CONFORMATIONAL PROPERTIES OF THE NEUROTOXINS AND CYTOTOXINS ISOLATED FROM ELAPID SNAKE VENOMS

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I. INTRODUCTION

Snakes of the Elapidae family (i.e., the cobras, kraits, mambas, tiger snakes, and seasnakes) have extremely toxic venoms which will produce flaccid paralysis and respiratory failure in animals.[1,2] Although this toxicity is based on a complex mixture of ingredients, one protein family stands out in having very pronounced pharmacological activities. Some members of this family are potent neurotoxins while others produce cardiotoxic and cytolytic effects. Further types seem to have no discernable toxicity whatsoever. Since these proteins are readily separated from the other venom toxins, enzymes, and peptides by gel filtration and ion-exchange techniques, much progress has been made in their study since the pioneer work in 1966.[3-7] The first amino acid sequence of a toxin of this type was reported by Eaker and Porath in 1967,[8] and subsequently research groups in Japan, Taiwan, Germany, South Africa, and the United Kingdom have contributed many more. In particular, Joubert and Tamiya with their co-workers have made major additions to the growing sequence data.[9]

The sequences show that the neurotoxins and cytotoxins are homologous with a characteristic positioning and linking of eight half-cystine residues.[10] As such, this conservation of a basic molecular organization, when associated with the variety of pharmacological activities, provides a largely unique opportunity to deduce the determinants of each mode of action. In order to achieve this, it is necessary to direct attention to the conformations that the toxins can adopt, especially as no enzymic activity is inherent. The first detailed crystallographic data on one of these toxins appeared in 1976 and this prompted interpretations of the extensive sequence and chemical modification data in terms of a three-dimensional framework.[11,12] Tentative interactive sites were identified and it became apparent that the conformation deduced for the one toxin was essentially appropriate for the other examples.[13-16] However, the insight as to how similar structures could produce such different modes of action (i.e., neurotoxicity, cardiotoxicity, cytolysis) was not immediately forthcoming. Recent developments now indicate that there are certain conformational characteristics involved which could have a great bearing on our future understanding. The purpose of this review is to detail these advances and to suggest their significance.

II. THE TOXIN FAMILY

From amongst the wide variety of sequence types, the majority so far determined can be classified under the three headings of "short neurotoxins", "long neurotoxins", and "cytotoxins" (Tables 1 to 3).[10] Those members of the family that fall outside these categories tend not to have any potent pharmacological activity, but often possess

Table 1
THE SEQUENCES OF THE SHORT NEUROTOXIN (SN) AND THE SHORT NEUROTOXIN HOMOLOGUES (SH)[a]

