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Unfolding stabilities of two paralogous proteins from *Naja naja naja* (Indian cobra) as probed by molecular dynamics simulations

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ABSTRACT

Structurally similar but functionally different two paralogous proteins, CTX1 (a cardiotoxin) and LNTX2 (an alpha-neurotoxin), from venom of *Naja naja naja* have been homology modeled and subjected to molecular dynamics (MD) simulations at four different temperatures (298 K, 310 K, 373 K & 473 K) under close quarters of physiological conditions. Each MD simulation was performed for 25 ns and trajectory structures stored at every 25 ps were used to probe various structural events occurring in the temperature-induced unfolding of the proteins. Notwithstanding their similar scaffolds, the two proteins are drastically differing in their unfolding stabilities from each other. The structural orders of flexibilities for the CTX1 and LNTX2 were found to be loop II > loop III > loop I > C-terminal and C-terminal > loop I > loop III > loop II, respectively. Based on the comprehensive analyses of the simulation data and studies on the various structural interactions of all cardiotoxins (CTXs) and alpha-neurotoxins (NTXs) for which three-dimensional structures determined by experimental techniques are available to date, we have herein proposed a hypothesis ('CN network') rationalizing the differential stabilities of the CTXs and NTXs belonging to a three-finger toxin superfamily of snake venoms.

1. Introduction

Protein toxins belonging to three-finger toxin (TFT) superfamily have been reported to exist in all families of snakes (Hegde et al., 2009). Most of the TFTs are single polypeptide chain consisting of 60–74 standard amino acids and all β-sheet proteins. Exempting a few of the TFTs that are reported to exist as dimers and to have a short helical segment in their loop II, all the TFTs depict similar protein folds: five anti-parallel strands, three loops, a globular head and an unstructured C-terminal segment (Kang et al., 2011; Kini and Doley, 2010). Notwithstanding the similar topology structures, the TFTs could be grouped into many families based on their unique biological activities (Kini, 2011): α-neurotoxins (target muscle nAChR – nicotinic acetylcholine receptor), β-cardiotoxins (target β_1/β_2 adrenergic receptors), cardiotoxins (target lipid membranes), calciseptines (block L-type calcium channels), dendroaspins (inhibit platelet aggregation), fasciculins (inhibit AChE – acetylcholine esterase), k-bungarotoxins (target neuronal nAChR) and muscarinic toxins (target muscarinic AChR; Doley and Kini, 2009; Rajagopalan et al., 2007; Kini, 2006, 2002; Nirthanan et al., 2003; Karlsson et al., 2000; Antil-Delbeke et al., 2000; Tsetlin, 1999; Menez, 1998; De Weille et al., 1991; Chiappinelli and Wolf, 1989). Since the TFTs have similar moulds with different functions, understanding the structure–function relationships of the protein toxins is an uphill task and the lacuna has not yet been unambiguously addressed. On the other hand, simple folds, compact in sizes, free of co-factors, well

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characterized structures and functions of the proteins make them as suitable candidates for uncovering the 'protein folding paradox' – a quest for correlating the relationships among the sequences, folds, stabilities and functions of proteins, in general.

Proteins belonging to the TFT superfamily of the snake venoms cause a wide array of physiological reactions leading to risk of death, when the venoms are injected into victims (Li et al., 2004). Of the many groups of the TFT superfamily, the CTXs and NTXs are most abundant and principal toxic components. Moreover, the CTXs and NTXs are well-characterized proteins among the TFT superfamily: to date, 82 and 115 authentically annotated primary structures of the CTXs and NTXs purified from various species of snakes have been reported in the literature, respectively. As on Jan 2013, 84 three-dimensional (3D) structures of the TFTs determined by experimental methods have been deposited in the 'protein data bank': of the 84 structures, 20 (3VTS, 1TGX, 1CDT, 1CVO, 1CXN, 2CCX, 1CRE, 2CDX, 2CRT, 1KBS, 1KXI, 1CCQ, 1CHV, 1FFJ, 1I02, 1H0J, 1UG4, 1XT3, 1ZAD and 2BHI) and 25 (1NXB, 3EBX, 1NTX, 5EBX, 6EBX, 1NEA, 1NOR, 1COD, 1ERA, 1QKE, 3ERA, 1G6M, 1JE9, 1ONJ, 1IQ9, 1V6P, 3NDS, 1CTX, 2CTX, 1NTN, 1LSI, 1TXA, 1LXH, 1W6B and 1YI5) are non-redundant structures of the CTXs and NTXs, respectively. The biological functions of the families have also been characterized to most extent vis-à-vis that of other families of the superfamily. The CTXs exhibit cytolysis, hemolysis and heart failures, whereas the NTXs cause paralysis by targeting nicotinic acetylcholine receptors (nAChRs) at the post-synaptic level of neuromuscular junctions (Kini, 2011; Servent et al., 2000). The NTXs are further classified as LNTXs (long-chain neurotoxins) and SNTXs (short-chain neurotoxins) based on their amino acid compositions and number of disulfide bonds. Interestingly, both the LNTXs and SNTXs showed similar affinities for binding on muscle nAChR, whereas the toxins drastically differed in their interactions with neuronal nAChR (Servent et al., 1997). These findings on the CTXs and NTXs suggest that the protein toxins adopt similar scaffolds with subtle but crucial differences among them to be versatile 3D folds executing various physiological processes on their target systems.

