

Characterization of a Partially Structured Intermediate of Cardiotoxin VI from *Naja naja atra* at High Temperature

Biswajit Gorai and Thirunavukkarasu Sivaraman*

Structural Biology Lab, ASK, School of Chemical and Biotechnology,
SASTRA University, Thanjavur – 613 401, Tamil Nadu, India.

Abstract

The unfolding kinetics of cardiotoxin analogue VI from Taiwan cobra (*Naja naja atra*) have been studied at 473 K, pH 7.0 in the presence of 0.1 M NaCl using molecular dynamics simulations for 50 ns. Trajectory structures stored at every 25 ps (2000 structures) were probed at molecular (RMSD and radius of gyration) and residue (RMSF, surface area accessibility and secondary structures) level resolutions by means of various computational strategies. Comprehensive analysis of the data suggested that the C-terminal tail of the protein, which is sandwiched in the cleft region, was first destabilized followed by double-stranded domain and strand IV of triple-stranded domain of the protein. Strikingly, it was found that the protein assumed a partially structured state consisting of a sheet composed of stand III and V at around 16 ns and the intermediate was also found to be stable in the rest of the dynamic scales at 473 K. The structural features of the partially structured intermediate and its implications on understanding the stability and folding dynamics of the protein have been brought into fore in detail.

Keywords: Cardiotoxin, intermediates, molecular dynamics and unfolding kinetics.

1. INTRODUCTION

The energetic pathways by which polypeptide chains fold to unique and well-defined three-dimensional structures have been shown to be engraved in their primary sequences [1]. However, the factors that are governing the stability and folding kinetics of proteins still remain elusive. Many phenomenological models (Hydrophobic collapse model, Framework model, Diffusion-collision model and Jigsaw puzzle model) have been proposed for describing events occurring in the unfolding and refolding of proteins [2-5]. Thus, there have been extensive efforts to detect, trap and characterize either native-like or non-native intermediates in the folding kinetics of proteins [6-7]. There are numerous reports on the structural characterization of intermediates accumulating in the folding kinetics of multi-domain and large sized (> 120 amino acids) proteins [8-9]. However, folding pathways of small (< 100 amino acids) and single domain proteins have been characterized in the absence of detectable partially folded or molten-globule states [10-11].

In the present study, we have extensively characterized the unfolding pathways of cardiotoxin analogue VI (CTX VI) from Taiwan cobra (*Naja naja atra*) at close quarters of physiological conditions (pH 7.0, 1 atmospheric pressure and 0.1 M ionic strength) using molecular dynamics (MD) simulations. CTX VI is an all beta-sheet, monomeric protein containing 60 amino acids with four disulfide bridges [12]. The high resolution three-dimensional (3D) structures of the protein and its membrane depolarization activities have been well characterized [13]. In general, cardiotoxins (CTXs) belong to the three-finger toxin (TFT) superfamily and they are one of the principle toxic components of all venomous snakes [14]. The CTXs exhibit various adverse biological activities to victims like hemolysis, cytotoxicity, depolarization of muscles and inhibition of key enzymes such as Na⁺/K⁺-ATPase enzymes and protein kinase C [15]. Interestingly, the CTXs may presumably serve as typical model systems of all beta-sheet protein as the 3D structures of the proteins are bereft of helical segments and also have negligible propensities to

adopt non-native helical segments in their backbone conformations [16]. In these backgrounds, we strongly believe that the temperature-induced partially structured intermediate of the CTX VI we demonstrated in the present study may pave a way to generalize the unfolding pathways of all beta-sheet proteins belonging to the three-finger toxin family, particularly CTXs.

