Perturbation Approaches for Exploring Protein Binding Site Flexibility to Predict Transient Binding Pockets

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ABSTRACT: Simulations of the long-time scale motions of a ligand binding pocket in a protein may open up new perspectives for the design of compounds with steric or chemical properties differing from those of known binders. However, slow motions of proteins are difficult to access using standard molecular dynamics (MD) simulations and are thus usually neglected in computational drug design. Here, we introduce two nonequilibrium MD approaches to identify conformational changes of a binding site and detect transient pockets associated with these motions. The methods proposed are based on the rotamERICALLY induced perturbation (RIP) MD approach, which employs perturbation of side-chain torsional motion for initiating large-scale protein movement. The first approach, Langevin-RIP (L-RIP), entails a series of short Langevin MD simulations, each starting with perturbation of one of the side-chains lining the binding site of interest. L-RIP provides extensive sampling of conformational changes of the binding site. In less than 1 ns of MD simulation with L-RIP, we observed distortions of the α-helix in the ATP binding site of HSP90 and flipping of the DFG loop in Src kinase. In the second approach, RIPlig, a perturbation is applied to a pseudoligand placed in different parts of a binding pocket, which enables flexible regions of the binding site to be identified in a small number of 10 ps MD simulations. The methods were evaluated for four test proteins displaying different types and degrees of binding site flexibility. Both methods reveal all transient pocket regions in less than a total of 10 ns of simulations, even though many of these regions remained closed in 100 ns conventional MD. The proposed methods provide computationally efficient tools to explore binding site flexibility and can aid in the functional characterization of protein pockets, and the identification of transient pockets for ligand design.

1. INTRODUCTION

Exploiting target flexibility in the discovery of protein inhibitors is an emerging and highly challenging goal in computer-aided drug design. The importance of protein motion in determining the selectivity and specificity of small molecule binding has recently become widely recognized, with an increasing number of publications being dedicated to the detection and analysis of protein motions required for pocket opening. MD simulation is often the method of choice, but its practical application is hindered by the computational costs of sampling the slow motions of proteins. Routine MD simulations, such as those usually employed in drug design, are generally limited to events on the 1–100 ns time scale, while opening of a binding pocket or subpocket often requires longer times. Microsecond time scale trajectories showing the opening of transient pockets or of tunnels along ligand binding pathways have been reported in only a few studies, see, for example, ref 3. To go beyond the time-scale of standard MD simulations, a number of enhanced sampling MD methods, such as replica-exchange methods,4,5 temperature accelerated MD,6 and hyper- or meta-dynamics,7,8 have been proposed. These methods have not, however, been adopted widely in the computer-aided drug design field because they are still computationally expensive, their efficiency is strongly dependent on user expertise, and their application often requires prior knowledge of the type of motion in the system studied and adaptations of the computational procedures to the particular system.

Several years ago, Ho and Agard9 proposed a nonequilibrium MD method, named rotamERICALLY induced perturbation (RIP), which enables protein flexibility that normally occurs on the microsecond or longer time scale (for example, distortion of secondary and tertiary structure) to be explored. RIP employs...
repeated perturbations of a single residue (specifically, transfer of the residue’s kinetic energy to one side-chain torsion angle), followed by short (0.1 ps) implicit solvent MD simulations under constant energy conditions. The final snapshots from each of these MD runs are combined in a RIP trajectory that consists of 100 such perturbation steps (pulses) and a total of 10 ps MD simulation in the original implementation reported in ref.1 The excitation of the torsional rotation of a single protein residue initiates kinetic energy transport through the protein structure, breaking unstable noncovalent bonds and leading to large (up to about 10 Å) conformational motions of flexible protein segments. A set of RIP trajectories, generated for each residue in the protein with a rotatable side-chain, provides a global map of the protein’s deformability.

A major advantage of the RIP approach in comparison to other enhanced MD methods is that it is completely unsupervised. Besides, RIP simulations are quite fast and can be trivially parallelized since they consist of many short, unsupervised. RIP simulations are quite fast and can be trivially parallelized since they consist of many short, unsupervised. Besides, RIP simulations are quite fast and can be trivially parallelized since they consist of many short, unsupervised. Moreover, RIP approaches are quite fast and can be trivially parallelized since they consist of many short, unsupervised. Besides, RIP simulations are quite fast and can be trivially parallelized since they consist of many short, unsupervised. Moreover, RIP approaches are quite fast and can be trivially parallelized since they consist of many short, unsupervised.

Motivated by the idea that a local protein motion might be initiated by kinetic perturbation of selected residues, we have modified the original RIP procedure with the aim of generating suitable tools for prediction of long-time scale (microsecond or millisecond) conformational variations of a designated binding site in a protein. To improve both the accuracy and the speed compared to the original RIP method, we developed two computational approaches: (1) RIP of a ligand, RIPlig, for fast prediction of flexible regions in a binding pocket, and (2) Langevin-RIP, L-RIP, for enhanced sampling of binding site conformations arising from the large-scale motions. In RIPlig, the perturbation procedure is applied not to each protein residue, but to an artificial molecule placed in the binding pocket with repetitions starting with different positions of the molecule. L-RIP is a computationally more expensive approach in which perturbation is applied to the side chains of the binding site residues, but the original RIP procedure is modified by using a Langevin thermostat in the simulations instead of the original constant-energy conditions. This ensures that the average protein temperature is preserved during the MD simulations. Additionally, the friction term enables the elements of the protein structure with fast relaxation times to equilibrate within each perturbation pulse. This makes it possible to increase the number of perturbation pulses and, thus, enhance sampling of slow conformational variations.

For evaluation of these methods, we considered four proteins that demonstrate different types of binding site flexibility: the heat shock protein 90 N-terminal domain (HSP90), Src tyrosine kinase (Src), interleukin-2 (IL2), and the progesterone receptor ligand binding domain (PR) (shown in Figure 1).

