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Understanding the Structure, Function and Folding of Cobra Toxins

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ABSTRACT

Cardiotoxins and neurotoxins derived from cobra venoms are small molecular weight (6.5-9.0 kDa) homologous proteins with a high degree of disulfide crosslinking. The lethality of the cobra venoms are attributed to the cardio- and neurotoxins. The three-dimensional structures of the members of these two classes of toxins show striking resemblance. Both cardio- and neurotoxins are 'three-finger' shaped proteins with three loops projecting from a globular head. The secondary structural elements in both of the toxins include an antiparallel triple and a double stranded β-sheet. Interestingly, despite the fact that the overall topologies of their three-dimensional structures are similar, cardio- and neurotoxins show drastically different biological properties. The molecular basis for the differential functional properties of these two classes of toxins is still not clear. The aim of this comprehensive review is to summarize and critically evaluate recent progress in research on the structure, function and folding aspects of cobra venom cardio- and neurotoxins.

Key Words: all β-sheet proteins, cobra toxins, folding, function, structure

I. Introduction

Venoms of snakes belonging to the *Elapidae* family are highly toxic and produce effects such as flabby paralysis and respiratory failure in higher animals (Dufton and Hider, 1983, 1991). Most often, these effects culminate in the death of the victim. The lethal effects of a snake bite are attributed to the presence of a variety of toxic principles in their venoms (Harvey, 1985). The most prominent of the lethal ingredients in the elapid venoms belong to the class of toxic polypeptides termed neurotoxins and cardiotoxins or cytotoxins (Kumar *et al*., 1994, 1997). Chemically, these neurotoxins and cardiotoxins are highly homologous (> 50% homology) proteins with identical positioning of the disulfide bridges (Dufton and Hider, 1983; Yu *et al*., 1994). Interestingly, despite the high degree of homology among the members belonging to these toxin classes, they differ drastically in their biological properties. Neurotoxins act on the acetylcholine receptor at the post-synaptic level of the neuromuscular junction (Zinn-Justin *et al*., 1992; Yang *et al*., 1969). In contrast, cardiotoxins exhibit a wide array of biological activities, such as (1) depolarization and contraction of muscular cells, (2) prevention of platelet aggregation and (3) lysis of cells like erythrocytes, epithelial cells, fetal lung cells and certain types of tumour cells, such as *Yoshida Sarcoma* cells (Kumar *et al*., 1997; Rees and Bilwes, 1993; Hinman *et al*., 1987). In addition, snake venom cardiotoxins are also known to inhibit the activity of enzymes, such as the Na^+, K^+-ATPase and protein kinase C (Kuo *et al*., 1983; Raynor *et al*., 1991; Chiou *et al*., 1993). It is still an enigma as to how and why snake venom neurotoxins and cardiotoxins, inspite of significant amino acid sequence similarities, exhibit entirely dissimilar functional properties. It is imperative that a detailed analysis of the structural features of these

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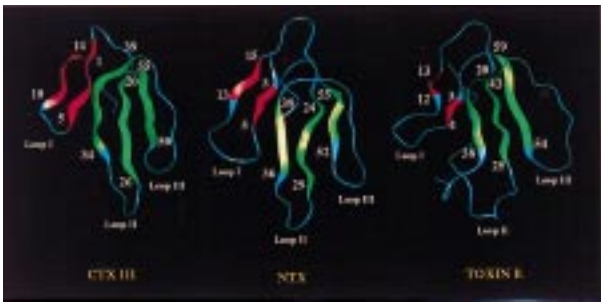

Fig. 1. Ribbon representation of CTX III (*Naja naja atra*), NTX (*Naja naja atra*) and toxin b (*Ophiophagus hannah*), depicting the overall backbone fold and the secondary structural (in red and green) elements.

neurotoxins and cardiotoxins be made to aid our understanding of the molecular basis of the disparity that exists in the biological properties of the members of these two toxin groups. In this review, while focusing on recent progress made in research on aspects of the structure, function and folding of cardiotoxins and neurotoxins from the Taiwan Cobra (*Naja naja atra*), we attempt to provide an overview of the current status and the future research directions in this field in general.

II. The Chemistry of Toxins

Cardiotoxins and neurotoxins are highly homologous proteins heavily crosslinked by disulfide bridges (Dufton and Hider, 1983). They are small molecular weight (6.5-9.0 kDa), all β-sheet proteins (Kumar *et al*., 1994) (Fig. 1). Characteristically, these proteins lack helical segments in their three-dimensional structures (Arunkumar *et al*., 1996; Sivaraman *et al*., 1997c; Jayaraman *et al*., 1996b).

Cardiotoxins, with few exceptions, are single chain, sixty amino acid, highly basic (*pI*>10.0), small molecular weight (6.5-7.0 kDa) proteins (Kumar *et al*., 1997; Hung *et al*., 1993). To date, the amino acid sequences of about 52 cardiotoxins are known (Kumar *et al*., 1997). Comparison of the primary sequences of the members belonging to this class reveals that they are highly conserved, sharing more than 90% amino acid sequence homology. In general, the disulfide bonds in cardiotoxins are located at positions 3-21, 14-38, 42-53 and 54-59. At the present time, six cardiotoxin analogues have been isolated and purified from the venom of the Taiwan Cobra (*Naja naja atra*) (Kaneda *et al*., 1977; Hayashi *et al*., 1975; Chiou *et al*., 1995) (Table 1). The amino acid sequence of cardiotoxins, in general, are characterized by a lack of glutamic acid. A closer look at the sequences of the cardiotoxin isoforms from the Taiwan Cobra shows that these toxin analogues also are bereft of histidine and tryptophan (Table 1). Interestingly, the presence of a tripeptide sequence, -I-D-V-, at residue positions spanning 39-41 is the characteristic feature of snake venom cardiotoxins. There is much controversy over the nomenclature of cardiotoxins (Sivaraman *et al*., 1997b). For example, highly basic, similar molecular weight (~7 kDa) proteins with amino acid sequences homologous to those of cardiotoxins have been recently isolated from many snake venom sources (Takechi *et al*., 1985). These proteins are collectively called the 'cardiotoxin-like basic proteins' (CLBPs) (Takechi *et al*., 1985). One such CLBP isoform has been reportedly isolated from the venom of the Taiwan Cobra (Takechi *et al*., 1985). Careful comparison of the amino acid sequence of CLBP with the cardiotoxin isoforms from the same source (*Naja naja atra*) reveals subtle but important differences. While the amino acid sequences of all cardiotoxins show the presence of the tripeptide signature sequence, -I-D-V-, extending between residues 39-41, CLBP lacks such a sequence. In addition, CLBP from *Naja naja atra* also lacks methionine residues, which are strongly implicated in the biological activity(ies) of cardiotoxins. It should be mentioned that CLBPs possess no cardiotoxin-related biological properties. Currently, the functional significance of the presence of CLBP in snake venoms is not understood, although *in vitro* studies indicate that these proteins promote the aggregation of defined lipid vesicles (Chien *et al*., 1994).

Neurotoxins, based on the length of the amino acid sequence, are conventionally classified as short and long neurotoxins (Dufton and Hider, 1983; Endo and Tamiya, 1987; Joubert, 1973). The short neurotoxins are mostly sixty two amino acids long and crosslinked by four disulfide bridges (Dufton and Hider, 1983). Long neurotoxins, on the other hand, consist of more than seventy amino acids and characteristically contain an extra disulfide bridge as compared to the short neurotoxin counterparts (Endo and Tamiya, 1991). It is still a mystery as to why two different types of neurotoxins should exist in the same venom source. So far, only a short neurotoxin, cobrotoxin, has been reported to occur in the venom of the Taiwan Cobra (Chiou *et al*., 1995). The amino acid sequence of the long neurotoxins further differ from the short neurotoxins with respect to the deletion of amino acid residues in the segment of residues 4 to 16 and the presence of additional amino acid residues 32, 34, 35 and 36 (Endo and Tamiya, 1991). In addition, amino acid sequences of long neurotoxins are characterised by the insertion between Cys45 and Cys49 of the sequence Ala46-Ala47-Thr48 instead of Gly48, which is present

