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Structural Stabilities of Two Homologous Proteins from *Naja naja atra* as Examined by Molecular Dynamics Simulations.

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ABSTRACT

Two homologous proteins, cardiotoxin (CTX III) and cobrotoxin (CBTX), from venom of Taiwan cobra (*Naja naja atra*) were subjected to molecular dynamics (MD) simulations for 25 nanoseconds under similar physiological conditions (0.1 M ionic strength, pH 7.0 and 1 atmospheric pressure) at four different temperatures (298 K, 310 K, 373 K, 473 K). Trajectory structures of simulations stored at every 5 picoseconds were used for analysis of different structural events occurring in unfolding of the proteins. CTX III retained its three-dimensional conformation throughout the simulations performed in all 4 different temperatures used in the study, whereas, CBTX underwent remarkable structural changes at 473 K. Data analysis of the MD revealed that C-terminal region of CBTX is much more unstable and flexible comparing the counterpart segments in CTX III. Moreover, the MD simulations analysis clearly showed that overall unfolding stability of CTX III was stronger than that of CBTX. These findings were consistent with structural stabilities of the proteins reported in the literature and also reinforcement the 'CN network' hypothesis, which is useful to predict thermodynamic stabilities of three-finger toxins super-family from snake venoms in qualitative manner.

Keywords: 'CN network' hypothesis, molecular dynamics, stability, three-finger toxins.

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INTRODUCTION

Natural toxins are produced from four primary kingdoms: bacteria, fungi, plants and animals. The natural toxins are extremely specific in their interaction with target molecules and provide impetus for the design and development of numerous therapeutic agents [1-7]. Particularly, the investigation on toxins from snake venoms has provided profound implications for the drug discovery process. Snake venoms are complex mixtures of bioactive compounds such as peptides and proteins. These proteins and peptides exhibit a broad spectrum of interesting biological activities. In several instances, the snake toxins themselves have been developed into therapeutic agents [8-10].

The cardiotoxins and neurotoxins (cobrotoxins) are belonging to three-finger toxins super-family of snake venoms. Members of the super-family are of non-enzymatic polypeptides containing 59-74 amino acid residues and have similar three-dimensional folds consisting of five β-strands and 4-5 disulfide bridges [11-13]. Notwithstanding similar 3D structures, they exhibit wide array of different biological activities [14-16]. For instance, cardiotoxins exhibit biological functionalities like muscle contraction and lysis of erythrocytes, whereas neurotoxins block nerve transmission by post-synaptic binding to the acetylcholine receptors. As the 3D folds, stabilities and functions of proteins, in general, are highly correlated to each other, in the present study, we have scrutinized unfolding stabilities of two structurally similar proteins by means of MD simulations in order to map-out residues that are essential for structural integrities and functional activities of the proteins.

Cardiotoxin III (CTX III) and cobrotoxin (CBTX) from *Naja naja atra* snake venom are belonging to three-finger toxin super-family and are single polypeptide chains made-up 60 and 62 amino acids (about 7 KDa), respectively. The three-dimensional structures, stabilities and biological activities of the proteins have been well-characterized by using various experimental methods [17-21]. In these backgrounds, the two proteins are ideal models to study by means of MD simulations and also to interpret outcomes of the MD in terms of structure-stability relationships of the structurally similar proteins. In the present study, temperature-induced unfolding events of the CTX III and CBTX have been studied at four different temperatures (273K, 310K, 373K and 473K) under near physiological conditions (0.1 M ionic strength, pH 7.0 and 1 atmospheric pressure) by molecular dynamics simulations for 25 ns. Extensive analysis carried out on the MD data suggested that the CTX III was higher in the structural stability than that of the CBTX. Moreover, the MD data paved a way to rationalize ('CN network' hypothesis) the differential stabilities of the two structurally similar proteins. We have also undertaken a comparative analysis between the MD data of present study with stabilities of the proteins examined by experimental methods at molecular and atomic level resolutions as reported in the literature.

METHODS

The 3D structures of CTX III (2CRT) [22] and CBTX (1COD) [23] were retrieved from protein data bank and were subjected to 25 ns molecular dynamics simulations using GROMACS 4.5.4 [24] at four different temperatures (298 K, 310 K, 373 K, 473 K) under unique physiological conditions (0.1 M ionic strength, pH 7.0 and 1 atmospheric pressure).