2. METHODS

Molecular dynamics and structural analysis

The 3D structures of CTX VI (1UG4) was retrieved from protein data bank (www.pdb.org) and subjected for MD simulations using GROMACS 4.5.4 molecular dynamics package with force field AMBER99SB-ILDN [17,18]. The protein was placed at center of an dodecahedron box maintaining minimum distance of 3.0 nm between the wall and the protein (the minimum distance was chosen such that the periodic replicas will not interact with each other during any time point of simulation) and then the system was solvated with desired amount of water (TIP3P explicit water molecules were used). Periodic boundary conditions and a minimum image were used to reduce edge effects. The steric clashes and bad geometry such as unrealistic bond distances, bond angles and torsion angles were successfully removed upon subjecting the system (in the presence of 0.1 M NaCl) to energy minimization using the steepest-descent algorithm down to a maximum gradient of 1000 kJ/mol/nm. The minimized system was then heated to 473 K for 2 ns under isothermal ensemble coupled with V-rescale (modified Brendsen) thermostat. Bonds involving hydrogen atoms were constrained according to the LINCS protocol and long range electrostatic interactions were calculated through the Particle Mesh Ewald (PME) approach [19]. Finally, molecular dynamic simulation was performed for 50 ns at a stretch. Coulomb interaction and van der Waals interactions were truncated at 10 Å. The non-bonded pair lists were updated every 10 steps and conformations were stored at every 25 ps. The trajectories files were analyzed using GROMACS scripts and VMD molecular visualization tool.

3. RESULTS AND DISCUSSION

The 3D structure of CTX VI is shown in Figure 1. The protein comprises of five beta-strands: the strands I and II constitute the double-stranded domain; strands III, IV and V constitutes the triple-stranded domain of the protein. The C- & N-termini of the protein are brought together and stabilized in the cleft region, which is also a part of globular head of the protein. The protein was subjected to all atom MD simulations for 50 ns in 473 K at a stretch and trajectories collected at every 25 ps were analyzed at many facets of structural contexts. Overall RMSD (root mean square deviation) of the backbone atoms of the target protein from the starting native structure over the course of simulation can be used as a measure of the conformational stability of a protein during the simulations. The backbone RMSD of the protein was gradually increasing and reached a maximum value of about 13 angstrom at 27 ns and showed negligible changes in rest of the dynamic timescale. Fluctuation of every backbone atoms of the protein was also analyzed throughout the simulation and the data revealed that loop I (comprising of 6-10 residues), loop II (comprising of 29-34 residues), N-and C-termini of the protein were very flexible and strand III and V, a portion of triple-stranded domain, of the protein were most intact at 473 K (data not shown). Radius of gyration (Rg) is defined as the root mean square distance of the collection of atoms from their common center of gravity and it describes the overall spread of the molecule and compactness of the structures at different time scales of simulations [20]. The native protein showed Rg of about 11 Å and the Rg values of the protein were higher than 11 Å throughout the dynamic time scale. However, the Rg values showed haphazard correlation with dynamic structures. Taken together, the dynamic data (RMSD, RMSF and Rg) of the protein imply that the overall stability of the protein could be drastically destabilized at around 16 ns of the dynamic process at 473 K.

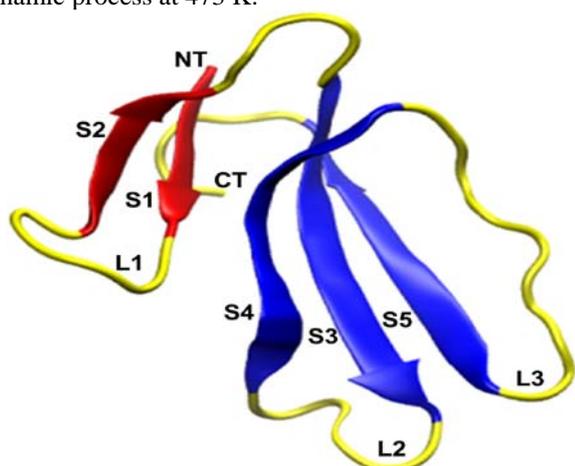


Figure 1: The 3D structure of CTX VI (1UG4) is depicted in cartoon model. The double stranded (S1&S2) domain and triple stranded (S3,S4&S5) domain are shown in red and blue colors, respectively. The loops (L1,L2&L3), N-terminal (NT) and C-terminal (CT) of the protein are represented in yellow color.