Table 1. Amino Acid Sequences of Selected Cardiotoxins and Neurotoxins

Source	Toxin	Reference
Neurotoxin from Taiwan Cobra (*Naja naja atra*) venom	**Cobrotoxin** LECHNQQSSQ TPTTTGCSGG ETNCYKKRWR DHRGYRTERG CGCPSVKNGI EINCCTTDRC NN	Yang *et al.* (1969)
Neurotoxin from King Cobra *(Ophiophagus hannah)* venom	**Toxinb** TKCYVTPDAT SQTCPDGQDI CYTKTWCDGF CSSRGKRIDL GCAATCPKVK PGVDIKCCST DNCNPFPTWK RKH	Joubert *et al.* (1973)
Cardiotoxins analogues from Taiwan Cobra (*Naja naja atra*) venom	**CTX I** **L**KCN**K**L**I**P**IA S**KTC**P**AGKNL CYKMFM**MSDL TI**PVKRGCID VCPK**NS**LLVK YVCCNTDRCN	Hayashi *et al.* (1975)
	CTX II **L**KCN**K**L**V**P**LF Y**KTC**P**AGKNL CYKMFM**VSNL TV**PVKRGCID VCPK**NSA**LVK YVCCNTDRCN	Kaneda *et al.* (1977)
	CTX III **L**KCN**K**L**V**P**LF Y**KTC**P**AGKNL CYKMFM**VATP KV**PVKRGCID VCPK**SS**LLVK YVCCNTDRCN	Narita *et al.* (1970)
	CTX IV **R**KCN**K**L**V**P**LF Y**KTC**P**AGKNL CYKMFM**VSNL TV**PVKRGCID VCPK**NSA**LVK YVCCNTDRCN	Kaneda *et al.* (1977)
	CTX V **L**KCN**K**L**V**P**LF Y**KTC**P**AGKNL CYKMFM**VSNK MV**PVKRGCID VCPK**SS**LLVK YVCCNTDRCN	Chiou *et al.* (1995)
	CTXn **L**KCN**Q**L**I**P**PF Y**KTC**A**AGKNL CYKMFM**VAAP KV**PVKRGCID VCPK**SS**LLVK YVCCNTDRCN	Hung *et al.* (1993)

Note: The variable amino acids among the different cardiotoxins are in bold type.

in short neurotoxins. Functionally, the important difference between the short and the long neurotoxins lies in the rates of association and dissociation with the cholinergic receptors. Long neurotoxins are generally found to associate and dissociate with the receptor more slowly than short neurotoxins (Dufton and Hider, 1983).

Neurotoxins, in general, like cardiotoxins, are all β-sheet proteins lacking helical conformation in their backbones (Dufton and Hider, 1983). As mentioned earlier, despite significant differences in their pharmacological properties, cardiotoxins and neurotoxins show a significant amount of homology (Dufton and Hider, 1991). Many invariant or type-invariant residues are conserved in both cardiotoxins and neurotoxins. Endo and Tamiya (1991) provided an accurate and critical comparison of the amino acid sequences of these two classes of toxins in their review. There are residues which are highly conserved among the neurotoxins but not among the cardiotoxins. These include Lys27, Trp29, Asp31, His/Phe/Trp33, Arg/Lys37, Gly38, Glu/Asp42, Val/Ala52 and Lys/Arg53 (as per the neurotoxin sequence numbering system). Interestingly, glutamic acid and histidine residues which are mostly not found in the cardiotoxin sequences are consistently found in the neurotoxin sequences.

Dufton and Hider (1983) systematically analysed differences in the distribution of the polar and nonpolar residues in the sequences of neurotoxins and cardiotoxins using hydrophathy index plots. They found that most of the neurotoxin sequences show a high density of hydrophilic residues. Hydropathy plots of cardiotoxin sequences, in contrast, show an asymmetric distribution of hydrophobic and hydrophilic amino acids. Kini and Evans (1989a, 1989b) showed that this unique spread of polarity of the residues among cardiotoxins is important for lytic activity exhibited by this class of toxins.

III. Three-Dimensional Structure of the Toxins

A quest to delineate the structural basis for the functional roles of cardiotoxins and neurotoxins resulted in the elucidation of solution and crystal structures of a great number of cardiotoxin and neurotoxin analogues, isolated from a variety of snake venom sources (Table 2). For convenience, we will discuss

Table 2. List of Cardiotoxin and Neurotoxin Analogues whose Three Dimensional Structures have been Solved

Toxin	Source	PDB Code	Reference
Cardiotoxins			
CTX I	*N. n. atra*	2CDX	Jahnke *et al.* (1994)[a]
CTX II	*N. n. atra*	1CRE, 1CRF	Bhaskaran *et al.* (1994b)[a]
			Jang *et al.* (1997)[a]
CTX IIB	*N. m. mossambica*	2CCX	O'Connell *et al.* (1993)[a]
CTX III	*N. n. atra*	2CRS, 2CRT	Bhaskaran *et al.* (1994a)[a]
			Sivaraman *et al.* (1998b)[a]
CTX IV	*N. n. atra*	1KBS, 1KBT	Jang *et al.* (1997)[a]
CTX V^{II_2}	*N. m. mossambica*	–	Otting *et al.* (1987)[a]
CTX V^{II_4}	*N. mossambica*	1CDT	Rees *et al.* (1990)[b]
CTX_{γ}	*N. pallida*	1CXO	Bilwes *et al.* (1994)[b]
Short neurotoxins			
NX1_DENPO	*D. p. polylepis*	1NTX	Brown and Wuthrich (1992)[a]
NXS1_LATSE	*L. semifasciata*	1FRA, 1ERA	Hatanaka *et al.* (1994)[a]
		5EBX	Corfield *et al.* (1989)[b]
NXS1_NAJAT	*N. n. atra*	1COD, 1COE	Yu *et al.* (1993)[a]
NXS1_NAJOX	*N. oxiana*	1NOR	Golovanov *et al.* (1993)[a]
NXS1_NAJPA	*N. pallida*	1NEA	Zinn-Justin *et al.* (1992)[a]
Long neurotoxins			
NXL1_BUNMU	*B. multicinctus*	2ABX	Love and Stroud (1986)[b]
		1ABT	Basus *et al.* (1993)[a]
NSL1_LATSE	*L. semifasciata*	1LSI	Connolly *et al.* (1996)[a]
NSL1_NAJKA	*N. n. kaouthia*	2CTX	Betzel *et al.* (1991)[b]
		–	Le Goas *et al.* (1992)[a]
NXL2_BUNMU	*B. multicinctus*	1KBA	Dewan *et al.* (1994)[b]
		1NBT	Oswald *et al.* (1991)[a]
Toxin b	*O. hannah*	1TXA, 1TXB	Peng *et al.* (1997)[a]

Note: The superscripts 'a' and 'b', in the references indicate that the structures have been solved by means of NMR and X-ray crystallography, respectively.

the salient features of the structures of cardio- and neurotoxins using the solution structures of cardiotoxin analogue III (CTX III) (Bhaskaran *et al.*, 1994a) and cobrotoxin isolated from Taiwan Cobra (Yu *et al.*, 1990, 1993) (*Naja naja atra*) venom.

1. Structure of Neurotoxins

The structure of neurotoxins present a picture of three loops emerging from a globular head (Yu *et al.*, 1990, 1993). When viewed from the front, the backbone of the neurotoxin molecule appears as a flat disc with a roughly round shape (Corfield *et al.*, 1989; Golovanov *et al.*, 1993; Hatanaka *et al.*, 1994). The three different loops contain five different strands and form a double and a triple stranded antiparallel β-sheet; Loop I involves two strands (Strand I and II) of the double stranded β-sheet (Yu *et al.*, 1993). The middle loop (Loop II) consists of strands III and IV of the triple stranded β-sheet and loop III contributes strand V. The double stranded β-sheet is characterized by backbone NH-O' hydrogen bonds 5-13, 15-3 and 13-5. The sheet in this region of the molecule has a relatively strong right-handed twist. The two β-strands involved in the double stranded β-sheet formation are connected by a three-turn (β-turn) segment (Gln7 to Glu10), which possesses a hydrogen bond between residues 7 and 10. Loop I on the surface of the protein has been shown to possess higher flexibility (Yu *et al.*, 1990, 1993).

The triple-stranded antiparallel β-sheet is formed among strand III (residues, 25-30), strand IV (residues, 36-40) and strand V (residues, 51-56). Strand III lies in between the other two. The triple stranded, antiparallel β-sheet is defined by backbone NH-O' hydrogen bonds between residues 27-38, 38-27, 29-36, 36-29, 55-24, 26-53, 53-26, 28-51 and 51-28 (Yu *et al.*, 1993). Strands III and IV are connected by a type II β-turn consisting of residues 31-34 (Turn II). The residues involved in this β-turn have been shown to have higher flexibility due to their greater exposure to solvent. The fourth and the fifth β-strands are connected by an eight residue long segment spanning residues 42-49 (Turn III). Similar to the β-turn con-

necting strands III and IV, this segment has high mobility. The higher mobilities observed in both of these two turns (Turns II and III) could be due to the presence of cationic groups (Arg33 and Lys47, respectively), which are thought to be functionally responsible for the neuromuscular blocking activity of the protein (Zinn-Justin *et al.*, 1992). The conformation of residues 55 to 62 in the carboxy terminal tail of the neurotoxin sequence appears to be involved in stabilization of the protein structure. This region is one of the well-defined portions in the short neurotoxin structures (Peng *et al.*, 1997). It has been reported that residues 57 to 60 form a distorted type II turn with a backbone NH-O' hydrogen bond between residues 60 and 57 in cobrotoxin isolated from the Taiwan Cobra (Yu *et al.*, 1993; Bhaskaran *et al.*, 1994c). This β-turn is further fortified by the presence of a hydrogen bond between the N- and C- terminal residues (a NH-O' hydrogen bond between residues 59 and 2) and the occurrence of a disulfide link between cysteine residues at 55 and 60. In addition, it is found that the side chain of residue 61 forms two hydrogen bonds to the backbone atoms of residues 4 and 25. The four disulfide bridges (3-24, 17-41, 43-54 and 55-60) are crucially positioned in the structure of the neurotoxins to fortify the structure (Yu *et al.*, 1993; Brown and Wuthrich, 1992). The double stranded β-sheet segment is stabilized by two disulfide bonds at positions 3-24 and 17-41. Additionally, the disulfide bridge located between residues 43 and 54 tethers the top of Loop III. The double and triple stranded β-sheet segments in the neurotoxin molecules are linked by an extended turn between residues 17 and 23.