Force field and water model used for all MD simulations performed in the study were AMBER99SB-ILDN [25] and TIP3P [26], respectively. Protein structures (2CRT and 1COD) retrieved from PDB server were pre-processed through PDB2PQR server [27] to obtain correct protonation states of residues at neutral pH, before subjecting them to MD simulations. The steps involved in MD simulations using GROMACS were topology generation, defining box, solvation, adding ions, energy minimization, equilibration and production MD. The protein molecules were placed at the centre of dodecahedron box maintaining 1.5 nm for 298 K and 310 K, 2.5 nm for 373 K and 3.0 nm for 473 K from the edges of the box. Equivalent amounts of sodium (Na^+) and chloride (Cl^-) ions were added to maintain ionic strength of 0.1 M concentration. The protein systems were then subjected to energy minimization using steepest descent algorithm [28] in order to remove steric clashes and bad geometry. After the energy minimization, the systems were equilibrated for 500 picoseconds under isothermal conditions to achieve the desired temperatures using Berendsen coupling algorithm [29] and the systems were also coupled with V-rescale barostat [30] for 1 nanoseconds to equilibrate them at 1 bar pressure. Once systems attained equilibrated states at desired temperature and pressure, production runs were performed for 25 nanoseconds with a time step of 2 femtoseconds. Bonds involving hydrogen atoms were constrained according to the LINCS [31] protocol, long range electrostatic interactions were calculated through the Particle Mesh Ewald (PME) approach [32] and Coulomb interaction and Van der Waals interactions were truncated at 10 Å. Trajectory structures stored at every 5 picoseconds were analyzed using GROMACS scripts and VMD molecular visualization tool [33].

RESULTS AND DISCUSSION

In order to map the structural determinants for the structural integrities of the CTX III and CBTX, the 3D structures of the proteins were subjected to 25 nanoseconds (ns) MD simulations at 298, 310, 373 and 473 K in near physiological conditions. Various structural events of the CTX III and CBTX in each MD simulations were examined using their trajectory structures (stored at every 5 picoseconds). Overall structural variations of the proteins were preliminarily monitored through the changes in backbone RMSD (root mean square deviation) of their structures in the dynamic timescale of 25 ns. The CTX III remained native-like structures with backbone RMSD fluctuating between 2 to 10 Å throughout 25 ns simulations at 4 different temperatures used in the present study (data not shown). While the CBTX maintained native-like structures at 298 K, 310 K and 373 K temperatures, the protein exhibited a set of breathing motions leading to remarkable conformational changes at 473 K (data not shown). Hence, the unfolding stabilities of the CTX III and CBTX have been compared at 473 K in a systematic manner. Root mean square flexibility (RMSF) resolved at individual amino acid of the CTX III and CBTX have been shown in Figure 1A and Figure 1B, respectively. From a quick inspection to the figures, one can easily observe that the structured regions (5 β-strands) of the proteins have less flexibility vis-à-vis respective unstructured regions (loops and termini regions) of the proteins. Moreover, maximum flexibilities of C-termini regions (the chain starting from the end of stand V) of the CTX III and CBTX were found to be around 0.4 nm and 1.1 nm, respectively, at 473 K. In addition, orders of flexibilities of the CTX III and CBTX were loop I > loop III > loop II > C-terminal and C-terminal > loop I > loop III > loop II, respectively (Fig. 1).

Figure 2A and 2B show secondary structural content of each residue with respect to simulation time of CTX III and CBTX at 473 K, respectively. As illustrated in the Fig. 2A, all five strands of CTX III were found to be intact throughout the simulations and no significant changes in overall secondary structural contents of the protein was observed at the temperature. However, transition of a few residues (31-33) in strand IV to coil was observed in the time span of 1-7 ns and as well around 15-25 ns. In contrary, double stranded domain composed of strand I & II of CBTX began to melt at around 9 ns and fully lost the secondary structures at around 17 ns implying that the protein adopted partial folded conformation at 473 K. Moreover, 3_{10}-helical structures were erupted randomly after 7 ns in the region comprising 7 to 22 residues located in the double stranded domain of the CBTX.

