Conformational transitions of caspase-6 in substrate-induced activation process explored by perturbation-response scanning combined with targeted molecular dynamics

Shuheng Huang⁎, Hu Mei⁎⁎, Laichun Lu⁎⁎, Zuyin Kuang⁎, Yu Heng⁎, Lei Xu⁎, Xiaoqi Liang⁎, Minyao Qiu⁎, Xianchao Pan⁎⁎

⁎ Key Laboratory of Biochemical Science and Technology (Ministry of Education), College of Bioengineering, Chongqing University, Chongqing 400045, China
⁎⁎ Department of Medicinal Chemistry, College of Pharmacy, Southwest Medical University, Luzhou, Sichuan 646000, China

A B S T R A C T

Caspase-6 participates in a series of neurodegenerative pathways, and has aroused widespread attentions as a promising molecular target for the treatment of neurodegeneration. Caspase-6 is a homodimer with 6 central-stranded β-sheets and 5 α-helices in each monomer. Previous crystallographic studies suggested that the 60's, 90's and 130's helices of caspase-6 undergo a distinctive conformational transition upon substrate binding. Although the caspase-6 structures in apo and active states have been determined, the conformational transition process between the two states remains poorly understood. In this work, perturbation-response scanning (PRS) combined with targeted molecular dynamics (TMD) simulations was employed to unravel the atomistic mechanism of the dynamic conformational transitions underlying the substrate-induced activation process of caspase-6. The results showed that the conformational transition of caspase-6 from apo to active states is mainly characterized by structural rearrangements of the substrate-binding site as well as the conformational changes of 60's and 130's extended helices. The H-bond interactions between L1, 130 helix and 90 helix are proved to be key determinant factors for substrate-induced conformational transition. These findings provide valuable insights into the activation mechanism of caspase-6 as well as the molecular design of caspase-6 inhibitors.

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1. Introduction

Caspase, also known as apoptotic protease Mch-2, is a cysteinyll aspartate-specific protease that plays essential roles in cell regulatory apoptosis and inflammation [1,2]. Up to now, a total of 11 functional caspase subtypes have been found in human, which are mainly divided into three classes: (i) apoptotic initiators (caspases-2, –8, –9, and –10); (ii) apoptotic executioners (caspases-3, –6, and –7); and (iii) inflammatory caspases (caspases-1, –4, –5 and –14) [3-5]. As an important member of caspase family, caspase-6 is involved in the process of axonal pruning and neuronal degeneration by cleaving neuronal substrates, e.g., microtubule-associated protein Tau [6], amyloid precursor protein (APP) [7], huntingtin protein (HTT) [8] and Parkinson disease protein 7 (PARK7) [9]. Recently, a series of substrate-mimic and allosteric molecules have been identified as caspase-6 inhibitors with nanomolar to micromolar potencies [10,11]. Thus, caspase-6 has become one of the most important therapeutic targets for the treatments of neurodegenerative diseases [9,12-19].

Caspase-6 is firstly expressed as an inactive zymogen. The zymogen can be activated in two ways: auto-activation or caspase-3-mediated activation [20]. In the process of auto-activation, caspases-6 zymogen is initially cleaved at Asp193 in the inter-subunit linker. Then, it sequentially goes through proteolytic cleavages at Asp23 in the pro-domain and Asp179 in the inter-subunit linker. At last, full maturation caspase-6 consists of 6 central-stranded β-sheets and 5 α-helices (Fig. 1) [21,22]. As a homodimer and each monomer consists of 6 central-stranded β-sheets and 5 α-helices (Fig. 2). The core structure of caspase-6 is comprised of a large subunit, a small subunit and an inter-subunit linker. The large subunit contains active-site catalytic dyad residues (i.e., Cys163-His121), which can cleave natively folded proteins at a specified aspartic acid residue for apoptosis [23]. The small subunit comprises most of the

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dimer interface and an allosteric site [22-24]. Four loops (L1, L2, L3, and L4) protruding from the central β-sheet form the substrate-binding groove. All of the caspase subtypes can adopt a canonical conformation, where the substrates bound to the β-strand platform of the 130's helix. Intriguingly, the lack of helix-breaking residues in the 130's helix of caspase-6 leads to a non-canonical conformation solely presented in caspase-6 [25]. Notably, the canonical conformation was observed in the zymogen, ligand free (apo) and active states of caspase-6, while the non-canonical conformations exist in the apo, zinc-mediated allosteric and irreversible inhibitor Z-VAD-FMK-bound state, etc. [22,24,26-28]. Different from the canonical conformation, the non-canonical conformation adopts two extended helices in atop of 60's (residues 57–70) and 130's (residues 128–142) regions. These conformational changes are also accompanied by an outward rotation of the 90's helix (Fig. 2) [23,24].