The binding site of the N-terminal ATP-binding domain of HSP90 is lined by the unstable α-helix3 which, according to available crystal structures, undergoes distortion in the middle, converting into two short helices, connected by a loop, as shown in Figure 1A. It is noteworthy that even in the structure with a complete α-helix3 (PDB 2UVD), the hydrogen-bond network is slightly distorted in the region between residues L103 and L107 and in the absence of a ligand, this region adopts a loop conformation (PDB: 1YER, 1YES). However, a transition from complete α-helix3 to loop conformation was not observed in a 0.4 μs standard MD trajectory (the simulation procedure is given in section 1 of Supporting Information and the results are shown in Figure S2) To the best of our knowledge, no simulation has been reported that reveals unwinding of α-helix3 or a transition between different conformations of the HSP90 binding site. On the other hand, the high flexibility of the ATP lid (which includes the C-terminal part of α-helix3 and the following loop up to residue 135) was shown to be associated with the chaperone function of bacterial and yeast HSP90s (reviewed, for example, in ref.11.)

The complete α-helix3 is associated with the most open binding pocket, including the ATP binding site and the transient pocket region under α-helix3. In the distorted state (for example, cocrystallized with adenosine-diphosphate, ADP; PDB 1BYQ shown in Figure 1A), the side chains of the loop part of α-helix3 move into the binding pocket and close its transient part. There are two main conformations of the distorted α-helix3: (i) loop-in, in which residues N106–I110 form a loop that is oriented toward the pocket which has its smallest size; (ii) loop-out, in which the distorted part is shifted to residues T109-G114 of α-helix3 and the pocket is slightly larger (structures are shown in Figure 1A in blue and red, respectively). The latter conformation is observed in human HSP90 bound with ATP or ADP (PDB 3T0Z and 1BYQ, respectively). Crystal structures of the unbound HSP90 reveal that both loop-in and loop-out conformations are possible, but a conformation with a complete helix has not been observed in an apo-form.

Src tyrosine kinase is a two-domain enzyme: the N-terminal lobe (N-lobe) includes a 5-stranded β-sheet and the Ca-helix, which are connected by a flexible linker (P-loop), and the C-terminal lobe (C-lobe) comprises α-helices from the Dα-helix to the Iα-helix and several short β-strands. One of the important flexible elements of the binding site is a catalytically crucial DFG loop (D404-F405-G406) located just before the activation loop (A-loop, shown in pink in Figure 1B). Its backbone motion is accompanied by rotation of F405 by 180° (blue and red structures in Figure 1B) and causes opening of a transient subpocket behind the ATP binding pocket (DFG-out), which has been used for inhibiting tyrosine kinases (so-called “type-II” inhibitors). Several computational studies of the DFG loop motion have been reported for different tyrosine
Figure 1. Four proteins used for the method validation. Left: Crystal structures of the four evaluation proteins, illustrating the variety of conformational changes observed. Right: The most flexible parts of the binding sites and parts often missing in crystal structures are shown in blue and pink, respectively; the rest of the protein structure is shown in gray cartoon. The residue numbering is as in the PDB files: 1UYD, 3U4W, 1M47, 1A28 for HSP90, Src, IL2, and PR, respectively. (A) HSP90: PDB structures 1UYD, 1BYQ, and 1YER are shown in blue, red, and blue; α-helix3 is unstable, its middle part transforms into a flexible loop 75−79 and the rotation of F78. (B) Src kinase: PDB structures 3L4W and 3G6H are shown in blue and red. The main structural changes observed include motion of two beta-sheets (in particular of the K295 side-chain), P-loop, Cα-helix, DFG loop (F405 moves to the center of the ATP binding pocket opening a hidden subpocket), and A-loop which may adopt a helical form. (C) IL-2: PDB structures 1M48, 1M4b, 1M47, 1PW6, 1NBP, are shown in blue, green, orange, magenta, and gray, respectively, along with a ligand from 1NBP and 1PW6 shown with carbon atoms in black and magenta, respectively; the protein binding site closes/opens by rotation of the side chains of F42, R38, L72. Additionally, an adjacent transient pocket opens due to the motion of the flexible loop 75−79 and the rotation of F78. (D) PR: PDB structures 3ZRA, 2OVH, and 1A28 are shown in blue, red, and gray, respectively, along with a ligand from 1A28. Displacement of the α-helix9 in the structure 2OVH does not affect the shape of the binding site, which is deeply buried. Structures were rendered in UCSF Chimera.

kinases. In P38 MAP kinase, the transition between the DFG-out and -in loop conformations has been observed in a 60 ns trajectory of explicit solvent MD simulations carried out at 1000 K\(^1\) and using accelerated MD driving the transition from the DFG-in to the DFG-out orientation.\(^13\) In insulin receptor kinase, DFG loop motion initiated by temperature accelerated MD was achieved in a 47 ns trajectory.\(^13\) In standard explicit solvent MD simulations on an Anton computer, the DFG-in–out transition has been observed in the Eph receptor after 32 μs of simulations.\(^14\) Besides the DFG loop motion, displacement and partial distortion of beta-sheet elements including the P-loop, are observed in crystal structures (shown in blue in Figure 1B, right panel) and cause additional changes of the binding pocket. Displacement of the Cα-helix, that presumably also contributes to the protein motion required for transition between DFG-in and DFG-out loop conformations, was also observed in simulations.\(^14\)

The cytokine hormone interleukin-2 (IL-2) is an example of a target with a solvent-exposed protein–protein binding site that is highly adaptive due to the flexibility of several side chains around R38, F42, and L72 (see Figure 1C). Multiple openings of the binding pocket have been observed in a 10 ns MD trajectory.\(^7\) Additionally, crystal structures show some conformational changes outside the main binding site, including partial unwinding of α-helix1 and motion of the loops formed by residues Y31−K35 and S75−R81 (shown in pink in Figure 1C). The latter loop motion together with rotation of the P78 side chain leads to closure/opening of a deeply buried transient pocket (adjacent to the protein–protein binding site) detected in titration experiments in ref 15 (the ligand, shown with carbon atoms in black in Figure 1C, is covalently bound to Y31C in the mutant structure PDB 1NBP). In this study, cooperative binding of one ligand to the protein binding site and another ligand to the transient pocket behind the loop D74-R81 was reported. As the pocket shape depends on the loop motion, one should expect that longer MD simulations might be required for its opening.

