampling technique.[37,38] In FEP calculations for drug design, perturbations of the initial lead compound are made using a coupling parameter during the simulations, driving a smooth mutation of the starting molecule to another close one. This cycle is then repeated a number of times, evaluating the relative binding free energies of several possible changes of the initial lead compound, which can be transformed into several close analogs. The relative free energies of binding of each compound are calculated according to the thermodynamic cycle in Figure 1. If needed, the standard binding free energy can be retrieved by appropriately modifying the cycle (Figure 2).[37,81,135] The final free-energy estimate suffers from the usual issues of FF-based calculations. These include the quality of the FF, missing polarization effects, and limited sampling. Notably, thermodynamic integration (TI) calculations could be used to compute free-energy changes in a way that is as formally rigorous as using FEP. Nevertheless, FEP-based calculations appeal more broadly to the drug discovery community.[136] Jorgensen et al. recently provided a thorough overview of the history, main contributors, and methodological development of FEP theory since its inception.[38]

In 1985, Jorgensen et al.[37] became the first to use FEP calculations to convert one molecule of ethane into methanol, in water. Just 1 year later, McCammon et al.[137] used FEP for protein−ligand binding calculations. More recently, however, increased computational power has allowed researchers to robustly validate FEP calculations for the rational design of small molecules. This rigorous approach is now endorsed for screening small libraries of close analogs for enzyme inhibition.[136] In the past, FEP calculations of protein−ligand systems were primarily used to reproduce known experimental data for a few small molecule inhibitors. Remarkably, prospective FEP calculations, within an MD framework (FEP/MD) for drug design, were already reported in 1989 by Merz and Kollman, who correctly predicted the binding free energy of a previously unreported inhibitor of the thermolysin endopeptidase.[138] Similarly, few years later, FEP/MD was again successfully employed to predict the affinity of a novel HIV-1 peptide inhibitor.[139] Interestingly, the design of HIV-1 inhibitors became an excellent testing ground for the whole FEP methodology. Several groups utilized such model system to rationalize structure−activity relationships and to suggest viable modifications of known compounds, using simulations of

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increasing complexity. Among these, we mention free energy calculations between two classes of HIV-1 protease inhibitors (amide and ester analogs) by Rao et al.[140] and the calculations of the free energy difference caused by the deletion of an entire valine residue from a peptidomimetic compound,[141] which represented a significant alchemical transformation relative to the computational resources available at the time.

Today, FEP calculations have demonstrated astonishing potential for driving lead optimization campaigns.[142] Those first early studies are now recognized as crucial in advancing this effective methodology for rational lead optimization. Coincidentally, in their initial prospective applications of FEP to drug design, Jorgensen's group mostly coupled FEP to MC (FEP/MC).[136] If the pose of a lead compound within the binding pocket is known with confidence (possibly based on the X-ray structure of the ligand−target complex), rigorous FEP/MC calculations have indeed proven to be highly accurate and informative in predicting what type of (small) modifications could generate a boost in ligand-binding affinity for the target.

One exceptionally successful application of FEP/MC-guided lead optimization is the rational design of what are currently the most potent non-nucleoside inhibitors of HIV-1 reverse transcriptase (NNRTIs). The FEP/MC-guided modifications of a few promising scaffolds have led to several potent NNRTIs inhibitors, as reported by Jorgensen et al.[143,144] These modifications include the optimization of a 5 μM docking hit into a 55 pM catechol diether NNRTIs inhibitor,[143] which was further optimized to improve druglikeness and resistance to mutations (Figure 5).[145] Other successful examples include inhibitors of the tautomerase activity of human macrophage migration inhibitory factor (MIF)[146−148] and others.[149] Taken together, these paradigmatic examples attest the impact of FEP calculations on lead optimization for drug discovery, with a clear demonstration that this approach, nowadays, offers great advantages and progress.

These numerous examples have also provided a way to test and standardize protocols for running FEP calculations efficaciously, via MC- or MD-based simulations.[150] It is only recently, however, that researchers designed tight-binding ligands using massive FEP/MD calculations.[142] Wang et al.[142] used over 200 ligands and 10 targets to validate, with retrospective studies, the high accuracy of FEP calculations (with an average error of ∼1 kcal/mol). They demonstrated the efficiency of these FEP/MD calculations, obtained using an improved force field (OPLS2.1 and 3), an improved enhanced sampling technique (i.e., FEP coupled to replica exchange with solute tempering or REST; see more on this in the below sections), and importantly, an automated user-friendly workflow running on graphics-processing units (GPUs).[142]

In accounting for the widespread applicability of the FEP approach in drug design, key factors include the improved accuracy of FEP results and the accelerated input setup and output generation. FEP/MD is now proposed for screening medium-to-large libraries of potential new compounds, where modifications of the initial compound can be quite substantial. FEP/MD can now generate the type of information that can pragmatically drive a drug design project, as Wang et al.'s encouraging study[142] suggests. In the coming years, we expect new prospective studies to further demonstrate FEP/MD's impact on the hit-to-lead and lead optimization phases of drug discovery. FEP/MD could gradually become a routinely applied method for guiding key medicinal chemistry decisions and ultimately accelerating the generation of promising lead and drug candidates.

5.2. Umbrella Sampling (US). Umbrella sampling (US) can be considered the forerunner of all the CV-based enhanced sampling methods. US was conceived by Torrie and Valleau in 1977 in the context of MC sampling.[39] Soon after, it was adapted to the framework of MD simulations. The idea is to enforce sampling along the chosen CV by performing staged simulations subject to an energetic bias, which traditionally takes the form of a harmonic potential (umbrella). In this way, high-energy regions along the reaction coordinate can be sampled exhaustively, and the unbiased distribution of states required to compute the potential of mean force (PMF) is recovered through suitable postprocessing methods. The weighted histogram analysis method (WHAM) is the most popular postprocessing method,[151] but other strategies, such as the umbrella integration, can also be used.[152] The key parameters for US simulations are (i) the force constants chosen for the umbrellas, which must be high enough to ensure uniform sampling along the entire CV space, and (ii) the separation in the CV space between different simulations (usually called "windows"), which must be small enough to provide sufficient overlap between neighboring windows. A more detailed introduction to the theory of US can be found elsewhere.[152]

Although US is one of the most accurate techniques for free-energy calculations, its practical application in drug design is mostly limited by its elevated computational cost. For example, many overlapping windows are needed to examine the free energy of a ligand unbinding event from the protein, where each window must be prepared, sufficiently equilibrated, and finally sampled. Moreover, simulations of ligand unbinding often require a few CVs, leading to a PMF reconstruction that is exponentially slower as the number of CV increases. Other enhanced sampling methods, such as metadynamics and adaptive biasing force (ABF),[153] are more efficient in exploring the free-energy space using multiple CVs, returning a multidimensional PMF. For this reason, they are more appealing for practical drug design (see below).

In 2006, Woo and Roux reported a practical US-based methodology for successfully computing the binding affinity of a peptide to the SH2 domain of human Lck kinase.[154] This approach describes the ligand (un)docking, using a one-dimensional PMF. This PMF is calculated from US simulations, using a well-defined axis as the CV to connect the protein and the ligand. Importantly, restraining potentials are used to appropriately control the transverse degrees of freedom that might change upon ligand (un)binding. These include ligand conformation, orientation, and radial translations along the CV. The absolute binding free energy is recovered by the equilibrium-binding constant, which is in turn obtained by integrating the PMF. This methodology was later successfully applied to other pharmaceutically relevant problems.[155] Interestingly, this approach has been thoughtfully discussed and compared with the double decoupling method (DDM),[81,89] with which it shares some resemblances.[156]

More recently, similar US-based strategies have been devised by Lee and Olson[157] and, later, by Doudou et al.,[158] who used these free-energy simulations to estimate ligand binding. The overall procedure, however, remains so elaborate that US-based methods for drug discovery are not yet routinely or widely used. We expect this to change once the procedure and related corrections for US-based simulations to relate the PMF to the

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binding affinity become less cumbersome. In particular, automated procedures, such as self-learning approaches and adaptive sampling, may be a viable solution for reducing computational costs and improving the method's general applicability.[159]

5.3. Steered MD. With this method, a time-dependent external potential is applied to the ligand to facilitate its unbinding from the target protein. Ligand undocking is therefore accelerated by acting on a descriptor (or CV), which is usually the protein−ligand distance or a vector describing the ligand exit pathway. To achieve this, the ligand is attached to a spring with a given force constant, and the center of the harmonic restraint is moved to a finite velocity along the descriptor so as to drive a smooth exit of the ligand from the pocket.[42,43] Steered MD is actually a nonequilibrium method, in which the pulling velocity and spring constant are key parameters for these simulations. Conceptually, steered MD is similar to performing atomic force microscope (AFM) experiments, although pulling in steered MD is typically performed at much higher velocities.

Importantly, researchers can use steered MD to calculate the exerted force and the external work performed on the system for the unbinding of each ligand considered. Grubmüller et al.[43] first demonstrated this in their pioneering investigation of the streptavidin−biotin complex. If the force constant is high enough (stiff-spring regime), the rupture force scales linearly with the amount of irreversible work (i.e., with the applied velocity). This provides important qualitative and semi-quantitative insights into the unbinding process. However, in 1997, Jarzynski[160] defined a fundamental relationship between irreversible and reversible work (i.e., the free-energy difference). The so-called "Jarzynski equality" (also known as "non-equilibrium work relation") was a milestone that allowed the PMF for the investigated process to be obtained through a series of pulling simulations, regardless of the speed applied. The equality is exact only in the limit of an infinite number of realizations. Therefore, Park et al.[161] devised an approximate formula, which holds in the limit of the stiff-spring regime. This provides better statistical results than the naive application of the Jarzynski equality. A theoretical description of steered MD can be found elsewhere.[162]

In the context of MD-based rational drug design, one prospective study investigated the binding of five structurally related flavonoids to the protein FabZ. This enzyme from the malaria-causing parasite *Plasmodium falciparum* is a potential target for antimalarial drugs.[134] Steered MD simulations were used in a similar way to single-molecule pulling experiments, where the force required to pull out each of the studied ligands from the target protein was computed and used to individuate the tighter binders (Figure 6). Strongly bound inhibitors gave profiles with higher peak forces than weakly bound ligands, which gave a flatter force profile. A new flavonoid was designed and predicted via steered MD, with experimental validation of the approach's reliability for computational drug discovery. This paradigmatic study clearly demonstrated that steered MD could distinguish active from inactive inhibitors, although the computed observables (forces required to extract each inhibitor from the targeted enzyme) remained only qualitative. This is mostly due to the poor efficiency in computing fully converged free-energy profiles during steered MD simulations. Later, this approach was used to examine different series of CDK5 inhibitors, confirming steered MD's ability to correctly discriminate binders from nonbinders, although the method

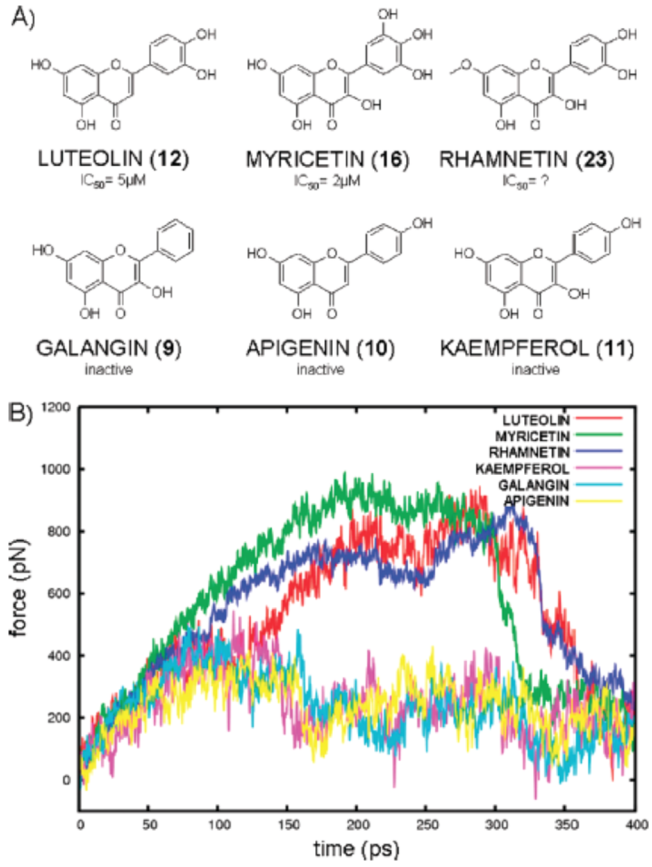

Figure 6. Comparison of the undocking force profiles of different flavonoid ligands. (A) Chemical structures of ligands under investigation. (B) Force profiles derived from pulling the ligands along the unbinding reaction coordinate. For each ligand, the plots show the resulting mean values from averaging the force profiles from five different SMD runs.[134]

failed to correctly rank series of inhibitors with similar inhibiting potencies.[163] Researchers may use the unbinding force profile to improve docking scoring function or alternatively as a postprocessing tool in structure-based virtual screening campaigns.

On the basis of these examples, steered MD seems particularly suited to discriminating active and inactive binders, which interact with the target through H-bonds that must be disrupted during the unbinding process. This was true for steered MD simulations used to characterize the unbinding of the clinical candidate F14512, which bears a spermine chain forming a complex H-bond network with the targeted topoisomerase enzyme.[164] The polyamine chain in F14512 generates high peak forces during the steered MD for drug unbinding, when compared to close analogs with no ability to form such a strong H-bond network. Conversely, hydrophobic interactions do not provide clear rupture forces, marginally affecting the resulting work profile and hampering a proper ranking of ligands with different potencies.[164] Notably, this is a further example where MD simulations have been used in a prospective manner to design analogs of F14512, which have been synthesized and tested to validate the MD-based results.

Patel et al. reported a different approach, devising a steered-MD-based protocol to capture relevant biological information from local work profiles obtained by protein−ligand pulling simulations in an unsupervised way. Interestingly, this protocol

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could in principle be exploited to tune affinity and kinetic observables.[165] However, steered MD is somewhat limited to qualitatively estimating the tightness of drug binding rather than accurately calculating the free energy of binding (as with FEP calculations). This suggests steered MD as suitable for postprocessing ligand screening during hit identification rather than for lead optimization. Steered-MD-based screening of large and diverse data sets of compounds would increase our understanding of this methodology's true potential and practical location within the drug discovery pipeline.