The secondary structural contents of 2000 trajectories were analyzed by means VMD. The regular backbone interactions of the protein were intact in the first 2.5 ns

simulations; after the timescale, structural integrity between the stand I and C-terminal begun to get weakened. At around 3 ns, C-terminal portion of the protein begun to move off the cleft region and double stranded domain of the protein started to melt; the strand I and II got completely melted in the time span of 11.3 ns. The stand IV, a part of triple stranded domain, begun to lose its structural contacts at 6.3 ns and it fully lost the structures at about 15.3 ns. Interestingly the anti-parallel β -sheet interactions between the strand III and V were highly rigid and their interactions were not at all disturbed in the entire dynamic scale of the present simulations. Hence, it seemed that the protein assumed a partially structured state composed of strand III and V at this high temperature. In these contexts, it is worthy to mention that the TFE-induced and TCA-induced equilibrium unfolding pathways of CTX III from the same source have been reported in the literature [21,22]. It has been shown, in both cases, that the protein molecules were unfolded by accumulating an intermediate, which were characterized to possess significant amount of triple-stranded beta-sheet domain. Similarly, temperature-induced unfolding pathways of the CTX III (from the same source) were also examined at different solution pH by using fluorescence and circular dichroism spectroscopic techniques [23]. Though the study could demonstrate possible existence of stable intermediates at 353 K, pH 4, the structure of the intermediate could not be characterized at residue level resolution due to various experimental restraints. In these backgrounds, the partially structured intermediate of the CTX VI characterized in the present study may pave a way for generalizing kinetic events in the temperature/denaturants-induced unfolding of CTXs. In general, it is believed that understanding the folding pathways and structural characterization of intermediates accumulating in the folding pathways of proteins may throw hints on designing *de novo* peptides/proteins.

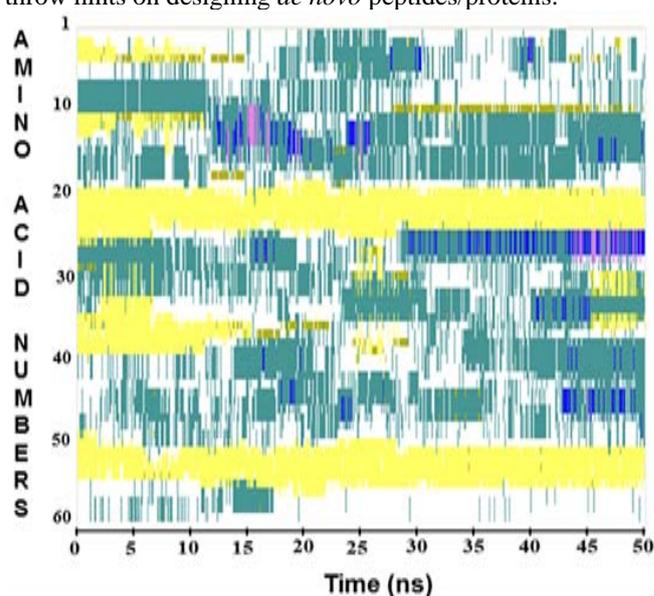


Figure 2: Various secondary structural contents of the CTX VI as estimated from the MD trajectories stored for the proteins at 473 K are shown with respect to the simulation time scales. Secondary structures such as strands, turns, α -helices, 3_{10} -helices and random coils are represented in yellow, green, magenta, blue and white colors, respectively.