Experimental studies on the thermodynamic stabilities, kinetic folding and equilibrium unfolding of a few CTXs and NTXs have been reported in the literature (Sivaraman et al., 2000, 1999a, 1999b, 1999c, 1998; Jayaraman et al., 1996). Also, interactions between the CTXs and lipid membrane surfaces have been examined by using experimental techniques and computational methods (Hung et al., 2012; Levtsova et al., 2009; Dubovskii et al., 2001; Raynor et al., 1991). However, the rationalization for the structural integrities of the CTXs and NTXs is still so as to understand how the proteins of similar moulds drastically differ in their flexibility regions and structural determinants. In the present study, we have studied temperature-induced unfolding events of cardiotoxin 1 (CTX1) and long neurotoxin 2 (LNTX2) from the venom of Indian cobra (*Naja naja naja*) at four different temperatures (298, 310, 373 and 473 K) in near physiological conditions (pH 7.0, 1 atmospheric pressure and aqueous solution of 0.1 M ionic strength) by means of MD simulations. Analysis of various structural parameters of trajectory structures obtained from the MD simulations of the proteins revealed that most flexible segments of the CTX1 and LNTX2 are loop II and C-terminal region, respectively. And most stable structural parts of the CTX1 and LNTX2 were found to be regions comprising of their globular heads and strands III, IV and V. However, the triple-stranded domain of the CTX1 showed greater structural rigidity comparing that of counterpart region of the LNTX2. Moreover, the CTX1 did not undergo any remarkable conformational changes even at high temperatures (373 and 473 K), whereas LNTX2 lost significant amount of its secondary structures at 473 K. Rationalizations for the differential structural stabilities of the homologous toxins have been attributed to the differences in the structural contacts between the C- and N-termini regions in their 3D structures. In addition, we have, herein, proposed a hypothesis, 'CN network', based on a comprehensive analysis of the data from MD simulations of the present study and structural parameters derived from experimental structures of all the CTXs and NTXs available to date, to qualitatively addressing the differential thermodynamic stabilities of the structurally similar CTXs and NTXs from the venoms of snakes.

2. Materials and methods

2.1. Retrieval of primary and tertiary structures of the TFTs

Primary structures of all the TFTs from Indian cobra, reported to date, have been retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) and UniProt (http://www.uniprot.org/) databases and the sequences were pre-processed by PrediSi (http://www.predisi.de/) and SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The tools, PrediSi and SignalP, were used to identify and to remove signal peptides and pro-peptides, if they found to present in the raw sequences, from the TFTs in the pre-processing. The pre-processed sequences were subjected to multiple sequence alignments (MSA) by means of ClustalX (Thompson et al., 1994) and the analyses resulted in 9 non-redundant TFTs of the snake venom. The literature annotations of the 9 sequences are as follows: 1 cytotoxin (CTX1, GI117667), 2 cytotoxin-like proteins (GI85687562 and GI1184523), 2 long neurotoxins (LNTX2 – GI128943 and LNTX4 – GI128950) and 4 weak toxins (GI136566, GI136565, GI136564 and GI136563). Moreover, predicted annotations for the sequences by TFTX 1.0 (Rajesh and Sivaraman, 2011), a sequence-based computational tool for classifying the CTXs and NTXs (http://sblab.sastra.edu/tftx.html), were also consistent with the literature annotations of the protein toxins and the analyses also revealed that the LNTX2 possessed more characteristic features to be a typical LNTX than that of the LNTX4. Based on the combined analyses of the data from the literature annotations, MSA and the TFTX tool, the CTX1 and LNTX2 were selected for investigating their structures–stabilities correlations in the present study. The experimental 3D structures of the CTXs, LNTXs and SNTXs were retrieved from the PDB (http://www.pdb.org/pdb/home/home.do) and their non-bonding intra-molecular interactions (residue–residue contacts and hydrogen bonds) were scrutinized by means