A growing body of evidence suggested that caspase-6 undergoes distinctive conformational transitions upon substrate binding. By comparing the circular dichroism (CD) spectra between apo and substrate-bound states of caspase-6, Vaidya et al. [23] observed that the molar ellipticity (θ) ratio between 208 and 222 nm signals increased consistently, indicating a loss in α-helical content upon substrate binding. Furthermore, Dagbay et al. [29] employed hydrogen/deuterium exchange mass spectrometry (H/DX-MS) to explore the conformational flexibility of caspase-6. The results suggested that caspase-6 underwent a distinct helix-strand interconversion in the initial substrate recruitment, and then reached to a dynamic equilibrium between the helix-extended and canonical strand conformations. Although crystallographic studies have determined the helix-extended apo and active (bound by a substrate-mimic inhibitor) conformations [22,28], the activation mechanism of caspase-6 is still unclear.

In this work, taking full advantages of the crystal structures of caspase-6 in apo and active states, perturbation-response scanning (PRS) combined with targeted molecular dynamics (TMD) simulations was employed to elucidate the conformational transition in...
caspase-6 (Fig. 3). Firstly, 100 ns MD simulations were carried out to explore the intrinsic dynamics of the caspase-6 in apo and active states, respectively. Then, based on the MD trajectories, a total of 22 crucial residues for conformational transition were identified by PRS method. Finally, TMD simulations based on the Cα atoms of the 22 crucial residues identified were performed to explore atomic-level details in the process of the conformational transition. Collectively, our findings can provide valuable insights into the activation mechanism of caspase-6.

2. Methods

2.1. Structural preparation

In this work, the crystal structures of caspase-6 in apo (PDB ID: 2WDP) and active states (PDB ID: 3OD5, co-crystallized with a substrate-mimic inhibitor: Ac-VEID-CHO) were used to explore the conformational transition [22,24]. The missing residues in L4 loop (residues 262–265) were modeled and refined by Modeller 9 software. The protonation states of Glu, Asp and His were assigned by using PDB2PQR web server at a pH value of 7.5 [30]. Glu135 was set to deprotonation state based on the experimental research [29]. After solvated by a TIP3P water box with the dimensions of 100 \times 100 \times 100 \text{Å}^3 and neutralized by Cl⁻ ions, 50 ps of steepest descent followed by 50 ps of conjugate gradient energy minimizations were performed before MD simulations.

2.2. Molecular dynamics simulations

MD simulations with periodic boundary conditions [31,32] were performed by NAMD 2.12 with CHARMM36 force field [33]. First, each system was gradually heated from 0 to 310 K within 5000 ps in the NVT ensemble, where the backbone of caspase-6 was harmonically constrained by a force of 50 kcal/mol Å². Then, a 100 ns MD simulation was performed in the NPT ensemble (310 K, 1 atm) without any constraint. The integration time step was set to 2 fs and the cutoff value of van der Waals and electrostatic interactions was set to 10 Å. The particle mesh Ewald (PME) method was used to calculate the long-range electrostatic interactions [34] and the SHAKE algorithm [35] was used to constrain the covalent bonds with H atoms. For the structure in active state, due to the Asp residue of Ac-VEID-CHO forms a covalent bond with the catalytic Cys163, the distance of C-S bond was thus harmonically constrained by a force of 250 kcal/mol Å² during MD simulations.

