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Ca^{2+} dissociation from the C-terminal EF-hand pair in calmodulin: A steered molecular dynamics study

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Abstract **We used steered molecular dynamics (SMD) to simulate the process of Ca^{2+} dissociation from the EF-hand motifs of the C-terminal lobe of calmodulin. Based on an analysis of the pulling forces, the dissociation sequences and the structural changes, we show that the Ca^{2+}-coordinating residues lose their binding to Ca^{2+} in a stepwise fashion. The two Ca^{2+} ions dissociate from the two EF-hands simultaneously, with two distinct groups among the five Ca^{2+}-coordinating residues affecting the EF-hand conformational changes differently. These results provide new insights into the effects of Ca^{2+} on calmodulin conformation, from which a novel sequential mechanism of Ca^{2+}-calmodulin dissociation is proposed.**

Keywords: Ca^{2+} dissociation; Calmodulin; EF-hand; Steered molecular dynamics; Conformational change

1. Introduction

Calmodulin (CaM) is a ubiquitous intracellular Ca^{2+} binding protein, which is composed of C- and N-termini globular domains that are linked by a central helix. Each domain contains a pair of EF-hand motifs and binds two Ca^{2+} ions [1,2]. The EF-hand structure, with its helix–loop–helix design, is the most common Ca^{2+}-binding motif. In a canonical EF-hand, the 1st, 3rd, 5th and 12th residues of the EF-hand loop are oriented so that their side-chain oxygen atoms can bind to Ca^{2+}, while backbone oxygen atom of the 7th residue coordinates Ca^{2+}. A short anti-parallel β-sheet, termed EFβ-scaffold, is created by two adjacent EF-hand loops, which normally exist as a pair in EF-hand proteins [3–5]. The EF-hands undergo large conformational changes upon Ca^{2+} binding. As shown in Fig. 1, the Ca^{2+}-bound EF-hands in CaM adopt an open conformation, while the Ca^{2+}-free EF-hands adopt a closed conformation [3,5–8].

In light of the critical regulatory role of CaM in binding many proteins to trigger their functions, it is critical to unravel the large CaM conformational change caused by Ca^{2+} association/dissociation. In the mid-1980s, the H-M-J model was first proposed to explain this process [9]; this model suggested the movement of a pair of helices in EF-hand causes an exposure of a hydrophobic patch. Grabarek put forward a possible two-step Ca^{2+}-binding mechanism based on the crystal structure of the postulated EF-hand intermediate state, which adopts a closed conformation with the Ca^{2+}-bound N-terminal domain of CaM locked via a disulfide bond [10,11]. However, the entire process of conformational change from the Ca^{2+}-bound to the Ca^{2+}-free EF-hand motif cannot be examined experimentally. Hence it is necessary to investigate the underlying mechanism of Ca^{2+} association/dissociation by computer simulation.

Previous theoretical studies of the EF-hand motif focused mainly on the equilibrium state simulations of the Ca^{2+}-free or the Ca^{2+}-bound structures [12–17]. These investigations did not deal with the entire process of structural transformation between the Ca^{2+}-bound and Ca^{2+}-free EF-hands. Besides, Goto et al. modified the structure of the Ca^{2+} binding sites in NMR-based Ca^{2+}-free EF-hand pairs, following the addition of Ca^{2+} ions, and performed MD to examine the change in the inter-helical angle [18]. However, this work does not represent the entire process of Ca^{2+}-EF-hand association. Kobayashi et al. conducted both umbrella sampling for the free-energy surface, and steered molecular dynamics for the pathway search of Ca^{2+} dissociation from the CaM loop, using a model of the loop region of the EF-hand motif (12 residues, and one Ca^{2+}) [19]. While this work is important the model chosen for MD investigation is suspect because the EF-hand motif is composed of a helix–loop–helix structure and the conformational change of the entire helix–loop–helix following by Ca^{2+} association/dissociation is critical to most EF-hand proteins [19].