	5 10 15 20 25 30 35 40 45 50 55 60	
SN 01	M I C H N Q Q S S Q R P T I K T C P — G E T N C Y K K R W R D H R G T I I E R G C G C P S V K K G V G I Y C C K T D K C N R	*Naja nivea:* β
SN 02	L E C H N Q Q S S E P P T T T R C S G G E T N C Y K K R W R D H R G Y R T E R C G C C P T V K K G I E L N C C T T D R C N N	*Naja mossambica mossambica:* I
SN 03	L N C H N Q M S A Q P P T T T R C S R W E T N C Y K K R W R D H R G Y K T E R G C G C P T V K K G I Q L H C C T S D N C N N	*Naja mossambica mossambica:* III
SN 04	M I C H N Q Q S S Q P P T I K T C P — G E T N C Y K K Q W R D H R G T I I E R G C G C P S V K K G V G I Y C C K T D K C N R	*Naja haje haje:* CM-10A
SN 05	L E C H N Q Q S S Q P P T T K T C P — G E T N C Y K K R W R D H R G S I T E R G C G C P S V K K G I E I N C C T T D K C N N	*Naja haje haje:* CM-6
SN 06	L Q C H N Q Q S S Q P P T T K T C P — G E T N C Y K K R W R D H R G S I T E R G C G C P S V K K G I E I N C C T T D K C N N	*Naja haje annulifera:* α and *Naja nivea:* δ
SN 07	M I C H N Q Q S S Q P P T I K T C P — G E T N C Y K K R W R D H R G T I I E R G C G C P S V K K G V G I Y C C K T N K C N R	*Naja haje annulifera:* CM-14
SN 08	M E C H N Q Q S S Q P P T T K T C P — G E T N C Y K K Q W S D H R G T I I E R G C G C P S V K K G V K I N C C T T D R C N N	*Naja melanoleuca:* d
SN 09	L E C H N Q Q S S Q P P T T K T C P — G E T N C Y K K V W R D H R G T I I E R G C G C P T V K P G I K L N C C T T D K C N N	*Naja nigricollis:* α
SN 10	L E C H N Q Q S S Q T P T T T G C S G G E T N C Y K K R W R D H R G Y R T E R G C G C P S V K N G I E I N C C T T D R C N N	*Naja naja atra:* Cobrotoxin
SN 11	L E C H N Q Q S S Q P P T T K T C — S G E T N C Y K K W W S D H R G T I I E R G C G C P K V K P G V N L N C C R T D R C N N	*Naja naja oxiana:* II
SN 12	L E C H N Q Q S S Q P P T T K S C P — G D T N C Y N K R W R D H R G T I I E R G C G C P T V K P G I N L K C C T T D R C N N	*Hemachatus hemachates:* II
SN 13	L E C H N Q Q S S Q T P T T Q T C P — G E T N C Y K K Q W S D H R G S R T E R G C G C P T V K P G I K L K C C T T D R C N K	*Hemachatus hemachates:* IV
SN 14	R I C Y N H Q S T T P A T T K S C — — G E N S C Y K K T W S D H R G T I I E R G C G C P K V K R G V H L H C C Q S D K C N N	*Dendroapsis viridis:* 4.11.3
SN 15	R I C Y N H Q S T T P A T T K S C — — G E N S C Y K K T W S D H R G T I I E R G C G C P K V K Q G I H L H C C Q S D K C N N	*Dendroaspis jamesoni:* Vn[1] 1
SN 16	R I C Y N H Q S T T R A T T K S C — — E E N S C Y K K Y W R D H R G T I I E R G C G C P K V K P G V G I H C C Q S D K C N Y	*Dendroaspis polylepis:* ∝
SN 17	R I C F N Q H S S Q P Q T T K T C P S G E S S C Y N K Q W S D F R G T I I E R G C G C P T V K P G I K L S C C E S E V C N N	*Laticauda semifasciata:* erabutoxin a
SN 18	R I C F N Q H S S Q P Q T T K T C P S G E S S C Y H K Q W S D F R G T I I E R G C G C P T V K P G I K L S C C E S E V C N N	*Laticauda semifasciata:* erabutoxin b
SN 19	R I C F N Q H S S Q P Q T T K T C P S G E S S C Y H K Q W S D F R G T I I E R G C G C P T V K P G I N L S C C E S E V C N N	*Laticauda semifasciata:* erabutoxin c
SN 20	R R C F N H P S S Q P Q T N K S C P P G E N S C Y N K Q W R D H R G T I T E R G C G C P T V K P G I K L T C C Q S E D C N N	*Laticauda laticauda:* laticotoxin a
SN 21	R R C F N H P S S Q P Q T N K S C P P G E N S C Y N K Q W R D H R G T I T E R G C G C P Q V K S G I K L T C C Q S D D C N N	*Laticauda laticauda:* laticotoxin a′
SN 22	R R C Y N Q Q S S Q P K T T K S C P P G E N S C Y N K Q W R D H R G S I T E R G C G C P T V K P G I K L R C C E S E D C N N	*Laticauda colubrina:* II
SN 23	M T C C N Q Q S S Q P K T T T N C — — A E S S C Y K K T W S D H R G T R I E R G C G C P Q V K S G I K L E C C H T N E C N N	*Enhydrina schistosa:* 5
SN 24	M T C C N Q Q S S Q P K T T T N C — — A E S S C Y K K T W S D H R G T R I E R G C G C P Q V K P G I K L E C C H T N E C N N	*Enhydrina schistosa:* 4 and *Lapemis Hardwickii*
SN 25	L T C C N Q Q S S Q P K T T T D C — — A D N S C Y K K T W Q D H R G T R I E R G C G C P Q V K P G I K L E C C K T N E C N N	*Aipysurus laevis:* a
SN 26	L T C C N Q Q S S Q P K T T T D C — — A D N S C Y K M T W R D H R G T R I E R G C G C P Q V K P G I K L E C C K T N E C N N	*Aipysurus laevis:* b
SN 27	L T C C N Q Q S S Q P K T T T D C — — A D N S C Y K K T W K D H R G T R I E R G C G C P Q V K P G I K L E C C K T N E C N N	*Aipysurus laevis:* c
SN 28	M T C C N Q Q S S Q P K T T T N C — — A E S S C Y K K T W S D H R G T R I E R G C G C P Q V K K G I K L E C C H T N E C N N	*Hydrophis cyanocinctus:* Hydrophitoxin B and *Pelamis platurus:* Pelamitoxin A
SN 29	M T C C N Q Q S S Q P K T T T N C — — A E S S C Y K K T W S D H R G T R I E R G C G C P Q V K K G I K L E C C H T N E C N N	*Hydrophis cyanocinctus:* Hydrophitoxin A
SN 30	M T C C N Q Q S S Q P K T T T N C — — A E S S C Y K K T W R D F R G T R I E R G C G C P Y V K P G I K L E C C H T N E C N N	*Hydrophis ornatus* 75 A
SN 31	M T C C N Q Q S S Q P K T T T N C — A G — N S C Y K K T W S D H R G T I I E R G C G C P Q V K S G I K L E C C H T N E C N N	*Astrotia stokesii:* a and *Hydrophis ornatus:* 73 A
SN 32	M Q C C N Q Q S S Q P K T T T T C P G G V S S C Y K K T W R D H R G T I I E R G C G C P R V K P G I R L I C C K T D E C N N	*Acanthopis antarcticus:* C