The overall topology of long neurotoxins appears to be very similar to that of the short neurotoxins described in the previous paragraph (Basus *et al.*, 1988, 1993; Betzel *et al.*, 1991; Connolly *et al.*, 1996; Love and Stroud, 1986; Martin *et al.*, 1983; Le Goas *et al.*, 1992; Oswald *et al.*, 1991; Dewan *et al.*, 1994). The recently solved solution structure of a long neurotoxin from the venom of the King Cobra (*Ophiophagus hannah*), Toxin b, shows that unlike the well defined and rigid C-terminal tail noticed in the solution structures of short neurotoxins, the structures of the long neurotoxins are characterised by a highly flexible C-terminal tail (Peng *et al.*, 1997). Chemical modification experiments on long neurotoxins, such as toxin a and α-bungarotoxin, have shown that excision of the C-terminal tail by means of treatment with trypsin and carboxypeptidase P has no effect on the structure of the long neurotoxin (Pillet *et al.*, 1993).

The high affinity acetylcholine receptor (AChR) binding sites of neurotoxins (Chicheportiche *et al.*, 1975) are thought to be located at the tip of Loop II, wherein the residues at positions 29 and 31 to 34 are strictly conserved or only conservatively substituted among short and long neurotoxins (Lin and Chang, 1991, 1992; Lin *et al.*, 1995). Although a number of proposals have been put forward to account for the possible acetylcholine binding regions in neurotoxins, no clear-cut experimental evidence is yet available to authenticate these hypotheses. The suggestion that the residues located at the tip of Loop II are solely responsible for AChR binding is untenable due to the fact that the residues at the tip of Loop II tend to have different local conformations in the three-dimensional structures of the different neurotoxins. Neibig and Cohen (1979), based on the relative binding efficiencies of the neurotoxins and their AChR antagonists, suggested that the AChR binding site in neurotoxins could comprise residues located in the concave surface made up of Loops I and II. It is found that most of the neurotoxins possess this type of concave surface. Glu38, Pro44 and Ile52 constitute one group and the other group is comprised of hydrophobic residues, such as Trp29, Ile36 and Val50 (Yu *et al.*, 1994). In certain cases, such as the α-bungarotoxin (a long neurotoxin), the toxin is reported to form a dimer in solution by means of intermolecular hydrogen bonding (Basus *et al.*, 1988). It has been proposed that such dimer formation plays a role in the binding of neurotoxins to the AChR. However, no direct experimental evidence exists to support this proposal. Endo and Tamiya (1991) suggest that the neurotoxins, upon binding to the AChR, undergo extensive conformational changes. Peng *et al.* (1997), after studying the solution conformation of toxin b (a long neurotoxin from the venom of King Cobra *Ophiophagus hannah*), found that the tip portion of Loop II is highly flexible, and it is contemplated that this high flexibility could have significant effect(s) on the thermodynamics and kinetics of ligand receptor binding of neurotoxins (Peng *et al.*, 1997). The rate of association of the toxin to the AChR has been proposed to increase since the high flexibility of a loop (Loop II) could favour complex formation with the acetylcholine receptor by lowering the free energy barrier. Peng *et al.* (1997) further suggested that the length of the loop is intricately connected with the binding affinity of the neurotoxins to the receptor. It is believed that the longer the length of Loop II in the toxin, the greater is the affinity to the receptor. It has been found that among the long neurotoxins (for which the structures have been solved), the length of Loop II in toxin b is the shortest. The structures of long neurotoxins show minor differences from those of the short neurotoxins. In both α-bungarotoxin (Basus *et al.*, 1993) and α-cobratoxin (Betzel *et al.*, 1991) (both long neurotoxins), there is a distinct short nascent helix

formed by residues in segment 29-35. In toxin b, although there is a local structure in the same region of Loop II, it is not a helical conformation. Peng *et al.* (1997) also provided an elaborate discussion of the AChR binding sites in neurotoxins. They proposed that the highly conserved tryptophan residue located at position 26 plays a critical role in orienting the side chains of Lys24 and Asp28 located at the tip of Loop II for effective binding to the acetylcholine receptor. They suggested that the charged and conserved residues located at the tip of Loop II form a 'cationic cluster', which in turn is believed to play a critical role in binding of the toxin to the acetyl choline receptor (Lin *et al.*, 1996; Chang *et al.*, 1993, 1995; Chang, 1994).

Ruan *et al.* (1991) in an elegant study attempted to delineate the short neurotoxin binding regions on the α-chain of human and *Torpedo californica* acetylcholine receptors. Five cobrotoxin (a short neurotoxin) binding regions were found to reside within peptides α1-16, α23-38/α34-39 overlap, α100-115, α122-138 and α194-210 on the *Torpedo* AChR. The erabutoxin b regions were located within peptides α23-38/α34-49/α34-39/α45-60 overlap, α100-115 and α194-200. The main binding activity for both of these toxins was found to be lodged within the α122-138 peptide region. In a related study, the same research group reported that the α-bungarotoxin binding region was found to reside in the α182-198 and the α122-138 peptide regions of the AChR (Ruan *et al.*, 1990). It was postulated that the region within residues α122-138 of the AChR constitutes a universal binding site for short and long neurotoxins on the AChR from various species (Mulac-Jericevic and Attasi, 1987). Basus *et al.* (1993) structurally characterized the α185-195 synthetic dodecapeptide and α-bungarotoxin complex. They found that the residues in the Loop II in α-bungarotoxin are components of the receptor recognition site. In addition, that study revealed a receptor binding cleft within the bungarotoxin structure, and the binding of the α185-195 synthetic peptide to this cleft was postulated to trigger a conformational change in the toxin molecule.

2. Structure of Cardiotoxins

The structures of cardiotoxins have been probed using a variety of biophysical techniques (Surewicz and Mantsch, 1988; Pezolet *et al.*, 1982). To date, the X-ray and NMR solution structures of eight cardiotoxins isolated from various snake venom sources have been elucidated (Table 2) (Kumar *et al.*, 1997, 1998; Bhaskaran *et al.*, 1994b; Bilwes *et al.*, 1994; Dauplais *et al.*, 1995; Gilquin *et al.*, 1993; Otting *et al.*, 1987; Grognet *et al.*, 1988; Rees *et al.*, 1990; Jahnke *et al.*, 1994). In the average solution structure of CTX III from the Taiwan Cobra (*Naja naja atra*), the molecule is 'three-finger shaped' with three loops projecting from a globular head (Kumar *et al.*, 1997; Bhaskaran *et al.*, 1994a; Sivaraman *et al.*, 1998b). Thus, the overall topology of cardiotoxins is very similar to that of short neurotoxins. The secondary structure of cardiotoxins is predominantly β-type, consisting of five β-strands protruding from the globular head or core. The head region of the molecule is excessively crosslinked by disulfide bridges. There are five β-strands in the protein which align themselves antiparallely into a double and a triple stranded β-sheet. The double stranded β-sheet segment in CTX III is formed at the N-terminal end of the molecule spanning residues 1 to 5 (Strand I, -Leu-Lys-Cys-Asn-Lys-) and 10 to 14 (Strand II, -Phe-Tyr-Lys-Thr-Cys-). The two β-strands are interconnected by a stretch of residues spanning 6 to 9. The double stranded β-sheet segment is built by means of hydrogen bonds formed between residues 3-12, 5-10, 12-3 and 14-1. Strand I shows a backbone NH-O' hydrogen bond between Lys5 and Phe10. The triple stranded β-sheet consists of three β-strands (Strands III, IV and V) juxtaposed side-by-side. Strand III, comprising residues 20-26 (-Leu-Cys-Tyr-Lys-Met-Phe-Met-), and Strand IV, consisting of residues 34-39 (-Val-Lys-Arg-Gly-Cys-Ile-), are located in the middle loop of the molecule (Bhaskaran *et al.*, 1994a; Sivaraman *et al.*, 1998b). The β-sheet secondary structure between these two strands is characterised by the presence of a β-bulge formed by Pro33 and Val34. This type of bulge has been categorized as G1 type of β-bulge according to the classification of Richardson (1981). The fifth β-strand comprising the triple stranded β-sheet domain, Strand V, is made up of residues extending from 50-55 (-Lys-Tyr-Val-Cys-Cys-Asn-) that are antiparallel to residues 20-26 (Strand III). The double and triple stranded β-sheet segments are tethered by two disulfide bonds located at positions 3-21 and 14-38. Strand V is linked to the C-terminal tail portion of the molecule through the disulfide bond, 42-53. There is a β-turn (type II) extending between Ser46 and Val49. It is interesting to note that seven out of the eight cysteine residues that are present in cardiotoxin III (CTX III) are part of the β-sheets or the C-terminal loop. The odd Cys42, however, is an exception. The high density of disulfide bridges in cardiotoxins probably explains the high structural stability (Bhaskaran *et al.*, 1994a; Sivaraman *et al.*, 1998b).