In this study, we have investigated the continuous dissociation process of Ca^{2+} ions from the C-terminal domain of CaM to elucidate the structural changes that occur when moving from the Ca^{2+}-bound to Ca^{2+}-free conformations of EF-hands using steered molecular dynamics (SMD). This method can induce ligand dissociation from biomolecule because the time-dependent external forces are applied to a system [20], and has been successfully used to research the process of ligand dissociation from protein in the past decade [21–24].

2. Methods

2.1. System preparation and equilibration

We have chosen the C-terminal globular domain of the Ca^{2+}-bound CaM since this domain has been reported to be a relative rigid body by essential dynamics and normal-mode analysis and have a higher affinity for Ca^{2+} and a higher affinity for the majority of CaM target

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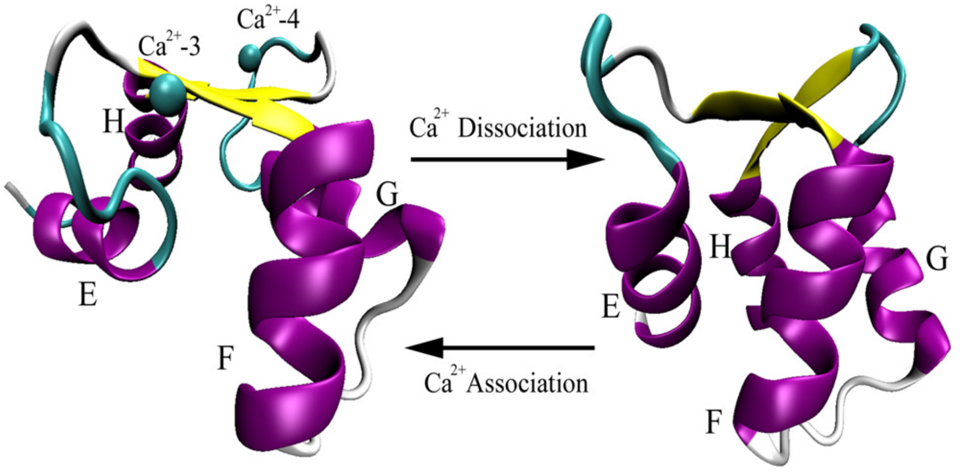

Fig. 1. Conformational transformation of the EF-hand pair between Ca^{2+}-bound and Ca^{2+}-free states. Left: the Ca^{2+}-bound structure of the C-lobe of holo-CaM (PDB code: 4CLN). Right: the Ca^{2+}-free structure of the C-lobe of apo-CaM (PDB code: 1CFD [29]). Helix E: residues 84–91, Helix F: residues 105–112, Helix G: residues 120–127, and Helix H: residues 139–146. The two β strands contain residues 98–102 and 134–138.

protein [25–27]. Our simulation studies have been based on a 2.2 Å-resolution X-ray structure of Ca^{2+}-bound CaM (PDB:4CLN [28], residues 83–148), because no residue in the C-terminus is missing and there are only three different residues between it and the NMR Ca^{2+}-free CaM (PDB:1CFC [29]), which would allow a better comparison between our resulting Ca^{2+}-free CaM structure from the SMD simulation and the experimental NMR structure.

After the addition of missing hydrogen atoms, the protein was immersed in a water box and the minimal distance from protein toward box edge is 16 Å. Sodium ions were included to bring the system to the neutrality. Finally, the system contained 20645 atoms in total, of which there were 1025 protein atoms, two Ca^{2+} ions, seven counter ions (Na^{+}) and 6537 TIP3P water molecules. Energy minimization was first performed until the maximum force in the system was less than 7 kcal mol^{-1} Å^{-1} using conjugate gradients method. The subsequent system equilibration period was divided into two steps: heating the system from 0 to 300 K (constant volume, Berendsen thermostat [30]) in 30 ps, followed by equilibrating the system for 8000 ps (300 K, Nose–Hoover thermostat [31,32]; 1 atm, Parrinello–Rahman barostat [33]). During the simulation, the particle mesh Ewald (PME) method was used to reduce the truncation error of electrostatic interactions. The SHAKE method (rigid bonds) was used on all hydrogen-containing bonds to allow a 2 fs time step in the MD simulations. Temperature coupling was performed on the protein and solvent, respectively, using 0.1 ps as the coupling time constant. Pressure coupling constant was set to 0.2 ps. The structure snapshots were saved every 1 ps.

