Coupling Supervised Molecular Dynamics (SuMD) with Entropy Estimations To Shine Light on the Stability of Multiple Binding Sites

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Supporting Information

ABSTRACT: Exploring at the molecular level, all possible ligand–protein approaching pathways and, consequently, identifying the energetically favorable binding sites is considered crucial to depict a clear picture of the whole scenario of ligand–protein binding. In fact, a ligand can recognize a protein in multiple binding sites, adopting multiple conformations in every single binding site and inducing protein modifications upon binding. In the present work, we would like to present how it is possible to couple a supervised molecular dynamics (SuMD) approach to explore, from an unbound state, the most energetically favorable recognition pathways of the ligand to its protein, with an enthalpic and entropic characterization of the most stable ligand–protein bound states, using the protein kinase CK2 α as a prototype study. We identified two accessory binding pockets surrounding the ATP-binding site having a strong enthalpic contribution but a different configurational entropy contribution, suggesting that they play a different role.

KEYWORDS: Ligand–protein binding, recognition pathway, multiple binding sites, molecular dynamics, supervised molecular dynamics, enthalpic and entropic contributions, protein kinase CK2

The complex network of ligand–protein interactions is very often summarized under the expression “molecular recognition” incorporating both thermodynamic aspects (quantified by the K_d) and kinetic aspects of ligand binding (described by the rate constants k_{on} and k_{off}).¹ Nowadays, one of the most valuable powers of structural biology is in translating knowledge coming from protein structures into insights about their ligand recognition mechanisms.² In parallel, structure-based computational approaches represent now a core complement of structural biology in helping to describe “the invisible part of the binding story”.³ In fact, it is reasonably understandable that a single molecular structure cannot accurately describe the experimental values of both ligand–protein K_d and k_{on}/k_{off} . In fact, from a molecular point of view, a ligand can recognize a protein in multiple binding sites, adopting multiple conformations in every single binding site; and inducing protein modifications upon binding. Moreover, conformational and solvation/desolvation entropy contributions can regularly play significant and, otherwise, unpredictable roles.^{4,5} This complex ensemble of events contributes to making less obvious the simple concept of ligand–protein binding affinity. However, structure-based computational approaches are helping to elucidate some crucial aspects of the binding story.⁶ In particular, exploring at the molecular level all possible ligand–protein approaching pathways and, consequently, identifying all possible energetically favorable binding sites is considered crucial to depict a picture of the whole scenario of a ligand–protein binding.^{6–8} In this framework, the supervised molecular dynamics (SuMD)

technique has been developed to simulate different possible binding pathways and to analyze them in a qualitative fashion. SuMD enables the investigation of binding events in a reduced nanosecond time-scale, without introducing any energetic bias.^{9–13}

In the present work, SuMD is used to predict different alternative bound states, and subsequently, those states are characterized by deriving both enthalpic and entropic contributions, with the aim to evaluate the relative energetic stability of different binding sites. The binding of human protein Casein Kinase 2 (CK2) and ellagic acid has been chosen as a prototype study. CK2 is a ubiquitous and constitutively active serine/threonine kinase (PK) that phosphorylates more than 300 substrates.¹⁴ It is involved in the regulation of numerous cellular processes such as cycle progression, apoptosis, transcription, and viral infection.¹⁵ Known inhibitors bind to CK2 mainly in the canonical ATP-binding region, but some ligands are reported to bind alternative sites. On this basis, CK2 constitutes an appropriate case to test the ability of SuMD to individuate multiple recognition sites, and to verify if the combination of enthalpy and configurational entropy may discriminate the binding ability of the different sites.