The human progesterone receptor ligand binding domain (PR) is a target for hormone therapy. The structure of the binding site is conserved and has the typical nuclear receptor fold.\(^16\) Though large-scale displacement of the α-helix outside the binding site has been observed in crystal structures (see structure colored in wheat in Figure 1D), variations of the binding site itself are quite modest and arise mainly due to side-chain rotation. Therefore, we have used PR to examine robustness of the proposed method with respect to the possible overestimation of the binding site distortions initiated by perturbation.

In the present study, we first compared the performance of the L-RIP approach with the original RIP method, using HSP90 and Src as test examples. We also explored the longer time scale dynamics of α-helix3 in HSP90, and motion of the DFG motif in the Src tyrosine kinase protein by performing short (up to 10 ns) explicit solvent MD equilibrium of excited structures generated in L-RIP simulations. Then, we compared binding site flexibility and transient binding pockets predicted using L-RIP and RIP simulations for the four test proteins with the results obtained from conventional 100 ns explicit solvent MD simulations and with the pockets observed in crystal structures. We demonstrate that the L-RIP and RIP simulations provide computationally efficient ways to explore binding site flexibility and to identify transient binding pockets.
2. METHODS

2.1. Protein Structure Preparation and Standard MD Simulations. The reference coordinates for starting the simulations were obtained from the following crystal structures in the Protein Data Bank: HSP90, 1UYD; Src, 3U4W; IL-2, 1M47; and PR, 1A28. Ligands and water molecules were removed. Missing loops (residues 75−76 and 99−102 in IL-2) were built using the Maestro software, version 9.4. 17 Hydrogen atoms were generated in the standard protonation state at pH 7 using the NAMD package. 18

Conventional explicit solvent MD simulations were performed using the NAMD software 18 and the CHARMM 27 force field. 19,20 The protein was solvated in a box of TIP3P water molecules extending at least 10 Å beyond the protein surface with periodic boundary using the VMD program; 21 counterions were added for system neutralization. A cutoff of 10 Å and the particle-mesh Ewald (PME) method were used for computing non-electrostatic forces and long-range electrostatics, respectively. A time step of 2 fs was employed and all bonds to hydrogen atoms were constrained using the SHAKE algorithm. 22 The system was minimized in 1000 steps and then gradually heated up to 300 K with a step of 25 K over 200 ps. Simulations were performed under NVT conditions with the Langevin thermostat (friction coefficient of 1 ps$^{-1}$) employed for temperature control. Trajectory data were written at 1 ps intervals in all simulations.

2.2. Definition of the Binding Site. For the proteins HSP90, Src, and PR, the position of the ligand in the reference protein crystal structure was used to define the binding pocket. Although more crystal structures are available for these proteins, we chose not to use them for the pocket definition for validation of the L-RIP and RIPlig procedures as, for many applications, only one reference structure with a ligand bound will be available. For IL2, however, we demonstrate how more than one reference crystal structure can be used by considering three different ligands, each bound to different transient pockets (from PDB structures 1M4A, 1NBP, and 1M48): the three holo-structures were aligned using their heavy atoms; the ligands were extracted and combined into one artificial molecule that was used later for defining the binding site in IL-2. All residues whose atoms were located within a threshold distance from any ligand atom were assigned to the binding site. The threshold distance (5.5−7.0 Å) was defined to be large enough to ensure that the complete active site is included in the simulation region, with the smallest value of 5.5 Å being used for the buried pocket in PR (the binding site residues for the four test targets are visualized in Figure S3; details of the setup of the L-RIP and RIPlig simulations are given in Table S1).

2.3. RIP and L-RIP Methods. The RIP method reported in ref 9 was implemented as a Python script (available at http://bosch.ch/rip/) using standard packages (in particular AMBER 23) for performing implicit solvent MD simulations. Here, we only give a short overview and details related to the implementation and modification of the method in the present study. Specifically, we modified the script in order to employ the freely available NAMD 2.9 software 18 with the generalized Born implicit solvent model (GB/SA) 24 and the CHARMM27 force field; 19,20 all the major MD simulation parameters and simulation procedure were kept the same. During the preparation step, the starting structures were energy minimized, gradually heated to 300 K, and equilibrated first under constant energy conditions and then with Langevin dynamics in NVT ensemble (damping coefficient of 10 ps$^{-1}$; each equilibration step is of 20 ps). The RIP simulation procedure consists of a set of perturbation pulses. In each pulse, the total kinetic energy of a single residue in the binding site with a rotatable side chain was applied to the rotational degrees of freedom of the torsion angle only. Then a short implicit solvent MD run of length 0.1 ps was performed to let the excess kinetic energy in the side-chain rotation be transferred to nearby residues. In the RIP method, 9 the MD simulations were performed under constant energy conditions, while in the L-RIP simulations, a Langevin thermostat (damping coefficient of 1 ps$^{-1}$) is employed. This allows us to increase the MD relaxation step from 0.1 ps (employed in the original RIP) to 0.3 ps or even longer without protein heating, which enables a larger conformational space to be sampled and short-time fluctuations to be damped out. The number of perturbation pulses can also be extended from the 100 used in RIP to 300 or even 1000, which enables slower motions to be explored. The last snapshots of each pulse were combined to make a RIP/L-RIP trajectory for further analysis. This is different from the original RIP procedure, 9 where only a single final snapshot from each trajectory was employed to explore protein flexibility. The L-RIP perturbation procedure was applied to each rotatable residue of a protein binding site, thereby providing us with a set of perturbation trajectories for each test protein.

2.4. RIPlig Method. In the RIPlig method, the perturbation was applied to an artificial molecule (referred to hereafter as a pseudoligand; we employed a phenylalanine, PHE, residue with H atoms capping the backbone atoms). The procedure was repeated several times, each time with the pseudoligand initially placed in a different part of the binding pocket. A protocol for selection of the pseudoligand position and orientation was designed to ensure that the perturbation is applied to different regions of the binding site (section S2). Consequently, each RIPlig trajectory reveals the flexibility of a particular region of the binding site (this is demonstrated in Figure S4), while a full set of RIPlig trajectories enables the complete binding site to be sampled.