The organization of the side-chains in cardiotoxins gives rise to two different faces: convex and concave surfaces (Bhaskaran *et al.*, 1994a; Sivaraman *et al.*,

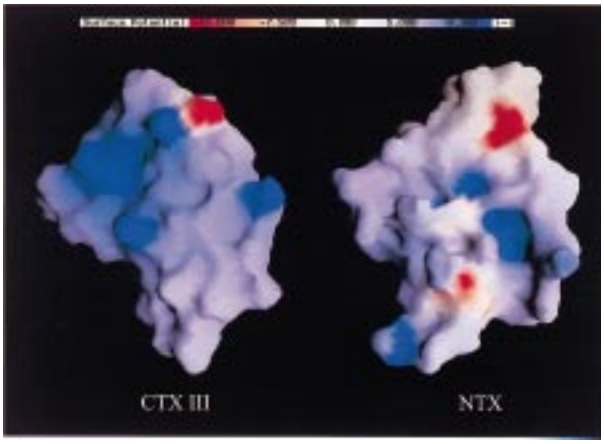

Fig. 2. Grasp representation of the distribution of the surface charges in CTX III and NTX (*Naja naja atra*).

1998b). The convex surface is located at a site in which the polypeptide chain crosses over the β-sheet between Loops II and III. The concave surface is lodged between the N- and C-terminal ends of the molecule. Large portion(s) of the convex surface of the molecule are occupied by vast hydrophobic patches (Fig. 2) contributed by residues belonging to the three loops (Marchot *et al*., 1988). Each of the two hydrophobic patches on the convex side of the molecule is organized around two tyrosine residues, Tyr22 and Tyr51 (Bhaskaran *et al*., 1994a; Sivaraman *et al*., 1998b). These two aromatic residues are well conserved in all the cardiotoxin sequences. Tyr22 is surrounded by residues Met24, Gly37, Ile39, Pro43 and Cys53. Met26, Val32, Val34 and Val49 encircle Tyr51. Both Tyr22 and Tyr51 lie very close in space in the three-dimensional structures of cardiotoxins. The phenolic rings are tilted to about 80° with respect to one another (Bhaskaran *et al*., 1994a; Sivaraman *et al*., 1998b). Both aromatic residues are buried deep inside the molecule. Studies based on chemical modification have shown that substantial loss in activity occurs when the conserved tyrosine residues are modified (Gatineau *et al*., 1987, 1990; Menez *et al*., 1990). The Van der Waals contacts between Loops I and II are exemplified by the identification of NOEs between the side chains of the conserved Leu6 and the alpha protons of Arg36 and Lys35 in the solution structures of cardiotoxins (Bhaskaran *et al*., 1994a; Sivaraman *et al*., 1998b). The concave surface of the molecule is stabilized by three disulfide bridges. The solution structure of cardiotoxin indicates that Leu1 presents several NOEs with residues at the C-terminal, such as Thr56, Asp57 and Arg58. Gatineau *et al*. (1987, 1990) carried out systematic chemical modification studies to ascertain the role of lysine residues in toxin γ from *Naja nigricollis*. They selectively modified lysine residues located in Loop I (Lys2 and Lys12) and Loop II (Lys18, Lys23 and Lys35) of the toxin molecule. It was found that nullification of the positive charge on the side chain ε-amino group of Lys12 (Loop I) resulted in substantial loss of cytotoxicity of the toxin (Fig. 3). It was ascertained that no global conformational changes occurred during the process of modification. Thus, it was concluded that Lys12 is crucial for the cytotoxicity of cardiotoxins. Acetylation of the ε-amino groups of Lys12, Lys18 and Lys23 did not produce similar pronounced cytotoxic effects (Menez *et al*., 1990). The cytotoxic potency of toxin γ declined by 33% when positively charged groups of Lys18 and Lys35 were modified. Based on these results, it appears that Lys18, Lys23 and Lys35 contribute significantly to the cytotoxic action of cardiotoxins.

Snake venoms contain multiple forms of cardiotoxins. However, the physiological/functional significance of the occurrence of various cardiotoxin isoforms in a single venom source is still not understood. We recently solved the solution structures of cardiotoxin analogues II (CTX II) and IV (CTX IV) from the Taiwan Cobra (*Naja naja atra*) (Jang *et al*., 1997). Interestingly, these two cardiotoxin isoforms, CTX II and CTX IV, differ in their amino acid sequences in the N-terminal. Leucine is the N-terminal amino acid in cardiotoxin II (CTX II) whereas, in CTX IV, it is replaced by a basic residue, arginine. Incidentally, CTX IV is the only cardiotoxin analogue (among the cardiotoxins whose amino acid sequences are known) which has a cationic residue as the N-terminal residue; all other cardiotoxin analogues start with a hydrophobic amino acid, either leucine or isoleucine. Comparison of the erythrocyte lytic activity of CTX II and CTX IV showed that the lytic activity

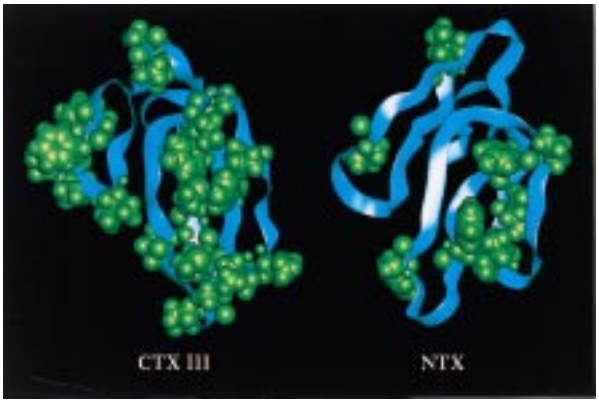

Fig. 3. Space filling model representing the distribution of the hydrophobic amino acid residues (in green) in the three-dimensional structures of CTX III and NTX from *Naja naja atra*.

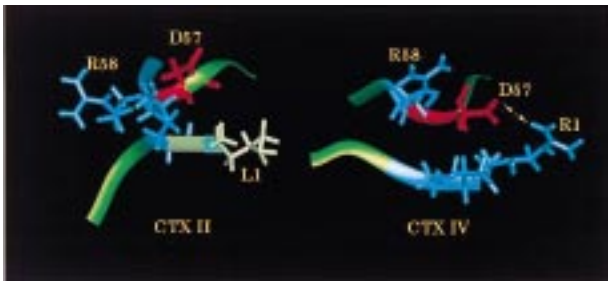

Fig. 4. Depiction of the salt bridge formation between the groups of Arg1 and Asp57 in CTX IV (*Naja naja atra*). This salt-bridge (double headed arrow, yellow) locks the N- and C-terminal ends and is considered to be crucial for the development of the cationic cluster responsible for the enhanced erythrocyte lytic activity of CTX IV. There is no such salt-bridge in CTX II.

of CTX IV was almost twice that of CTX II. Solution structures of these two toxins revealed that the residues constituting the β-sheet segments are the same in both toxin isoforms. Comparison of the spatial distribution of the side chain groups in CTX II and CTX IV revealed the distribution of the cationic residues in both toxin isoforms. Interestingly, in CTX IV, the charged side chain guanido groups of Arg1 in CTX IV are found to salt bridge to the carboxyl group contributed by the side chain of Asp57 (Fig. 4). In addition, the carbonyl and the imino groups of Lys2 and Cys59 (in the backbone, at the C-terminal) are hydrogen bonded in CTX IV. As a consequence of the occurrence of the salt bridge, a 'dense' cationic cluster is formed at the N-terminal end, comprising Arg1, Lys2, Lys5, Lys23, Lys50 and Arg58. This cationic cluster is located on the concave side of the molecule (Jang *et al.*, 1997). The presence of this positively charged cluster in CTX IV is thought to be responsible for the higher erythrocyte lytic activity of CTX IV over CTX II. Cardiotoxins have been shown to bind to membranes through their positively charged residues. The 'dense' cationic cluster in CTX IV is believed to intensify the binding of the toxin molecules to the negatively charged erythrocyte membrane. In contrast to CTX IV, there is no salt bridge in CTX II at its N-terminal end, and hydrogen bonding between the carbonyl group of Lys2 and the imino group of Cys59 is found to be lacking. These factors do not favor the development of a 'cationic cluster' at the N-terminal (in CTX II) as observed in CTX IV (Jang *et al.*, 1997).