2.3. Perturbation-Response scanning

PRS is a linear response theory-based computational technique, which can be used to determine the residue-level effects of perturbed forces on the residues of a given protein [36,37]. By monitoring the responses of the perturbed protein, PRS can depict dynamic couplings and link the directionality of the exerted forces to the protein response in functional movement [38-40]. In PRS, the motions of caspase-6 were fully defined by a 3N \times 3N covariance matrix (C), derived from the equilibrated MD trajectories. The coordinates of Cα atoms of each residue were used to represent a node of the network. The idea of PRS technique is to exert an external force on each residue, and record the response of each node in the network. The vector of exerted force and the corresponding response of each residue are represented as:

\[
\Delta F_i = [(000 \cdots F_i^0 F_i^1 F_i^2 \cdots 000)_{1 \times 3N}]^T
\]

\[
\Delta R_i = (R_i)_1 - (R_i)_0 \approx \frac{1}{k_B T} (\Delta R_i \Delta R_i^T) / \Delta F_i = \frac{1}{k_B T} C \Delta F_i
\]

where, (R_i)_0 and (R_i)_1 denote residue i conformations in caspase-6 initial (apo state) and perturbed coordinates; k_B and T represent the Boltzmann constant and the temperature, respectively; \Delta F_i vector contains the coordinates of the force vectors applied on the residues. C represents the cross-correlation matrix of the fluctuations of the nodes of the caspase-6 in apo state.

![Fig. 3.](image-url) The flowchart for exploring the conformational transition during caspase-6 activation.
Then, the Pearson correlation coefficients $C_i$ for each perturbed residue $i$ was used to quantify the correlation between the theoretical and experimental conformational displacements averaged over all the affected residues $j$ (Eq. 3):

$$C_i = \frac{\sum_{j=1}^{N} (\Delta R_{ij} - \bar{\Delta R}_j)(\Delta S_j - \bar{\Delta S})}{(N-1)\sigma_t \sigma_s}$$

where, $\Delta R_{ij}$ is the theoretical prediction of the displacement of residue $j$ affected by perturbations; and $\Delta S_j$ is the experimental displacements of residue $j$ between the caspase-6 structures in the apo and active states. The overbar represents the average; $\sigma_t$ and $\sigma_s$ represent as the standard deviations of theoretical and experimental displacements, respectively. The $C_i$ value close to 1 means that a good agreement with the experimental conformational change, while the $C_i$ value close to 0 means that the lack of correlation between theoretical prediction and experimental displacements.

In this work, the $C_i$ thresholds of PRS are selected from the 100 perturbations based on following rules: (i) Firstly, rank and list the residues in descending order based on its $C_i$ value; (ii) If there is a sharp decrease in the correlation values, the top residues will be listed until that gap; (iii) If there is a smooth decrease in the correlation values, define the threshold equal to the $C_{imax}$ value observed over all experiments less than 0.1 ($C_{imax}$-0.1) [41,42].

In this work, the crystallized caspase-6 structures in apo and active states were used as inputs to perform PRS, and the equilibrated MD trajectory of caspase-6 in apo state was used to construct cross-correlation matrix of the fluctuations of the nodes. The PRS and correlation analysis for each residue of caspase-6 were carried out by MD-TASK [43].

### 2.4. Targeted molecular dynamic

As an enhanced sampling method, TMD can efficiently explore the conformational transition between two conformational states of a given biological system [44]. In this paper, caspase-6 structures in apo and active states were used as the initial and targeted conformations for the TMD simulation, respectively. Then, a steering force was applied to the $C_{a}$ atoms of crucial residues identified by PRS, which accelerates the conformational transition from the initial apo state to the target active state. At each time step, the root-mean-square deviation (RMSD) between the current and target conformations was calculated, which was then used to determine the instantaneous force on each atom at next time step (Eq. 4).

$$E_{TMD} = \frac{k_{TMD}}{2M}[\text{RMSD}(t) - \text{RMSD}^*(t)]^2$$

Where $E_{TMD}$ represents the instantaneous potential applied to a given atom; $k_{TMD}$ is the steering force constant; $M$ represents the number of steered atoms; RMSD($t$) is the instantaneous RMSD between the current and the target coordinates; RMSD($t$) evolves linearly from the initial RMSD at the first TMD step to the final RMSD at the last TMD step.