In the RIPlig procedure, energy minimization and equilibration were carried out for the pseudoligand-protein complex as for the original RIP procedure described above. The backbone atoms of the pseudoligand were restrained with a harmonic potential with a force constant of 20 kcal mol$^{-1}$ Å$^{-2}$ to prevent the pseudoligand from moving out of the pocket. If the perturbed pseudoligand had close contacts with the atoms of a highly mobile part of the binding site, the pseudoligand tended to move away from the protein, resulting in less perturbation in subsequent pulses. For this reason, Langevin dynamics was not used as it would further diminish the perturbation effect. Accordingly, a 0.1 ps MD relaxation step under constant energy condition was employed in RIPlig as in the original RIP procedure.

2.5. Computation of the Pocket Shape and Identification of Transient Pocket Regions. For the identification of transient pocket regions, the generated structures were superimposed on the reference structure using only the backbone atoms of the binding site. Then the TRAPP (transient pockets in proteins) 25 protocol was employed to compute the pocket shape and identify transient regions of the binding pocket. The pocket shape was described by a distribution function mapped onto a 3D cubic grid (a grid spacing of 0.75 Å) centered on the pocket and accommodating all the binding site residues. Grid points were assigned either to
the pocket or to the protein or to the bulk solvent using the procedure described in ref 25.

To identify transient pocket regions, we did not use all snapshots of the generated MD or L-RIP/RIPlig trajectories, as this would require multiple simulations of very similar pocket conformations. Instead, we employed clustering of the generated structures by similarity of their binding site conformations (see details of the clustering procedure in section S3). Representative structures for each cluster were combined in an ensemble that was then used for detection of transient pocket regions. For each structure in an ensemble, the transient pocket regions were defined as protein cavities that appear or disappear relative to the position of the binding pocket of a reference structure (i.e., a grid point was assigned a value of 1 if the point belongs to a pocket in a structure, but is inside the protein in a reference structure, and a value of −1 for the opposite situation). Then a transient pocket distribution in an ensemble of structures was obtained by averaging of transient regions over all conformations in an ensemble, which roughly defines the fraction of structures in which a particular pocket region is found, but does not exist in the reference structure (or vice versa).

3. RESULTS AND DISCUSSION

3.1. Effect of Langevin dynamics: Comparison of RIP and L-RIP Methods. To examine the effect of the Langevin damping on the RIP simulation results, we assessed the RIP and L-RIP procedures using two test targets: Src kinase and HSP90, both characterized by backbone transitions between different conformations of their binding sites. Specifically, we focused on the DFG loop motion in Src kinase starting from the DFG-in conformation (PDB 3U4W) and the distortion of α-helix3 in HSP90 starting with the complete α-helix3 (PDB 1UYD). As noted in the Introduction, in the both cases, conformational transitions are expected to occur on the μs time-scale or longer, although there are some structural elements with shorter time scale motions.

For clarity, we consider for each target only RIP/L-RIP trajectories generated by perturbing one residue that reveals pronounced mobility of the structural elements analyzed. To explore the effect of the length of the MD relaxation step on the range of conformations sampled, two trajectories (each consisting of 300 perturbation pulses) were generated by each method (RIP/L-RIP). These had MD simulation times of either 0.1 ps (as in the original RIP procedure9) or 0.3 ps in each pulse, referred to as (L-)RIP-0.1 ps and (L-)RIP-0.3 ps, respectively, hereafter. The backbone RMSD variations along the corresponding RIP and L-RIP trajectories are illustrated in Figures 2 and 3 for HSP90 and SRC, respectively.

HSP90. For comparative analysis of the L-RIP and RIP methods, we employed perturbation of the L107 residue which is located in an unstable part of the α-helix3 and whose perturbation causes the most pronounced distortion of α-helix3 (RMSD plots for L107 and several other residues of the α-helix3 are shown in Figure S5). The protein flexibility pattern observed in the first 100 pulses of the RIP simulations is quite similar to that revealed by MD simulations (Figure 2A and E, respectively). However, during the next 200 pulses, the backbone fluctuations extend to the whole of α-helix3, leading to its unwinding (Figure 2G). Besides, the N-terminal part of α-
helix2 (residues 60–70), as well as the N-terminal region of the protein, undergo notable conformational changes. Increasing the length of the MD relaxation in each RIP pulse from 0.1 to 0.3 ps further extends distorted regions whereby the RMSD of the complete protein structure increases from 4 Å up to 6 Å after 200 pulses (shown in Figure S6). The average temperature of the protein rises from about 280 K up to 320 K after 300 pulses of the RIP-0.3 ps trajectory (Figure S1A). One can therefore conclude that the RIP simulations reveal the most flexible regions of the protein structure, but the number of perturbation steps must be limited to about 100 for HSP90 in order to keep the protein in a state reasonably close to the native folded one.

The use of Langevin dynamics in the L-RIP procedure enables the average protein temperature to be maintained along the perturbation trajectory (Figure S1B), thus reducing protein distortion as illustrated in Figure 2C,H. Increasing the relaxation time from 0.1 to 0.3 ps causes the perturbation to spread along α-helix3, giving rise to a number of different conformations of the loop part of α-helix3, while keeping the fluctuations of the rest of the protein within the values observed in standard MD simulations (Figure 2D,E). The total RMSD of the protein structure is maintained within 2–2.5 Å (in contrast to 4–6 Å in RIP simulations, see Figure S6). The pattern of the flexible regions observed in L-RIP and the extent of conformational changes are, in general, very similar to those revealed by MD simulations, only the mobility of the α-helix3 is essentially missing in the MD trajectory. This result demonstrates that, in contrast to RIP, different time-scale motions can be successfully combined in one L-RIP trajectory without extensive artificial distortions of loosely packed elements of the protein structure.

**Src Kinase.** In L-RIP simulations with a 0.3 ps MD relaxation step, the motion of the F405 side-chain, particularly flipping from DFG-in to DFG-out conformations, was observed upon perturbation of F405 itself (perturbation trajectories for neighboring residues show only a minor effect on the F405 orientation as illustrated in Figure S7). Therefore, we used F405 perturbation trajectories for assessment of the RIP and L-RIP methods.