	5	10	15	20	25	30	35	40	45	50	55	60	
SH 01	M I C Y K Q Q S L Q F P I T T V C P — G E K N C Y K K Q W S G H R G T I I E R G C G C P S V K K G I — E I N C C T T — D K C N R												*Naja haje annulifera:* CM-10
SH 02	M I C Y K Q R S L Q F P I T T V C P — G E K N C Y K K Q W S G H R G T I I E R G C G C P S V K K G I — E I N C C T T — D K C N R												*Naja haje annulifera:* CM-12
SH 03	R I C Y N H Q S N T P A T T K S C — — V E N S C Y K S I W A D H R G T I I K R G C G C P R V K S — — — K I K C C K S — D N C N L												*Dendroaspis jamesoni kaimose:* S_5C_{10}
SH 04	R I C Y N H L G T K P P T T E — C T — Q E D S C Y K N I W R N I T E D N I R R G C G C F T P R G D M P G P Y C C E S — D K C N L												*Dendroaspis jamesoni kaimose:* S_5C_1
SH 05	M I C Y S H K T P Q N S A T I T C — — E E K T C Y K K F V T N V P G V I L A R G C G C P K K E I F R — S I H C C R S — D K C N E												*Dendroaspis jamesoni kaimose:* S_5C_4
SH 06	R I C Y T H K S L Q A K T T K S C — — E G N T C Y K M F I R T S R E Y I S E R G C G C P T A M W P Y — Q T E C C K G — D R C N K												*Dendroaspis jamesoni kaimose:* S_4C_8
SH 07	T M C Y S H T T T S R A I L T N C — — G E N S C Y R K S R R H P P K M V L G R G C G C P P G D D N L — E V K C C T S P D K C N Y												*Dendroaspis angusticeps:* F7
SH 08	M I C Y S H K T P Q P S A T I T C — — E E K T C Y K K S V R K L P A V V A G R G C G C P S K E M L V — A I H C C R S — D K C N E												*Dendroaspis angusticeps:* F8
SH 09	R I C Y S H K A S L P R A T K T C — — V E N S C Y K M F I R T S P N Y I S N R G C G C P T A M W P Y — Q T A C C K G — D R C N K												*Dendroaspis angusticeps:* $C_{10}S_2C_2$
SH 10	R I C Y S H K L L Q A K T T K T C — — E E N S C Y K R S L P K I P — L I I G R G C G C P L T L P F L — R I K C C T S — N K C N												*Dendroaspis angusticeps:* $C_{13}S_1C_1$
SH 11	T I C Y S H T T T S R A I L K D C — — G E N S C Y R K S R R H P P K M V L G R G C G C P P G D D Y L — E V K C C T S P D K C N Y												*Dendroaspis polylepis:* C
SH 12	R I C Y S H K A S L P R A T K T C — — V E N T C Y K M F I R T H R Q Y I S E R G C G C P T A M W P Y — Q T E C C K G — D R C N K												*Dendroaspis polylepis:* FS2
SH 13	M I C Y S H K T P Q N S A T I T C — — E E K T C Y K — F V T K L P G V I L A R G C G C P K K E I F R K S I H C C R S — D K C N E												*Dendroaspis viridis:* 4.9.6
SH 14	L I C F N Q E T Y R P E T T T T C P D G E N — C Y S T F W H N D G H V K I E R G C G C P R V N P P I — S I — C C K T — D K C N N												*Ophiophagus hannah:* DE-1

[a] The one letter notation for amino acids is used throughout this review (*Eur. J. Biochem.*, 5, 152, 1968). The selection of the short neurotoxin homologues is based on the possession of the sequence CGC (41 to 43) and LD_{50} values of above 0.25 mg Kg^{-1} (i.v. mouse). Note that the short neurotoxins and the short neurotoxin homologues have different schemes.

From Kim and Tamiya[17] with the exception of SH 03,[18] SH 04,[18] SH 06,[19] and SH 10.[20]

Table 2
THE SEQUENCES OF THE LONG NEUROTOXINS (LN) AND THE LONG NEUROTOXIN HOMOLOGUES (LH)[a]