IV. Dynamics of Cardiotoxins

Recently, extensive studies have been carried out to understand the influence of protein structures and the dynamics of protein stability on their function. Until recently, very little information existed on the internal dynamics of snake venom toxins-neurotoxins and cardiotoxins. However, recently, this lacunae was filled by investigation of the main-chain dynamics of a cardiotoxin analogue II (CTX II) from Taiwan Cobra (*Naja naja atra*) venom using two-dimensional heteronuclear NMR spectroscopy at natural abundance (Lee *et al.*, 1998). Cardiotoxins, as stated previously, exhibit a broad spectrum of biological properties, but the exact mode of action of cardiotoxins is still a mystery. The main-chain internal dynamics of CTX II were studied based on T_1, T_2 and NOE values calculated for 50 out of 58 non-glycine residues (Fig. 5). It was found that more than 50% of the residues present very fast internal motion (<20 ps), and that only three residues (Leu30, Val32 and Tyr51) exhibit very slow internal motions in the nano second time scale. This corresponds to increased flexibility at the tip of Loop II of this protein, which could be related to its biological activity. The generalized order parameter, which is a direct measure of the amplitude of internal motion, ranges from 0.78-0.87 for residues involved in the β-sheet segments. The β-strands located in the segment

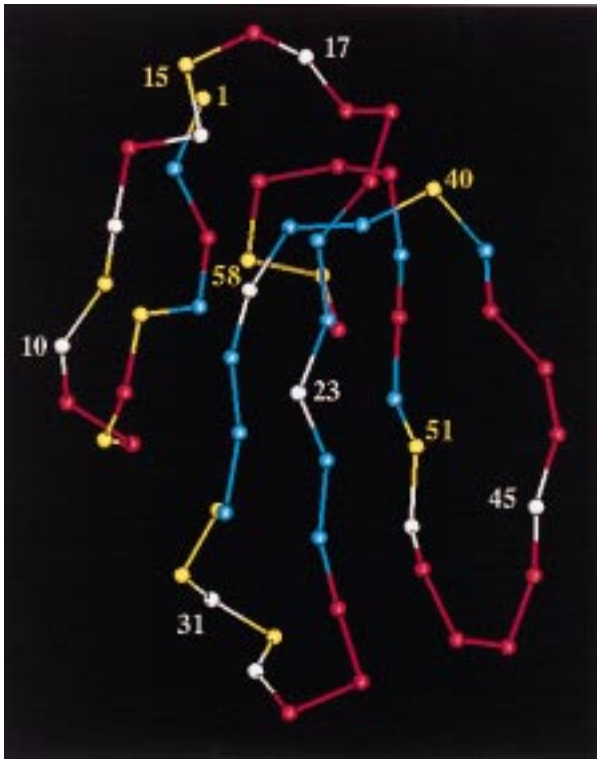

Fig. 5. Classification of the order parameter (S^2) values as measured from the C^α dynamics for 50 non-glycine residues of CTX II. $S^2 \geq 0.85$–blue; $0.85 > S^2 \geq 0.75$–red; $S^2 < 0.75$–yellow and residues for which S^2 values could not be evaluated - white.

spanning residues 20-25 and 35-39 have higher S^2 values (Lee *et al*., 1998). These β-strands form a part of the triple-stranded β-sheet segment and are well conserved in all cardiotoxin analogues isolated from the venom of the Taiwan Cobra. The study of the dynamics of CTX II have provided useful insight into the flexibility of residues, which have been implicated in the biological activity of the toxin. Although the structural basis for the erythrocyte lytic activity of this class of toxins is still not well understood, specific 'receptors' are believed to be involved in the selective binding of cardiotoxins. It is proposed that the residues located at the tips of Loops II and III are less flexible and, hence, are not involved in the receptor binding. Batenburg *et al*. (1985) predicted that the putative receptor binding regions of snake cardiotoxins are spread out among residues located at the tips of three loops. These authors contemplate that the flexibility of the residues located at the tips of the three loops facilitate interaction of the cardiotoxin molecules with their receptor sites on the erythrocyte membrane. Interestingly, the order parameters (S^2) at the tips of the loops in CTX II have been found to be 0.78, 0.77 and 0.79 for Loops I, II and III, respectively. Thus, it appears that the residues located at the tips of the loops are relatively more flexible and could possibly constitute the multipoint receptor binding site(s).

V. Mode of Action of Cardiotoxins

Cardiotoxin exhibits a wide variety of biological activities. It is reasonably well documented that cardiotoxins exhibit their primary mode of action through binding with the membrane surface(s) (Dufourcq *et al*., 1982, 1986; Hodges *et al*., 1987; Lauterwein and Wuthrich, 1978; Carbone and Macdonald, 1996). On account of their positively charged nature, they bind with high affinity to membranes/vesicles composed of negatively charged lipids. Liposomes prepared from phosphotidyl serine/ phosphotidyl choline mixtures have been shown to interact weakly as compared to the lipid vesicles formed by only phosphotidyl serine (Kumar *et al*., 1997). It has been reported that binding of a cardiotoxin analogue from *Naja mossambica mossambica* significantly increases the relative intrinsic fluorescence of the protein (Vincent *et al*., 1978). The association constant of this binding was found to be greater than 10^6 M^{-1}. Batenburg *et al*. (1985) in a classical study demonstrated that cardiotoxin molecules penetrate into the cardiolipin vesicles and trigger the fusion of unilamellar vesicles. It has been shown that cardiotoxin-induced liposome (prepared from phosphotidyl choline) lysis is dependent on the pH of incubation, and that it is maximal under pH conditions greater than 9.0. The increased lysis is facilitated by the tighter binding of the toxin to the negatively charged phosphatidic acid (in phosphatidyl choline). Bougis *et al*. (1981), using fluorescence spectroscopy to study phospholipid-cardiotoxin monomolecular films, showed that the cardiotoxin molecule(s) orient themselves in a 'flat' orientation from an 'edgewise' orientation, depending on the surface pressure developed. The 'edgewise' to 'flat' orientation begins at 30 mNm^{-1}, which is the normal surface pressure expected at the surface of the erythrocytes. It is thought that the drift in the orientation of the cardiotoxin molecules is responsible for the lytic activity of the snake venom cardiotoxins. Although the penetration of CTX molecules into the membrane surface depends on the lipid composition of the membrane, there is growing evidence that the first hydrophobic loop of the CTX molecule penetrates the lipid phase of the membrane in the 'edgewise' orientation. Using trapped spin labels inside liposomes, Hsia *et al*. (1978) demonstrated that cardiotoxins could also cause lysis of vesicles comprised of neutral phospholipids with no net charge. Direct binding of the toxin molecules to calcium ions is postulated to switch the cardiotoxin molecules from an active to an inactive conformation. However, the binding of calcium ions to cardiotoxins has been extensively debated. It has been reported that cardiotoxin analogues from the Indian Cobra (*Naja naja naja*) bind to calcium ions (Kumar *et al*., 1990). However, Zusman *et al*. (1984) observed that the biological activity of cardiotoxins from *Naja naja siamensis* is not critically dependent on the calcium ion concentration. The protein-lipid interaction is reported to be inhibited at high Ca^{+2} concentrations.

Cardiotoxins, as stated earlier, exhibit a broad spectrum of biological activity. However, the lysis of erythrocytes by cardiotoxins is a ready and reproducible test for assessing the biological activity of cardiotoxins. Though numerous hypotheses have been put forward to account for the lytic activity of cardiotoxins (Kumar *et al*., 1997), the most convincing of them is the '*Binding and Penetration Model*' (Fig. 6). The basic tenet of this model is that penetration of the CTX molecules into the membrane triggers the lysis due to the disruption of the osmolyte balance. The surface activity reported by Bougis *et al*. (1981) for cardiotoxins offers strong evidence for the ability of cardiotoxins to adsorb/penetrate an interface. However, the extent to which the cardiotoxin molecules penetrate into the membrane is an open question. Lauterwein and Wuthrich (1978) proposed that all the three hydrophobic loops of the cardiotoxin molecules are inserted into the membrane interface. These authors suggest that the middle loop penetrates into the

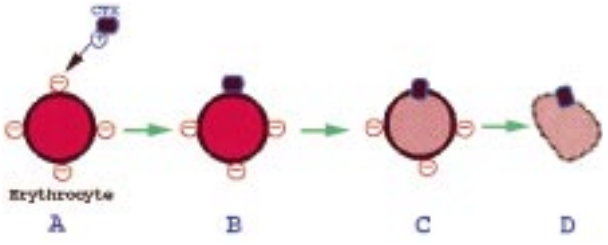

Fig. 6. Graphic representation of the binding-penetration model. According to this model, cardiotoxin, owing to their highly basic nature (*pI*>10), bind to the negatively charged centers [A] (indicated in red) located on the RBC membrane surface (indicated by the filled circle). These negative charges are proposed to be contributed by the phospholipids and negatively charged amino acids in the membrane protein, 'glycophorin'. After initial charge recognition through electrostatic interaction(s), CTX molecules penetrate into the membrane in an 'edgewise' orientation [B] through hydrophobic interactions. Upon penetration, CTX molecules change from an 'edgewise' to a 'flat' orientation [C]. This change in orientation of the CTX molecules is thought to induce membrane disorganization, leading to RBC lysis [D].