RIP perturbation of F405 with a short 0.1 ps relaxation results mostly in distortion of the Cα-helix and mobility of the P-loop with the first and second β-strands (see Figure 1B), which appear to be flexible in MD simulations as well (Figure 3A, E). Increasing the relaxation time in each pulse to 0.3 ps leads to some movement of the F405 side-chain, but at the same time causes significant distortion of the protein structure: unwinding of the Cα-helix as illustrated in Figure 3G (colored in green), a hinge motion of the N- and C-lobes that leads to a displacement of the Fa-helix (shown in pink in Figure 3G), and the overestimated mobility of the β-strand and P-loop (residues A259-S303, whose RMSD values exceeded 13 Å, Figure 3B). On the other hand, the applied perturbation was not large enough for the generation of the expected flipping of F405, as can be seen from the typical conformations visualized in the inset in Figure 3G.
Employment of the Langevin thermostat in the L-RIP procedure results in reduction of protein conformational distortions for both L-RIP simulations, with 0.1 and 0.3 ps relaxation time (see Figure 3A-D; the protein backbone RMSD from the starting structure does not exceed 2.5 Å, Figure S8 A, B). Specifically, the hinge motion of the C- and N-lobes of the protein is greatly diminished (i.e., motion of the Fα-helix is negligible) and the Cα-helix preserves its structure (Figure 3H). On the other hand, in contrast to RIP and standard MD simulations, the DFG loop reveals motion accompanied by rotation of the F405 side-chain, as illustrated in the inset in Figure 3H. Thus, similar to HSP90 example, the single L-RIP trajectory of Src reveals both short-time scale fluctuations (i.e., β-sheet and P-loop, as observed in 100 ns MD) and slower conformational changes (such as the DFG loop movement).

3.2. Exploring Binding Site Conformations Using L-RIP Trajectories. In the previous section, we demonstrated that using Langevin dynamics in the RIP procedure makes it possible to increase the relaxation time in each pulse and the number of pulses, while simultaneously preserving the protein...
temperature and maintaining the overall protein structure close to its original state. The longer relaxation step increases the variety of conformations sampled. At the same time, the perturbation of the degrees of freedom that correspond to slow protein motions cannot be equilibrated in such short MD runs, and multiple perturbation steps that gradually lead the system to higher energy states along the slow motion coordinates are required. This may lead the system into alternative locally stable states that are inaccessible on the nano- or microsecond time-scale of standard MD simulations. Thus, generation of multiple L-RIP trajectories enables extensive sampling of protein conformational space, although each conformation generated is not energetically validated and therefore, most likely does not correspond to any local energy minimum.

To transfer a protein from a perturbed L-RIP conformation into a nearby locally equilibrated state, standard MD simulations can be performed. Obviously, whether an MD run enables the global or a local energy minimum of the protein to be reached depends on the length of the MD simulations and the complexity of the protein’s energy landscape. One can expect, however, that the less stable, high energy protein conformations will be eliminated faster than the locally stable

Figure 5. Simulations of the DFG loop motion in Src kinase. Distribution of DFG loop conformations mapped onto the \( \psi \) dihedral angles of D404 and F405 as observed in (A) 100 ns explicit MD simulations; (B) two L-RIP trajectories of 1000 pulses each (0.3 ps relaxation MD step after each pulse); (C) the interval between 5 ns and 10 ns of MD trajectories started from L-RIP conformations. Red triangles indicate positions of crystal structures that represent the transition of the DFG loop from DFG-in to DFG-out conformations (the PDB identifiers are given in Table S4). The insets show the orientations of D404, F405, and K295 for a representative L-RIP structure before and after equilibration and the shape of the binding pocket for corresponding conformations obtained from simulations (B, C) and observed in crystal structures with cocrystallized ligands (ball-and-stick representation) (A, C). Population densities are computed and represented as in Figure 4.
ones. Therefore, the resultant ensemble of MD trajectories starting from L-RIP conformations may provide insight into the conformational space of locally stable states and the transition pathways between them. We tested this hypothesis using several L-RIP trajectories for HSP90 and Src kinase, consisting of 1000 perturbation pulses each (by perturbation of residues L107 in HSP90 (4 trajectories) and F405 in SRC (2 trajectories). We performed up to 10 ns MD simulations

Figure 6. Illustration of protein flexibility observed in simulations and in crystal structures. Residue-wise backbone RMSD relative to the reference structure is shown for the four test targets (A) HSP90, (B) Src, (C) IL2, and (D) PR, (reference structure PDB codes are 1UYD, 3U4W, 1M47, 1A28, respectively; they are shown on the right-hand side in cartoon representation colored by increasing B-factor from blue to red) in the crystal structures (X-ray) and in the simulations: 100 ns standard explicit solvent MD trajectories and L-RIP and RIPlig simulations (see simulation details in the text). For the crystal structures, the RMSD values were computed for each structure (see the list of PDB codes in Table S3), while for the simulation results, all generated structures were clustered according to the similarity of the binding site conformations and only representative structures of each cluster were then analyzed. Accordingly, each column of the RMSD plot represents one cluster representative, while rows represent residues in the protein sequence). The most flexible protein segments observed in simulations or in crystal structures are denoted by numbers in the RMSD plots and in the protein structures.
starting from about 20 snapshots from each L-RIP trajectory selected with a constant stride. To assess the conformational changes observed in simulations, we mapped the relative density distributions of protein conformations onto the 2D space of $\psi$ dihedral angles (defined by backbone atoms N–C$^\alpha$–C$'$–N) of several residues located in the flexible region of interest. Specifically, the dihedral angles of N105, I110, and K112 from the $\alpha$-helix3 of HSP90 and N404 and F405 of the DFG-loop in SRC kinase were used. The distributions generated from 100 ns MD trajectories, L-RIP simulations, and after equilibration of the L-RIP structures with standard MD simulation are shown in Figures 4 and 5, and compared to diverse crystal structures.

HSP90. In the crystal structures with an unperturbed $\alpha$-helix3 (indicated by yellow triangles in Figure 4), all three $\psi$ dihedral angles are about $-50^\circ$, while the conformations of the distorted $\alpha$-helix3 are characterized by larger values of two $\psi$ dihedral angles: N105/I110 in the case of loop-In conformations, or I110/K112 for loop-out conformations (the positions

Figure 7. Transient binding pocket regions detected in the four proteins. In panels A–D, a reference structure and a cross-section plane are shown for each target, and in the lower rows, the cross-section of the opening and closing transient regions (color variation from yellow/cyan to red/blue indicates increasing number of structures in which a particular pocket is open/closed) are shown along with the protein cross-section, as observed in crystal structures (E-H), obtained in explicit solvent MD (I-L), RIPlig (M-P), and L-RIP-0.3 ps (Q-T). Arrows indicate positions of transient binding pockets. The protein van der Waals surface is shown in gray, the structure in cartoon representation, and the ligand bound is shown with carbon atoms colored green. Structures were rendered in UCSF Chimera.
of loop-in and loop-out conformations are indicated in Figure 4 by red and blue triangles, respectively.