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Figure 1. (A) CK2 α major structural regions. The catalytic α subunit is composed of two lobes connected by a small loop called “hinge region” (magenta ribbon). The N-terminal lobe (cyan ribbon) presents five β -strands and the helix α C involved in the substrate recognition. The C-terminal lobe (white ribbon) is composed of mostly α helices. Other relevant elements are reported: $\beta 4/\beta 5$ loop (purple ribbon), glycine-rich loop (green ribbon), activation segment (yellow ribbon), and N-terminal segment (orange ribbon). Secondary structure elements (SSEs) of CK2 α are labeled. (B) Representative position of ellagic acid during the last 1.2 ns of the 12 SuMD replicas (R₁–R₁₂, each replica is coded with a different color, see panel D legend). The ligand centers of mass obtained by hierarchical clustering are shown as spheres over the CK2 α surface. (C) MM-GBSA binding energy profile for 12 SuMD replicas. MM-GBSA binding energy for frames coming from final two steps for each of 12 replicas, i.e., 600 frames for each replica. (D) MM-GBSA binding energy profile against the distance of the center of mass (CoM) from the CoM of ATP-binding site residues.

The procedure consisted of the use of SuMD for exploring the potential metastable binding sites of ellagic acid on CK2. All the arisen metastable states were clustered to identify unique ones and finally, the more interesting states were characterized by focusing on the enthalpic and conformation entropic components.

SuMD of Acid Ellagic-CK2: Metastable States Detection. SuMD was used to explore possible CK2 α -ellagic acid recognition pathways starting from unbound and unbiased starting geometries. CK2 α structural elements important for the ligand recognition and protein function are summarized in Figure 1A. We started disassembling the crystallographic complex (PDB code: 2ZJW; resolution: 2.4 Å) in which ellagic

acid (2,3,7,8-tetrahydroxychromeno[5,4,3-cde]chromene-5,10-dione) binds to CK2 α in the ATP-binding region.¹⁴

The ligand was placed at around 40 Å from the protein binding site to avoid any intermolecular interaction. This distance was measured considering the centers of mass of the ligand atoms and the CK2 α binding site. Then, the system was equilibrated and 12 SuMD simulations were collected (replicas labeled R₁ to R₁₂). A summary of the replica is reported (see SI Table S1). Since SuMD simulations rely on a stochastic engine, the resulting 12 trajectories explored different regions of the simulation box (Video S1). This was evident, in particular, for the first part of the simulation and, as a consequence, it leads to pathways approaching different surface regions of CK2 α . Three of them were able to reach the ATP-

Figure 2. Comparison of conformation obtained by SuMD with three different CK2 α -ligand X-ray complex structures. CK2 α ribbon is reported in gray and corresponds to the representative conformation of three different replicas. (A) R₃ representative conformation is superposed to the complex containing ellagic acid (PDB ID: 2ZJW). (B) Ellagic acid pose R₄ with the crystallized pose of DRB (PDB: 3H30, white ribbon); two DRB molecules were found in crystal structure: DRB1 at the ATP binding site and DRB2 in the protrusion formed by leaning of the β 4– β 5 loop over N-lobe beta sheets. (C) R₁ vs CK2 α -ANP complex (PDB: 3U87); hydrogen-bonds between CK2 α and ANP are shown in dotted-line and CK2-ellagic acid (R₁) in dashed line.

Table 1. Summary of Enthalpy Results for All 12 Conformational States Explored by SuMD Simulation Replicas^a

replica #	top contributing residues	ΔH (kcal/mol) using MM-GBSA ^b				
		vdW	EEL	GB	surf.	total
replica-1	K49, Y50, K77, H154, D156, K158, N161, D175, W176, K178, A179	−27.5	−55.0	61.0	−3.7	−25.2 ± 2.7
replica-2	R47, Y50	−22.1	−23.1	36.7	−3.0	−11.8 ± 3.9
replica-3	L45, G46, R47, G48, V53, F113, E114, I116, I174	−34.9	−25.6	38.6	−4.7	−26.6 ± 3.3
replica-4	Q36, 39, K41, F54, V67, I69, P104, V101, V105, S106, A110	−28.8	−11.4	25.9	−3.6	−17.7 ± 2.3
replica-5	R191, D205, N238, Y239	−19.2	−70.0	67.0	−2.8	−25.1 ± 2.6
replica-6	D237, Y239, D266, R268, F269, I272	−15.0	−54.7	50.7	−2.5	−21.4 ± 3.0
replica-7	R47, Y50, S51, E52	−13.2	−57.4	54.3	−2.2	−18.5 ± 2.6
replica-8	K44, L45, G46, R47, N118, T119, D120, F121, H160, M163	−24.3	−10.2	25.3	−2.9	−12.1 ± 2.4
replica-9	Q36, K41, K44, E54, I69, T108	−18.9	−19.6	29.7	−3.0	−11.8 ± 2.6
replica-10	R47, G48, L49, Y50, S51, E52, I69, K71, P72, T108	−29.8	−1.0	19.0	−3.0	−14.6 ± 2.4
replica-11	L45, G46, R47, G48, V53, V66, K68, V116, N117, N118, M163, I174, D175	−32.5	−30.1	46.1	−4.5	−21.1 ± 3.5
replica-12	L45, G46, V53, V66, K68, I95, E113, N118, M163, I174, D175	−26.9	−35.6	42.4	−4.2	−24.3 ± 3.2