	5 10 15 20 25 30 35 40 45 50 55 60 65 70	
LN 01	I R C F — — I T P D V T S Q I C A D G — H V C Y T K T W C D N F C A S R G K R V D L G C A A T C P T V K P G V N I K C C S T D N C N P F F P T R N R P	*Naja melanoleuca:* b
LN 02	I R C F — — I T P D V T S Q A C P D G — H V C Y T K M W C D N F C G M R G K R V D L G C A A T C P K V K P G V N I K C C S R D N C N P F F P T R K R S	*Naja nivea:* α
LN 03	I R C F — — I T P D I T S K D C P N G — H V C Y T K T W C D A F C S I R G K R V D L G C A A T C P T V K T G V D I Q C C S T D N C N P F F P T R K R P	*Naja naja siamensis* (Kaouthia): 3 α-Cobratoxin
LN 04	I R C F — — I T P D I T S K D C P N G — H V C Y T K T W C D G F C S I R G K R V D L G C A A T C P T V R T G V D I Q C C S T D N C N P F F P T R K R P	*Naja naja naja:* 3
LN 05	I R C F — — I T P D I T S K D C P N G — H V C Y T K T W C D G F C S S R G K R V D L G C A A T C P T V R T G V D I Q C C S T D N C N P F F P T R K R P	*Naja naja naja:* 4
LN 06	I R C F — — I T P D I T S K D C P N G — H V C Y T K T W C D G F C S I R G K R V D L G C A A T C P T V R T G V D I Q C C S T D D C D P F F P T R K R P	*Naja naja:* A
LN 07	I R C F — — I T P D I T S K D C P N G — H V C Y T K T W C D G F C S S R G K R V D L G C A A T C P T V R T G V D I Q C C S T D D C D T F F P T R K R P	*Naja naja:* B
LN 08	I R C F — — I T P D I T S K D C P N G — H V C Y T K T W C D A F C S I R G K R V D L G C A A T C P T V K T G V D I Q C C S T D D C D T F F P T R K R P	*Naja naja:* C
LN 09	I R C F I T P D I T S K D C P N G — H V C Y T K T W C D G F C R I R G E R V D L G C A A T C P T V K T G V D I Q C C S T D D C D P F F P T R K R P	*Naja naja:* D
LN 10	I R C F — — I T P D I T S K D C P N G — H V C Y T K T W C D G F C S R R G E R V D L G C A A T C P I V K T G V D I Q C C S T D D C D P F F P T R K R P	*Naja naja:* E
LN 11	I R C F — — I T P D V T S Q A C P D G Q N I C Y I K T W C D N F C G M R G K R V D L G C A A T C P T V K P G V D I K C C S T D N C N P F F P T R E R S	*Naja haje annulifera:* III
LN 12	I R C F — — I T P D V T S Q A C P D G — H V C Y T K M W C D N F C G M R G K R V D L G C A A T C P T V K P G V D I K C C S T D N C N P F F P T R K R S	*Naja haje haje:* CM-5
LN 13	T K C Y — — V T P D V K S Q T C P A G Q D I C Y T E T W C D A W C T S R G K R V D L G C A A T C P I V K P G V E I K C C S T D N C N P F F P T W R K R P	*Ophiophagus hannah:* a
LN 14	T K C Y — — V T P D A T S Q T C P D G E N I C Y T K T W C D G F C S S R G K R I D L G C A A T C P K V K P G V D I K C C S T D N C N P F F P T W K R K H	*Ophiophagus hannah:* b
LN 15	R T C N — — K T F S D Q S K I C P P G E N I C Y T K T W C D A W C S Q R G K R V E L G C A A T C P K V K A G V E I K C C S T D D C D K F Q F G K P R	*Dendroaspis polylepis:* V^1N
LN 16	R T C N — — K T P S D Q S K I C P P G E N I C Y T K T W C D A W C S W R G K I V E L G C A A T C P K V K A G V E I K C C S T D N C N K F K F G K P R	*Dendroaspis polylepis:* V^2N
LN 17	R T C N — — K T F S D Q S K I C P P G E N I C Y T K T W C D A W C S R R G K I V E L G C A A T C P K V K A G V G I K C C S T D N C N L F K F G K P R	*Dendroaspis polylepis:* V^N2
LN 18	R T C X Y — K T Y S D K S K T C P R G E N I C Y T K T W C D G F C S Q R G K R V E L G C A A T C P K V K T G V E I K C C S T D Y C N P F F P V W N P R	*Dendroaspis jamesoni Kaimose:* V^NIII-1
LN 19	R T C Y — — K T P S V K P E T C P H G E N I C Y T E T W C D A W C S Q R G K R E E L G C A A T C P K V K A G V G I K C C S T D N C D P F F P V K N P R	*Dendroaspis viridis:* 4.9.3
LN 20	R T C Y — — K T P S V K P E T C P H G E N I C Y T E T W C D A W C S Q R G K R V E L G C A A T C P K V K A G V G I K C C S T D N C N P F F P V W N P R G	*Dendroaspis viridis:* 1
LN 21	R T C Y — — K T P S V K P E T C P H G E N I C Y T E T W C D A W C S Q R G K R V E L G C A A T C P K V K A G V G I K C C S T D N C N P F F P V W N P R	*Dendroaspis viridis:* V
LN 22	R E C Y — — — L N P H D T Q T C P S G Q E I C Y V K S W C N A W C S S R G K V L E F G C A A T C P S V N T G T E I K C C S A D K C N T Y P	*Laticauda semifasciata:* LS III
LN 23	L S C Y — — — L G Y K H S Q T C P P G E N V C F V K T W C D G F C N T R G E R I I M G C A A T C P T A K S G V H I A C C S T D N C N I Y A K W G S — NH_2	*Astrotia stokesii:* b
LN 24	L S C Y — — — L G Y K H S Q T C P P G E N V C F V K T W C D A F C S T R G E R I V M G C A A T C P T A K S G V H I A C C S T D N C N I Y T K W G S G R — NH_2	*Astrotia strokesii:* c
LN 25	K R C Y — — R T P N L K S Q T C P P G E D L C Y T K K W C D A W C T S R G K V I E L G C V A T C P K V K P Y E Q I T C C S T D N C N P H P K M K P	*Nama melanoleuca:* 3.9.4
LN 26	I T C Y — K T P I P I T S E T C A P G Q N L C Y T K T W C D A W C G S R G K V I E L G C A A T C P T V E S Y Q D I K C C S T D D C N P H P K Q K R P	*Naja naja oxiana:* α
LN 27	I V C H T T A T C I P S S A V T C P P G E N L C Y R K M W C D A F C S S R G K V V E L G C A A T C P S K K P Y E E V T C C S T D K C N H P P K R Q G	*Bungarus multicinctus:* α-Bungarotoxin
LN 28	L I C Y — — — M G P K T P R T C P R G Q N L C Y T K T W C D A F C S S R G K V V E L G C A A T C P I A K S Y E D V T C C S T D N C N P F F P V R P R H P P	*Notechis scutatus scutatus:* III-4
LN 29	V I C Y — — — R G Y N N P Q T C P P G E N V C F T R T W C D A F C S S R G K V V E L G C A A T C P I V K S Y N E V K C C S T D N C N P F F P V R P R R P P	*Acanthopis antarcticus:* b
LH 01	R I C Y — — — L A P R D T Q I C A P G Q E I C Y L K S W D D G T G F L K G N R L E F G C A A T C P T V K P G I D I K C C S T D K C N P H P K L A	*Laticauda colubrina:* a
LH 02	R I C Y — — — L A P R D T Q I C A P G Q E I C Y L K S W D D G T G S I R G N R L E F G C A A T C P T V K R G I H I K C C S T D K C N P H P K L A	*Laticauda colubrina:* b