lipid membrane, and that the charged residues located at the tip of Loop II are salt-bridged to the polar region of the lipid head groups. Although this model accounts for the high affinity stoichiometry and specificity of binding of the cardiotoxin molecules, there is obvious inconsistency with respect to the length of the hydrophobic region of the protein and the thickness of the hydrophobic core of the lipid bilayer of the membrane. Interestingly, in contrast, Dufourcq *et al*. (1982) proposed that only one of the β-pleated loops of the cardiotoxin molecules interacts with the nonpolar interior of the membrane. Irrespective of the *pros* and *cons* of these two proposals, it is reasonable to envisage that cardiotoxins penetrate into the erythrocyte membrane upon initial binding. Obviously, the initial binding is favoured by the electrostatic interaction(s) between the negatively charged membranes and the cationic clusters on the cardiotoxin molecules. Bougis *et al*. (1981) suggested that following the electrostatic interaction step, the first hydrophobic loop of the CTX molecule penetrates the lipid phase of the membrane with the molecule in the 'edgewise' orientation. Such an interaction is believed to bring about a decrease in the surface pressure, which consequently is thought to facilitate a rapid transition from the 'edgewise' to a 'flat' orientation. Such flipping of the molecule is believed to amplify a structural perturbation of the erythrocyte membrane, resulting in disorganization of the cell. The weakening of the erythrocyte membrane upon cardiotoxin-erythrocyte interaction during the nonlytic phase supports the 'flipping' model proposed by Bougis *et al*. (1981).

VI. Aspects of Protein Folding

Snake venom toxins, despite their small size and unique structural feature of possessing all β-sheet proteins, still have not caught the attention of the protein folding community. Little information is available on the pathways of folding and unfolding of the snake venom toxins. Until recently, no significant study was done with the goal of understanding the folding/unfolding pathways(s) of various cardiotoxin analogues from the Taiwan Cobra (*Naja naja atra*). In this review we attempt to comprehensively discuss recent advance(s) in understanding aspects of protein folding of cardiotoxin analogues.

One of the questions that remains to be answered in molecular biology is the problem of protein folding (Anfinsen, 1973; Baldwin, 1994; Carlsson and Jonsson, 1995; Evans and Radford, 1994; Fink, 1995; Dill, 1985, 1990; Dobson, 1992, 1994). It is generally believed that proteins follow a fixed pathway(s) enroute to their attainment of unique native states from the completely unfolded state(s) (Privalov, 1996). The folding of small, single-domain proteins, in the majority of cases, is highly cooperative (Jamin and Baldwin, 1996; Itzhaki *et al*., 1995; Khorasanizadeh *et al*., 1996; Agashe *et al*., 1995; Bai *et al*., 1993; Elove *et al*., 1992; Lu *et al*., 1997; Kataoka *et al*., 1995). Stable, partially folded intermediates that might provide insight into the folding process are rarely observed (Kuwajima *et al*., 1996; Hagihara *et al*., 1994). Transient intermediates occasionally accumulate during folding, and techniques have recently been developed that could aid in probing their structures (Roder *et al*., 1988; Sosnick *et al*., 1994; Varley *et al*., 1993). The presence of multiple folded forms present during the folding process renders structural studies technically difficult to execute. The inability to detect stable folding intermediates is often believed to be due to the inherent stability of the native protein and the fact that conditions severe enough to unfold the native state generally destabilize the partially folded states as well. Stable intermediates have been identified and structurally characterized in several small molecular weight proteins under a variety of denaturing conditions (Buck *et al*., 1993, 1995; Bychokova *et al*., 1996; Hughson *et al*., 1990). One of the most significant and popular of the stable intermediates is the 'molten globule' state (Kuwajima, 1990, 1992; Kuroda *et al*., 1992; Liu *et al*., 1994; Ptitsyn, 1987; Ptitsyn *et al*., 1993; Uversky and Ptitsyn, 1994a, 1994b; Jeng *et al*., 1990). The salient structural features of a 'molten globule' state include: (1) hydrogen-bonded native-like secondary structure(s) is/are

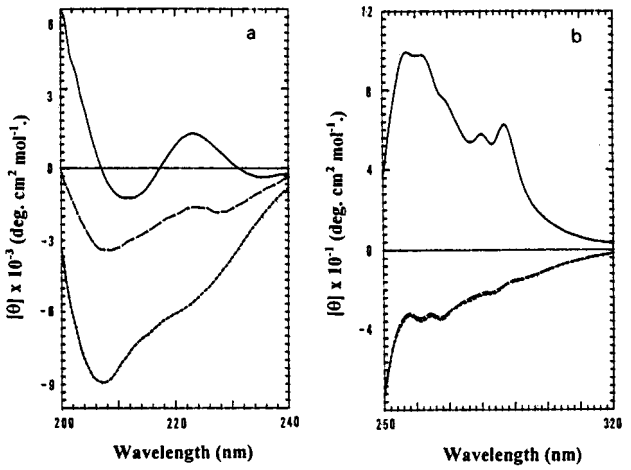

Fig. 7. Circular dichroism spectra of CTX III in the (a) far- and (b) near-UV region at 25 °C (solid line); 90 °C (broken line) and at 90 °C in 4 M urea (dotted line).

formed in the same regions as in the native three-dimensional structure of the protein and (2) loss of native tertiary structural contacts. It is currently believed that the occurrence of the 'molten globule' intermediate is necessary for successful folding of globular proteins (Uversky and Ptitsyn, 1994a, 1994b). Although there have been many reports on the identification of 'molten globule' intermediates in the folding/unfolding pathways of several proteins, it is still not possible to generalize the concept of 'molten globule' as a universal protein folding intermediate. This lacuna is due to the lack of sufficient evidence concerning the occurrence of 'molten globule' like intermediates in the folding/unfolding pathway of all β-sheet proteins (Kumar *et al.*, 1995). To fill the void in this research area, there has been a spurt in research activity focused on investigation of the protein folding of all β-sheet proteins, such as the snake venom cardiotoxins (Kumar *et al.*, 1996a). The small size of this class of proteins (6.5-7.5 kDa) and their all β-sheet secondary structure render these proteins ideal choices for protein folding studies. In addition, the solution structures of these proteins are available at high resolution, which could also facilitate employment of the hydrogen-deuterium quench-flow technique (complemented by two-dimensional NMR techniques) to probe into the structural features of the kinetic intermediate(s) occurring during the folding of cardiotoxins.

Thermal denaturation experiments were recently carried out by Jayaraman *et al.* (1996a) to understand the thermal unfolding pathway(s) of cardiotoxin analogue III (CTX III) from the Taiwan Cobra (*Naja naja atra*). The protein was subjected to thermal unfolding in the pH range of 2.0 to 10.0. It was found that protein is quite stable, and that it only melts at temperatures greater than 80 °C. The authors identified a stable, partially structured intermediate at a high temperature (90 °C) at pH 4.0 (Fig. 7). There are few examples of the detection of partially structured states at high temperatures in the thermal unfolding pathway(s) of proteins. Snake venom cardiotoxins, in general, are resistant to denaturation by urea under neutral pH conditions. They unfold only in highly concentrated (>5.0 M) solutions of guanidinium hydrochloride. The extraordinary stability of the cardiotoxin molecules is postulated to arise due to the fact that the stable β-sheet segments are tied together by the four disulfide bridges. Thus, these proteins could, in principle, serve as useful models to understand the physical forces governing the stability of a protein.