^aEnthalpy calculations are performed using MM-GBSA method over the last two SuMD steps, i.e., final 1.2 ns simulation trajectory. CK2 α residues found within 4.0 Å of ellagic acid and contributing more than 0.2 kcal/mol (absolute value) are reported. ^bCalculated from final 1.2 ns of the SuMD trajectory of the replica.

binding site reaching an RMSD value of 1.7 Å from the experimental structure (Figure 2A). The remaining replicas explored other surface regions possibly bumped into metastable states. To investigate the presence of metastable binding sites along the trajectories and to compare them, we performed an analysis of the energies characterizing such states. Having collected an unusual number of recognition trajectories, we first focused our attention to the last two steps (corresponding to 1.2 ns) of each replica since the simulation is concluded when the ligand is not able to get closer to the supposed binding site and usually this happens due to a certain stability that cannot be further explored by classical MD in the nanosecond time scale.

First, we pick the conformation closest to the mean center of mass ($_{\text{MEAN}}\text{CM}$) of each replica to have a simplified picture of the position of the ligand during the last 1.2 ns. The $_{\text{MEAN}}\text{CM}$ s are reported in Figure 1B. From this simple representation, it is easy to highlight the location of sites involved in such states and to clarify that different replicas can be grouped into a reduced number of binding sites. As anticipated, the mean $_{\text{MEAN}}\text{CM}$ R₃, R₁₁, and R₁₂ are located within the ATP-binding site. R₄ and R₉ $_{\text{MEAN}}\text{CM}$ lie in a pocket at the N-terminal lobe and are formed by β 1, β 2, β 3, and β 4 β -sheets. The C-terminal lobe exhibits a site within helix α G, α H, and α I where the R₅ and R₆ $_{\text{MEAN}}\text{CM}$ s are hosted. The remaining $_{\text{MEAN}}\text{CM}$, R₁, R₂,

R₇, R₈, and R₁₀ are more spread around the cleft formed by the two lobes, in the proximity of the activation segment.

It is very interesting to underline that R₄ and R₉ occupy a binding site that has been previously described¹⁶ by Raaf et al. in which a small molecule 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) bound CK2 α . In this crystal structure, two DRB molecules were found to occupy two distinct binding sites: the first one corresponds to the canonical ATP binding site, and the second one, the same site explored by R₄ and R₉, has been called “allosteric site or remote cavity” (PDB ID: 3H30). A superposition of DRB with the representative conformation of R₄ is reported in Figure 2B.

More interestingly, before reaching the ATP-binding site, the ellagic acid passes through transient interactions with the protein segment Glu230–Aps240 of the C-terminal lobe that corresponds to the region explored by R₅ and R₆. R₃ indeed represents a nice example of a transition from a metastable state toward a more stable binding site suggesting a possible connection pathway that connects such energetically favorable states (further details in SI Figure S2 and Video S2).