Figure 3A and 3B represents the trajectory structures of the CTX III and CBTX evolved at different time scales of MD simulations at 473 K. Structural changes induced by temperature were analyzed at every 5 picoseconds. Figure 3A implies that structures of CTX III were stable throughout the MD simulations at the defined temperature. On the other hand, significant changes evolved in CBTX during molecular dynamics simulations were shown as snapshots diagrams in Figure 3B and order of destabilizing events can also be viewed through the snapshots: at around 7 ns time scale, C- and N-termini of the protein were fully departed from each other; both strand I and II of double stranded domain of the protein were lost at around 10 ns; at about 15.7 ns, a region spanning residues from 13 to 15 adopted non-native 3_{10}-helical segment; the protein remained with partial structured conformation (with intact triple-stranded domain only) in the rest of MD simulation carried out for 25 nanoseconds. Taken together the analyses on the MD data of the two proteins, it is obvious that the unfolding stability of CTX III is stronger than that of CBTX.

Structural stabilities of the CTX III and CBTX examined by experimental methods have been reported in the literature at molecular and as well at atomic level resolution. Guandinium hydrochloride (GdmHCl)-induced free energy of unfolding (ΔG_U) of CTX III and CBTX were documented to be 4.9 kcal/mol and 2.3 kcal/mol, respectively, at pH 3.2 and 298 K temperature [18, 20, 21, 34]. The unfolding stabilities of the CTX III and CBTX have also been resolved at residue level by using hydrogen-deuterium (H/D) exchange studies in conjunction with NMR techniques [20, 21]. Based on the H/D exchange studies, overall free energy of exchange (ΔG_{HX}) for double-stranded domain, triple-stranded domain and C-termini of CTX III were reported to be 2.97, 4.17 and 4.00 kcal/mol, respectively; similarly, overall free energy of exchange (ΔG_{HX}) for double-stranded domain, triple-stranded domain and C-termini of CBTX were reported to be 3.09, 3.10 and 2.26 kcal/mol, respectively. The free energies of exchange analysis clearly suggested that differential stabilities of the structurally similar proteins were due to differences in the inter-residual non-covalent interactions of amino acids present in the triple-stranded domain and C-terminal regions of the proteins. These results are in excellent agreements with the data derived from MD simulations studies on the proteins as demonstrated above in the present work, wherein we showed rigidities of triple-stranded domain and C-terminal in CTX III were significantly higher than their counterparts in CBTX (Fig.2 and Fig.3). It is worth mentioning that we have recently proposed a 'CN network' hypothesis to qualitatively rationalize the differences in structural stabilities of three-finger toxins [35]. According to the hypothesis, network interactions between C- and N-termini of the proteins are positively correlated with their unfolding stabilities. The 'CN network' values of the CTX III (2CRT) and CBTX (1COD) were

calculated as 7 and 2, respectively, implying that the CTX III should be higher in stability than that of CBTX. Strikingly, the present MD studies on the proteins endorse the reliability of the hypothesis. Moreover, the present study unambiguously showed that the CTX III is stronger in structural stability than that of CBTX. And, the MD data clearly uncovered that network interactions between the C-terminal and N-terminal of CBTX were weaker comparing to their counterparts of CTX III. Taken together, the 'CN network' hypothesis and thermodynamic stabilities of the three-finger proteins examined by experimental (optical and H/D exchange studies reported in the literature) and computational (MD simulations of the present study) methods are consistent to each other and the experiments unanimously divulge that differences in the 'CN network' of the proteins are prime structural factors to account the differential unfolding or thermodynamic stabilities of the structurally similar three-finger toxin family proteins.