5.4. Metadynamics-Based Methods. These include a broad family of enhanced sampling techniques, which allow fast exploration of the underlying free-energy landscape of rare events. They use a set of order parameters, usually referred to as collective variables (CVs), that approximate the true reaction coordinate of the process. These CVs have a broad range, including coordination numbers, the number of hydrogen bonds, relative molecule orientation/rotation, and bond lengths, angles, or torsions. Once the most suitable CVs have been identified, the central idea in metadynamics is to bias the dynamics of the system along those CVs, using a history-dependent repulsive potential. To achieve this, at regular time intervals, a relatively small Gaussian-shaped potential is added to the bias at the current position of the CVs. This discourages the system from revisiting already explored regions of space.[41,166] The history-dependent potential thus builds up until it counterbalances the projection of the free energy along the chosen CVs. This allows the system to escape via a saddle point to a nearby local minimum, where the procedure is repeated. When all minima are "filled" with Gaussians, the system moves in a barrier-free manner among the different states. The bias potential can then be used as an unbiased estimator of the PMF by simply changing sign (see Figure 7). Since the bias potential changes over time during the simulations, metadynamics is considered to be a nonequilibrium enhanced sampling method.[41,167]

In metadynamics, the reconstruction of the free-energy surface can easily exceed two dimensions. This key feature distinguishes it from other CV-based algorithms. In addition, unlike umbrella sampling, metadynamics requires no additional postprocessing analysis step such as WHAM. The crucial parameters for reliably reconstructing the free energy in metadynamics are (i) the Gaussians height, (ii) the Gaussians width, and (iii) the deposition time. Briefly, the error approximately scales with the square root of the ratio between the Gaussians height and the deposition time. This implies that to achieve a reasonably accurate free-energy profile, the height of the Gaussians must be sufficiently small compared to the main free-energy barrier. They should also not be added too frequently in time. Conversely, the choice of the Gaussians width is much less straightforward. It should be small enough to provide a good resolution of the PMF.[168] A number of recent review articles provide a more thorough description of this method.[166,169,170]

Metadynamics has significantly evolved since its introduction, with several modifications aimed at improving its convergence behavior or efficiency. Researchers have also focused on better describing the investigated event and have occasionally extended the conventional metadynamics formulation to achieve this. The multiple walkers metadynamics is one notable variation, wherein multiple simulations share the same bias potential, which improves the parallel performances of sampling. However, the most important improvement is the

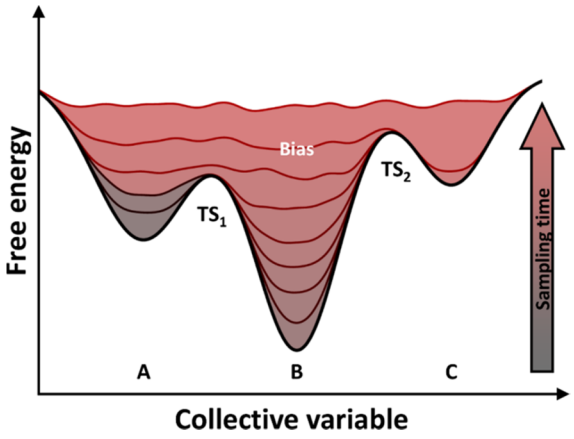

Figure 7. Pictorial representation of a metadynamics simulation. The underlying free energy, which is unknown beforehand, consists of three minima, A, B, and C, separated by two transition states. It is depicted as a black thick curve. The bias introduced by metadynamics is represented by filled curves with a dark-gray to red color gradient indicating the progression of the simulation. Starting the calculation in minimum A, the Gaussian bias released in time by metadynamics gently pushes the system toward the first transition state (TS_1) and leads to the identification of the global minimum B. Then, by continuation of the simulation, TS_2 and the metastable state C are also visited. Finally, when the underlying free energy is completely filled by the Gaussian potentials, the system evolves with diffusive motion along the collective variable, and the simulation can be stopped. At that time, with conventional metadynamics, the free energy can be recovered from the bias by simply changing the sign.

so-called "well-tempered metadynamics" (WTmetaD). In WTmetaD, the underlying bias and a given energy threshold govern the height of the added Gaussians, generating a more efficient and unbiased estimate of the free energy of the system.[171] In contrast to conventional metadynamics, the bias in WTmetaD does not fully compensate the underlying PMF, but it converges to a well-defined value. In a recent variation of metadynamics, researchers explored the possibility of using adaptive Gaussians with on-the-fly modified width and orientation with respect to the CV space. This variation showed better accuracy and convergence properties than previous formulations.[172] Eventually, combining WTmetaD and adaptive hills may relieve the user of the delicate choice of parameters, controlling the shape of the Gaussians.

Branduardi et al.[173] developed the path collective variables (PCVs) method to improve the definition of CVs. The PCVs method allows a nonlocal exploration of complex multidimensional processes, using a predefined pathway on a low-dimensional (2D) space. Notably, several metrics can be used to define the path, including the rmsd or the contact map distance (CMAP).[174] So far, PCVs have been successfully used to address several biological problems ranging from large protein conformational transitions to ion permeation. Their use is not strictly limited to metadynamics sampling.[175,176] More recently, to improve the unsupervised definition of CVs, significant effort has been devoted to developing "smarter" variables based on dimensional reduction techniques, such as nonlinear multidimensional scaling.[177] From a different perspective, when a suboptimal choice of CVs adversely affects the reconstructed free energy, this can be mitigated by coupling metadynamics to replica-exchange-like methods. This can effectively improve the sampling along transverse degrees of freedom.[178] In this context, another metadynamics formulation

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used the potential energy as a CV, thus enhancing the energy fluctuations required for an efficient replica exchange setup (well-tempered ensemble).[179] Bias-exchange metadynamics (BEmetaD)[180] is a popular extension of the conventional metadynamics method, which still relies on a replica-exchange-like scheme. In this case, a series of metadynamics simulations, biasing different CVs, are run in parallel and exchanged at fixed times according to a Metropolis-like criterion. This reduces the problem of choosing a priori a small number of CVs for the investigated event. Along the same lines, reconnaissance metadynamics is a sophisticated scheme for automatically detecting a set of CVs through machine-learning techniques. It has been introduced and successfully applied to biologically related problems.[181]

In the past few years, metadynamics, in its various implementations, has been applied to a number of ligand−target complexes, demonstrating its ability to characterize binding and unbinding paths, to treat conformation flexibility, and to compute free-energy profiles. Since Gervasio et al.[133] first applied metadynamics to ligand−target complexes, several other informative studies have been reported. Two representative examples are the recent investigation of the (un)binding of a nonsteroidal anti-inflammatory ligand 4-[5-(4-bromophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (SC-558) to COX-1 and COX-2 isoforms,[182] and the recognition of cortisone from the 11β-hydroxysteroid dehydrogenase enzyme obtained by combining steered MD and metadynamics.[183] Both studies used PCVs to describe the (un)binding process, with the former using the CMAP metric and the latter using the rmsd metric. In a paper investigating the recognition mechanisms of naloxone to the δ opioid receptor, Provasi et al.[184] provided a notably elegant way of recovering the standard binding free energy from (un)binding metadynamics simulations. This was analogous to the methods discussed above in the context of US. Another reported strategy more closely resembled Woo and Roux's method,[154] using a reverse funnel-shaped potential that limits the space available to the ligand once it has undocked ("funnel metadynamics").[185] Other studies have described drug binding to protein kinases, or the association mechanism of molecules binding to DNA and RNA, such as the interaction of ligands with DNA G-quadruplexes and the unbinding of the anticancer drug distamycin from the DNA minor groove.[170,186] "Coarse metadynamics" uses a combination of docking, cluster analysis, and metadynamics confined within the internal binding cavity. When applied to drug design and compound screening, it has shown the best performance in conventional structure-based endeavors.[187] Nevertheless, if one is interested in the relative free energy of a set of congeneric compounds with a similar binding pose, enhanced sampling methods based on unphysical pathways (i.e., methods such as FEP and TI, which compute the difference of free energy between the bound and the unbound state) are often more efficient and rigorous than methods that compute the free energy along a hypothetical association pathway. For this reason, metadynamics has so far mostly been used in retrospective studies of ligand binding. These informative applications of metadynamics-based simulations suggest that the time has now come to use metadynamics in prospective drug design efforts, with a particular focus on lead optimization. If these studies were successful, then metadynamics simulations would offer an intrinsic added value over FEP and TI. This is because the entire physical path of protein−ligand binding and/or unbinding could be accurately computed and revealed, together with the associated thermodynamic and kinetic profiles.

5.5. Other Approaches and Method Combinations. Several other MD-based enhanced sampling techniques exist and have recently been used to estimate the free energy of drug binding and to rationalize the experimental drug−target affinity. These include the adaptive biasing force (ABF)[153] and accelerated MD.[44] While these approaches can correctly reproduce experimental data and rationalize target affinity for different drugs/ligands, their predictive applicability for practical drug design is in its infancy. Their high computational cost is the most likely hindrance. Nonstandardized protocols and parameters, which can affect the final results, may also be a factor at times.

Of these many methods, the replica exchange class of methods is gaining popularity for drug design. This is mostly due to their flexibility and the embarrassingly parallel nature of computations, which makes them ideal for coupling to several enhanced sampling techniques. In addition, they almost linearly scale on multicore supercomputers, making them the methods of choice for CPU-intensive or GPU-intensive simulations. Replica exchange methods use several copies of the system evolving, in parallel, under different simulating conditions, such as the temperature (T-REMD).[40] Then, at regular intervals, swapping between a pair of replicas is attempted according to a Metropolis MC acceptance criterion. Thus, a random walk along the temperature space provides the required enhancement in sampling, while the coldest (or physical) replica ensures the correct distribution of states in the reference statistical ensemble. However, a large number of replicas are required for the method to be effective. This is because the probability of accepting MC moves depends exponentially on the difference in potential energy between replicas. Moreover, because the potential energy grows with the number of degrees of freedom, this issue is more pronounced in large systems. A valid alternative is the H-REMD variant (or REST),[93,95] in which, rather than increasing temperature, the potential energy function is gradually scaled down along the progression of the replicas. By doing so, it is possible to affect only selected (relevant) degrees of freedom (i.e., the binding site and the ligand), greatly improving the acceptance probability and, in turn, the efficiency compared to the conventional temperature version. Interestingly, H-REMD has been used to accelerate the sampling to obtain the correct protein−ligand binding modes for implicit or explicit solvent simulations.[188,189] This mimics the search algorithms of docking programs within an MD framework.

Actually, the combination of MD sampling with MC has a long tradition in theoretical physics, which goes back to the 80s when the hybrid Monte Carlo (HMC) method consisted of evolving the equations of motions, accepting or rejecting the advancement in positions according to the Metropolis criterion.[190] Later, this method was further developed, like in the case of adaptive temperature HMC by Fischer at al.,[191] where transitions to higher temperatures were used to overcome relevant energy barriers. Then, an appropriate reweighting scheme was employed to assess the correct populations of states at the target temperature. Interestingly, the same method was utilized as sampling engine for one of the earliest applications of Markov state model (MSM) to biomolecular relevant problems (see below), such as the conformational dynamics of *n*-pentane and a triribonucleotide fragment.[192] Concerning more drug-design-oriented methods,

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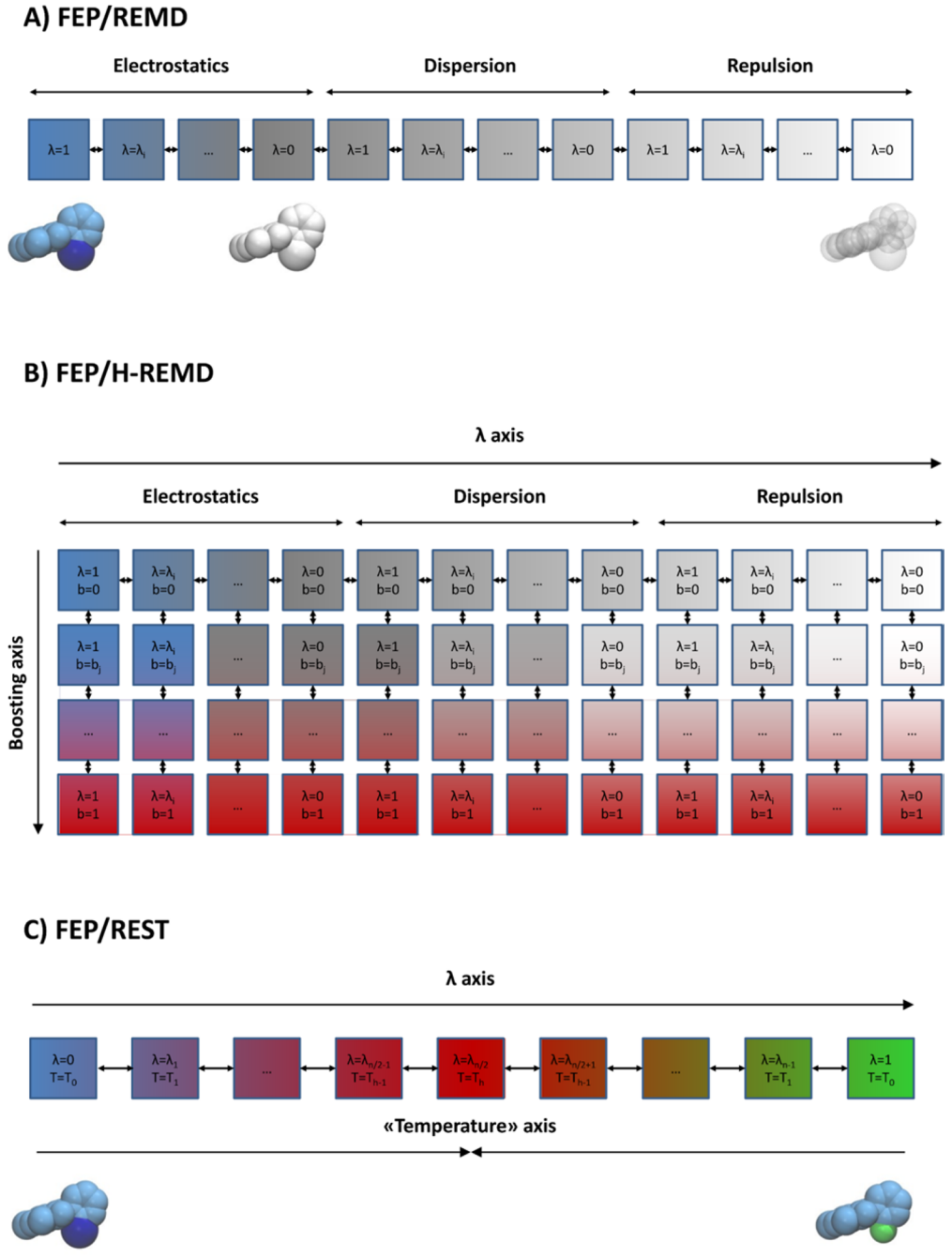

Figure 8. Comparison of three strategies to couple FEP with replica exchange. In panel A, the FEP/REMD method implements replica exchanges between the staged alchemical transformation in the context of the double decoupling method (DDM). Here, the unphysical transformation is performed in three steps: charges, dispersive, and repulsive van der Waals interactions are turned off separately and in sequence. For simplicity, the inclusion of rototranslational and conformational restraints, as envisioned in the original paper, is not considered. The axis controlling the alchemical transformation (λ-axis) is retained in the FEP/H-REMD method (B), but it is coupled to a boosting axis where the potential energy of selected dihedrals is scaled down, mimicking the effect of high temperature. In the context of relative binding free-energy calculations, a fictitious high temperature is also used in the FEP/REST method (C). In this case, the alchemical transformation is performed together with the potential energy rescaling. Thus, the "hottest" replicas are located in the middle of the ladder, and physical states are only found at the end points.

we also mention the mixed MC/SD scheme proposed by Guarnieri and Still.[193] Here, different from HMC implementations, SD sampling is periodically interrupted by ordinary MC moves performed in the dihedral space of molecules. As such, the MC/SD approach samples effectively the conformational space of relatively small druglike molecules in implicit solvent.