Enthalpic Analysis of Acid Ellagic-CK2 α Multiple Binding Sites. To investigate the enthalpy component characterizing the binding sites earlier described, all the conformations explored by the last 1.2 ns of each replica were subjected to MM-GBSA calculations. The results are

Table 2. Summary of Binding Enthalpy and Relative Configurational Entropy^a

conformation	ΔH (kcal/mol) using MM-GBSA					$-T\Delta\Delta S$ (kcal/mol)
	vdW	EEL	GB	surf.	total	
ATP-site (R_3)	−32.9	−22.1	37.3	−4.6	−22.4 ± 4.8	reference
allosteric site (R_4)	−29.3	−15.5	28.7	−3.6	−19.8 ± 4.2	4.61
conformation (R_1)	−24.6	−45.0	50.0	−3.3	−22.9 ± 3.6	15.20

^aResults for three chosen stable conformations R_3 , R_4 , and R_1 during the 5 μ s simulations for each conformation

Figure 3. (A) Contributions to configurational entropy changes $-T\Delta\Delta S$ for replica-1 (R_1) and replica-4 (R_4) conformations relative to replica-3 conformation (R_3): bonds (B), angles (A), torsions (T), six-pseudo DoFs appearing due to defining ligand relative to receptor, BAT construction (P1), $S1 = B + A + T$; bond–bond (BB), bond–angle (BA), bond–torsion (BT), angle–angle (AA), angle–torsion (AT), torsion–torsion (TT), $I2 = BB + BA + BT + AA + AT + TT$; pseudopseudo (P2), $S2 = S1 + P1 + I2 + P2$. (B) Second order configurational entropy change $-T\Delta\Delta S^{(2)}$ for states R_1 and R_4 with reference to ATP-site state (R_3).

summarized in Table 1 and Figure 1C,D. R_3 , R_{11} , and R_{12} conformations showed a strong enthalpic contribution in agreement with their localization within the ATP-binding site. Among them, R_3 showed the lowest value (−26.6 kcal/mol). It is intriguing to observe that R_1 and R_5 also showed negative values in the same range of those in the ATP-binding site. Indeed, R_5 and R_1 reached −25.1 and −25.2 kcal/mol, respectively. In addition, R_4 showed slightly lower values, −17.7 kcal/mol, as well as R_7 , −18.5 kcal/mol. Interestingly, the remaining replicas show MM-GBSA values consistent with the ones lying in the same binding site. For instance, R_{12} and R_{11} confirm the stability of the R_3 within the ATP-binding site. The enthalpic contribution of each residue involved in the binding is reported on Table S2. The picture that comes out from MM-GBSA analysis suggests the existence of a variegated set of stable and metastable binding sites. The stability profile of replicas that lie in the same binding site is consistent among them.

Configurational Entropic Analysis of Acid Ellagic-CK2 α Multiple Binding Sites. To examine more in-depth the stability of ellagic acid in the binding site around the canonical ATP binding site and which role they can play, we focused our attention on the entropic component of corresponding states. In particular, the configurational entropy of the three-selected ligand binding states R_3 (the canonical ATP binding site), R_4 (the allosteric site or “remote cavity”), and R_1 , it has been calculated according to A-MIST method¹⁷ (Experimental Procedures). Configurational entropy accounts change in flexibility of the molecular system in the bound state with reference to the unbound state. An increase in fluctuation in the bound state in comparison to a free state is entropically favorable, while any decrease opposes the bound state. Configurational entropy estimation requires large MD

simulation, in the range of microseconds and obviously much longer than SuMD trajectory. Consequently, for those selected states we collected a series of short classical MD simulation starting from the reference conformation obtained by SuMD for a total of 5 μ s each. To compare the states corresponding to R_4 and R_1 , we calculated the configurational entropy difference between each state and state R_3 used as a reference. The energy associated with this component will be indicated as $-T\Delta\Delta S$ hereafter. The results are summarized in Table 2. The first observation that stands out is about the sign and the different values that assume the term $-T\Delta\Delta S$ in the two states.