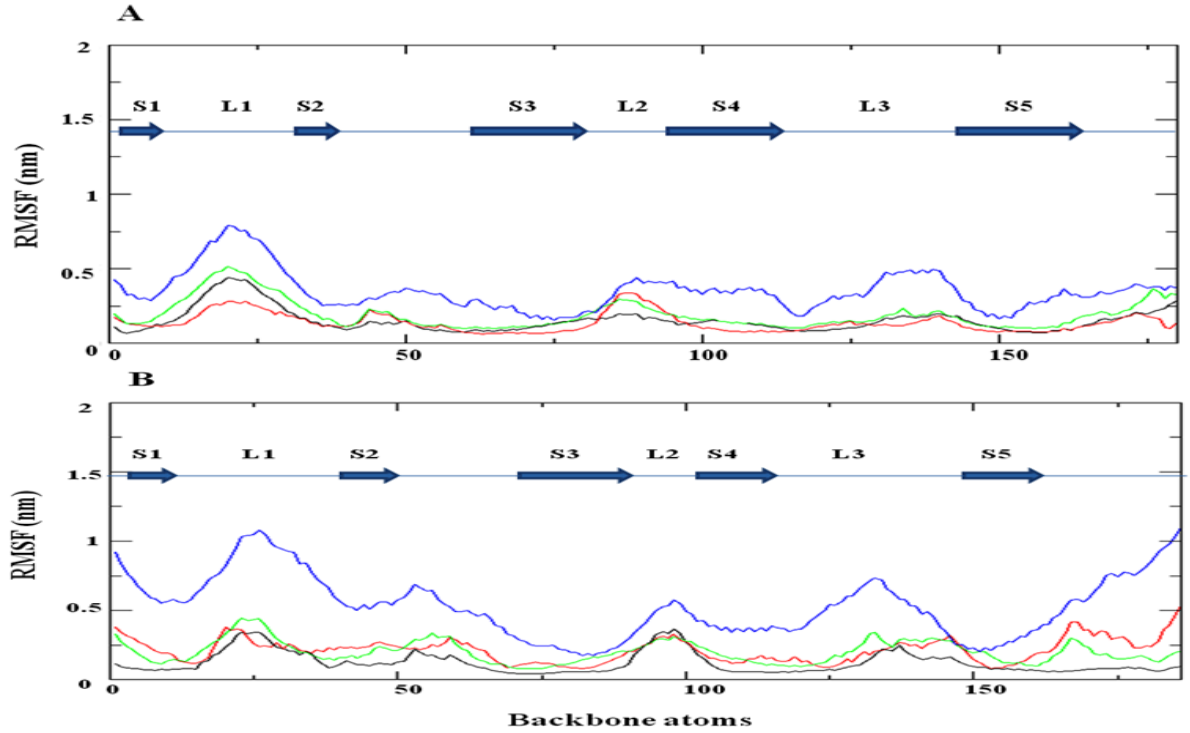

Figure 1: Root mean square fluctuations (RMSF) of backbone atoms in (A) CTX III and (B) CBTX are plotted with respect to dynamic time scales at four different temperatures. The RMSF data at 298 K, 310 K, 373 K and 473 K are shown in black, red, green and blue colours, respectively. Various structural contexts of the proteins are represented by lines (L1 - L3: Loop I, Loop II and Loop III) and horizontal-arrows (S1 – S5: Strand I, Strand II, Strand III, Strand IV and Strand V).

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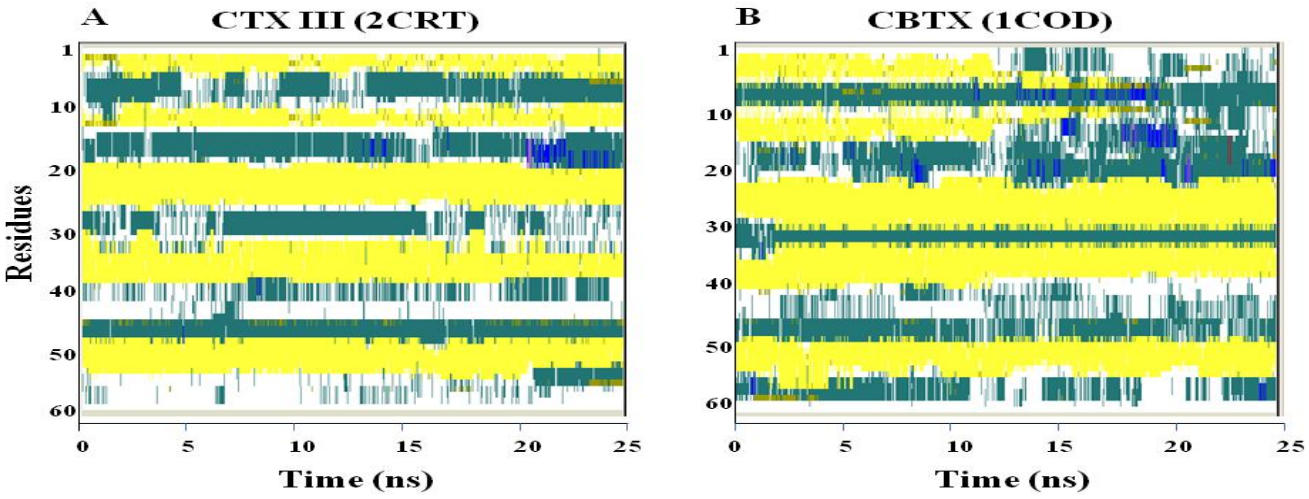