As can be seen from Figure 4A, the helical orientation of the three selected residues is retained in the 100 ns MD simulations (and even in 0.4 μs MD, see Figure S2). In contrast, the first 300 pulses of L-RIP-0.3 ps simulations readily result in an increase of the N105 dihedral angle and then, starting from about 400 pulses, α-helix3 undergoes distortions in the region of the N105–I110 residues, forming perturbed states that are similar to the loop-in conformation in the space of the three dihedral angles (Figure 4B). Interestingly, the loop-out conformation, which is associated with flipping of the K112 side-chain (see Figure 1A), was not observed as K112 seems to be insensitive to the L107 perturbation. Additional analysis, however demonstrated that motion of K112 can be initiated by perturbation of several residues outside the binding pocket, particularly those located at the N-terminus of the α-helix3 (including K112 itself) and the rest of the ATP-loop (residues 111–138; see Figure S9A). This result is consistent with the mobility of HSP90 observed in bacterial and yeast structures, where α-helix3 can be divided into two regions whose motion is practically uncorrelated: N-terminal, which remains stable, and C-terminal, which belongs to the ATP loop and undergoes complete unfolding during the ATPase cycle, see Figure S9B.

As expected, a subsequent 10 ns explicit solvent MD equilibration of the L-RIP structures reduces the diversity of conformations, making the conformational distribution more compact (Figure 4C). Specifically, a high population of conformations that are close to the loop-In structure is formed, though many perturbed structures tend to be restored back to the helical conformation of α-helix3. A possible explanation of the latter effect is that the CHARMM 2.720 force field employed may introduce some bias toward the helical conformation.

One of the generated structures that clearly converges to a loop-in conformation is displayed in the inset to Figure 4C (the corresponding per-residue RMSD from the loop-in conformation is shown in Figure S10). Although the backbone RMSD of the α-helix3 between L-RIP and the loop-in crystal structure is reduced from 4.2 to 2.6 Å, some deviation from the loop-in crystal structure is still observed after 10 ns of the MD equilibration. This might be caused by the roughness of the energy landscape and by additional stabilization of the loop region of α-helix3 in the loop-in conformation due to direct crystal packing contacts between α-helix3 and α-helix2 (particularly between D102 and Y61).

Src Kinase. Similarly to the HSP90 analysis, we mapped the relative density distribution of the DFG-loop conformations in Src kinase onto the 2D space of the ψ dihedral angles of D404 and F405 (Figure 5). Points in this space that correspond to 16 crystal structures with DFG-out and DFG-in conformations are shown by triangles. In the 100 ns standard MD trajectory started from the PDB structure 3U4W, the DFG-in state is quite well preserved during the whole simulation (Figure 5A). In the first hundreds of L-RIP perturbation pulses, however, D404 and F405 rotate (Figures 5B and S11). Increasing the number of perturbation pulses initiates not only further mobility of the F405 side-chain, but also breaking of the D404 and K295 salt-link, which leads to formation of DFG-out loop conformations, whose population increases with the number of perturbation pulses applied (see Figure S12).

Curiously, after 10 ns of MD equilibration, the conformational distribution becomes more widely spread over the D404 ψ dihedral angle when F405 is in the DFG-in orientation (∼150°). This is due to strong interactions between the K295 and D404 side chains, which keep the K295 orientation restrained while the DFG loop, specifically the F405 side-chain, adopts different orientations (as shown in Figure 5C and Figure S11). This indicates the complexity of the corresponding energy landscape. In contrast, the orientation of the K295 and D404 side chains in the DFG-out orientation is well-localized, which agrees with the compact distribution of DFG-out dihedral angles observed in crystal structures (Figure 5).

3.3. Benchmarking of the L-RIP and RIPlig Approaches on the Four Test Proteins. We performed L-RIP and RIPlig simulations with 0.3 and 0.1 ps MD relaxation steps, respectively, and 300 perturbation pulses in each trajectory for all four test proteins, considering this as a basic framework for fast exploration of the flexible elements in a binding site. The number of trajectories generated for each protein depends on the size of the binding site in L-RIP and on the pocket size in RIPlig (see Table S1 and Figure S2). Additionally, 100 ns standard MD simulations were carried out for each target starting from the same reference structures as used in perturbation MD simulations.

All frames in a simulated trajectory were clustered according to the binding site similarity (details of the clustering procedure are given in section S3). Residue-wise backbone RMSD values for each cluster representative relative to the starting structure are shown for each target and simulation method used in Figure 6, along with a similar plot for a set of crystal structures.

Finally, the same set of representative conformations for each target was employed for computation of the binding pocket variations and for identification of transient pocket regions. To visualize the detected transient regions, we selected a plane for each protein that crosses the most important transient regions (see Figures 7A–D) and represented pocket occurrence in this plane by color (yellow to red for rarely to most often opening pocket regions, and cyan to blue for rarely to most often closing regions) in Figures 7E–T. This view obviously cannot fully represent pocket similarity in the 3D space, but gives an overview of the positions of the transient pocket regions detected.

HSP90. RIPlig and L-RIP simulations, starting from a conformation with a complete α-helix3 (PDB: 1UYD), yield two main regions of the binding site with high flexibility (see Figure 6A): (1) The middle part of α-helix3 that is partially transformed into a loop in several structures generated by L-RIP, and also strongly distorted in RIPlig simulations, though without a clear helix-loop transition. In the MD trajectory only backbone fluctuations in the neighborhood of L107 indicate that such motion might be feasible on a longer time scale. (2) The ATP-lid that comprises the C-terminal part of α-helix3 and the following loop (residues 112–138). Perturbation MD simulations reveal notably higher flexibility of the ATP-lid than the standard MD simulations and the crystal structures of human HSP90. Notably, unfolding of the ATP-lid has been shown to be a prerequisite for the ability of yeast HSP90s to form a protein binding face for the substrate during the ATPase cycle. Therefore, the high flexibility of the ATP-lid predicted in the perturbation simulations indicates that the dynamics and structural properties of the human HSP90 might be similar. This suggestion is in agreement with the results of experiment,27 in which a high similarity in the underlying enzymatic mechanism of human and yeast HSP90 was observed. The regions (3) C-terminus of α-helix2 and (4) α-helix1, do not

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directly affect the binding pocket; they are flexible in all simulations and have quite large temperature factors in the crystal structures, although they display little variation in conformation.