2.2. Steered molecular dynamics

In the Ca^{2+}-bound EF-hand structure, five residues bind Ca^{2+} at the vertices of a pentagonal pyramid, and the only opening for Ca^{2+} dissociation would be at the direction shown in Fig. 2A and B. Prior to SMD simulation of Ca^{2+} dissociation, several snapshot structures of the equilibration period were chosen as the initial structures. For each initial structure, three ∼2000 ps SMD simulations were performed to pull Ca^{2+} ions out from the EF-hands. In one case, two Ca^{2+} ions were pulled out from EF-3 and EF-4 together; in another two cases only one Ca^{2+} ion was pulled out from EF-3 or EF-4, respectively. A dummy spring was attached to Ca^{2+} and an external force was applied to the Ca^{2+} ion. The spring force constant (k) was set at 36 kcal mol^{-1} Å^{-1} (∼250 pN Å^{-1}) and the moving velocity was fixed at 0.01 Å ps^{-1}. This velocity is very close to those used in similar SMD studies [21–24]. Finally, a 3500 ps MD simulation (300 K, 1 atm) was carried out in order to relax the system for each SMD simulation. In the simulation, center of mass (COM) translation and rotation were reduced every 0.1 ps for the protein.

For comparison purposes, we investigated two other dissociation directions, as shown in Fig. 2C. From our simulations, for the direction II and direction III, we found that SMD simulations led to the collapse of the secondary structure, and moreover, Ca^{2+} did not dissociate from the EF-hand even when the secondary structure of the EF-hand was lost completely during simulation. We therefore conclude that these two dissociation directions are not relevant for Ca^{2+} dissociation. Only the results of SMD simulation along the direction I will be discussed.

2.3. Tools and software

All preparations of systems, energy minimizations and molecular dynamics simulations were carried out using the Gromacs 3.3.1 software package [34]. Amber99 force field was used in molecular dynamics simulation [35]. All graphics analysis and illustrations were performed using the VMD program [36].

3. Results and discussion

3.1. Systems equilibration and relaxation analysis

In the equilibration period, the RMSD between protein backbone atoms and the crystal structure versus equilibration time is shown in Fig. 3. The RMSD values reflect a relatively large conformational change in the first 5000 ps before subsequent convergence around 3.0 Å after 5000 ps, demonstrating that the system structure had stabilized after 30 ps heating and 5000 ps equilibration. From the MD trajectory, it is clear the system structure does not change significantly and the relative positions of the four helices do not deviate significantly from the crystal structure. The main structural change is localized in the loop region, as shown by the overlay of average structures from 1 to 5 ns and 5 to 8 ns periods with the X-ray structure (Supplementary Material, Fig. S1). All five residues that coordinate Ca^{2+} in the EF-hand still maintain their positions at the vertices of a pentagonal pyramid despite the fact that the loop region of EF-hand experiences some movement. The distances of Ca^{2+} and its coordination residues are 2.4–2.7 Å during the equilibration period, similar to the crystal structure. Consequently, we felt comfortable using the snapshot structures from the 5000–8000 ps equilibration as the starting points for SMD simulation of Ca^{2+} dissociation.

3.2. Force profiles during Ca^{2+} dissociation

In each SMD simulation, there is always a maximal pulling force, which indicates that there is maximal restriction on Ca^{2+} in its dissociation process. The maximal pulling forces and the