[a] Long neurotoxins have LD_{50} values < 1.0 mg Kg^{-1} (i.v. mouse). Sequences LN 25 to 29 are considered to be a subgroup since they possess a tyrosine at position 54. Long neurotoxins homologues are distinguished by the lack of the 29 to 33 disulphide bridge.

Sequences adopted from Mebs[9] with the exceptions of LN 09,[21] LN 10,[22] LN 12,[23] LN 28,[24] LN 29[25] and LH 01 and LH 02.[26]

Table 3
THE SEQUENCES OF THE CYTOTOXINS (CT) AND THE CYTOTOXIN HOMOLOGUES (CH)[a]

	5	10	15	20	25	30	35	40	45	50	55	60	
CT 01	L K C N K L I P L A Y K T C P A G K N L C Y K M Y M V S T P K V P V K R G C I D V C P K N S L V L K Y E C C N T D R N N	*Naja naja:* CM-XI
CT 02	L K C N K L I P L A Y K T C P A G K N L C Y K M Y M V S N K T V P V K R G C I D V C P K N S L V L K Y E C C N T D R C N	*Naja naja naja:* I Cobramine A
CT 03	L K C N K L V P L F Y K T C P A G K N L C Y K M Y M V A T P K V P V K R G C I D V C P K S S L V L K Y V C C N T D R C N	*Naja naja naja:* II Cobramine B
CT 04	L K C N K L I P L A Y K T C P A G K N L C Y K M F M V S N K T V P V K R G C I D V C P K N S L V L K Y V C C N T D R C N	*Naja naja naja:* II A
CT 05	L K C N K L I P I A S K T C P A G K N L C Y K M F M M S D L T I P V K R G C I D V C P K N S L L V K Y V C C N T D R C N	*Naja naja siamensis (Kaouthia):* CM-6
CT 06	L K C N K L I P L A Y K T C P A G K N L C Y K M F M V S N K T V P V K R G C I D A C P K N S L L V K Y V C C N T D R C N	*Naja naja siamensis (Kaouthia):* CM-7
CT 07	L K C N K L I P L A Y K T C P A G K N L C Y K M F M V S N K T V P V K R G C I D V C P K N S L L V K Y V C C N T D R C N	*Naja naja siamensis (Kaouthia):* CM-7A
CT 08	L K C K K L V P L F S K T C P A G K N L C Y K M F M V A A P H V P V K R G C I D V C P K S S L L V K Y V C C N T D K C N	*Naja naja oxiana*
CT 09	L K C N K L I P I A S K T C P A G K N L C Y K M F M M S D L T I P V K R G C I D V C P K S N L L V K Y V C C N T D R C N	*Naja naja atra:* I
CT 10	L K C N K L V P L F Y K T C P A G K N L C Y K M F M V S N L T V P V K R G C I D V C P K N S A L V K Y V C C N T D R C N	*Naja naja atra:* II
CT 11	L K C N K L V P L F Y K T C P A G K N L C Y K M F M V A T P K V P V K R G C I D V C P K S S L L V K Y V C C N T D R C N	*Naja naja atra:* Cardiotoxin
CT 12	R K C N K L V P L F Y K T C P A G K N L C Y K M F M V S N L T V P V K R G C I D V C P K N S A L V K Y V C C N T D R C N	*Naja naja atra:* IV
CT 13	L E C N K L V P I A H K T C P A G K N L C Y Q M Y M V S K S T I P V K R G C I D V C P K S S L L V K Y V C C N T D R C N	*Naja melanoleuca:* $V^{II}1$
CT 14	I E C N K L V P I A H K T C P A G K N L C Y Q M Y M V S K S T I P V K R G C I D V C P K S S L L V K Y V C C N N D R C N	*Naja melanoleuca:* $V^{II}1A$
CT 15	L K C N Q L I P P F W K T C P K G K N L C Y K M T M R A A P M V P V K R G C I D V C P K S S L L I K Y M C C N T N K C N	*Naja mossambica mossambica:* $V^{II}1$
CT 16	L K C N Q L I P P F W K T C P K G K N L C Y K M T M R G A S K V P V K R G C I D V C P K S S L L I K Y M C C N T D K C N	*Naja mossambica mossambica:* $V^{II}2$
CT 17	L K C N R L I P P F W K T C P E G K N L C Y K M T M R L A P K V P V K R G C I D V C P K S S L L I K Y M C C N T N K C N	*Naja mossambica mossambica:* $V^{II}3$
CT 18	L K C N K L I P I A Y K T C P E G K N L C Y K M M L A S K K M V P V K R G C I N V C P K N S A L V K Y V C C S T D R C N	*Naja mossambica mossambica:* $V^{II}4$