Distortion of α-helix3 leads to partial closing of the binding pocket in HSP90. Specifically, the transient subpocket beneath α-helix3 presented in the reference structure was identified as a disappearing transient region in experimental as well as in simulated structures (indicated by the arrow in Figure 7A, E, I), with the smallest transient region identified in standard MD simulations. In addition to the transient pocket, the ATP pocket is partially closed in some snapshots due to the flexibility of the ATP lid and side-chains of α-helix2. In contrast, in crystal structures the shape of the ATP pocket seems to be very stable, possibly because in the majority of structures it is occupied by ligands.

Src kinase. One can distinguish at least six mobile regions of Src kinase indicated in Figure 6B. Four of them belong to the binding site and are observed in crystal structures and in perturbation MD simulations: (1) DFG and A-loop; (2) a part of the beta-strand with a key residue K295 that makes a salt-link with DFG loop residue D404, and P-loop; (3) a hinge loop Y340-G344 between the C- and N-lobes with residue M341 pointing to the pocket; (4) Cα-helix, residues P304-L317. As has been discussed above, motion of the DFG loop, presumably the slowest motion in the Src structure among those mentioned above, is observed upon L-RIP perturbation of F405, though the complete flipping of F405 from DFG-in to DFG-out positions is not always achieved in a 300 pulse L-RIP trajectory. In the RIPlig simulations, F405 changed its orientation without notable movement of the backbone when a pseudoligand was placed directly behind the DFG loop (see Figure S4). In standard MD, the mobility of the DFG loop is essentially missing.

The largest RMSD values in the region (1) for crystal structures in Figure 6B correspond to residues of the mobile A-loop that are missing in many crystal structures (and were modeled for the analysis). In the reference structure, (PDB 3U4W), the A-loop is folded into a short helix (see Figure 1B) that is very weakly coupled with the rest of the protein structure, which explains its low sensitivity to the perturbation of the binding site residues. Accordingly, no clear correlation between the A-loop and the binding site conformations (particularly, the position of the DFG loop) is observed in the crystal structures. Additionally, fluctuation of the regions 5 (G453-L360) and 6 (V467-G476), located outside the binding site, were observed in all simulations. These elements have quite large B-factors but little variation in conformation in the crystal structures.

The first three mobile regions of the binding site mentioned above manifest themselves as transient regions in the binding pocket simulations: regions 1 and 3 in Figure 6 are denoted in Figure 7B, F by DFG-loop and M341, respectively, while protein motion in region 2 gives rise to two transient pocket regions, labeled K295 and P-loop in Figure 7 B,F. Displacement of the Cα-helix, (region 4) affects the pocket shape as well, but the corresponding transient region cannot be projected on the plane used for visualization in Figure 7. Analysis of binding pocket fluctuations in the standard MD trajectory shows only two transient regions, arising from the side-chain rotation (particularly K295) and motion of the P-loop (Figure 7J). The same regions are observed in L-RIP and RIPlig trajectories (Figure 7N, R). Additionally, a part of the transient subpocket behind the DFG loop is detected in RIPlig and L-RIP trajectories, though complete opening of the subpocket was not observed. Also, two transient regions arising from the motion of the hinge loop and rotation of residue M341 are detected in general agreement with the crystal structures.

IL-2. For simulation of the binding site flexibility in IL-2, we used a structure (PDB 1M47) where the adjacent buried binding pocket (denoted as I in Figure 7G, see also Figure 1C) was partially closed by the G74-L80 loop and the F78 side chain. Perturbation simulations reveal high flexibility of the loop and opening of the corresponding transient pocket region, while in the standard MD trajectory it is permanently closed by the F78 side-chain (see Figure 7, O, S, and K).

The solvent exposed binding site (residues K35—Y45; region 2 in Figure 6 C) changes its conformation mainly due to side-chain rotation. This kind of protein flexibility is not clearly pronounced in the backbone RMSD plot, but manifests itself as transient pocket regions in the X-ray structures (Figure 7G, pocket denoted by number 2) as well as in all simulations (Figure 7, K, O, S). Both the RMSD plot in Figure 6 and the detected transient pockets illustrated in Figure 7 show that the backbone mobility and therefore transient pocket size is somewhat overestimated in perturbation simulations and underestimated in standard MD simulations relative to the crystal structures. Two further flexible loops that do not belong to the binding site (denoted as 3 and 4 in Figure 6) are observed in the crystal structures and simulations.

PR. The binding pocket in PR is buried and, in the majority of crystal structures, only two flexible regions of the binding site are observed, which arise from rotation of the M909 and F794 side-chains located in α-helix9 and α-helix5, on opposite sides of the binding pocket (see Figure 1C). However, there are several mobile elements observed in the PR crystal structures that are adjacent to the binding site of cocrystallized ligands (denoted as regions 1–3 in Figure 6D). Specifically, in some structures a part of α-helix5 and α-helix6, (residues G778-Y795, region 1 in Figure 6D), is displaced or distorted (see Figure 1D); α-helix9 (R900-A922, region 2) is completely displaced (with its part missing in PDB 2OVH shown in Figure 1D or PDB 1A52); α-helix2 (region 3) is mobile, showing displacement by several Å. All these regions have high B-factors in the reference PDB structure and are successfully identified by perturbation MD simulations as shown in Figure 6D. Their mobility, however, is not observed in 100 ns standard MD simulations since it arises from slow backbone motion and distortion of the secondary structure of the protein.