Figure 2: Secondary structural contents from MD simulations performed for (A) CTX III and (B) CBTX at 473 K are shown with respect to simulation time scales. Strands, turns, α-helices, 3_{10}-helices and random coils are represented by yellow, green, magenta, blue and white colours, respectively.

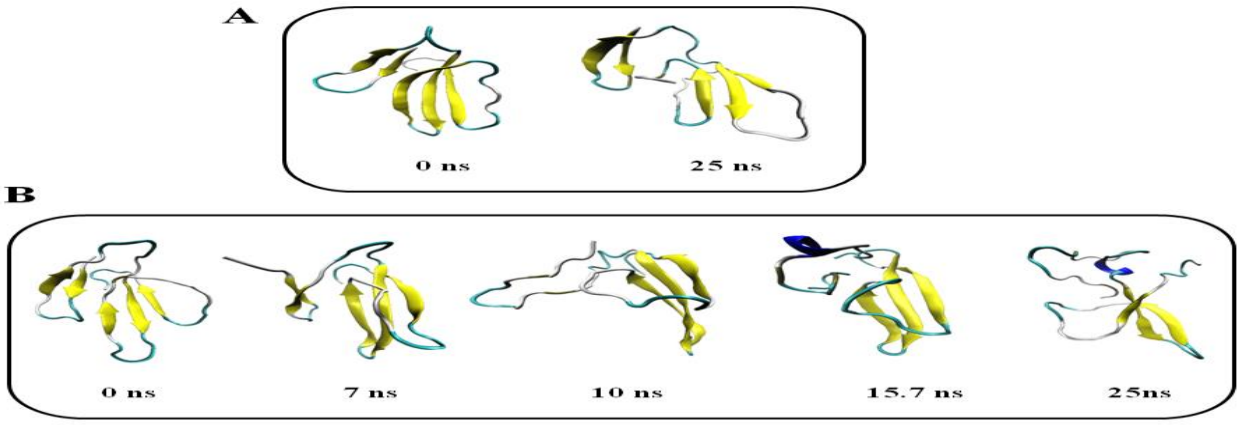

Figure 3: Snapshots of MD structures of CTX III and CBTX. Trajectory structures of (A) CTX III and (B) CBTX evolved at different time scales of molecular dynamics simulations carried out for the proteins at 473 K are illustrated in cartoon models.

CONCLUSION

In the present study, we have studied the structural stabilities of two paralogous proteins of three-finger toxin family from Taiwan cobra (*Naja naja atra*) by means of all-atom MD simulations in near physiological conditions at 473 K. Thorough analysis on the structural events encountered by the proteins were scrutinized through their trajectory structures stored at every 5 picoseconds in the 25 nanoseconds MD simulations and the analysis clearly demonstrated that inter-residues non-covalent interactions among the amino acids located in the C- and N-termini regions of the proteins play significant roles on governing stabilities of the proteins. The data presented in the paper are consistent with unfolding stabilities of the proteins determined by experimental methods and also endorse the 'CN network' hypothesis proposed to qualitatively analyse the thermodynamic stabilities of the three-finger toxin family proteins from snake venoms. It is believed that more detailed MD simulations on exploring structural stabilities of a large number of three-finger

toxins would be conducted in near future, which in turn may be helpful to precisely predict thermodynamic stabilities of the proteins in qualitative and as well quantitative manner.

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