CT 19	L K C N Q L I P P F W K T C P K G K N L C Y K M T M R A A P M V P V K R G C I D V C P K S S L L I K Y M C C N T D K C N	*Naja nigricollis:* cardiotoxin
CT 20	L K C H K L V P P V W K T C P E G K N L C Y K M F M V S T S T V P V K R G C I D V C P K D S A L V K Y V C C S T D K C N	*Naja nivea:* $V^{II}1$
CT 21	L K C H Q L I P P F W K T C P E G K N L C Y K M Y M V A T P M I P V K R G C I D V C P K N S A L V K Y M C C N T D K C N	*Naja nivea:* $V^{II}2$
CT 22	L K C N Q L I P P F W K T C P K G K N L C Y N M Y M V S T S T V P V K R G C I D V C P K N S A L V K Y V C C N T D R C N	*Naja nivea:* $V^{II}3$
CT 23	L K C H Q L V P P F W K T C P E G K N L C Y K M Y M V A T P M I P V K R G C I D V C P K N S A L V K Y M C C N T D K C N	*Naja haje haje:* CM-7
CT 24	L K C H Q L V P P F W K T C P E G K N L C Y K M Y M V S S S T V P V K R G C I D V C P K N S A L V K Y V C C N T D K C N	*Naja haje haje:* CM-8
CT 25	L K C H Q L V P P F W K T C P A G K N L C Y K M Y M V A T P M I P V K R G C I D V C P K N S A L V K Y M C C N T D K C N	*Naja haje haje:* CM-9
CT 26	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V A T P M I P V K R G C I D V C P K N S A L V K Y V C C N T D K C N	*Naja haje haje:* CM-10B
CT 27	L K C H K L V P P V W K T C P E G K N L C Y K M F M V S T S T V P V K R G C I D V C P K N S A L V K Y V C C S T D K C N	*Naja haje annulifera:* $V^{II}1$
CT 28	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V A T P M L P V K R G C I D V C P K D S A L V K Y M C C N T D K C N	*Naja haje annulifera:* $V^{II}2$
CT 29	L K C H K L V P P F Y K T C P E G K N L C Y K M Y M V A T P M L P V K R G C I D V C P K D S A L V K Y V C C S T D K C N	*Naja haje annulifera:* $V^{II}2A$
CT 30	L E C N K L V P I A H K T C P E G K N L C Y K M F M V S T S T V P V K R G C I D V C P K D S A L V K Y V C C N T D R C N	*Naja haje annulifera:* CM-2E
CT 31	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V A T P M L P V K R G C I D V C P K D S A L V K Y M C C N T N K C N	*Naja haje annulifera:* CM-2H
CT 32	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V A T P M L P V K R G C I D V C P K D S A L V K Y V C C S T N K C N	*Naja haje annulifera:* CM-2HA
CT 33	L E C N Q L I P I A H K T C P E G K N L C Y K M F M V S T S T V P V K R G C I D V C P K N S A L V K Y V C C N T D R C N	*Naja haje annulifera:* CM-4A
CT 34	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V A T P M L P V K R G C I N V C P K D S A L V K Y M C C N T N K C N	*Naja haje annulifera:* CM-4B
CT 35	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V A T P M L P V K R G C I N V C P K D S A L V K Y V C C S T N K C N	*Naja haje annulifera:* CM-4BA
CT 36	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V A T P M I P V K R G C I D V C P K N S A L V K Y M C C N T N K C N	*Naja haje annulifera:* CM-6
CT 37	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V S T L T V P V K R G C I D V C P K N S A L V K Y V C C N T N K C N	*Naja haje annulifera:* CM-7
CT 38	L K C Y K L V P P F W K T C P E G K N L C Y K M Y M V S T L T V P V K R G C I D V C P K N S A L V K Y V C C N T D K C N	*Naja haje annulifera:* CM-8
CT 39	L K C H K L V P P F W K T C P E G K N L C Y K M Y M V S T L T V P V K R G C I D V C P K N S A L V K Y V C C N T D K C N	*Naja haje annulifera:* CM-8A
CT 40	L K C N K L I P P F W K T C P K G K N L C Y K M Y M V S T L T V P V K R G C I D V C P K N S A L V K Y V C C N T N K C N	*Naja haje annulifera:* CM-11