Organic solvents such as 2,2,2-trifluoroethanol (TFE) are useful in realizing stable structured intermediates in the folding/unfolding pathways of proteins. TFE has been successfully used to trap stable, partially structured states in the unfolding of several proteins (Baum *et al.*, 1989; Fan *et al.*, 1993). Kumar *et al.* (1995) studied the effect of TFE on cardiotoxin analogue III (CTX III) from the Taiwan Cobra (*Naja naja atra*) using a variety of biophysical techniques and demonstrated the existence of a 'molten globule'-like intermediate at high concentrations of TFE (>80% TFE). The circular dichroism spectrum of the protein in the 'molten globule' like state showed the presence of helical segments in the protein in the intermediate state (Fig. 8). Structural elucidation of the 'molten globule' intermediate, using two-dimensional NMR spectroscopy, revealed that the native β-structural elements (Fig. 9) in the protein are intact in the intermediate. Interestingly, the NMR data failed to identify any helical

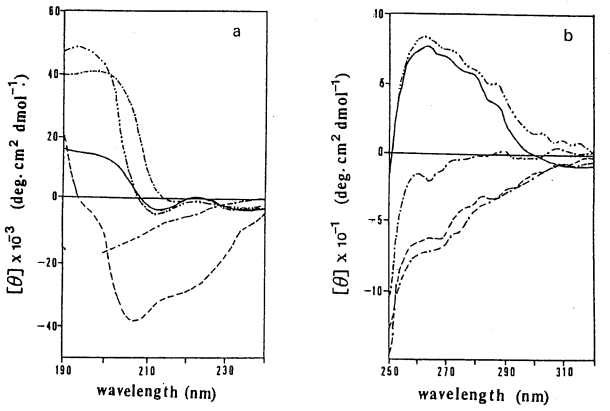

Fig. 8. Far (a) and near (b) UV CD spectra of TFE untreated (—) and treated CTX III (*Naja naja atra*). The alcohol concentrations used in the treatment of CTX III were 20% (-•••), 50% (-•-•-), 80% (---) and 80% TFE containing 2M urea (--•--). All the spectra were recorded at pH 2.5.

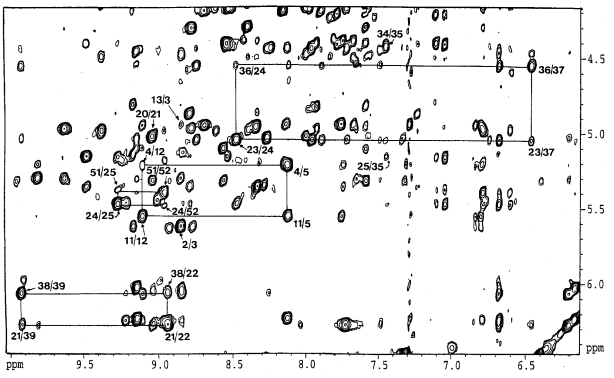

Fig. 9. NOESY spectrum of CTX III (*Naja naja atra*) in 80% TFE-d_3/H_2O (v/v), depicting the NOEs in the NH-Hα (finger print) region. Assigned Hα-NH crosspeaks corresponding to the double and triple stranded β-sheet regions are indicated. CTX III in 80% TFE is shown to exist in the molten globule state.

segments in the 'molten globule' state.

An essential step in the folding of a protein, from a random coil to the well-defined native conformation, is the formation of a local secondary structure(s). Detection of cryptic structural propensities in the amino acid sequence would be useful in understanding the molecular basis of formation of such local structures during folding (Dyson *et al.*, 1992; Hamada *et al.*, 1995; Lehman *et al.*, 1990; Nelson and Kallenbach, 1986, 1989; Shiraki *et al.*, 1995; Sonnichsen *et al.*, 1992). It is in this context that characterization of the conformational tendencies of isolated peptide fragments of proteins is being pursued very actively (Segawa *et al.*, 1991). However, the tendencies of these small peptide fragments to adopt one particular conformation in aqueous medium is often very weak. One of the techniques used to enhance and stabilize the conformational tendencies of peptides is the addition of TFE as a cosolvent. Numerous investigations employing isolated peptide fragments of proteins have demonstrated that TFE can only induce helix in those peptide fragments that are either helical in the native state or predicted to possess a propensity to adopt a helical conformation (Dyson *et al.*, 1992; Hamada *et al.*, 1996). Very little information exists on the effects of TFE on other secondary structural elements. Recently, Shiraki *et al.* (1995), studying the TFE-induced conformational changes in the native structure of the predominantly β-sheet proteins, such as β-lactoglobulin and concanavalin A, showed that TFE could induce a high percentage of helicity in these proteins. Interestingly, secondary structure prediction analysis of these two predominantly β-sheet proteins revealed that these proteins have a high sequence propensity to adopt a helical conformation. Based on these results, it was argued that despite the fact that the protein does not possess a helical structure in its native state in water, TFE could successfully induce helical conformation if the polypeptide backbone merely shows propensity to form helix. In this context, Jayaraman *et al.* (1996b) attempted to understand the effect(s) of TFE on an all β-sheet cardiotoxin analogue II (CTX II) from the Taiwan Cobra (*Naja naja atra*). They studied the effects of TFE on the three different states of the protein: the native (nCTX II), denatured (dCTX II) and the denatured and reduced (rCTX II) states (Fig. 10). TFE were found to induce helical conformations in nCTX II only at high concentrations (>80% v/v). Even in dCTX II (wherein the helix disulfide bonds in the protein are intact), helix-induction occurred only at concentrations of TFE greater than 80% v/v. The results of that study imply that hydrophobic and ionic interactions stabilizing the native state of the protein do not make a significant contribution to the induction of helix by TFE. Interestingly, in rCTX II (wherein the disulfide bonds are disrupted), TFE induces helical conformation even at very low concentrations (~20% v/v). This result is quite intriguing since CTX II is an all β-sheet protein and has no sequence propensity to adopt helical conformation. Based on the results obtained in their study, Jayaraman *et al.* (1996b) opined that the effects of TFE on proteins are, to an extent, non-specific. The results of that study clearly caution against the indiscriminate use of structure-inducing cosolvents in structural studies of proteins/peptides. In addition, it appears that the stabilization of the native structure of CTX II by disulfide bonds significantly influences the induction of helix by TFE.

2,2,2-Trichloro acetic acid (TCA) is a commonly used protein-precipitation reagent. Sivaraman *et al.* (1997a) recently attempted to understand the mechanism underlying the precipitation action of TCA on

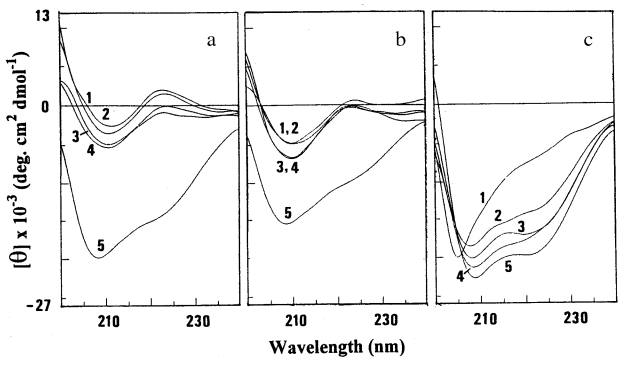

Fig. 10. Far UV CD spectrum of (a) nCTX II, (b) dCTX II and (c) rCTX II. Curves 1-5 represent spectra in 0%, 20%, 40%, 60% and 80% TFE (v/v) at 298 K, respectively.

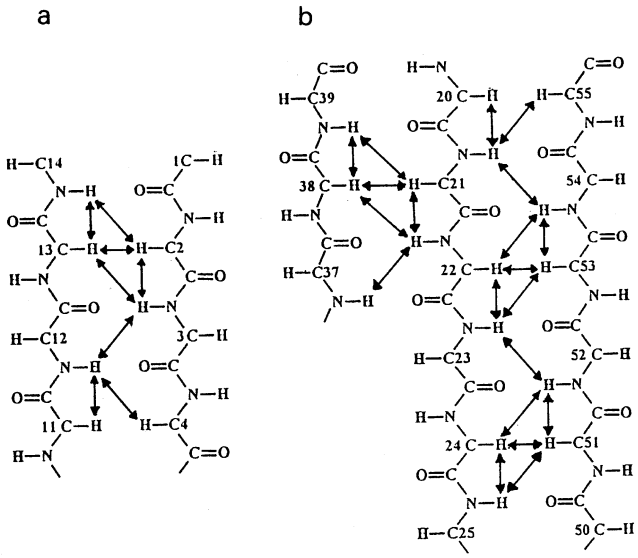

Fig. 11. Schematic representation of the through-space NOE interactions in CTX III in 3% (w/v) TCA of (a) eight NOEs in the double stranded region and (b) 20 NOEs in the triple stranded region. Proton pairs that correspond to the cross peak in the NOESY spectrum are connected by double headed arrows. In total, 28 NOEs are observed for CTX III in the β-sheet region.

cardiotoxin analogue III from *Naja naja atra*. They found that the precipitation curve is U-shaped, with protein precipitation beginning at 3% w/v of TCA, and that at concentrations of the acid greater than 3% w/v, most of the protein is completely in solution with no visible precipitation. Study of the structural features of the protein in 3% w/v (the concentration of acid above which protein precipitation commences) revealed that the protein CTX III exists in a partially structured state (with features similar to the molten globule state) at this (3% w/v) concentration of the acid. The protein showed substantial loss in the tertiary structural contacts in the acid-induced intermediate state. However, some of the native hydrophobic interactions in the protein were found to be preserved in the intermediate state. The aromatic side-chains appeared to be mostly flexible as indicated by the line broadening effects observed in the 1D-NMR spectra of the protein in the 3% w/v TCA induced-intermediate state. The β-sheet segments that are disrupted in the TCA-induced partially structured state are located in the end portions of the loops (Fig. 11), which are not stabilized by disulfide bonds. In general, it is felt that the four disulfide bonds in cardiotoxins are crucial for protection of the β-sheet secondary structural elements in the protein 'molten globule' like state identified along the acid-induced unfolding pathway of CTX III from the Taiwan Cobra.