Recently, replica exchange methods have been efficaciously coupled to conventional FEP methods to improve the sampling. Jiang et al.[194] reported one way of coupling replica exchange to FEP, by including swaps along the staged alchemical transformation (FEP/REMD scheme, see Figure 8A). This improved the convergence properties of the free-energy calculations. In this context, the Roux group took full advantage of the H-REMD potentiality only later, when a second ladder of replicas controlling the torsional energy of selected rotamers on the binding site was added, thus leading to

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a two-dimensional space of exchanges (FEP/H-REMD scheme, Figure 8B).[195] However, the large number of replicas required by the FEP/H-REMD scheme probably counterbalances its effective benefits. Recently, researchers proposed a very efficient implementation of FEP augmented with replica exchange, named FEP/REST.[96] As shown in Figure 8C, FEP/REST uses a one-dimensional ladder of replicas, where both the alchemical transformation on the ligand and the potential energy scaling of the binding site (amino acids as well as ligand torsions) occur. Thus, intermediate points along the progression represent unphysical states of the ligand bound to "hot" conformations of the protein−ligand complex, while the end points correspond to physical states of ligand analogs, each bound to the corresponding "cold" protein conformation.

While FEP has been coupled to enhanced sampling techniques other than replica exchange,[196] the coupling of replica-exchange-like methods is not limited to alchemical transformations only. Indeed, it is possible to use a replica exchange framework to alleviate convergence issues related to CV-based enhanced sampling methods, such as US or metadynamics. For example, the Roux group adopted the previously reported FEP/H-REMD methodology for exchanges along US windows on a two-dimensional CV space (US/H-REMD).[197] Furthermore, in the context of metadynamics, Sutto and Gervasio[198] studied the conformational free energy of EGFR kinase, using WTmetaD coupled to T-REMD. In this case, the replica exchange strategy helps in relaxing all the transverse degrees of freedom that are not explicitly accounted for in the definition of CVs. Moreover, the energy overlap among replicas, required for an optimal usage of resources, was greatly improved using the bias obtained in preliminary metadynamics simulations where the potential energy was used as an additional CV (well-tempered ensemble sampling).

Finally, we mention two other methods that inherit the idea of alchemical transformations augmented with a replica exchange framework, even though they are rather different in formulation from all the techniques discussed above. The first is Gallicchio et al.'s BEDAM (binding energy distribution analysis method) approach,[199] which uses the AGBNP2 solvation model to perform replica exchanges along the staged alchemical transformation. By virtue of the implicit solvent model used, the alchemical transformation in BEDAM involves the effective ligand−environment interactions. Thus, the end points of the progression of replicas consist of physical states representing the ligand interacting with the solvent and the protein (uncoupled and coupled states, respectively). The absolute binding free energy is then recovered from the sampled energy distributions by exploiting statistical mechanics arguments. From this standpoint, BEDAM shares some resemblance with the MM-PB/GBSA method and with DDM. Procacci et al. have recently proposed another interesting approach (called EDU-HREM[200]) to compute the standard binding free energy. This approach can be thought of as an explicit-solvent variant of BEDAM. During EDU-HREM, an unphysical transformation is achieved with an H-REMD strategy whereby protein−ligand interactions as well as torsional and intramolecular nonbonded potentials are progressively *decreased* along replicas, while, at the same time, the protein−solvent and ligand−solvent interactions are gradually *increased*. This scheme undocks the ligand from the binding site without the need to specify any CV in advance, so the binding free energy can be recovered accordingly. Taken together, it is clear that the field of MD coupled to enhanced sampling schemes has evolved remarkably over the past 10−15 years. This is particularly true with reference to protein−ligand binding, opening up novel avenues and scenarios for computational medicinal chemists.

6. MD-DERIVED KINETICS OF DRUG BINDING AND UNBINDING

Kinetics represents the physicochemical description of association and dissociation rates of a drug binding to and unbinding from its target. While high affinity for a target is the basic requirement for any potential drug candidate, thermodynamics alone is not enough to comprehensively characterize drug−target binding. Association and dissociation rates depend on transient interactions between the ligand and the surroundings (i.e., protein and solvent), which cannot be captured by a state function such as the binding free energy. In addition, high-affinity ligands can sometimes show unexpectedly poor pharmacological efficacy in vivo, where the equilibrium conditions underlying binding potency are not necessarily met.[201] In this context, researchers recently found MD-based methods to be potentially useful in evaluating ligand-binding association (k_{on}) and dissociation (k_{off}) rates.[202] These constants (particularly the k_{off}) return a direct measure of how long a ligand is likely to remain bound to its receptor, thus generating the desired pharmacological effects. Ligands that remain bound to their receptor for a longer time are pharmacologically more appealing than those characterized by a short-lived complex. The use of MD to estimate k_{on} and k_{off} is at the forefront of computational drug discovery. Several different MD-based approaches have been developed to calculate these key observables.[202]

Considering the reaction scheme for the noncovalent association shown in eq 6, we see that the rate of forward reaction is of second order in reactant concentrations, whereas the reverse process (i.e., the dissociation of the protein−ligand complex) is of first order. When these rates are combined, we obtain the phenomenological rate equation for the protein−ligand complex:

$$\frac{d[PL]}{dt} = k_{on}[P][L] - k_{off}[PL] \tag{12}$$

Equation 12 represents the law of mass action; at the equilibrium (i.e., when $d[PL]/dt = 0$), the binding constant can be expressed as the ratio of k_{off} over k_{on}. Thus, the following expression connects the thermodynamic observable K_d with the kinetics observables k_{off} and k_{on} at equilibrium conditions:

$$K_d = \frac{k_{off}}{k_{on}} \tag{13}$$

From a microscopic point of view, the (un)binding process can be described as a double-welled one-dimensional PMF (see Figure 3). The barrier separating the two minima is assumed to be high enough that the transitions from one basin (i.e., the bound state) to the other (i.e., the unbound state) can be considered as rare events compared to the intrabasin dynamics. According to transition state theory, the rate constant for these processes is expressed as[203]

$$k = k_0 e^{-\Delta G^{\ddagger}/(k_B T)} \tag{14}$$

where $\Delta G^{\ddagger}$ is the activation free energy (or free-energy barrier), whereas k_0 is a proportionality constant (i.e., the pre-exponential factor, or Arrhenius constant), which takes into account the frequency of transition attempts as well as the

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probability of recrossing events from the transition state. Equation 14 shows the exponential relationship between kinetic constant and activation free energy. This makes any computational prediction of kinetics particularly challenging.

Shan et al.[31] and Buch et al.[30] conducted the first computational studies wherein the entire binding process was revealed and analyzed at the atomic level from MD simulations. Notably, Buch et al.[30] characterized the trypsin−benzamidine complex formation. They applied Markov state model (MSM)[204] analysis, a mathematical approach borrowed from protein-folding studies, to calculate both the thermodynamics and kinetics observables related to drug−target complex formation. The key idea is to discretize the configurational space of the system under investigation using some structural metric (usually the rmsd) and traditional clustering techniques. Then, the stochastic jumps between states are modeled by counting the number of transitions observed in the simulation trajectories during a certain lag time. This generates the transition probabilities matrix, which includes both the structural transitions (eigenvectors) of the investigated event and the corresponding time scale (eigenvalues). This indicates possible pathways between initial and final states and reactive fluxes between them. Thus, MSM can predict the equilibrium distribution of states and kinetic quantities for events occurring on time scales longer than those reached through the ensemble of the MD simulations that are actually performed. This returns an understandable picture of the investigated event through a simplified kinetic model, which does not rely on physical reaction coordinates. Several informative reviews have provided a more thorough and exhaustive introduction to MSM theory.[204,205]

Recently, researchers using MSM to resolve ligand-binding kinetics have begun to include the role played by protein conformational transitions in the entire ligand recognition process. By studying the binding of choline to ChoX and using a flux analysis, Gu et al. quantified that a contribution of about 90% of conformational selection over induced fit is expected to operate under experimental conditions.[206] Platter and Noé investigated the trypsin−benzamidine system, identifying a combination of conformational selection and induced-fit mechanisms in the binding kinetics.[207] Notably, an advantage of MSM over standard analysis of MD trajectories is the possibility of identifying undersampled states along the (un)binding process and thus of lowering the statistical uncertainty on the calculated observables by rerunning simulations starting from the more relevant configurations (seeding).[204] This strategy, which is opposed to the brute force extension of previous simulations, is usually referred to as adaptive sampling. To this regard, the on-the-fly learning method devised by Doerr and De Fabritiis seems to be particularly appealing not only because the adaptive sampling is totally unsupervised but also because the iterative seeding allows one to converge thermodynamic quantities at about 1 order of magnitude faster than conventional approaches.[208] There is a growing number of software packages aimed at constructing and visualizing MSM-derived kinetic models, such as MSMBuilder and pyEMMA. These will most likely increase the popularity of this technology in more application-oriented investigations too.

Still relying on extensive and unbiased MD simulations, Decherchi et al.[32] provided a novel approach to calculating k_{on}. Using available experimental thermodynamic (K_i) and kinetic (k_{off}) data, they estimated the time needed for the very first molecule of a ligand to move from the bulk of the solvent into the target binding pocket (the "experimental time for first binding"). Under their simulation conditions, the experimental time for first binding was estimated to be about 250 ns. Then, using all the trajectories that led to a binding event (11 out of 13 MD simulations of about 1 μs each), they obtained the "calculated time for first binding". This was about 220 ns, in very good agreement with the experimental estimate. For prospective studies, this may offer a further way of estimating k_{on} and comparing computational predictions with experimental data.

However, from a drug discovery standpoint, it is more relevant to estimate and optimize the unbinding kinetics, k_{off}. This is because the dissociation of protein−ligand complexes, which follows an exponential decay with the characteristic time $\tau_R = 1/k_{off}$, is quite a reliable indicator of in vivo drug efficacy.[201,209] This is the average time required for the complex to dissociate and is usually referred to as "residence time". The long time scales involved in the dissociation of protein−ligand complexes can last from milliseconds to seconds (or even more), which makes it remarkably difficult to simulate dissociation events through brute force MD. In this respect, MSM applied to long and unbiased MD trajectories has been reported to provide estimations of unbinding kinetics rates.[205] However, in several notable examples reported, the error on the k_{off} estimation was quite high. This is most likely because unbiased MD simulations can perform very limited sampling in the region around the unbinding transition states.[30,31] As mentioned above, the exponential relationship between k_{off} and activation free energy amplifies the error in the k_{off} prediction. This is mainly because sampling around transition states remains poor and the unbinding activation free energies are rather approximate.

Mollica et al. recently suggested a practical solution for screening the residence time of a congeneric series of compounds.[210] The authors used scaled MD, an enhanced sampling method with which the potential energy of the whole system is reduced by an arbitrary λ factor. In doing so, they observed several unbinding events and acquired enough statistics to correctly rank the dissociation constants in pharmacologically relevant case studies. Notably, this methodology is CV-free, requiring no preidentification or predefinition of a reaction coordinate or of collective variables. Therefore, the protocol is fully unsupervised, as no (or just a little) a priori information about the unbinding path must be known. In addition, the good correlation between computed residence times and experimental k_{off} values favors this approach for kinetics prediction. But prospective application studies are needed to definitively assess its robustness and actual applicability for drug discovery. Indeed, unbinding kinetics is emerging as a crucial parameter for fine-tuning during lead optimization for drug discovery so that compounds can be prioritized during chemical synthesis campaigns. Conversely, even fast approaches applied to binding kinetics estimation can be much slower than conventional virtual screening methodologies, which limits their applicability to hit identification for drug discovery.

Researchers have proposed and successfully applied other approaches to evaluating ligand-binding kinetics within the framework of CV-based enhanced sampling methods. Once the free-energy barriers are determined, the kinetic constant can in principle be estimated through eq 14 only after assessing the pre-exponential factor. For example, in their seminal work, Bui

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et al.[211] estimated rate constants for tetramethylammonium (un)binding to AChE using US to reconstruct the PMF. They then derived the pre-exponential factor through transition state theory arguments. Despite this important result, to the best of our knowledge, this and related approaches are mostly effective for simpler computational problems. This is mostly due to difficulties with estimating the pre-exponential factor with reasonable accuracy for complex processes such as protein−ligand associations/dissociations. Marinelli et al.[212] conceived an elegant procedure to address this. They first used BEmetaD[180] to reconstruct a multidimensional free-energy landscape for the investigated process. They then used a discrete-state kinetic-MC simulation to build a consistent kinetic model.[212] They later used this approach to successfully compute the binding free energy and corresponding association/dissociation rates for a peptide substrate to the HIV-1 protease.[213] Conversely, Tiwary and Parrinello[214] devised a way to recover unbiased rates from metadynamics-biased simulations, bypassing the problems related to the pre-exponential factor.[215] Their approach assumed that as long as the Gaussian deposition frequency is sufficiently small, the well-to-well dynamics is not affected by the bias potential. Unbiased rate constants could therefore be estimated after assessing the acceleration factor introduced by metadynamics. Notably, the authors showed a faster convergence of rates rather than the full free-energy reconstruction, which makes this approach particularly appealing for drug discovery.

7. OTHER CHALLENGES FOR MD IN SBDD

Above, we discussed how MD-based methods can be used to investigate and understand binding affinity and kinetics for rational drug design. Now, we briefly touch upon the use of MD to tackle two additional topics: allosteric mechanisms and modulation, and the role of water for ligand binding and optimization. Both topics have been actively investigated using MD-based approaches over the past decade, as they have become major research areas for computationally driven drug discovery. This has led to several methodological advances, increasing our comprehension of these complex biological phenomena, which could play a role in drug discovery.

7.1. MD and Allosteric Modulation in Drug Design. Allostery occurs when distant binding pockets of biological macromolecules, mostly proteins, communicate and so modulate their activity. Interfering with this allosteric process can thus regulate target function acting far from the catalytic (orthosteric) site of proteins. This offers a new strategy for tuning target activity through allosteric ligands (Figure 9).

Since Monod and Jacob first introduced the term "allostery" in 1961, two major conceptual models have emerged for describing this communication between distinct binding sites: the Monod, Wyman, and Changeux model (MWC) and the Koshland, Nemethy, and Filmer model (KNF).[216] These phenomenological descriptions mirror the more familiar concepts of population shift (or conformational selection) and induced fit, respectively. Allostery is usually associated with long-range propagation of large conformational movements (domain motions, hinge-bending movements, etc.). Notably, however, it can sometimes be related to alterations in dynamics between distinct binding sites with no major conformational changes. Indeed, while structural changes are largely driven by enthalpy, alterations in dynamics due to an allosteric binding (such as changes in frequencies and amplitudes of thermal fluctuations) are primarily entropic in nature.[217]

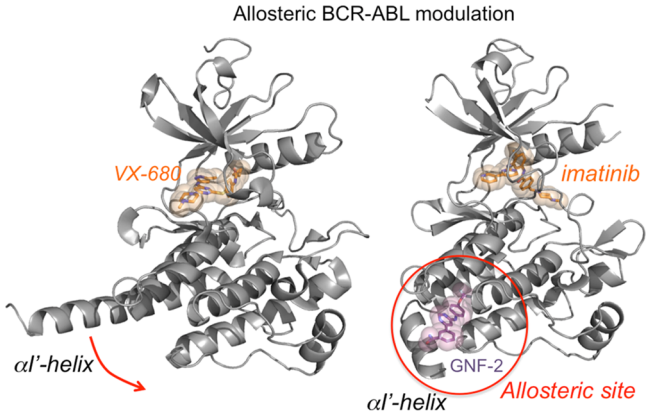

Figure 9. Allosteric modulation in BCR-ABL (PDB codes 2f4j and 3k5v). The ligand GNF-2 binds to the myristate pocket (red circle, allosteric site) concomitantly to the bending of the αI′-helix and induces a c-ABL-like autoinhibited conformation of BCR-ABL that has reduced kinase activity.