Both contributions oppose binding but with different magnitude; R_4 (allosteric) and R_1 are respectively associated with 4.6 and 15.2 kcal/mol. It should be noted that this remarkable difference (three times) is obtained by reference to those states of R_3 to help understand the underlying importance of the configurational entropy contribution in discriminating the stability of ellagic acid in such binding sites. A more complete picture can be argued considering the contributing terms (Figure 3A) of configurational entropy and from the per-residue $-T\Delta\Delta S$ decomposition (Figure 3B and SI Figure S1). It is interesting to note that MM-GBSA component was recalculated on those 5 μ s long simulations (Table 2) resulting in values consistent with those arising from 1.2 ns of the last two steps of SuMD (Table 1).

Configurational Entropy Analysis for Conformation R_1 . In the state R_1 , bonds and angle DoFs (hard degrees of freedom) fluctuate less than torsion DoFs (soft degree of freedom); this fact is evident from configurational entropy changes, and bonds, angles, and torsions DoFs, respectively, contribute by −0.23, 1.21, and 14.58 kcal/mol. No remarkable difference can be ascribed to the difference in translational and

rotational freedom, which contributes 2.97 kcal/mol (Figure 3A). Since R_3 (the canonical ATP binding site) cavity volume is larger than ellagic acid, the ligand can retain its rotational and translational freedom to a larger extent, in comparison to R_1 state, where the ligand is involved in strong electrostatic interactions with CK2 α . Moreover, correlations among DoFs, which tend to decrease the effective configurational entropy value of first-order estimate (using SI eq 6 assumes DoF motions are uncorrelated). The changes in correlations of DoFs in two states contributes -3.32 kcal/mol to entropy. Finally, total second order configurational entropy (SI eq 9) of the state R_1 relative to state R_3 (SI eq 2) comes to be 15.20 kcal/mol. N-lobe residues (1–114) contribute 0.60 kcal/mol indicating that flexibility in N-lobe region for the R_1 state has not changed much from R_3 state, but 12.08 kcal/mol entropy contribution from C-lobe residues (121–332) strongly oppose binding entropically. P-loop residues (46–50) contribute -1.47 kcal/mol favorable entropy, while $\beta 4$ – $\beta 5$ loop (103–109) strongly opposes binding with entropy value 4.65 kcal/mol.

Configurational Entropy Analysis for Conformation

R4. Soft degrees of freedom (torsions), here again, emerge as major entropy contributors with a value of 8.60 kcal/mol to the total first order entropy 9.21 kcal/mol. In R_4 state, ellagic acid is having a similar degree of rotational and translational freedom, and hence contribution from pseudo-DoFs is only -0.01 kcal/mol (first order), see Figure 3. Ellagic acid is a rigid planar molecule and thus contributes less than 0.1 kcal/mol in both R_1 and R_4 states relative to R_3 . N-lobe residues (1–114) contribute -9.47 kcal/mol to entropy favoring binding in the R_4 state relative to the R_3 state, with alone P-loop residues (46–50) contributing -3.47 kcal/mol. However, the favorable contribution of N-lobe residues is outweighed by the opposing 14.76 kcal/mol entropy contribution made by C-lobe residues; hinge region (115–120) and activation segment (176–199) residues make nominal favorable entropy contributions with values -0.49 and -0.27 kcal/mol, respectively.

In conclusion, we have presented a novel application to evaluate the stability of metastable binding sites by coupling SuMD as a computational tool to explore, from an unbound state, energetically favorable approaching pathways and entropy calculation to refine the relative energetic stability of multiple binding states that were not exhaustively described by only the enthalpic point of view. We use the protein kinase CK2 α as a prototype study. In fact, this kinase presents at least two distinct ligand binding sites as experimentally demonstrated by X-ray crystallography and, consequently, represents a valuable key study to test the capability of SuMD in identifying them *ab initio*. As described, the proposed approach not only was able to recognize the two expected ligand binding sites but it was also able to identify other possible sites, all accessible from an energy point of view by the specific ligand. Interestingly, we also observe in certain cases possible transitions connecting metastable states. However, to energetically discriminate the relative stabilities of this ensemble of putative ligand binding states, an enthalpic and entropic (configurational entropy) characterization has been carried out. In this specific case study, it has been computationally inferred that the crystallographic binding state represents the state where both enthalpic and entropic factors favorably converge to determine the binding event. Interestingly, the coupling of the SuMD approach for the identification of possible ligand binding sites with the

calculation of thermodynamic parameters of the most relevant ligand binding state represents a potential starting point for characterizing accurately and in its entirety the recognition process between a ligand and a protein.