Folding to the native conformation is a highly cooperative process for small, single domain proteins (Privalov, 1996). Although transient intermediates have been observed during the folding process, it is still not clear in what stage cooperation in the folding process originates. Characterization of early folding intermediates has provide insight into the cooperativity phenomenon observed in the folding of small sized proteins. Recently, with the advent of powerful techniques, such as the quenched-flow deuterium-hydrogen exchange, in conjunction with 2D NMR experiments, it has become possible to characterize the transient intermediates that occur in the early stages of folding (Baldwin, 1995). The quenched-flow hydrogen-deuterium exchange technique is particularly powerful because it permits many specific sites within a protein to be probed on the millisecond time scale. This technique has been successfully used to study the folding pathway of several proteins (Jacobs and Fox, 1994). Sivaraman *et al.* (1998a) using various biophysical techniques, including the quenched-flow hydrogen-deuterium technique (Fig. 12), and to completely characterize the kinetic folding pathway of cardiotoxin analogue III (CTX III) from the Taiwan cobra venom. The authors found that the folding is complete within 200 ms. Using stopped-flow circular dichroism studies, they determined that secondary and tertiary structural folding occurs almost simultaneously. In-

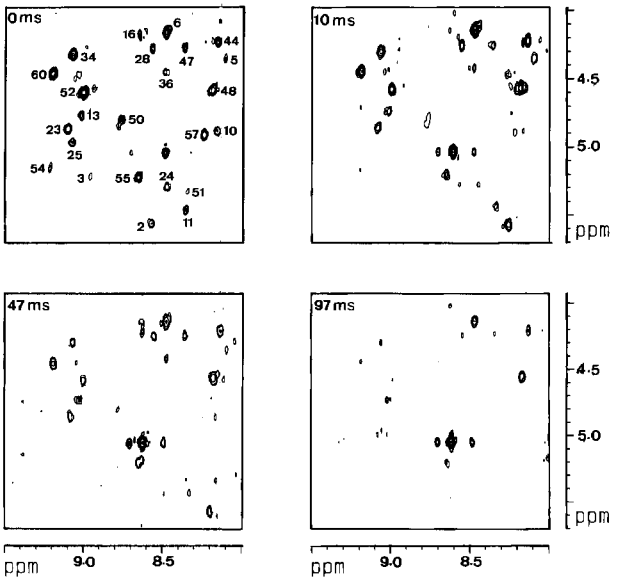

Fig. 12. Magnitude COSY spectra of CTX III samples prepared using quenched-flow hydrogen exchange methods at various pulse-labeling time periods. The labeled NH-CαH cross peaks represent residues which have undergone amide deuterium-proton exchange during various refolding time periods.

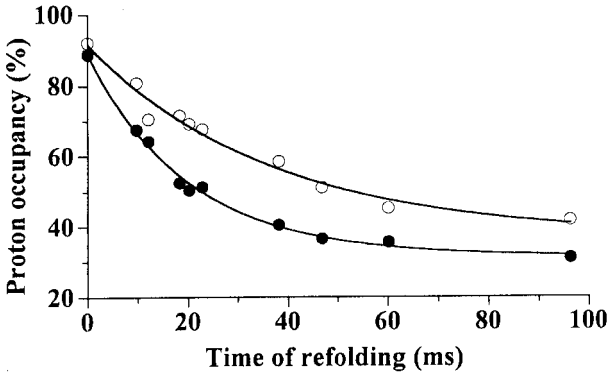

Fig. 13. Time courses for the protection of amides through exchange of residues involving double and triple stranded β-sheets during the refolding of CTX III. The curve represents the average of a single exponential fit to the time course of individual amides in the double and triple stranded β-sheet segments. Non-linear least square fits of the data give values of 19.7 ms (filled circle) and 35.0 ms (open circle) for the average time constants of folding of the triple and double stranded β-sheet domains, respectively. Time courses of five and nine residues were averaged to trace the kinetic curves for the double and triple stranded β-sheets, respectively.

terestingly, they deduced that the folding of CTX III proceeds through a 'hydrophobic collapse'. The formation of a nascent hydrophobic core was traced out and found to be one of the earliest events in the folding of this protein. Sivaraman *et al.* (1998a), using quenched-flow kinetics data, determined that the segment spanning residues Tyr51-Asn55 along with residues Lys23, Ile39, Val49, Tyr51 and Val52 constitute the 'hydrophobic cluster' which forms in the early stages of the folding of the protein. The formation of the 'hydrophobic cluster' was also identified through observation of a steep increase in the 1-anilino-8-naphthalene-sulphonic acid emission signal within the burst phase of folding of the protein. For the first time, a protein of the size of CTX III (6.5 kDa) was observed to fold via a 'hydrophobic collapse'. Sivaraman *et al.* (1998a), tracing the formation of the various 'domains' in CTX III, demonstrated that the triple stranded, antiparallel β-sheet segment, which is located in the central part of the CTX III molecule, folds faster than the double stranded β-sheet segment (Fig. 13). A figurative representation of the events in the secondary structure formation in CTX III is shown in Fig. 14. Sivaraman *et al.* (1998a) observed that the time scale(s) of formation of the hydrophobic cluster and formation of certain portions of the secondary structure in the protein are similar. Hence, it is not clear if the hydrophobic collapse precedes or succeeds the formation of the secondary structure elements in CTX III. Detailed sub-millisecond kinetics are needed to obtain a definitive answer to this question (Balbach *et al.*, 1995). Recently, CTX III has been cloned and expressed in high yields (Kumar *et al.*, 1996b). Detailed folding studies with appropriate mutants could provide useful insight into the structural interactions that come into play during the folding of CTX III.

VII. Future Perspectives

Snake toxins, owing to their varied and interesting properties, are bound to attract the attention of researchers working in various areas of modern biology. Although the modes of action of snake venom neurotoxin are better understood than are those of their counterpart cardiotoxin molecules, we believe that more detailed structural analysis of the neurotoxin-AChR complex will be carried out to elucidate the molecular basis of the action of neurotoxins. This aspect could pave the way for the formulation of useful therapeutic agents for auto-immune neuromuscular diseases such as *Myasthenia gravis*.

Cardiotoxins, by virtue of the broad spectrum of biological activities which they exhibit, are expected to remain the subject(s) of extensive multidisciplinary research in the future. In recent years, cloning and expression of cardiotoxins have been accomplished. This aspect is expected to pave the way for the generation of a variety of site-directed mutants of this class of toxins, which in-turn would aid complete delineation of the 'active site' in cardiotoxins. Recently, snake venom cardiotoxins have been shown to act as potent inhibitors of coagulation and platelet aggregation (Kumar *et al.*, 1997). We forsee intensified efforts among researchers to develop potential medical applications of snake venom cardiotoxins. Cardiotoxins have been recently shown to possess ion-channel activity. We foresee hectic research activity in the area

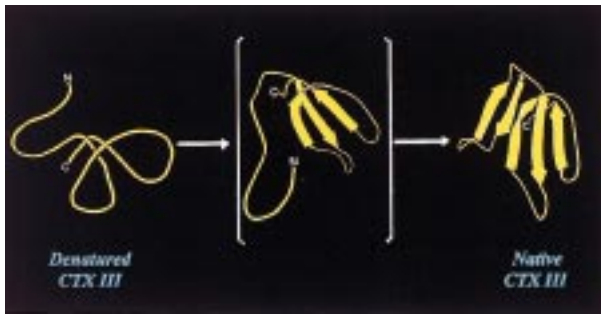

Fig. 14. Figurative representation of the refolding pathway of CTX III (*Naja naja atra*). In the time course of refolding, the triple stranded β-sheet forms initially, followed by formation of the double stranded β-sheet.

of neurobiology which will aim to understand the properties of the cardiotoxin-induced ion-channels. Ironically, despite their small size, snake venom toxins have not been adopted as popular model molecules for understanding protein folding. The recent study of Sivaraman *et al*. (1998a) is expected to stimulate the interest of the 'protein folders' the world over in snake venom toxins. We predict that a great deal of exciting research will be carried out on snake venom toxins in the near future.

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眼鏡蛇毒蛋白的結構、功能及其蛋白質摺疊的研究

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摘 要

眼鏡蛇毒中含有心臟毒及神經毒蛋白兩種毒素，它們的分子量約在6500至9000道爾頓之間，二者皆有多數的雙硫鍵。它們的三維結構都相當類似，為三手指狀。其二級結構皆含二股及三股反平行板塊。有趣的是這兩類蛇毒雖然有類似的三維結構，但其生化活性卻大不相同。這篇回顧文章的主要目的在評論及總結心臟毒及神經毒蛋白近來在結構、功能及摺疊路徑的研究進展。