For drug design, targeting allosteric binding sites offers several potential advantages over traditional orthosteric sites. These can be summarized as (i) improved selectivity, (ii) improved druggability, (iii) activity rescuing, and (iv) activity potentiation.[218] The potential for improved selectivity is because orthosteric inhibition is often associated with poor specificity toward a given target, such as protein kinases or GPCRs. This is mainly due to different targets within the same family sharing similarities in their orthosteric sites. Allosteric ligands thus offer a viable strategy for targeting binding sites that are topologically diverse and often less conserved, which could in principle allow better target selectivity. In addition, allosteric modulation expands the druggability of a given target to include different pockets of the same target protein. Furthermore, it offers the possibility of rescuing the activity of dysregulated proteins caused by disease-related mutations, which often lead to drug-resistance issues. Mutation of key amino acids can prevent the binding and efficacy of drugs targeting the orthosteric site. In this case, the allosteric effector can restore the protein function by canceling or surmounting the conformational change associated with the dysregulated activity of mutated/resistant forms of the targeted proteins. Finally, allosteric ligands could potentiate the target activity, with examples including benzodiazepines for GABAA[219] and galantamine for nAChRs.[220]

The potential advantages are, however, counterbalanced by some serious challenges in effectively discovering and optimizing small molecule allosteric ligands. These are mainly related to the structural and dynamic nature of allosteric pockets, which are usually rather shallow, superficial, and highly flexible or even transient. These difficult features are then reflected in allosteric ligands with poorer affinity (in the low μM range) for the target than orthosteric inhibitors, which usually reach low nM potency. It can also be particularly challenging to experimentally verify allosteric modulation by new compounds. Sophisticated functional assays must be put in place to verify and confirm the allosteric mode of action of new ligands. Finally, poor target affinity makes it more difficult to structurally resolve allosteric ligand/target complexes by X-ray crystallography.

In this context, MD-based approaches could be used to detect and characterize allosteric binding sites. The current time scales of MD simulations allow the formation of transient

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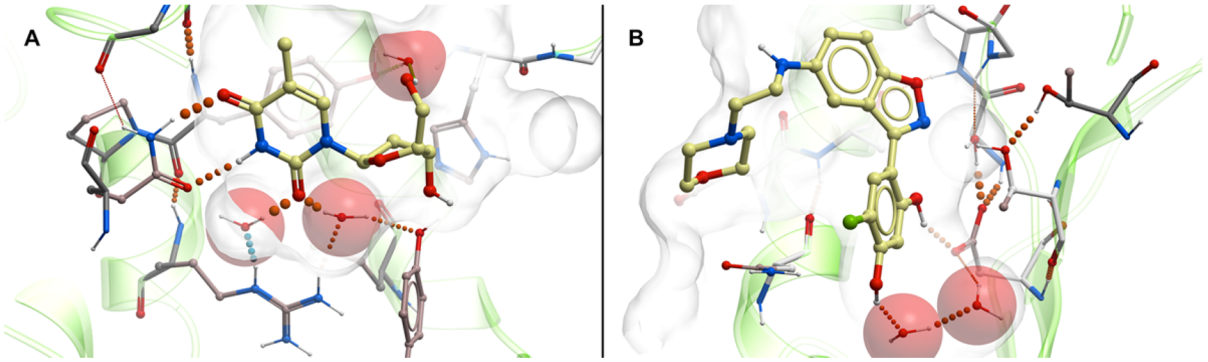

Figure 10. Water-mediated protein−ligand interactions from crystallographic structures. (A) Bound pose of deoxythymidine at the binding site of thymidine kinase (PDB code 1KIM) and (B) bound pose of a benzisoxazole-based derivative at the binding site of HSP90 (PDB code 3BMY). Ligands, relevant protein residues, and bridging water molecules are reported in ball and stick representation. Water molecules are highlighted by red spheres. The boundaries of the binding pocket are represented by a transparent white mesh. Dotted red lines represent the most relevant hydrogen bonds.

pockets to be observed.[221,222] Enhanced sampling techniques for MD-based conformational sampling further increase the ability of MD simulations to detect allosteric pockets, as recently reported for accelerated MD.[223] Through MD simulation runs, researchers can also identify communication pathways between putative allosteric sites and the active site of the targeted protein. This challenging task has been addressed by new methods, which borrow from the concepts on which the MWC allosteric model is based. That is, most MD data analysis methods rely on identifying correlated motions through which the structural and/or dynamic information can be transmitted between distal pockets. Well-established methods are based on calculating the covariance matrix of atomic fluctuations:[224]

$$c_{ij} = \langle (\mathbf{r}_i - \langle \mathbf{r}_i \rangle)(\mathbf{r}_j - \langle \mathbf{r}_j \rangle) \rangle \quad (15)$$

where $\mathbf{r}_i$ and $\mathbf{r}_j$ are the instantaneous position vectors of atoms i and j, respectively, in the reference frame of the protein, and the brackets stand for ensemble average. The diagonal elements of the matrix correspond to mean-squared fluctuation of atoms and are related to their *B*-factors. Upon diagonalization of the covariance matrix (or principal component analysis, PCA), one can obtain a set of orthogonal modes of motions that maximize the fluctuation amplitudes along each mode and that represent a quasi-harmonic approximation of the free-energy surface of the protein.[224] The "essential subspace" of protein dynamics can also be used to visualize correlated motions with common graphical software. Moreover, achieving the essential subspace corresponds to extracting relevant CVs, along which the conformational free energy of the protein can be projected and, if needed, resampled. Similarly, the cross-correlation matrix can be calculated by normalizing the elements of the covariance matrix:

$$C_{ij} = \frac{c_{ij}}{c_{ii}^{1/2} \cdot c_{jj}^{1/2}} \quad (16)$$

The values of the elements of the cross-correlation matrix range from −1 for fully anticorrelated motions to +1 for fully correlated motions. They correspond to the Pearson correlation coefficient. In contrast to the elements of covariance matrix, however, the magnitude of the correlated motion is lost.

Both analyses, however, suffer from two major drawbacks. The first limitation is because atomic correlation is only detected for parallel motions. Due to the form of eq 15, fully correlated motions of two atoms oscillating along perpendicular directions will return a null correlation. Moreover, the Pearson coefficient only treats linear correlations, excluding nonlinear or higher-order correlations. To overcome this, Lange and Grubmüller devised a generalized metric, called mutual information (MI),[225] which quantifies the correlation between random variables as the deviation of their joint probability distributions from the hypothetical distributions of independent random variables. Thus, MI returns a null value only in the case of fully uncorrelated motions but properly detects any kind of correlation. In analogy to PCA, the same authors developed a consistent way to extract collective degrees of freedom based on the MI metric (full correlation analysis, FCA).[226]

The second limitation that affects metrics based on atomic fluctuations (including the previously reported implementation of MI) is that they are based on Cartesian coordinates. Hence, they are appropriate for detecting major conformational changes that are typical of classical allosteric sites. Conversely, the MutInf approach developed by McClendon et al.[227] is related to MI, but the analysis is performed on the internal degrees of freedom (backbone and side chains). Moreover, relying on an entropy-based metric (as well as MI), this approach seems to be particularly suited to detecting minor or subtle alterations in dynamics, such as those involved in entropic-driven allosteric modulations. A robust statistical analysis based on Bayesian filtering and sampling penalties further strengthens an unambiguous detection of relevant correlated motions. Notably, these correlation methods are often complemented by graph-theory and community network analysis in order to characterize communication channels between distal binding sites. For example, Sethi et al.[228] identified signaling pathways in different tRNA−protein complexes by building a dynamical network where the nodes represent amino acids or nucleotides, whereas the weight of the edges (w_{ij}) is proportional to the information transfer between the two nodes i and j quantified in terms of the Pearson coefficient ($w_{ij} = -\log(|C_{ij}|)$). Community network analysis[229] is thus used to identify loosely interconnected but locally densely intraconnected substructures. The allosteric signal is then detected as a function of the number of shortest paths between critical nodes belonging to different communities. Similarly, Rivalta et al.[230] identified the allosteric pathways in imidazole glycerol phosphate synthase, using the same theoretical framework, although operating with MI as a

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measure of the information transfer between nodes. There are also complementary approaches that focus on energy couplings rather than on correlated motions alone. One group recently proposed an elegant combined approach based on structural fluctuation analysis and pairwise energy decomposition to characterize the allosteric mechanism for the homologous PDZ2 and PDZ3 proteins.[231]

Nowadays, extended MD simulations can be combined with a growing number of methods for analyzing correlated motions. This has generated several informative MD-based studies of allosteric modulation in different target families. These include Foda et al.'s study on allosteric network and binding cooperativity in Src protein kinases[128] and Morra et al.'s integrated MD/dynamic pharmacophore approach for identifying and targeting allosteric hot-spots on the N-terminal domain of the molecular chaperone Hsp90.[232] Here too, the field is in its infancy. Future MD applications and analysis methods are needed to definitively assess the reliability of these protocols for prospective drug discovery, in particular for contributing to the search for novel allosteric modulators as drug candidates in different therapeutic areas.

7.2. MD To Investigate the Role of Waters in Drug Binding. Water molecules can influence the binding affinity of a ligand to its targeted biomolecule in different ways (Figure 10). First, interfacial waters can mediate the initial approach of the ligand to the pocket, with an active role in determining ligand−protein binding or rejection. Desolvation of the binding pocket is then necessary for drug binding. During this process, a network of waters could affect ligand binding, raising complex considerations of whether or not the displacement of ordered waters can produce an entropy gain, ultimately aiding binding affinity.[233,234] For example, it can be very challenging to determine from structural data alone whether a key bridging water, which mediates the ligand−target interaction in the crystal structure of the complex, should be maintained or displaced during ligand optimization.[235,236] The entropic reward derived from releasing an ordered water molecule into the bulk should be coupled to an enthalpic gain generated by forming tight protein−ligand interactions. Clearly, this area presents major scientific challenges related to better understanding of the fundamental principles that govern drug binding. Thus, rational drug design could be greatly impacted by methods and approaches to understand and quantify the crucial entropic/enthalpic balance due to water displacement or solvent reorganization upon ligand binding.

Over the past decade, MD simulations have been extensively used to characterize waters located in the binding sites, with a focus on those buried waters with long residence times in protein structures. These often raise questions as to whether or not water-mediated interactions should be retained to improve lead compound potency. Major progress has been made in locating water molecules in the targeted binding pocket and identifying and classifying specific water molecules that should be displaced and retained to improve binding affinity. Thus, there are now several computational methods for better quantifying the enthalpy−entropy compensation in protein−ligand binding.[237]

Nowadays, long MD trajectories can serve to sample the solvation network of binding sites, revealing hydration patterns within the binding pocket that can complement or support structural data. For example, MD-based approaches have been used to characterize the role of the interfacial waters during ligand approach and binding.[238−240] From MD simulations, one can identify and analyze water sites by the peaks in water density or averaging over water molecule locations during the collected trajectories. Also, from short MD runs, Lazaradis et al.'s inhomogeneous fluid solvation theory (IFST)[241] conveniently allows quantification of the entropic and enthalpic contributions and overall thermodynamics of water molecules in the binding site. More recently, researchers have developed methods like WaterMap, which uses IFST, to map the locations and thermodynamic properties of water molecules that solvate protein binding sites, indicating which should be removed or retained to improve ligand binding affinity.[242] Similarly, researchers have reported, several other methods, such as waterFLAP, for characterizing waters in the binding sites. These work by coupling (short) MD trajectories to other methods/approaches such as docking and scoring, or MM-PB/GBSA calculations.[243] This field is vast and increasing, with the potential to more directly link the in-depth description of thermodynamics of binding site waters with the rational design of new, more efficient ligands.

8. SUMMARY AND PERSPECTIVES

As extensive molecular dynamics (MD) becomes ever more affordable, it promises to impact fast-paced drug discovery programs. Mainly because of the advent of GPUs and software codes that can fully exploit these innovative hardware architectures, it is nowadays possible to run MD simulations in the time frame of microseconds up to a few milliseconds. This allows a thorough sampling of the conformational space, including that of large biomolecules. This can include, for example, the complete description of the pathway of the ligand binding to its target protein. These long MD trajectories can then be coupled to free-energy methods to provide the free-energy profile of protein−ligand binding, with the thermodynamic and kinetic data being crucial for drug discovery.

Although brute force MD-based approaches can be quite powerful, they are computationally very demanding. This limits their practical use for drug discovery to just a small number of ligands. In the past few decades, researchers have reported several different strategies for overcoming this by enhancing the sampling of relevant regions of the free-energy surface. Of these, free-energy perturbation (FEP) has best demonstrated its great potential to impact drug discovery. FEP is ready for prime time. We expect that an increasing number of MD-based FEP prospective studies will be published, ultimately proving FEP to be an efficient tool for optimizing a variety of new leads. The very successful MC-based FEP studies reported recently are one such example.[143] However, FEP does not describe the route of binding and unbinding, which instead can be depicted by other pathway-dependent enhanced sampling methods. Here, for example, we have extensively covered metadynamics and steered MD, which are among the first methods used to dissect routes for protein−ligand binding and unbinding. Nevertheless, metadynamics depends on the correct definition of the collective variables used to describe the chemical process under investigation. The CVs must be properly identified in order for metadynamics to accurately predict the free-energy landscape related to the ligand-binding process and so aid lead optimization campaigns. This is particularly true given its recent evolution (e.g., WTmetaD). Alternatively, more accurate and expensive protocols of metadynamics could be used to thoroughly investigate binding routes, providing thermodynamic and kinetic profiles associated with drug binding and unbinding. In analogy, when using a single distance-dependent

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reaction coordinate, steered MD can evaluate binding affinity quickly but only in a qualitative manner. For this reason, we can envision a scenario where steered MD could be used as a postprocessing tool for virtual ligand screening in order to improve the enrichment factor of active hits from among the best-ranked compounds.

Binding kinetics, and in particular residence time (i.e., the inverse of k_{off}), is nowadays considered to be one of the key parameters in lead optimization for potency and efficacy in vivo. In this context, other methods (including scaled MD and similar smoothed-potential approaches) are emerging as suitable for unbinding-kinetics investigations and k_{off} predictions. Prospective applications will likely appear in the literature in the near future, further testing the actual applicability of this quite novel approach to kinetics predictions.

From the above, it is clear that MD-based methods can nowadays help in several key drug discovery steps. The aforementioned methods are just a few of the many MD-based approaches to studying ligand binding. Each deserves attention. Rational drug design will be majorly impacted by the inclusion of full flexibility and entropic effects in studying protein−ligand recognition processes, allosteric modulation, and the thermodynamics and kinetics of binding-site waters. This will ultimately increase understanding of ligand binding, returning a more accurate and quantitative description of this crucial event for drug discovery and development.