### 3. Results and discussion

#### 3.1. Structural flexibility of Caspase-6

In this paper, a 100-ns MD simulation was performed for caspase-6 structures in apo and active states, respectively. Both of the caspase-6 systems reached to equilibrium states in the last 80 ns, of which the mean RMSDs were $2.35 \pm 0.18$ Å for the apo...
state and 1.41 ± 0.09 Å for the active state (Fig. S1). The root-mean-square fluctuations (RMSFs) revealed that the structural flexibility of caspase-6 in active state is, in general, lower than that in apo state (Fig. 4a), which suggests a reduced structural flexibility upon the ligand binding. Also, it can be observed that the 90° helix in active state exhibits smaller fluctuations than that in apo state. Moreover, it can be seen that, in both apo and active states, the fluctuations of the residues located in L1-L4 loops, 90° and 130° helices are more significant than other residues.

Based on the last 80 ns equilibrium trajectories, principal component analysis (PCA) was carried out to investigate the structural variations and motion modes of caspase-6 in apo and active states. Fig. 4b shows the predominant movements of caspase-6 in the first PC mode. For the caspase-6 in apo state, it can be clearly observed that, the structural variance along the first PC mode is dominated by the motion of 90° helix, the top half of 130° helix, and loop residues. By contrast, the structural variance of the caspase-6 in active state is relatively small with the exception of residues in L1-L4 loops. Fig. 4c shows the residue cross-correlation map between the apo and active states. Notably, it can be seen that, in both apo and active states, the fluctuations of the residues located in L1-L4 loops, 90° and 130° helices are more significant than other residues.

3.2. PRS reveals 22 crucial residues in conformational transition

In comparison with apo state, the caspase-6 in active state undergoes significant structural changes: conformational transitions in L1-L4; β-turn formation in the top of 130° helix (residues 125–142); rearrangements in the top of the extended 60° helix (residues 57–70), and a 21° inward rotation of the 90° helix (Fig. 5a) [24,25]. Based on the last 80 ns MD trajectories of caspase-6 in apo state, the PRS with 100 times of perturbations was performed to detect the crucial residues affecting conformational transition. Fig. 5b shows the correlation coefficients ($C_i$) profiles between theoretical prediction and experimental displacements for each perturbed residue. According to the maximum $C_i$ value obtained ($C_{i_{max}} = 0.56$), the threshold of $C_i$ was set to 0.46 (threshold = $C_{i_{max}} - 0.1$) for the following analysis. Herein, a total of 22 crucial residues with significant $C_i$ were identified, which are mainly located in substrate-binding loops (L1-L3) and the top of 130° helix (Table 1 and Fig. 5c).
Previous studies revealed that there are strong inter-molecular interactions between the substrate-mimic inhibitor Ac-VEID-CHO and His121, Gly122, Tyr217, Ser218, His219, Arg220, Glu221 and Thr222 of the substrate-binding groove, especially the H-bond interactions with Arg64, His121, Ser218, Arg220, Glu221 [22,24]. Recent researches suggested that residues located far from the substrate-binding groove, e.g. Gly122, Gly123, Gly124, Asn125, His126, Ile127 and Tyr128 in the top of 130’s helix, also played important roles in the substrate-induced conformational transition process [23,25]. Table 1 summarizes the recently reported hot residues affecting the conformational transition of caspase-6. It has been observed that most of the crucial residues determined by PRS are consistent with the identified functionally/allosterically relevant residues. For instance, His121-Tyr128 in 130’s helix and Ser211-Arg220 in L3 are pivotal to the binding of substrate-mimic inhibitors. In addition, it is worthy to note that Gly122, Gly123, His126, Gly214 and Tyr217 are located in allosteric sites for binding allosteric peptide pep419/Z-VAD-FMK [26,45]. Collectively, the 22 crucial residues identified by PRS method agree well with the known hot residues, implying that PRS technique can provide useful information for exploring the key residues affecting conformational transition process.

3.3. The atom-level details in conformational transition by TMD analysis

3.3.1. kTMD optimization

TMD simulation was performed to investigate the dynamic activation process of caspase-6 according to the 22 crucial residues determined by PRS (Table 1). In TMD simulations, the steering forces were only imposed on the 22 crucial residues affecting the conformational transition of caspase-6. It has important roles in the substrate-induced conformational transition during caspase-6 activation process and the molecules targeting these residues may stabilize the non-canonical conformation.