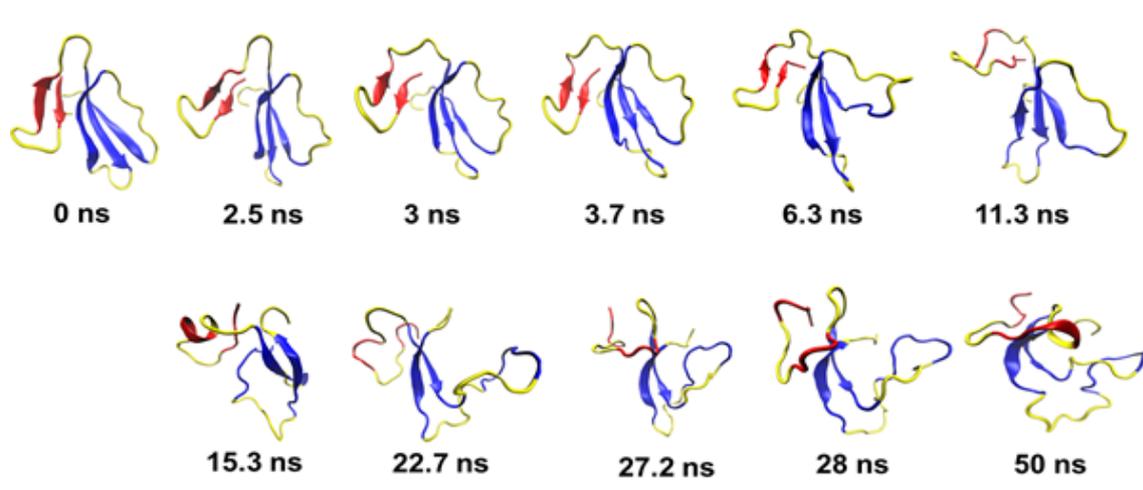


Figure 3: Snap-shots of trajectory structures of the CTX VI evolved at different time scales of the MD simulations carried out at 473 K are depicted in cartoon representations. The double-stranded domain, triple-stranded domain and unstructured portions of the CTX VI are represented in red, blue and yellow colours, respectively.

Figure 3 depicts the snap-shots of the 3D structures of the CTX VI at various simulation time scales, wherein the protein adopted remarkable conformational changes. From a quick inspection to the figure, one can unambiguously observe that the protein was unfolded to its partially structured intermediate around 16 ns at 473 K. Other than the structural features of intermediates, the dynamic data of the protein have also helped to uncover a few segments showing propensities to assume non-native helical conformations in their backbone conformations. Segments spanning residues from 9-14, 26-29, 33-36 and 45-48 adopted 3_{10} helical conformations at various dynamic periods (Figure 2). However, except the region comprising of 26-29, the other segments assumed helical structure for a short time span of a few ns only. The region comprising of residues 26-29 adopted helical structure at 28 ns and the structure could be well stabilized till the 50 ns simulation. Interestingly, the region (26-29) is well conserved in all CTXs reported (82 sequences and 20 3D structures) to date implying that the region should be responsible for non-native helical structures of the proteins in the presence of helix-inducing solvents such as ethanol, trifluoro ethanol (TFE) and hexafluoro isopropanol (HFIP). Since the solvents are membrane mimicking potential, the region undergoing drastic conformational changes (from strand/turn to helix) of the protein must be responsible for its interaction on the membrane surfaces. Experimental validations for the significant findings (partially structured intermediates and membrane spanning regions of CTX VI) reported by means of the MD simulations in the present research work are right now under progress in our laboratory.

4. CONCLUDING REMARKS

In the present study, we have examined the temperature-induced unfolding of CTX VI from *Naja naja atra* at 473 K in close quarters of physiological conditions (pH 7.0, 1 atmospheric pressure and 0.1 M ionic strength) using molecular dynamic simulations. The comprehensive structural analysis of 2,000 trajectory files obtained from 50 ns simulations of the protein revealed that the protein

adopted a partially unfolded state at high temperature of 473 K. We have, herein, showed that the partially structured intermediate of the protein was found to be possession of the triple-stranded domain constituting of stands III & V. Though the temperature-induced unfolding of many cardiotoxins from snake venoms have been experimentally characterized and reported in the literature, structural characterizations of the intermediate states accumulating in temperature-induced unfolding pathways of the proteins have not been successful at residue levels resolutions due to various experimental limitations (heterogeneity and structural flexibility). In these backgrounds, we strongly believe that the results reported in the present study may particularly be helpful to generalize our understanding on the temperature-induced unfolding pathways of the cardiotoxins. Moreover, the MD studies on CTX VI have also brought a few hints on identifying regions that may presumably interact with biological membrane surfaces.

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