Although the recent results from MD-based drug discovery studies are very encouraging, we should nevertheless remember that major challenges must be overcome to deepen the impact of MD-based methods on drug design. Improvements in the current force field are expected (and most probably needed) to further progress the accuracy of free-energy predictions. The currently available molecular mechanics force fields partially or fully neglect polarization effects, charge transfer, and many electronic-based interactions ($\pi-\pi$, cation$-\pi$, halogen bonds, etc.). In this respect, polarizable force fields or quantum mechanical calculations might be used in future to help refine free-energy estimations for increasingly accurate predictions. The limits of force field and MD-based methods should be pushed to correctly treat other challenging target families (such as metalloproteins), which include many drug discovery targets that can still only be studied with limited accuracy.

One additional key challenge in drug discovery comes from the fact that potency, although essential, is only the very first step toward the discovery of a promising drug candidate. Once a potent inhibitor is discovered, this must be tuned into a druglike compound with a favorable ADMET profile, during the lead optimization phase. Most of the time, this is the very key step, and the real bottleneck, in drug discovery. The ability of predicting thermodynamics and kinetics of binding through MD-based methods has the potential to impact lead optimization as well, indicating which compounds and modifications are the most favorable ones. If this will be demonstrated by prospective studies, MD-based methods will take more standard, and static, SBDD approaches to a new level. Toward this goal, we expect and call for research efforts proving that thermodynamics and kinetics of binding, retrieved from MD-based methods, can pragmatically impact the overall complex lead optimization phase of a drug discovery project.

We conclude that the time has come for an operational use of MD and related methods for fast-paced drug discovery, which would offer savings in time and money. Novel methods, novel software, and novel hardware have boosted the widespread diffusion of MD-based methods within the pharmaceutical community. Indeed, MD is on the verge of joining the already large arsenal of computational tools routinely applied in biopharmaceutical drug discovery. Despite the limitations and major challenges in using MD simulations for drug discovery, we therefore conclude by calling for more MD-based prospective studies. Prospective studies will serve as the ultimate proof that MD can indeed be used to assist the costly and highly challenging drug discovery process.

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Notes

The authors declare the following competing financial interest(s): Andrea Cavalli and Giovanni Bottegoni are cofounders of BiKi Technologies, a startup company that develops tools for drug discovery based on molecular dynamics. Giovanni Bottegoni is CEO of the same company.

Biographies

Marco De Vivo obtained an M.Sc. in Chemistry and a Ph.D. in Pharmaceutical Chemistry in 2004 from the University of Bologna. Then, for 3 years, he was a postdoctoral researcher in the group of Prof. M. L. Klein at the University of Pennsylvania, before joining the structure-based drug design group at Rib-X Pharmaceuticals. In 2009, he joined the Istituto Italiano di Tecnologia (IIT), where he leads the Laboratory of Molecular Modeling and Drug Discovery. He is also a Research Associate at the IAS-5/INM-9 Computational Biomedicine Institute, Forschungszentrum Jülich. His research interests include the computational investigation of pharmaceutically relevant enzymes. In his group, computational insights are fully integrated with experiments to discover promising new compounds potentially endowed with the desired beneficial effect.

Matteo Masetti received his Ph.D. in Medicinal Chemistry in 2007 from the University of Bologna under the supervision of Maurizio Recanatini. He is now a postdoctoral researcher at the Department of Pharmacy and Biotechology at the same university. His research interests include the study of pharmacotoxicologically relevant membrane proteins and the computational prediction of free energies related to biomolecular interactions.

Giovanni Bottegoni obtained his Doctorate in Medicinal Chemistry from the University of Bologna in 2006. After a postdoctoral experience at the Scripps Research Institute in La Jolla, CA, U.S., he joined the Istituto Italiano di Tecnologia (IIT), where he was team leader at CompuNet. In 2015, he completed a Master program in International Health Care Management, Economics and Policy (MIHMEP) offered by SDA Bocconi (Milan, IT), graduating cum laude. Currently, he is cofounder and CEO of a start-up company, BiKi Technologies. His main research interests are in in silico polypharmacology and structure-based drug design. In 2015, he was awarded the DCF prize for medicinal chemistry.

Andrea Cavalli is a Professor in Medicinal Chemistry at the University of Bologna and Head of CompuNet at the Istituto Italiano di Tecnologia. His research interests are in the field of computational drug discovery. In particular, he has developed and applied computational approaches to accelerate the discovery of druglike compounds in several therapeutic areas, including cancer, neurodegenerative diseases, and neglected tropical diseases. Prof. Cavalli is

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the author of more than 170 publications in high-ranked journals, co-inventor of several PCT international patents and has delivered numerous invited lectures and seminars. In 2003, he was awarded the "Farmindustria Prize for Pharmaceutical Research". He is co-founder of the high-tech startup company, BiKi Technologies.

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■ ABBREVIATIONS USED

SBDD, structure-based drug design; GPU, graphical processor unit; MM, molecular mechanics; FF, force field; QM, quantum mechanics; SD, stochastic dynamics; PBC, periodic boundary conditions; PME, particle-mesh Ewald; FEP, free-energy perturbation; TI, thermodynamic integration; DDM, double decoupling method; DAM, double annihilation method; CV, collective variable; PMF, potential of mean force; US, umbrella sampling; T-REMD, temperature replica exchange MD; H-REMD, Hamiltonian replica exchange MD; MRC, multiple receptor conformations; PARP-1, poly (ADP-ribose) polymerase 1; rmsd, root-mean-square deviation; MM-PB, molecular mechanics Poisson−Boltzmann; GBSA, generalized Born surface area; MC, Monte Carlo; NNRTI, non-nucleoside inhibitor of HIV-1 reverse transcriptase; MIF, migration inhibitory factor MIF; REST, replica exchange with solute tempering; WHAM, weighted histogram analysis method; ABF, adaptive biasing force; AFM, atomic force microscope; FabZ, β-hydroxyacylacyl carrier protein dehydratase; WTmetaD, well-tempered metadynamics; PCV, path collective variable; CMAP, contact map distance; BEmetaD, bias-exchange metadynamics; HMC, hybrid Monte Carlo; MSM, Markov state model; BEDAM, binding energy distribution analysis method; PCA, principal component analysis; MWC, Monod, Wyman, and Changeux; KNF, Koshland, Nemethy, and Filmer; MI, mutual information; FCA, full correlation analysis; PDZ, PSD-95/SAP-90, disc large, zonulla occludens-1; IFST, inhomogeneous fluid solvation theory

■ REFERENCES

(1) Jorgensen, W. L. The many roles of computation in drug discovery. *Science* **2004**, *303*, 1813−1818.
(2) De Vivo, M. Bridging quantum mechanics and structure-based drug design. *Front. Biosci., Landmark Ed.* **2011**, *16*, 1619−1633.
(3) Durrant, J.; McCammon, J. A. Molecular dynamics simulations and drug discovery. *BMC Biol.* **2011**, *9*, 71.
(4) Harvey, M. J.; De Fabritiis, G. High-throughput molecular dynamics: the powerful new tool for drug discovery. *Drug Discovery Today* **2012**, *17*, 1059−1062.
(5) Boehr, D. D.; Nussinov, R.; Wright, P. E. The role of dynamic conformational ensembles in biomolecular recognition. *Nat. Chem. Biol.* **2009**, *5*, 789−796.
(6) Changeux, J.-P.; Edelstein, S. Conformational selection or induced fit? 50 years of debate resolved. *F1000 Biol. Rep.* **2011**, *3*, 19.
(7) Vogt, A. D.; Di Cera, E. Conformational selection or induced fit? A critical appraisal of the kinetic mechanism. *Biochemistry* **2012**, *51*, 5894−5902.
(8) Fischer, M.; Coleman, R. G.; Fraser, J. S.; Shoichet, B. K. Incorporation of protein flexibility and conformational energy penalties in docking screens to improve ligand discovery. *Nat. Chem.* **2014**, *6*, 575−583.
(9) Abagyan, R.; Totrov, M. High-throughput docking for lead generation. *Curr. Opin. Chem. Biol.* **2001**, *5*, 375−382.
(10) Borhani, D. W.; Shaw, D. E. The future of molecular dynamics simulations in drug discovery. *J. Comput.-Aided Mol. Des.* **2012**, *26*, 15−26.
(11) Frenkel, D.; Smit, B. *Understanding Molecular Simulation*; Academic Press, Inc.: San Diego, CA, 2001; p 638.
(12) Allen, M. P.; Tildesley, D. J. *Computer Simulation of Liquids*; Oxford University Press: Oxford, U.K., 1989.
(13) McCammon, J. A.; Gelin, B. R.; Karplus, M. Dynamics of folded proteins. *Nature* **1977**, *267*, 585−590.
(14) Levitt, M.; Warshel, A. Computer simulation of protein folding. *Nature* **1975**, *253*, 694−698.
(15) Carlson, H. A. Protein flexibility and drug design: how to hit a moving target. *Curr. Opin. Chem. Biol.* **2002**, *6*, 447−452.
(16) Cozzini, P.; Kellogg, G. E.; Spyrakis, F.; Abraham, D. J.; Costantino, G.; Emerson, A.; Fanelli, F.; Gohlke, H.; Kuhn, L. A.; Morris, G. M.; Orozco, M.; Pertinhez, T. A.; Rizzi, M.; Sotriffer, C. Target Flexibility: An emerging consideration in drug discovery and design. *J. Med. Chem.* **2008**, *51*, 6237−6255.
(17) Totrov, M.; Abagyan, R. Flexible ligand docking to multiple receptor conformations: a practical alternative. *Curr. Opin. Struct. Biol.* **2008**, *18*, 178−184.
(18) Bottegoni, G.; Kufareva, I.; Totrov, M.; Abagyan, R. Four-dimensional docking: a fast and accurate account of discrete receptor flexibility in ligand docking. *J. Med. Chem.* **2009**, *52*, 397−406.
(19) Bottegoni, G. Protein-ligand docking. *Front. Biosci., Landmark Ed.* **2011**, *16*, 2289−306.
(20) Rauh, D.; Klebe, G.; Stubbs, M. T. Understanding protein-ligand interactions: the price of protein flexibility. *J. Mol. Biol.* **2004**, *335*, 1325−41.
(21) Damm, K. L.; Carlson, H. A. Exploring experimental sources of multiple protein conformations in structure-based drug design. *J. Am. Chem. Soc.* **2007**, *129*, 8225−8235.
(22) Alonso, H.; Bliznyuk, A. A.; Gready, J. E. Combining docking and molecular dynamic simulations in drug design. *Med. Res. Rev.* **2006**, *26*, 531−568.
(23) Morra, G.; Genoni, A.; Neves, M.; Merz, K., Jr.; Colombo, G. Molecular recognition and drug-lead identification: what can molecular simulations tell us? *Curr. Med. Chem.* **2010**, *17*, 25−41.
(24) Pang, Y.-P.; Kozikowski, A. Prediction of the binding sites of huperzine A in acetylcholinesterase by docking studies. *J. Comput.-Aided Mol. Des.* **1994**, *8*, 669−681.
(25) Ivetac, A.; McCammon, J. A. Molecular recognition in the case of flexible targets. *Curr. Pharm. Des.* **2011**, *17*, 1663−1671.
(26) Lin, J.-H.; Perryman, A. L.; Schames, J. R.; McCammon, J. A. The relaxed complex method: accommodating receptor flexibility for drug design with an improved scoring scheme. *Biopolymers* **2003**, *68*, 47−62.
(27) Tian, S.; Sun, H.; Pan, P.; Li, D.; Zhen, X.; Li, Y.; Hou, T. Assessing an ensemble docking-based virtual screening strategy for kinase targets by considering protein flexibility. *J. Chem. Inf. Model.* **2014**, *54*, 2664−2679.
(28) Tarcsay, A.; Paragi, G.; Vass, M.; Jojart, B.; Bogar, F.; Keseru, G. M. The impact of molecular dynamics sampling on the performance of virtual screening against GPCRs. *J. Chem. Inf. Model.* **2013**, *53*, 2990−2999.
(29) Klepeis, J. L.; Lindorff-Larsen, K.; Dror, R. O.; Shaw, D. E. Long-timescale molecular dynamics simulations of protein structure and function. *Curr. Opin. Struct. Biol.* **2009**, *19*, 120−127.
(30) Buch, I.; Giorgino, T. Complete reconstruction of an enzyme-inhibitor binding process by molecular dynamics simulations. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (25), 10184−10189.
(31) Shan, Y.; Kim, E. T.; Eastwood, M. P.; Dror, R. O.; Seeliger, M. A.; Shaw, D. E. How does a drug molecule find its target binding site? *J. Am. Chem. Soc.* **2011**, *133*, 9181−9183.