EXPERIMENTAL PROCEDURES

The procedure for the preparation of CK2 α -ellagic complex and the details for the SuMD simulation are reported in SI S1.

The last 1.2 ns of each SuMD replica were analyzed in terms of the average center of mass of the ligand and MM-GBSA interaction energy, computed with the MMPBSA.py¹⁸ tool (see SI S2 for further details).

Three SuMD final states were used as a starting point for three 5 μ s-long MD simulations (see SI S1 and S3 for further details). These simulations were analyzed in terms of MM-GBSA and configurational entropy, using a modified version of the MIST¹⁹ (see SI S3 for further details) method. In particular, the so-called Neighbor Approximated MIST (A-MIST)¹⁷ was used (see eq 1):

$$S_{\text{MIST}_2}^{N_b(C)}(r) = \sum_{i=1}^n S_1(r_i) - \max_{(i,j) \in N_b(C)} \{I_2(r_i; r_j)\} \quad (1)$$

A-MIST calculates the mutual information (MI) (see SI S3.1) only for those pairs of degrees of freedom (DoFs) whose smallest average distance among the constituent atoms is less than a chosen distance cutoff. In other words, MI is calculated only for the pairs of DoFs whose constituent atoms belong to a neighborhood $N_b(C)$ of a defined cutoff distance, C . The distance cutoff-based neighborhood can be constructed using the expression

$$N_b(C) = \{(r_i, r_j): \exists d_e(a_x, a_y) \leq C; x \in ar_i \& y \in ar_j\} \quad (2)$$

where ar_i and ar_j are atoms involved in DoFs r_i and r_j , respectively, and $d_e(x,y)$ is the mean Euclidian distance between atoms x and y during the simulation (see SI Figure S3).

The A-MIST configurational entropy with a 14 Å was computed compared with MIST (see SI Figure S4) and deconvoluted in a per-residue scale as reported in SI S4.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00490.

Supporting data, schematic illustration of A-MIST, and details of methods (PDF)

Pathways taken by ellagic acid from initial position 40–50 Å distant from ATP-binding site of CK2 α to reach the representative conformation of replica, as captured in each of twelve SuMD simulation replicas is shown, for video frames are rendered every 10 ps of actual simulation time. All the trajectories obtained from all replica are superposed to state R_3 over backbone atoms of the receptor. Ellagic acid molecules are colored according to the color used for a replica in Figure 1D (main text), images used for creating video are rendered using visual molecular dynamics (VMD version 1.9.3). (AVI)

Recognition pathway followed by ellagic acid to reach and bind into ATP-cleft of CK2 α as explored by replica-3 of SuMD simulation. CK2 α is shown in the surface view, crystallized ellagic acid pose (PDB code: 2ZJW) is shown in stick representation and ellagic acid positions as observed in the simulation are shown in CPK (ball and stick) representation. (AVI)

Recognition pathway followed by ellagic acid to reach and bind into allosteric-site or remote cavity involving a $\beta 4$ - $\beta 5$ loop of CK2 α as explored by replica-4 of SuMD simulation. CK2 α is shown in the surface view, crystallized DRB (PDB code: 3H30) pose is shown in stick representation and ellagic acid positions as observed in the simulation are shown in CPK (ball and stick) representation. (AVI)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CK2, casein kinase 2; IE, interaction energy; K_d , equilibrium dissociation constant; k_{off} , dissociation rate constants; k_{on} , association rate constants; MD, molecular dynamics; PDB, protein data bank; CoM, center of mass; SuMD, supervised molecular dynamics; DoF, degree of freedom; BAT, bond-angle-torsion; MIST, maximum information spanning tree; A-MIST, neighbor approximated MIST

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