Two flexible regions of the binding site mentioned above (from rotation of the M909 and F794 side chains), appear as transient subpockets (Figure 7H, denoted as regions 1 and 2) with all sampling methods, albeit with some shifting in the position of the subpockets because of the slight displacement of α-helices during equilibration in both explicit solvent (standard MD) and implicit solvent (L-RIP, RIPlig) MD simulations. Both transient regions identified from the standard MD trajectory (Figure 7L) arise from side-chain fluctuations and are notably smaller than in the X-ray structures and in perturbation simulations. Interestingly, L-RIP and RIPlig reveal two additional flexible regions merged with the region 2 (denoted by 3 and 4 in Figure 7T). These transient regions do not appear in the crystal structures analyzed. However, this apparent overestimation of the protein flexibility by perturbation MD methods in fact reveals a possible opening of adjacent pockets due to motion of α-helix2 and α-helix5, which is
present in some crystal structures that were not used for the transient pocket analysis because of their incompleteness. Indeed, both flexible regions 3 and 4 can be observed as small transient pockets adjacent to the ligand binding pocket in the crystal structure PDB 2OVH (see Figure S13; this structure has part of α-helix3 missing).

4. CONCLUSIONS

In the present work, we introduce and evaluate the applicability of two perturbation MD methods that provide computationally efficient and inexpensive ways to explore protein binding site flexibility.

For all test targets, the simulation results demonstrate that the proposed L-RIP and RIPlig procedures reveal all flexible elements of the binding sites observed in experimental structures, although they are not equally well pronounced. The same is true for the positions of the transient pocket regions identified, which demonstrate very good correlations with those identified in crystal structures. For all test proteins, the computational time required to reveal binding site flexibility is less than 7 ns of MD simulations if the L-RIP method is used and several times less for RIPlig (see section S4). In contrast, in 100 ns standard explicit solvent MD simulations, only side chain fluctuations and the motion of loosely bound elements in protein structures are observed, while the long-time scale backbone motion or distortions of the secondary structure are not sampled.

A further important advantage of both proposed protocols is that they do not require a priori knowledge of system behavior, which often appears to be a bottleneck for enhanced MD methods.28 We have demonstrated that the same protocol is applicable for different types of proteins, and that it can be used to explore pocket variations arising from elements of protein structure characterized by quite different degrees of flexibility—from large-scale backbone motions to side chain rotations.

Additionally, we have demonstrated with the examples of HSP90 and Src kinase that the L-RIP procedure can be used to obtain insights into the conformational space of the binding site and not just to find regions of high flexibility as the original RIP method was designed to do. Starting standard MD simulations from perturbed (high-energy) states makes the sampling of the energy landscape much faster than in simulations started from the global energy minimum, although complete sampling of the energy landscape may not be achieved either. However, local thermodynamic minima separated by high energy barriers that are hardly accessible in conventional MD simulations can be efficiently accessed in this procedure. We demonstrated that microsecond time-scale motions, such as DFG loop motion and unwinding of the α-helix, observed in Src and HSP90 proteins, respectively, are elucidated in less than 1 ns of L-RIP simulations.

One should note here some limitations of the proposed methods. First, the conformational changes observed in RIPlig may depend on the initial placement of the pseudoligand and the pocket space available for motion of the pseudoligand. Particularly, the pocket flexibility observed in RIPlig may be overestimated for small buried pockets and underestimated for open solvent-exposed pockets. Furthermore, because of the constant energy equilibration procedure used in RIPlig, the average protein temperature may rise with increasing number of perturbations, as well as with the length of the subsequent implicit solvent MD simulations (as in the original RIP method). This makes RIPlig more useful for a rapid, though less accurate, evaluation of pocket flexibility. Then, as both the L-RIP and RIPlig approaches are based on perturbation of protein structures, they are unlikely to be able to predict folding of protein segments (for example, the rebuilding of the α-helix in HSP90). For the same reason, they provide only sampling of protein conformations, not the kinetics or thermodynamics of the protein motion. One should also take into account, that although the position of the transient pocket regions can be revealed from a single run, it is advisable to generate several perturbation trajectories for each perturbed residue if better sampling of protein conformational space is required. Finally, an overestimation of the pocket mobility cannot be ruled out because both simulation methods, RIPlig and L-RIP, provide nonequilibrated structures. To obtain the accurate conformations of a binding site required for ligand docking, additional explicit solvent MD equilibration of L-RIP structures is advisable. We demonstrated for two example cases that MD equilibration effectively stabilizes the system after perturbation simulations, by eliminating the most energetically unfavorable conformations. Importantly, L-RIP simulations combined with explicit solvent equilibration are several-fold faster than standard MD simulations to explore such conformational changes.

ASSOCIATED CONTENT

Supporting Information

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Variation of protein temperature in L-RIP and RIP simulations, procedure for the 400 ns standard MD simulations of HSP90, procedure for the placement of the pseudoligand used in RIPlig simulations, clustering procedure, computation time required for perturbation simulations, sets of PDB structures and parameters of the binding sites for the four test targets, comparison of protein flexibility generated by perturbation of different residues in HSP90 and Src, variation in RMSD of HSP90 along RIP and L-RIP trajectories, RMSD of Src kinase as observed in three different L-RIP trajectories upon perturbation of F405, RMSF of α-helix3 and the ATP lid observed in L-RIP trajectories obtained by perturbation of different residues, per-residue RMSD of the L-RIP conformation in HSP90 as observed before and after MD equilibration, α-helix3 RMSD before and after equilibration, evaluation of the Src kinase DFG loop conformations along L-RIP trajectories, and opening of an adjacent transient pocket in PR (PDF)

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Notes

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A modified RIP package including L-RIP and RIPlig functionalities is available in a command line python application that uses the NAMD package for performing MD simulations. The software is licensed under the GNU General Public License v2.0 and is freely available at http://www.mcm.h-its.org/rip-riplig/. L-RIP and RIPlig simulations are also implemented in a TRAPP (transient pockets in proteins) web server http://www.mcm.h-its.org/trapp2.

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■ ABBREVIATIONS

HSP90, human heat-shock protein 90; Src, cellular and sarcoma tyrosine protein kinase; IL-2, interleukin-2; PG, progesterone receptor ligand binding domain; RIP, rotametrically induced perturbation; L-RIP, Langevin rotametrically induced perturbation; RIPlig, rotametrically induced perturbation of a ligand

■ REFERENCES