DOI: 10.1021/acs.jmedchem.5b01684
J. Med. Chem. 2016, 59, 4035−4061

(32) Decherchi, S.; Berteotti, A.; Bottegoni, G.; Rocchia, W.; Cavalli, A. The ligand binding mechanism to purine nucleoside phosphorylase elucidated via molecular dynamics and machine learning. *Nat. Commun.* **2015**, *6*, 6155.
(33) Dror, R. O.; Jensen, M. O.; Borhani, D. W.; Shaw, D. E. Exploring atomic resolution physiology on a femtosecond to millisecond timescale using molecular dynamics simulations. *J. Gen. Physiol.* **2010**, *135*, 555−562.
(34) Lane, T. J.; Shukla, D.; Beauchamp, K. A.; Pande, V. S. To milliseconds and beyond: challenges in the simulation of protein folding. *Curr. Opin. Struct. Biol.* **2013**, *23*, 58−65.
(35) Lu, H.; Tonge, P. J. Drug-target residence time: critical information for lead optimization. *Curr. Opin. Chem. Biol.* **2010**, *14*, 467−474.
(36) Abrams, C.; Bussi, G. Enhanced sampling in molecular dynamics using metadynamics, replica-exchange, and temperature-acceleration. *Entropy* **2014**, *16*, 163−199.
(37) Jorgensen, W. L.; Ravimohan, C. Monte-carlo simulation of differences in free-energies of hydration. *J. Chem. Phys.* **1985**, *83*, 3050−3054.
(38) Jorgensen, W. L.; Thomas, L. L. Perspective on free-energy perturbation calculations for chemical equilibria. *J. Chem. Theory Comput.* **2008**, *4*, 869−876.
(39) Torrie, G. M.; Valleau, J. P. Nonphysical sampling distributions in monte carlo free-energy estimation: umbrella sampling. *J. Comput. Phys.* **1977**, *23*, 187−199.
(40) Sugita, Y.; Okamoto, Y. Replica-exchange molecular dynamics method for protein folding. *Chem. Phys. Lett.* **1999**, *314*, 141−151.
(41) Laio, A.; Parrinello, M. Escaping free-energy minima. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 12562−12566.
(42) Isralewitz, B.; Gao, M.; Schulten, K. Steered molecular dynamics and mechanical functions of proteins. *Curr. Opin. Struct. Biol.* **2001**, *11*, 224−230.
(43) Grubmuller, H.; Heymann, B.; Tavan, P. Ligand binding: molecular mechanics calculation of the streptavidin biotin rupture force. *Science* **1996**, *271*, 997−999.
(44) Hamelberg, D.; Mongan, J.; McCammon, J. A. Accelerated molecular dynamics: a promising and efficient simulation method for biomolecules. *J. Chem. Phys.* **2004**, *120*, 11919−11929.
(45) Faradjian, A. K.; Elber, R. Computing time scales from reaction coordinates by milestoning. *J. Chem. Phys.* **2004**, *120*, 10880−10889.
(46) Bolhuis, P. G.; Chandler, D.; Dellago, C.; Geissler, P. L. Transition path sampling: Throwing ropes over rough mountain passes, in the dark. *Annu. Rev. Phys. Chem.* **2002**, *53*, 291−318.
(47) Ellingson, S. R.; Miao, Y.; Baudry, J.; Smith, J. C. Multi-conformer ensemble docking to difficult protein targets. *J. Phys. Chem. B* **2015**, *119*, 1026−1034.
(48) Surade, S.; Blundell, T. L. Structural biology and drug discovery of difficult targets: the limits of ligandability. *Chem. Biol.* **2012**, *19*, 42−50.
(49) Bowman, G. R.; Bolin, E. R.; Hart, K. M.; Maguire, B. C.; Marqusee, S. Discovery of multiple hidden allosteric sites by combining markov state models and experiments. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 2734−2739.
(50) Lu, S.; Huang, W.; Zhang, J. Recent computational advances in the identification of allosteric sites in proteins. *Drug Discovery Today* **2014**, *19*, 1595−1600.
(51) Lodola, A.; De Vivo, M. The increasing role of QM/MM in drug discovery. *Adv. Protein Chem. Struct. Biol.* **2012**, *87*, 337−362.
(52) Palermo, G.; Rothlisberger, U.; Cavalli, A.; De Vivo, M. Computational insights into function and inhibition of fatty acid amide hydrolase. *Eur. J. Med. Chem.* **2015**, *91*, 15−26.
(53) Cavalli, A.; Carloni, P.; Recanatini, M. Target-related applications of first principles quantum chemical methods in drug design. *Chem. Rev.* **2006**, *106*, 3497−3519.
(54) Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Gould, I. R.; Merz, K. M.; Ferguson, D. M.; Spellmeyer, D. C.; Fox, T.; Caldwell, J. W.; Kollman, P. a. A 2nd generation force-field for the simulation of proteins, nucleic-acids, and organic-molecules. *J. Am. Chem. Soc.* **1995**, *117*, 5179−5197.
(55) MacKerell, A. D.; Bashford, D.; Bellott, M.; Dunbrack, R. L.; Evanseck, J. D.; Field, M. J.; Fischer, S.; Gao, J.; Guo, H.; Ha, S.; Joseph-McCarthy, D.; Kuchnir, L.; Kuczera, K.; Lau, F. T. K.; Mattos, C.; Michnick, S.; Ngo, T.; Nguyen, D. T.; Prodhom, B.; Reiher, W. E.; Roux, B.; Schlenkrich, M.; Smith, J. C.; Stote, R.; Straub, J.; Watanabe, M.; Wiorkiewicz-Kuczera, J.; Yin, D.; Karplus, M. All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J. Phys. Chem. B* **1998**, *102*, 3586−3616.
(56) Jorgensen, W. L.; Tiradorives, J. The Opls Potential Functions for proteins - energy minimizations for crystals of cyclic-peptides and crambin. *J. Am. Chem. Soc.* **1988**, *110*, 1657−1666.
(57) Wang, J.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A. Development and testing of a general amber force field. *J. Comput. Chem.* **2004**, *25*, 1157−1174.
(58) Vanommeslaeghe, K.; Hatcher, E.; Acharya, C.; Kundu, S.; Zhong, S.; Shim, J.; Darian, E.; Guvench, O.; Lopes, P.; Vorobyov, I.; MacKerell, A. D. CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. *J. Comput. Chem.* **2010**, *31*, 671−690.
(59) Wang, J.; Wang, W.; Kollman, P. A.; Case, D. A. Automatic atom type and bond type perception in molecular mechanical calculations. *J. Mol. Graphics Modell.* **2006**, *25*, 247−260.
(60) Betz, R. M.; Walker, R. C. Paramfit: automated optimization of force field parameters for molecular dynamics simulations. *J. Comput. Chem.* **2015**, *36*, 79−87.
(61) Hopkins, C. W.; Roitberg, A. E. Fitting of dihedral terms in classical force fields as an analytic linear least-squares problem. *J. Chem. Inf. Model.* **2014**, *54*, 1978−1986.
(62) Huang, L.; Roux, B. Automated force field parameterization for non-polarizable and polarizable atomic models based on target data. *J. Chem. Theory Comput.* **2013**, *9*, 3543−3556.
(63) Wang, L. P.; Martinez, T. J.; Pande, V. S. Building force fields: an automatic, systematic, and reproducible approach. *J. Phys. Chem. Lett.* **2014**, *5*, 1885−1891.
(64) Ryckaert, J.-P.; Ciccotti, G.; Berendsen, H. J. C. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *J. Comput. Phys.* **1977**, *23*, 327−341.
(65) Hopkins, C. W.; Le Grand, S.; Walker, R. C.; Roitberg, A. E. Long-time-step molecular dynamics through hydrogen mass repartitioning. *J. Chem. Theory Comput.* **2015**, *11*, 1864−1874.
(66) Hünenberger, P. Thermostat algorithms for molecular dynamics simulations. In *Advanced Computer Simulation*; Holm, C., Kremer, K., Eds.; Springer: Berlin, Germany, 2005; Vol. *173*, pp 105−149, DOI: 10.1007/b99427.
(67) Darden, T.; York, D.; Pedersen, L. Particle mesh Ewald: An N·log(N) method for ewald sums in large systems. *J. Chem. Phys.* **1993**, *98*, 10089−10092.
(68) Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen, L. G. A smooth particle mesh ewald method. *J. Chem. Phys.* **1995**, *103*, 8577−8593.
(69) Sagui, C.; Darden, T. A. Molecular dynamics simulations of biomolecules: long-range electrostatic effects. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 155−179.
(70) Masetti, M.; Rocchia, W. Molecular mechanics and dynamics: numerical tools to sample the configuration space. *Front. Biosci., Landmark Ed.* **2014**, *1* (19), 578−604.
(71) Shi, Y.; Ren, P.; Schnieders, M.; Piquemal, J.-P. Polarizable force fields for biomolecular modeling. In *Reviews in Computational Chemistry*; John Wiley & Sons, Inc, 2015; Vol. *28*, pp 51−86, DOI: 10.1002/9781118889886.ch2.
(72) Jiao, D.; Golubkov, P. A.; Darden, T. A.; Ren, P. Calculation of protein-ligand binding free energy by using a polarizable potential. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 6290−6295.
(73) Warshel, a.; Levitt, M. Theoretical studies of enzymic reactions - dielectric, electrostatic and steric stabilization of carbonium-ion in reaction of lysozyme. *J. Mol. Biol.* **1976**, *103*, 227−249.

DOI: 10.1021/acs.jmedchem.5b01684
J. Med. Chem. 2016, 59, 4035−4061

(74) Raha, K.; Peters, M. B.; Wang, B.; Yu, N.; WollaCott, A. M.; Westerhoff, L. M.; Merz, K. M. The role of quantum mechanics in structure-based drug design. *Drug Discovery Today* **2007**, *12*, 725−731.
(75) Zhou, T.; Huang, D.; Caflisch, A. Quantum mechanical methods for drug design. *Curr. Top. Med. Chem.* **2010**, *10*, 33−45.
(76) van Duin, A. C. T.; Dasgupta, S.; Lorant, F.; Goddard, W. A. ReaxFF: A reactive force field for hydrocarbons. *J. Phys. Chem. A* **2001**, *105*, 9396−9409.
(77) Goh, G. B.; Hulbert, B. S.; Zhou, H. Q.; Brooks, C. L. Constant pH molecular dynamics of proteins in explicit solvent with proton tautomerism. *Proteins: Struct., Funct., Genet.* **2014**, *82*, 1319−1331.
(78) Swails, J. M.; York, D. M.; Roitberg, A. E. Constant pH replica exchange molecular dynamics in explicit solvent using discrete protonation states: implementation, testing, and validation. *J. Chem. Theory Comput.* **2014**, *10*, 1341−1352.
(79) Donnini, S.; Tegeler, F.; Groenhof, G.; Grubmuller, H. Constant pH molecular dynamics in explicit solvent with lambda-dynamics. *J. Chem. Theory Comput.* **2011**, *7*, 1962−1978.
(80) Grossfield, A.; Zuckerman, D. M. Quantifying uncertainty and sampling quality in biomolecular simulations. *Annu. Rep. Comput. Chem.* **2009**, *5*, 23−48.
(81) Gilson, M. K.; Given, J. A.; Bush, B. L.; McCammon, J. A. The statistical-thermodynamic basis for computation of binding affinities: a critical review. *Biophys. J.* **1997**, *72*, 1047−1069.
(82) General, I. J. A note on the standard state's binding free energy. *J. Chem. Theory Comput.* **2010**, *6*, 2520−2524.
(83) De Jong, D. H.; Schafer, L. V.; De Vries, A. H.; Marrink, S. J.; Berendsen, H. J. C.; Grubmuller, H. Determining equilibrium constants for dimerization reactions from molecular dynamics simulations. *J. Comput. Chem.* **2011**, *32*, 1919−1928.
(84) Seco, J.; Luque, F. J.; Barril, X. Binding site detection and druggability index from first principles. *J. Med. Chem.* **2009**, *52*, 2363−2371.
(85) Bakan, A.; Nevins, N.; Lakdawala, A. S.; Bahar, I. Druggability assessment of allosteric proteins by dynamics simulations in the presence of probe molecules. *J. Chem. Theory Comput.* **2012**, *8*, 2435−2447.
(86) Jorgensen, W. L.; Buckner, J. K.; Boudon, S.; Tiradorives, J. Efficient computation of absolute free-energies of binding by computer-simulations - application to the methane dimer in water. *J. Chem. Phys.* **1988**, *89*, 3742−3746.
(87) Hermans, J.; Wang, L. Inclusion of loss of translational and rotational freedom in theoretical estimates of free energies of binding. Application to a complex of benzene and mutant T4 lysozyme. *J. Am. Chem. Soc.* **1997**, *119*, 2707−2714.
(88) Hamelberg, D.; McCammon, J. A. Standard free energy of releasing a localized water molecule from the binding pockets of proteins: Double-decoupling method. *J. Am. Chem. Soc.* **2004**, *126*, 7683−7689.
(89) Wang, J. Y.; Deng, Y. Q.; Roux, B. Absolute binding free energy calculations using molecular dynamics simulations with restraining potentials. *Biophys. J.* **2006**, *91*, 2798−2814.
(90) Helms, V.; Wade, R. C. Computational alchemy to calculate absolute protein-ligand binding free energy. *J. Am. Chem. Soc.* **1998**, *120*, 2710−2713.
(91) Woods, C. J.; Malaisree, M.; Hannongbua, S.; Mulholland, A. J. A water-swap reaction coordinate for the calculation of absolute protein-ligand binding free energies. *J. Chem. Phys.* **2011**, *134*, 054114.
(92) Rocchia, W.; Masetti, M.; Cavalli, A. Enhanced sampling methods in drug design. In *Physico-Chemical and Computational Approaches to Drug Discovery*; The Royal Society of Chemistry, 2012; Chapter 11, pp 273−301, DOI: 10.1039/9781849735377-00273.
(93) Liu, P.; Kim, B.; Friesner, R. A.; Berne, B. J. Replica exchange with solute tempering: A method for sampling biological systems in explicit water. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 13749−13754.
(94) Sinko, W.; Miao, Y. L.; de Oliveira, C. A. F.; McCammon, J. A. Population based reweighting of scaled molecular dynamics. *J. Phys. Chem. B* **2013**, *117*, 12759−12768.
(95) Wang, L.; Friesner, R. A.; Berne, B. J. Correction to "Replica exchange with solute scaling: a more efficient version of replica exchange with solute tempering (REST2)". *J. Phys. Chem. B* **2011**, *115*, 11305−11305.
(96) Wang, L.; Berne, B. J.; Friesner, R. A. On achieving high accuracy and reliability in the calculation of relative protein-ligand binding affinities. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 1937−1942.
(97) Lionta, E.; Spyrou, G.; Vassilatis, D. K.; Cournia, Z. Structure-based virtual screening for drug discovery: principles, applications and recent advances. *Curr. Top. Med. Chem.* **2014**, *14*, 1923−1938.
(98) Matter, H.; Sotriffer, C. Applications and success stories in virtual screening. *Virtual screening: principles, challenges, and practical guidelines* **2011**, *48*, 319−358.
(99) Kitchen, D. B.; Decornez, H.; Furr, J. R.; Bajorath, J. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat. Rev. Drug Discovery* **2004**, *3*, 935−949.
(100) Jiang, F.; Kim, S. H. Soft Docking - Matching of molecular-surface cubes. *J. Mol. Biol.* **1991**, *219*, 79−102.
(101) Leach, A. R. Ligand docking to proteins with discrete side-chain flexibility. *J. Mol. Biol.* **1994**, *235*, 345−356.
(102) Meiler, J.; Baker, D. Rosettaligand: protein-small molecule docking with full side-chain flexibility. *Proteins: Struct., Funct., Genet.* **2006**, *65*, 538−548.
(103) Teague, S. J. Implications of protein flexibility for drug discovery. *Nat. Rev. Drug Discovery* **2003**, *2*, 527−541.
(104) Abagyan, R. Computational chemistry in 25 years. *J. Comput.-Aided Mol. Des.* **2012**, *26*, 9−10.
(105) Gane, P. J.; Dean, P. M. Recent advances in structure-based rational drug design. *Curr. Opin. Struct. Biol.* **2000**, *10*, 401−404.
(106) Hopkins, A. L.; Groom, C. R. The druggable genome. *Nat. Rev. Drug Discovery* **2002**, *1*, 727−730.
(107) Schlessinger, A.; Geier, E.; Fan, H.; Irwin, J. J.; Shoichet, B. K.; Giacomini, K. M.; Sali, A. Structure-based discovery of prescription drugs that interact with the norepinephrine transporter, NET. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 15810−15815.
(108) Rueda, M.; Bottegoni, G.; Abagyan, R. Consistent improvement of cross-docking results using binding site ensembles generated with elastic network normal modes. *J. Chem. Inf. Model.* **2009**, *49*, 716−725.
(109) Campbell, A. J.; Lamb, M. L.; Joseph-McCarthy, D. Ensemble-based docking using biased molecular dynamics. *J. Chem. Inf. Model.* **2014**, *54*, 2127−2138.
(110) Lin, J. H.; Perryman, A. L.; Schames, J. R.; McCammon, J. A. Computational drug design accommodating receptor flexibility: The relaxed complex scheme. *J. Am. Chem. Soc.* **2002**, *124*, 5632−5633.
(111) Spyrakis, F.; BidonChanal, A.; Barril, X.; Luque, F. J. Protein flexibility and ligand recognition: challenges for molecular modeling. *Curr. Top. Med. Chem.* **2011**, *11*, 192−210.
(112) Antolin, A. A.; Carotti, A.; Nuti, R.; Hakkaya, A.; Camaioni, E.; Mestres, J.; Pellicciari, R.; Macchiarulo, A. Exploring the effect of PARP-1 flexibility in docking studies. *J. Mol. Graphics Modell.* **2013**, *45*, 192−201.
(113) McGovern, S. L.; Shoichet, B. K. Information decay in molecular docking screens against holo, apo, and modeled conformations of enzymes. *J. Med. Chem.* **2003**, *46*, 2895−2907.
(114) Rueda, M.; Bottegoni, G.; Abagyan, R. Recipes for the selection of experimental protein conformations for virtual screening. *J. Chem. Inf. Model.* **2010**, *50*, 186−193.
(115) Barril, X.; Morley, S. D. Unveiling the full potential of flexible receptor docking using multiple crystallographic structures. *J. Med. Chem.* **2005**, *48*, 4432−4443.
(116) Nichols, S. E.; Baron, R.; McCammon, J. A. On the Use of molecular dynamics receptor conformations for virtual screening. *Methods Mol. Biol.* **2012**, *819*, 93−103.
(117) Amaro, R. E.; Baron, R.; McCammon, J. A. An improved relaxed complex scheme for receptor flexibility in computer-aided drug design. *J. Comput.-Aided Mol. Des.* **2008**, *22*, 693−705.