Table 3 (continued)
THE SEQUENCES OF THE CYTOTOXINS (CT) AND THE CYTOTOXIN HOMOLOGUES (CH)[a]

	5	10	15	20	25	30	35	40	45	50	55	60	
CH01	I K C H N T L L P F I Y K T C P E G Q N L C F K G T L K F — P K K T T Y N R G C A A T C P K S S L L V K Y V C C N T N K C N												*Naja melanoleuca:* $V^{II}2$
CH02	I K C H N T L L P F I Y K T C P E G Q N L C F K G T L K F — P K K T T Y K R G C A A T C P K S S L L V K Y V C C N T N K C N												*Naja melanoleuca:* $V^{II}3$
CH03	I K C H N T P L P F I Y K T C P E G N N L C F K G T L K F — P K K I T Y K R G C A D A C P K T S A L V K Y V C C N T D K C N												*Naja melanoleuca:* 3.20
CH04	L K C H N T Q L P F I Y K T C P E G K N L C F K T T L K K L P L K I P I K R G C A A T C P K S S A L L K S V C C N T D K C N												*Naja haje annulifera:* CM-13A
CH05	L I C H N R P L P F L H K T C P E G Q N I C Y K M Y L K K T P M K L S V K R G C A A T C P S E R P L V Q V E C C K T D K C N W												*Hemachatus hemachates:* 9B
CH06	L I C H N R P L P F L H K T C P E G Q N I C Y K M T L K K T P M K L S V R R G C A A T C P S A R P L V Q V E C C K T D K C N W												*Hemachatus hemachates:* 9BB
CH07	L K C H N K L V P F L S K T C P D G K N L C Y K M S M E V T P M — I P I K R G C T D T C P K S S L L V K V V C C K T D K C N												*Hemachatus hemachates:* 11
CH08	L K C H N K L V P Y L S K T C P D G K N L C Y K M S M E V T P M — I P I K R G C T D T C P K S S L L V K V V C C K T D K C N												*Hemachatus hemachates:* 11A
CH09	L K C H N K V V P F L S K T C P E G K N L C Y K M T L K K V P — K I P I K R G C T D A C P K S S L L V N V M C C K T D K C N												*Hemachatus hemachates:* 12A
CH10	L K C H N K L V P F L S K T C P E G K N L C Y K M T M L K M P — K I P I K R G C T D A C P K S S L L V K V V C C N K D K C N												*Hemachatus hemachates:* 12B DLF

[a] The selection of the cytotoxin homologues is based on the lack of the sequence INV or IDV (39 to 41) and the presence of an additional residue between half cystines 3 and 14. Note that the cytotoxins and cytotoxin homologues have different alignment schemes.

Sequences adopted from Mebs[9] with the exception of CT 04 to 06[27] and CT 23 to 26.[28]

structural features that associate them with one group or another. Thus further subdivisions of the data can be made into "short neurotoxin homologues" (Table 1), "long neurotoxin homologues" (Table 2), and "cytotoxin homologues" (Table 3). An impromptu class of "miscellaneous types" (Table 4) can be used for whatever sequences remain.

Since most work has been concentrated on the three major groups, the following account seeks only to discuss these and their intercomparison. It is important, however, not to neglect the minority types because their apparent lack of pharmacological properties might be due to either inappropriate assays or their separation from synergistic partners.[34]

In sequence comparisons between the toxin groups, it is often considered useful to devise a numbering system that identifies a residue according to its position in an overall homology line-up. Thus, in neurotoxins, although tryptophan is the 29th residue in erabutoxin b (a short type) and the 25th residue in α-cobratoxin (a long type), reference to the homology position (29) will reveal the location in both neurotoxin groups simultaneously (Table 5). However, in order to obtain a common numbering scheme, a satisfactory homology line-up is required, and this becomes subjective where there are major differences in local chain length. Therefore, use of a homology position number does not necessarily mean that the residues so identified in different toxin groups are actually equivalent, even though this is the clear impression given to the reader. As an example, consider the CGC (41 to 43) sequence of short neurotoxins, which must be matched with the CAATC (44 to 48) sequence of long neurotoxins in any homology line-up. A widely used system by Karlsson suggests that G and T are the homologous residues and introduces gaps to correspond with AA in the short neurotoxin sequences (Table 5). This implies that the dipeptide AA is an evolutionary insertion or deletion and that the T of long neurotoxins is functionally equatable with the G of short neurotoxins. A contrary view would be that G should be lined up with A because the minimum base mutation distance is less, but again this is not absolute evidence. In fact, a more appropriate view would be that in molecular terms, the G of short neurotoxins is equivalent to the AAT of long neurotoxins and not to any one residue therein.

In view of this, the numbering systems used in this review are unique to each major group of toxins. The homology gaps required in the groups themselves are relatively few in number and less open to subjective placement.

A. Short Neurotoxins

These toxins produce respiratory and peripheral paralysis in the prey by binding strongly to post synaptic cholinergic receptors located at nerve-nerve and nerve-muscle junctions.[35] Most examples have dissociation constants in the range $10^{-10} - 10^{-11}$ M, indicating an extremely high affinity for these membrane associated complexes.[36,37] Typical LD_{50} values for mice lie between 50 and 150 $\mu g\ kg^{-1}$.