Previous research suggested that the protonation state of Glu135 is important for the conformational transition of the extended 130’s helices. At basic pH condition, deprotonated Glu135 can destabilize the 130’s extended helix by leading to electrostatic interactions with neighboring His52, Arg54 and Glu131 [29,51]. In the substrate-induced conformational transition process, it can be observed that the deprotonated Glu135 can facilitate the formation of the β-turn through H-bond interactions with Lys133 and Ile134 (Fig. 6d).

3.3.2. Structural rearrangements in active site facilitate the reorganization of 60’s and 130’s extended helices

Accumulated evidence suggested that 60’s and 130’s extended helices of caspase-6 must undergo structural reorganizations before substrate binding. Fig. 6b shows the snapshots of 60’s helix during the TMD simulation. It can be observed that Arg64 is important for structural rearrangement of the 60’s extended helix, which facilitates the conformational transition by forming a H-bond interaction with Thr67. Previous researches suggested that Arg64 is located in the primary specificity pocket and responsible for the strict substrate specificity of caspase-6 (Fig. S2) [50].

By contrast, the reorganization of the 130’s helix is more complicated. Ser120 and His121 located in the 130’s helix can form strong polar interactions with the inhibitor Ac-VEID-CHO by the formation of β turn motif, implying that Ser120 and His121 play important roles in the substrate-induced conformational rearrangement of 130’s helix (Fig. 6c and S3) [22,23]. Besides, it can be also observed that the rearrangement of the extended 130’s helix involves a series of ruptures and formations of inter-helix H-bonds (Fig. 6c), e.g., the H-bonds interactions of Glu123, His126 and Lys133, etc. It should be noted that Glu123, His126 and Lys133 are located in the allosteric site and maintain the helix-extended conformations of caspase-6 by forming polar interactions with inhibitor Z-VAD-FMK [26]. Thus, it can be deduced that the polar residues in 130’s helix contribute to the conformational transition during caspase-6 activation process and the molecules targeting these residues may stabilize the non-canonical conformation.

Previous research suggested that the protonation state of Glu135 is important for the conformational transition of the extended 130’s helices. At basic pH condition, deprotonated Glu135 can destabilize the 130’s extended helix by leading to electrostatic interactions with neighboring His52, Arg54 and Glu131 [29,51]. In the substrate-induced conformational transition process, it can be observed that the deprotonated Glu135 can facilitate the formation of the β-turn through H-bond interactions with Lys133 and Ile134 (Fig. 6d).

3.3.3. The 90’s helix undergoes an inward rotation during TMD simulation

Crystallographic studies revealed that the 90’s helix of caspase-6 undergoes an inward rotation of 21° and thus moves close to the 130’s helix in the activation process (Fig. 5a) [23-25]. However, the conformational transition process of 90’s helix and determinant factors are still unclear.

Fig. 7 shows the key snapshots of 90’s helix during the TMD simulations. It can be seen that Asp90 (90’s helix) forms stable H-bond interactions with His52 and Arg54 (L1 loop) during the TMD simulations. At the same time, Leu91 and Ala93 tend to gradually approach to 130’s helix by forming H-bond interactions with Tyr130 and Asp131 (130’s helix) at 300 ps. Moreover, intrinsic H-

Table 1

| Location | Crucial residues | Refs |
|----------|-----------------|------|
| L1/60’s helix | Glu53, Phe55, Phe56, Trp57, His58, Pro60, Glu63, Arg54*, Arg55* | [22,27,29] |
| L2/L2’ | Glu161*, Lys163*, Arg164*, Gly165, His168, Asp179, Asp193, Leu200 | [22,23] |
| L3 | Ser211, Val212, Glu214, Gly215, Tyr217*, Ser218*, His219*, Arg220, Glu221, Thr222, Val223, Ser226, Trp227* | [22,24,26,46] |
| 130’s helix | His121*, Gly122*, Gly123, Gly124, Asn125, His126, Ile127, Tyr128, Arg137*, Lys133* | [26,29,45] |
| Others | Asp23*, Arg42*, Arg43*, Arg46*, Lys36*, Arg55*, Gly66*, Ser242, Glu244, Thr250, Leu251, Val261*, Leu282, His287*, Ser257 | [22,23,47-49] |