DOI: 10.1021/acs.jmedchem.5b01684
J. Med. Chem. 2016, 59, 4035−4061

(118) Bottegoni, G.; Rocchia, W.; Rueda, M.; Abagyan, R.; Cavalli, A. Systematic exploitation of multiple receptor conformations for virtual ligand screening. *PLoS One* **2011**, *6*, e18845.
(119) Swanson, J. M. J.; Henchman, R.; McCammon, J. A. Revisiting free energy calculations: One step closer to rigorous scoring functions and one step beyond MM/PBSA. *Biophys. J.* **2004**, *86*, 67−74.
(120) de Beer, S.; Vermeulen, N. P. E. The role of water molecules in computational drug design. *Curr. Top. Med. Chem.* **2010**, *10*, 55−66.
(121) Cavalli, A.; Bottegoni, G.; Raco, C.; De Vivo, M.; Recanatini, M. A computational study of the binding of propidium to the peripheral anionic site of human acetylcholinesterase. *J. Med. Chem.* **2004**, *47*, 3991−3999.
(122) Kacker, P.; Masetti, M.; Mangold, M.; Bottegoni, G.; Cavalli, A. Combining dyad protonation and active site plasticity in BACE-1 structure-based drug design. *J. Chem. Inf. Model.* **2012**, *52*, 1079−1085.
(123) Rastelli, G.; Degliesposti, G.; Del Rio, A.; Sgobba, M. Binding estimation after refinement, a new automated procedure for the refinement and rescoring of docked ligands in virtual screening. *Chem. Biol. Drug Des.* **2009**, *73*, 283−286.
(124) Sabbadin, D.; Ciancetta, A.; Moro, S. Bridging molecular docking to membrane molecular dynamics to investigate GPCR-ligand recognition: the human A(2)A adenosine receptor as a key study. *J. Chem. Inf. Model.* **2014**, *54*, 169−183.
(125) Lauro, G.; Ferruz, N.; Fulle, S.; Harvey, M. J.; Finn, P. W.; De Fabritiis, G. Reranking docking poses using molecular simulations and approximate free energy methods. *J. Chem. Inf. Model.* **2014**, *54*, 2185−2189.
(126) Shirts, M.; Pande, V. S. Computing: screen savers of the world unite! *Science* **2000**, *290*, 1903−1904.
(127) Dror, R. O.; Pan, A. C.; Arlow, D. H.; Borhani, D. W.; Maragakis, P.; Shan, Y.; Xu, H.; Shaw, D. E. Pathway and mechanism of drug binding to G-protein-coupled receptors. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 13118−13123.
(128) Foda, Z. H.; Shan, Y.; Kim, E. T.; Shaw, D. E.; Seeliger, M. A. A dynamically coupled allosteric network underlies binding cooperativity in Src kinase. *Nat. Commun.* **2015**, *6*, 5939.
(129) Shan, Y.; Eastwood, M. P.; Zhang, X.; Kim, E. T.; Arkhipov, A.; Dror, R. O.; Jumper, J.; Kuriyan, J.; Shaw, D. E. Oncogenic mutations counteract intrinsic disorder in the EGFR kinase and promote receptor dimerization. *Cell* **2012**, *149*, 860−870.
(130) Palermo, G.; Favia, A. D.; Convertino, M.; De Vivo, M. The molecular basis for dual fatty acid amide hydrolase (FAAH)/cyclooxygenase (COX) inhibition. *ChemMedChem* **2015**, DOI: 10.1002/cmdc.201500507.
(131) Palermo, G.; Branduardi, D.; Masetti, M.; Lodola, A.; Mor, M.; Piomelli, D.; Cavalli, A.; De Vivo, M. Covalent inhibitors of fatty acid amide hydrolase: a rationale for the activity of piperidine and piperazine aryl ureas. *J. Med. Chem.* **2011**, *54*, 6612−6623.
(132) Palermo, G.; Stenta, M.; Cavalli, A.; Dal Peraro, M.; De Vivo, M. Molecular simulations highlight the role of metals in catalysis and inhibition of type II topoisomerase. *J. Chem. Theory Comput.* **2013**, *9*, 857−862.
(133) Gervasio, F. L.; Laio, A.; Parrinello, M. Flexible docking in solution using metadynamics. *J. Am. Chem. Soc.* **2005**, *127*, 2600−2607.
(134) Colizzi, F.; Perozzo, R.; Scapozza, L.; Recanatini, M.; Cavalli, A. Single-molecule pulling simulations can discern active from inactive enzyme inhibitors. *J. Am. Chem. Soc.* **2010**, *132*, 7361−7371.
(135) Bash, P. A.; Singh, U. C.; Brown, F. K.; Langridge, R.; Kollman, P. A. Calculation of the relative change in binding free energy of a protein-inhibitor complex. *Science* **1987**, *235*, 574−6.
(136) Jorgensen, W. L. Efficient drug lead discovery and optimization. *Acc. Chem. Res.* **2009**, *42*, 724−733.
(137) Wong, C. F.; Mccammon, J. A. Dynamics and design of enzymes and inhibitors. *J. Am. Chem. Soc.* **1986**, *108*, 3830−3832.
(138) Merz, K. M.; Kollman, P. A. Free energy perturbation simulations of the inhibition of thermolysin: prediction of the free energy of binding of a new inhibitor. *J. Am. Chem. Soc.* **1989**, *111*, 5649−5658.
(139) Ferguson, D. M.; Radmer, R. J.; Kollman, P. A. Determination of the relative binding free energies of peptide inhibitors to the HIV-1 protease. *J. Med. Chem.* **1991**, *34*, 2654−2549.
(140) Rao, B. G.; Tilton, R. F.; Singh, U. C. Free energy perturbation studies on inhibitor binding to HIV-1 proteinase. *J. Am. Chem. Soc.* **1992**, *114*, 4447−4452.
(141) Reddy, M. R.; Viswanadhan, V. N.; Weinstein, J. N. Relative differences in the binding free energies of human immunodeficiency virus 1 protease inhibitors: a thermodynamic cycle-perturbation approach. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 10287−10291.
(142) Wang, L.; Wu, Y.; Deng, Y.; Kim, B.; Pierce, L.; Krilov, G.; Lupyan, D.; Robinson, S.; Dahlgren, M. K.; Greenwood, J.; Romero, D. L.; Masse, C.; Knight, J. L.; Steinbrecher, T.; Beuming, T.; Damm, W.; Harder, E.; Sherman, W.; Brewer, M.; Wester, R.; Murcko, M.; Frye, L.; Farid, R.; Lin, T.; Mobley, D. L.; Jorgensen, W. L.; Berne, B. J.; Friesner, R. A.; Abel, R. Accurate and reliable prediction of relative ligand binding potency in prospective drug discovery by way of a modern free-energy calculation protocol and force field. *J. Am. Chem. Soc.* **2015**, *137*, 2695−2703.
(143) Bollini, M.; Domaoal, R. A.; Thakur, V. V.; Gallardo-Macias, R.; Spasov, K. A.; Anderson, K. S.; Jorgensen, W. L. Computationally-guided optimization of a docking hit to yield catechol diethers as potent anti-HIV agents. *J. Med. Chem.* **2011**, *54*, 8582−8591.
(144) Lee, W. G.; Gallardo-Macias, R.; Frey, K. M.; Spasov, K. A.; Bollini, M.; Anderson, K. S.; Jorgensen, W. L. Picomolar inhibitors of HIV reverse transcriptase featuring bicyclic replacement of a cyanovinylphenyl group. *J. Am. Chem. Soc.* **2013**, *135*, 16705−16713.
(145) Frey, K. M.; Puleo, D. E.; Spasov, K. A.; Bollini, M.; Jorgensen, W. L.; Anderson, K. S. Structure-based evaluation of non-nucleoside inhibitors with improved potency and solubility that target HIV reverse transcriptase variants. *J. Med. Chem.* **2015**, *58*, 2737−2745.
(146) Dziedzic, P.; Cisneros, J. A.; Robertson, M. J.; Hare, A. A.; Danford, N. E.; Baxter, R. H. G.; Jorgensen, W. L. Design, Synthesis, and Protein Crystallography of Biaryltriazoles as Potent Tautomerase Inhibitors of Macrophage Migration Inhibitory Factor. *J. Am. Chem. Soc.* **2015**, *137*, 2996−3003.
(147) Hare, A. A.; Leng, L.; Gandavadi, S.; Du, X.; Cournia, Z.; Bucala, R.; Jorgensen, W. L. Optimization of N-benzyl-benzoxazol-2-ones as receptor antagonists of macrophage migration inhibitory factor (MIF). *Bioorg. Med. Chem. Lett.* **2010**, *20*, 5811−5814.
(148) Cournia, Z.; Leng, L.; Gandavadi, S.; Du, X.; Bucala, R.; Jorgensen, W. L. Discovery of human macrophage migration inhibitory factor (MIF)-CD74 Aantagonists via virtual screening. *J. Med. Chem.* **2009**, *52*, 416−424.
(149) Acevedo, O.; Ambrose, Z.; Flaherty, P. T.; Aamer, H.; Jain, P.; Sambasivarao, S. V. Identification of HIV inhibitors guided by free energy perturbation calculations. *Curr. Pharm. Des.* **2012**, *18*, 1199−1216.
(150) Cole, D. J.; Tirado-Rives, J.; Jorgensen, W. L. Molecular dynamics and Monte Carlo simulations for protein-ligand binding and inhibitor design. *Biochim. Biophys. Acta, Gen. Subj.* **2015**, *1850*, 966−971.
(151) Kumar, S.; Bouzida, D.; Swendsen, R. H.; Kollman, P. A.; Rosenberg, J. M. The weighted histogram analysis method for free-energy calculations on biomolecules 0.1. The Method. *J. Comput. Chem.* **1992**, *13*, 1011−1021.
(152) Kastner, J. Umbrella sampling. *WIREs Comput. Mol. Sci.* **2011**, *1*, 932−942.
(153) Comer, J.; Gumbart, J. C.; Henin, J.; Lelievre, T.; Pohorille, A.; Chipot, C. The adaptive biasing force method: everything you always wanted to know but were afraid to ask. *J. Phys. Chem. B* **2015**, *119*, 1129−1151.
(154) Woo, H. J.; Roux, B. Calculation of absolute protein-ligand binding free energy from computer simulations. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 6825−6830.
(155) Ge, X. X.; Roux, B. Absolute Binding Free Energy Calculations of Sparsomycin Analogs to the Bacterial Ribosome. *J. Phys. Chem. B* **2010**, *114*, 9525−9539.