| Location | Crucial residues | Refs |
|----------|-----------------|------|
| L1/60’s helix | Glu53, Phe55, Phe56, Trp57, His58, Pro60, Glu63, Arg54*, Arg55* | [22,27,29] |
| L2/L2’ | Glu161*, Lys163*, Arg164*, Gly165, His168, Asp179, Asp193, Leu200 | [22,23] |
| L3 | Ser211, Val212, Glu214, Gly215, Tyr217*, Ser218*, His219*, Arg220, Glu221, Thr222, Val223, Ser226, Trp227* | [22,24,26,46] |
| 130’s helix | His121*, Gly122*, Gly123, Gly124, Asn125, His126, Ile127, Tyr128, Arg137*, Lys133* | [26,29,45] |
| Others | Asp23*, Arg42*, Arg43*, Arg46*, Lys36*, Arg55*, Gly66*, Ser242, Glu244, Thr250, Leu251, Val261*, Leu282, His287*, Ser257 | [22,23,47-49] |

Table 2

| No. | kTMD/(kcal/mol Å²) | Time (ps) | RMSD between the last TMD snapshot and initial/targeted conformations (Å) | RMSD between the last TMD and targeted conformations (Å) |
|-----|----------------|----------|-------------------------------------------------|--------------------------------------------------|
| 1   | 35            | 500      | 5.38                                            | 2.98                                             |
| 2   | 50            | 500      | 5.86                                            | 2.71                                             |
| 3   | 70            | 500      | 6.28                                            | 2.59                                             |
| 4   | 100           | 500      | 6.56                                            | 2.46                                             |
| 5   | 120           | 500      | 6.91                                            | 2.96                                             |

1. The optimal kTMD.
bond networks are also observed between L1 and 130's helix during the TMD simulation.

Table 3 summarizes the pivotal residues involved in caspase-6 conformational transition. It can be seen that the overall conformational transitions of caspase-6 are mainly divided into three stages: (i) substrate-induced residue rearrangements in the active site (L1 and L3); (ii) the rearrangements of the active site facilitate the β-turn formation in 130's extended helix;
(iii) complicated H-bond network formed among L1, 130°s and 90°s helices result in the inward rotation of 90°s helices.

4. Conclusions

Although intense efforts have been devoted to the treatment of neurodegenerative diseases over the past decades, the neurodegenerative diseases are clinically unmanageable due to the lack of success. Caspase-6 is involved in processes of axonal pruning and neuronal degenerations by cleaving a series of neuronal substrates, and thus has been considered as one of the most promising therapeutic targets. Accumulated evidences suggested that caspase-6 undergoes a distinctive conformational transition upon substrate binding. However, the conformational transition and activation mechanism of caspase-6 remains poorly understood, which limits the active-site oriented drug development, as well as the discovery of potential allosteric sites.

Herein, taking advantages of crystallized caspase-6 structures in apo and active states, PRS combined with TMD simulations was used to elucidate the conformational transition of caspase-6 at the atomic level. On the basis of MD simulations, a total of 22 residues were identified as crucial residues in caspase-6 activation process by PRS, which agree well with the functional/allostery residues reported previously. Then, TMD simulation was performed to explore the conformational transition process of caspase-6. The results showed that the conformational transition of caspase-6 is mainly driven by the structural rearrangement of the active site as well as the 60°s and 130°s extended helices, which results in the inward rotation of 90°s helix. Generally, this work provides a dynamic perspective for the conformational transition of caspase-6 from the apo to the active states. However, the conformational transition of caspase-6 is an extremely complicated process and the biased TMD simulations in this paper only characterize some representative features in the conformational transition process. The precise mechanism and details require further validated by exploring free-energy landscapes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author statement

All of the authors have seen and approved the final version of the manuscript being submitted. We warrant that the article is
the authors’ original work, hasn’t received prior publication and isn’t under consideration for publication elsewhere.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.07.017.

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