DOI: 10.1021/acs.jmedchem.5b01684
J. Med. Chem. 2016, 59, 4035−4061

(156) Deng, Y. Q.; Roux, B. Computations of standard binding free energies with molecular dynamics simulations. *J. Phys. Chem. B* **2009**, *113*, 2234−2246.
(157) Lee, M. S.; Olson, M. A. Calculation of absolute protein-ligand binding affinity using path and endpoint approaches. *Biophys. J.* **2006**, *90*, 864−877.
(158) Doudou, S.; Burton, N. A.; Henchman, R. H. Standard free energy of binding from a one-dimensional potential of mean force. *J. Chem. Theory Comput.* **2009**, *5*, 909−918.
(159) Wojtas-Niziurski, W.; Meng, Y. L.; Roux, B.; Berneche, S. Self-learning adaptive umbrella sampling method for the determination of free energy landscapes in multiple dimensions. *J. Chem. Theory Comput.* **2013**, *9*, 1885−1895.
(160) Jarzynski, C. Nonequilibrium equality for free energy differences. *Phys. Rev. Lett.* **1997**, *78*, 2690−2693.
(161) Park, S.; Khalili-Araghi, F.; Tajkhorshid, E.; Schulten, K. Free energy calculation from steered molecular dynamics simulations using Jarzynski's equality. *J. Chem. Phys.* **2003**, *119*, 3559−3566.
(162) Xiong, H.; Crespo, A.; Marti, M.; Estrin, D.; Roitberg, A. E. Free energy calculations with non-equilibrium methods: applications of the Jarzynski relationship. *Theor. Chem. Acc.* **2006**, *116*, 338−346.
(163) Patel, J. S.; Berteotti, A.; Ronsisvalle, S.; Rocchia, W.; Cavalli, A. Steered molecular dynamics simulations for studying protein-ligand interaction in cyclin-dependent Kinase 5. *J. Chem. Inf. Model.* **2014**, *54*, 470−480.
(164) Palermo, G.; Minniti, E.; Greco, M. L.; Riccardi, L.; Simoni, E.; Convertino, M.; Marchetti, C.; Rosini, M.; Sissi, C.; Minarini, A.; De Vivo, M. An optimized polyamine moiety boosts the potency of human type II topoisomerase poisons as quantified by comparative analysis centered on the clinical candidate F14512. *Chem. Commun.* **2015**, *51*, 14310−14313.
(165) Patel, J. S.; Branduardi, D.; Masetti, M.; Rocchia, W.; Cavalli, A. Insights into ligand-protein binding from local mechanical response. *J. Chem. Theory Comput.* **2011**, *7*, 3368−3378.
(166) Ensing, B.; De Vivo, M.; Liu, Z. W.; Moore, P.; Klein, M. L. Metadynamics as a tool for exploring free energy landscapes of chemical reactions. *Acc. Chem. Res.* **2006**, *39*, 73−81.
(167) Bussi, G.; Laio, A.; Parrinello, M. Equilibrium free energies from nonequilibrium metadynamics. *Phys. Rev. Lett.* **2006**, *96*, 090601.
(168) Laio, A.; Rodriguez-Fortea, A.; Gervasio, F. L.; Ceccarelli, M.; Parrinello, M. Assessing the accuracy of metadynamics. *J. Phys. Chem. B* **2005**, *109*, 6714−6721.
(169) Laio, A.; Gervasio, F. L. Metadynamics: a method to simulate rare events and reconstruct the free energy in biophysics, chemistry and material science. *Rep. Prog. Phys.* **2008**, *71*, 126601.
(170) Cavalli, A.; Spitaleri, A.; Saladino, G.; Gervasio, F. L. Investigating Drug-Target Association and dissociation mechanisms using metadynamics-based algorithms. *Acc. Chem. Res.* **2015**, *48*, 277−285.
(171) Barducci, A.; Bussi, G.; Parrinello, M. Well-tempered metadynamics: A smoothly converging and tunable free-energy method. *Phys. Rev. Lett.* **2008**, *100*, 020603.
(172) Branduardi, D.; Bussi, G.; Parrinello, M. Metadynamics with Adaptive Gaussians. *J. Chem. Theory Comput.* **2012**, *8*, 2247−2254.
(173) Branduardi, D.; Gervasio, F. L.; Parrinello, M. From A to B in free energy space. *J. Chem. Phys.* **2007**, *126*, 054103.
(174) Bonomi, M.; Gervasio, F. L.; Tiana, G.; Provasi, D.; Broglia, R. A.; Parrinello, M. Insight into the folding inhibition of the HIV-1 protease by a small peptide. *Biophys. J.* **2007**, *93*, 2813−2821.
(175) Berteotti, A.; Cavalli, A.; Branduardi, D.; Gervasio, F. L.; Recanatini, M.; Parrinello, M. Protein conformational transitions: the closure mechanism of a kinase explored by atomistic simulations. *J. Am. Chem. Soc.* **2009**, *131*, 244−250.
(176) Ceccarini, L.; Masetti, M.; Cavalli, A.; Recanatini, M. Ion Conduction through the hERG potassium channel. *PLoS One* **2012**, *7*, e49017.
(177) Tribello, G. A.; Ceriotti, M.; Parrinello, M. Using sketch-map coordinates to analyze and bias molecular dynamics simulations. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 5196−5201.
(178) Bussi, G.; Gervasio, F. L.; Laio, A.; Parrinello, M. Free-energy landscape for beta hairpin folding from combined parallel tempering and metadynamics. *J. Am. Chem. Soc.* **2006**, *128*, 13435−13441.
(179) Bonomi, M.; Parrinello, M. Enhanced sampling in the well-tempered ensemble. *Phys. Rev. Lett.* **2010**, *104*, 190601.
(180) Piana, S.; Laio, A. A bias-exchange approach to protein folding. *J. Phys. Chem. B* **2007**, *111*, 4553−4559.
(181) Tribello, G. A.; Ceriotti, M.; Parrinello, M. A self-learning algorithm for biased molecular dynamics. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 17509−17514.
(182) Limongelli, V.; Bonomi, M.; Marinelli, L.; Gervasio, F. L.; Cavalli, A.; Novellino, E.; Parrinello, M. Molecular basis of cyclooxygenase enzymes (COXs) selective inhibition. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 5411−5416.
(183) Favia, A. D.; Masetti, M.; Recanatini, M.; Cavalli, A. Substrate binding process and mechanistic functioning of type 1 11 beta-hydroxysteroid dehydrogenase from enhanced sampling methods. *PLoS One* **2011**, *6*, e25375.
(184) Provasi, D.; Bortolato, A.; Filizola, M. Exploring molecular mechanisms of ligand recognition by opioid receptors with metadynamics. *Biochemistry* **2009**, *48*, 10020−10029.
(185) Limongelli, V.; Bonomi, M.; Parrinello, M. Funnel metadynamics as accurate binding free-energy method. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 6358−6363.
(186) Di Leva, F. S.; Novellino, E.; Cavalli, A.; Parrinello, M.; Limongelli, V. Mechanistic insight into ligand binding to G-quadruplex DNA. *Nucleic Acids Res.* **2014**, *42*, 5447−55.
(187) Masetti, M.; Cavalli, A.; Recanatini, M.; Gervasio, F. L. Exploring complex protein-ligand recognition mechanisms with coarse metadynamics. *J. Phys. Chem. B* **2009**, *113*, 4807−4816.
(188) Wang, K.; Chodera, J. D.; Yang, Y. Z.; Shirts, M. R. Identifying ligand binding sites and poses using GPU-accelerated hamiltonian replica exchange molecular dynamics. *J. Comput.-Aided Mol. Des.* **2013**, *27*, 989−1007.
(189) Luitz, M. P.; Zacharias, M. Protein-ligand docking using hamiltonian replica exchange simulations with soft core potentials. *J. Chem. Inf. Model.* **2014**, *54*, 1669−1675.
(190) Duane, S.; Kennedy, A.; Pendleton, B.; Roweth, D. Hybrid monte carlo. *Phys. Lett. B* **1987**, *195*, 216−222.
(191) Fischer, A.; Cordes, F.; Schütte, C. Hybrid monte carlo with adaptive temperature in mixed-canonical ensemble: efficient conformational analysis of RNA. *J. Comput. Chem.* **1998**, *19*, 1689−1697.
(192) Schütte, C.; Fischer, A.; Huisinga, W.; Deuflhard, P. A Direct Approach to conformational dynamics based on hybrid monte carlo. *J. Comput. Phys.* **1999**, *151*, 146−168.
(193) Guarnieri, F.; Still, W. C. A rapidly convergent simulation method: mixed monte carlo/stochastic dynamics. *J. Comput. Chem.* **1994**, *15*, 1302−1310.
(194) Jiang, W.; Hodoscek, M.; Roux, B. Computation of absolute hydration and binding free energy with free energy perturbation distributed replica-exchange molecular dynamics. *J. Chem. Theory Comput.* **2009**, *5*, 2583−2588.
(195) Jiang, W.; Roux, B. Free energy perturbation hamiltonian replica-exchange molecular dynamics (FEP/H-REMD) for absolute ligand binding free energy calculations. *J. Chem. Theory Comput.* **2010**, *6*, 2559−2565.
(196) Mobley, D. L.; Chodera, J. D.; Dill, K. A. The confine-and-release method: obtaining correct binding free energies in the presence of protein conformational change. *J. Chem. Theory Comput.* **2007**, *3*, 1231−1235.
(197) Jiang, W.; Luo, Y.; Maragliano, L.; Roux, B. Calculation of free energy landscape in multi-dimensions with hamiltonian-exchange umbrella sampling on petascale supercomputer. *J. Chem. Theory Comput.* **2012**, *8*, 4672−4680.
(198) Sutto, L.; Gervasio, F. L. Effects of oncogenic mutations on the conformational free-energy landscape of EGFR kinase. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 10616−10621.

DOI: 10.1021/acs.jmedchem.5b01684
J. Med. Chem. 2016, 59, 4035−4061

(199) Lapelosa, M.; Gallicchio, E.; Levy, R. M. Conformational transitions and convergence of absolute binding free energy calculations. *J. Chem. Theory Comput.* **2012**, *8*, 47−60.
(200) Procacci, P.; Bizzarri, M.; Marsili, S. Energy-driven undocking (EDU-HREM) in solute tempering replica exchange simulations. *J. Chem. Theory Comput.* **2014**, *10*, 439−450.
(201) Copeland, R. A.; Pompliano, D. L.; Meek, T. D. Opinion - Drug-target residence time and its implications for lead optimization. *Nat. Rev. Drug Discovery* **2006**, *5*, 730−739.
(202) Pan, A. C.; Borhani, D. W.; Dror, R. O.; Shaw, D. E. Molecular determinants of drug-receptor binding kinetics. *Drug Discovery Today* **2013**, *18*, 667−673.
(203) Zhou, H. X. Rate theories for biologists. *Q. Rev. Biophys.* **2010**, *43*, 219−293.
(204) Pande, V. S.; Beauchamp, K.; Bowman, G. R. Everything you wanted to know about markov state models but were afraid to ask. *Methods* **2010**, *52*, 99−105.
(205) Chodera, J. D.; Noe, F. Markov state models of biomolecular conformational dynamics. *Curr. Opin. Struct. Biol.* **2014**, *25*, 135−144.
(206) Gu, S.; Silva, D. A.; Meng, L.; Yue, A.; Huang, X. Quantitatively characterizing the ligand binding mechanisms of choline binding protein using Markov state model analysis. *PLoS Comput. Biol.* **2014**, *10*, e1003767.
(207) Plattner, N.; Noe, F. Protein conformational plasticity and complex ligand-binding kinetics explored by atomistic simulations and Markov models. *Nat. Commun.* **2015**, *6*, 7653.
(208) Doerr, S.; De Fabritiis, G. On-the-Fly Learning and sampling of ligand binding by high-throughput molecular simulations. *J. Chem. Theory Comput.* **2014**, *10*, 2064−2069.
(209) Lu, H.; Tonge, P. J. Drug-target residence time: critical information for lead optimization. *Curr. Opin. Chem. Biol.* **2010**, *14*, 467−474.
(210) Mollica, L.; Decherchi, S.; Zia, S. R.; Gaspari, R.; Cavalli, A.; Rocchia, W. Kinetics of protein-ligand unbinding via smoothed potential molecular dynamics simulations. *Sci. Rep.* **2015**, *5*, 11539.
(211) Bui, J. M.; Henchman, R. H.; McCammon, J. A. The dynamics of ligand barrier crossing inside the acetylcholinesterase gorge. *Biophys. J.* **2003**, *85*, 2267−2272.
(212) Marinelli, F.; Pietrucci, F.; Laio, A.; Piana, S. A kinetic model of trp-cage folding from multiple biased molecular dynamics simulations. *PLoS Comput. Biol.* **2009**, *5*, e1000452.
(213) Pietrucci, F.; Marinelli, F.; Carloni, P.; Laio, A. Substrate binding mechanism of hiv-1 protease from explicit-solvent atomistic simulations. *J. Am. Chem. Soc.* **2009**, *131*, 11811−11818.
(214) Tiwary, P.; Parrinello, M. From Metadynamics to Dynamics. *Phys. Rev. Lett.* **2013**, *111*, 230602.
(215) Salvalaglio, M.; Tiwary, P.; Parrinello, M. Assessing the Reliability of the dynamics reconstructed from metadynamics. *J. Chem. Theory Comput.* **2014**, *10*, 1420−1425.
(216) Changeux, J. P. Allostery and the monod-wyman-changeux model after 50 years. *Annu. Rev. Biophys.* **2012**, *41*, 103−133.
(217) Tsai, C. J.; Nussinov, R. A Unified view of "how allostery works". *PLoS Comput. Biol.* **2014**, *10*, e1003394.
(218) Lee, G. M.; Craik, C. S. Trapping moving targets with small molecules. *Science* **2009**, *324*, 213−215.
(219) Conn, P. J.; Christopoulos, A.; Lindsley, C. W. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug Discovery* **2009**, *8*, 41−54.
(220) Chatzidaki, A.; Millar, N. S. Allosteric modulation of nicotinic acetylcholine receptors. *Biochem. Pharmacol.* **2015**, *97*, 408−417.
(221) Desdouits, N.; Nilges, M.; Blondel, A. Principal component analysis reveals correlation of cavities evolution and functional motions in proteins. *J. Mol. Graphics Modell.* **2015**, *55*, 13−24.
(222) Kokh, D. B.; Richter, S.; Henrich, S.; Czodrowski, P.; Rippmann, F.; Wade, R. C. TRAPP: a tool for analysis of transient binding pockets in proteins. *J. Chem. Inf. Model.* **2013**, *53*, 1235−1252.
(223) Kappel, K.; Wereszczynski, J.; Clubb, R. T.; McCammon, J. A. The binding mechanism, multiple binding modes, and allosteric regulation of staphylococcus aureus sortase a probed by molecular dynamics simulations. *Protein Sci.* **2012**, *21*, 1858−1871.
(224) Amadei, A.; Linssen, A. B.; Berendsen, H. J. Essential dynamics of proteins. *Proteins: Struct., Funct., Genet.* **1993**, *17*, 412−425.
(225) Lange, O. F.; Grubmuller, H. Generalized correlation for biomolecular dynamics. *Proteins: Struct., Funct., Genet.* **2006**, *62*, 1053−1061.
(226) Lange, O. F.; Grubmuller, H. Full correlation analysis of conformational protein dynamics. *Proteins: Struct., Funct., Genet.* **2008**, *70*, 1294−1312.
(227) McClendon, C. L.; Friedland, G.; Mobley, D. L.; Amirkhani, H.; Jacobson, M. P. Quantifying correlations between allosteric sites in thermodynamic ensembles. *J. Chem. Theory Comput.* **2009**, *5*, 2486−2502.
(228) Sethi, A.; Eargle, J.; Black, A. A.; Luthey-Schulten, Z. Dynamical networks in tRNA: protein complexes. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 6620−6625.
(229) Newman, M. E. J.; Girvan, M. Finding and evaluating community structure in networks. *Phys. Rev. E* **2004**, *69*, 026113.
(230) Rivalta, I.; Sultan, M. M.; Lee, N. S.; Manley, G. A.; Loria, J. P.; Batista, V. S. Allosteric pathways in imidazole glycerol phosphate synthase. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, E1428−E1436.
(231) Morra, G.; Genoni, A.; Colombo, G. Mechanisms of differential allosteric modulation in homologous proteins: insights from the analysis of internal dynamics and energetics of pdz domains. *J. Chem. Theory Comput.* **2014**, *10*, 5677−5689.
(232) Morra, G.; Neves, M. A. C.; Plescia, C. J.; Tsustsumi, S.; Neckers, L.; Verkhivker, G.; Altieri, D. C.; Colombo, G. Dynamics-based discovery of allosteric inhibitors: selection of new ligands for the c-terminal domain of hsp90. *J. Chem. Theory Comput.* **2010**, *6*, 2978−2989.
(233) de Beer, S. B. A.; Vermeulen, N. P. E.; Oostenbrink, C. The role of water molecules in computational drug design. *Curr. Top. Med. Chem.* **2010**, *10*, 55−66.
(234) Athri, P.; Wilson, W. D. Molecular dynamics of water-mediated interactions of a linear benzimidazole-biphenyl diamidine with the dna minor groove. *J. Am. Chem. Soc.* **2009**, *131*, 7618−7625.
(235) Biela, A.; Betz, M.; Heine, A.; Klebe, G. Water Makes the Difference: Rearrangement of Water Solvation Layer Triggers Non-additivity of functional group contributions in protein-ligand binding. *ChemMedChem* **2012**, *7*, 1423−1434.
(236) Michel, J.; Tirado-Rives, J.; Jorgensen, W. L. Prediction of the water content in protein binding sites. *J. Phys. Chem. B* **2009**, *113*, 13337−13346.
(237) Henchman, R. H.; McCammon, J. A. Extracting hydration sites around proteins from explicit water simulations. *J. Comput. Chem.* **2002**, *23*, 861−869.
(238) Li, S.; Bradley, P. Probing the role of interfacial waters in protein-DNA recognition using a hybrid implicit/explicit solvation model. *Proteins: Struct., Funct., Genet.* **2013**, *81*, 1318−1329.
(239) Ladbury, J. E. Just add water! The effect of water on the specificity of protein-ligand binding sites and its potential application to drug design. *Chem. Biol.* **1996**, *3*, 973−980.
(240) Huang, D. Z.; Rossini, E.; Steiner, S.; Caflisch, A. Structured water molecules in the binding site of bromodomains can be displaced by cosolvent. *ChemMedChem* **2014**, *9*, 573−579.
(241) Lazaridis, T. Inhomogeneous fluid approach to solvation thermodynamics. 1. Theory. *J. Phys. Chem. B* **1998**, *102*, 3531−3541.
(242) Breiten, B.; Lockett, M. R.; Sherman, W.; Fujita, S.; Al-Sayah, M.; Lange, H.; Bowers, C. M.; Heroux, A.; Krilov, G.; Whitesides, G. M. Water Networks contribute to enthalpy/entropy compensation in protein-ligand binding. *J. Am. Chem. Soc.* **2013**, *135*, 15579−15584.
(243) Maffucci, I.; Contini, A. Explicit ligand hydration shells improve the correlation between MM-PB/GBSA binding energies and experimental activities. *J. Chem. Theory Comput.* **2013**, *9*, 2706−2717.

DOI: 10.1021/acs.jmedchem.5b01684
J. Med. Chem. 2016, 59, 4035−4061