In terms of primary structure, short neurotoxins are 60, 61, or 62 amino acid residues in length (Table 1) and have their eight half cystinyl residues linked to form four disulphide bridges. The folding pattern of the polypeptide chain has been elucidated for one example (erabutoxin b) by X-ray crystallography and it is depicted in Figure 1. Although the relevance of this crystal structure to the solution conformation is a major point of discussion in this review, it is useful here as a means of displaying an aggregate short neurotoxin amino acid sequence with an indication of how each position is conserved throughout the known sequences. Furthermore, it gives valuable information on the general molecular environment of each residue. It should be pointed out, however, that the sequence shown in the diagram may not correspond to any one short neurotoxin per se.

Table 4
THE SEQUENCES OF THE MISCELLANEOUS TOXIN TYPES (MT)

	5	10	15	20	25	30	35	40	45	50	55	60	65	
MT 01	F T C F T T P S — — — D T S E T C P D G Q N I C Y E K R W N S H — Q G V E I — K G C V A S C P E F E S K F R Y L L C C R I D N C N K													*Naja haje haje:* CM-2
MT 02	L T C V T D K S F G G V N T E E C A A G Q K I C F K N W K K M G P K L Y D V K R G C T A T C P K A D D D G — C V K C C N T D K													*Dendroaspis jamesoni kaimose:* S_2C_4
MT 03	L T C V T G K S I G G I S T E E C A A G Q K R C F K K W T K M G P K L Y D V S R G C A A T C P K A D E Y G — C V K C C N T D K													*Dendroaspis angusticeps:* C_8S_2
MT 04	L T C V T G K S I G G I S T E E C A A G Q K I C F K K W T K M G P K L Y D V S R G C T A T C P K A D E Y G — C V K C C N T D R — N													*Dendroaspis angusticeps:* C_9S_3
MT 05	L T C L I C P E K Y C N K V H T C R N G E N I C F K R F Y E G N L L G K R Y P R G C A A T C P E A K — P R E I V E C C S T D K C N H													*Naja melanoleuca:* S_4C_{11}
MT 06	L T C F N C P E V Y C N R F H T C R N G E K I C F K R F N E R K L L G K R Y P T G C A A T C P V A K — P R E I V E C C S T D R C N H													*Naja haje annulifera:* CM-13b
MT 07	L T C F I C P E K Y C N K V H T C R N G E N Q C F K R F N E R K L L G K R Y T R G C A A T C P E A K — P R E I V E C C T T D R C N K													*Naja haje haje:* CM-11
MT 08	L R C L N C P E V F C R N F H T C R N G E K I C F K R F D Q R K L L G K R Y T R G C A V T C P V A K — P R E I V E C C S T D G C N R													*Naja nivea:* CM-10
MT 09	L R C L N C P E M F C G K F Q I C R N G E K I C F K K L H Q R R P L — S R Y I R G C A D T C P V G Y — P K E M I E C C S T D K C N R													*Naja naja siamensis (Kaouthia):* CM-9A
MT 10	L E C Y — Q K S — — — — K V V T C Q P E Q K F C Y S D T M T F F P N H P V Y L S G C T — F C — R T D E S G E — — R C C T T D R C N K													*Hemachatus hemachates:* CM-1B
MT 11	L E C Y — Q M S — — — — K V V T C K P E E T F C Y S D V F M P F R N H I V Y T S G C S S Y C — R — D G T G E — — K C C T T D R C N G A R G G													*Naja haje annulifera:* CM-2A
MT 12	L E C Y — Q M S — — — — K V V T C K P E E K F C Y S D V F M P F R N H — V Y T S G C S S Y C — R — D G T G E — — K C C T T D R C N G A R G G													*Naja haje annulifera:* CM-3

Sequences adopted from Mebs[9] with the exception of MT 01,[29] MT 02,[30] MT 03,[31] MT 04,[38] MT 07,[29] MT 08,[32] MT 09,[32] and MT 10.[33]

Table 5
THE COMMON NUMBERING SYSTEM DEVISED FOR SHORT NEUROTOXINS, LONG NEUROTOXINS, AND CYTOTOXINS BY KARLSSON[11]

```
                  10                  20                  30                  40                  50                  60                  70
1   R I C F N Q H S S Q P Q T T K T C P S G E S S C Y H K Q W S D — F — — — R G T I I E R G C — — G C P T V K — P G I K L S C C E S — E V C N N
2   I R C F — — — I T P D I T S K D C P N G — H V C Y T K T W C D A F C S I R G K R V D L G C A A T C P T V K — T G V D I Q C C S T — D N C N P F P T R K R P
3   L K C — N — — K L I P I A Y K T C P E G K N L C Y K M M L A S — K — — — K M V P V K R G C I N V C P K N S — A L V K Y V C C S T — D R C N
```

[1] *Laticauda semifasciata* erabutoxin b (SN 18).
[2] *Naja naja siamensis:* α-Cobratoxin (LN 03).
[3] *Naja mossambica mossambica:* $V^{II}4$ Cytotoxin (CT 18).

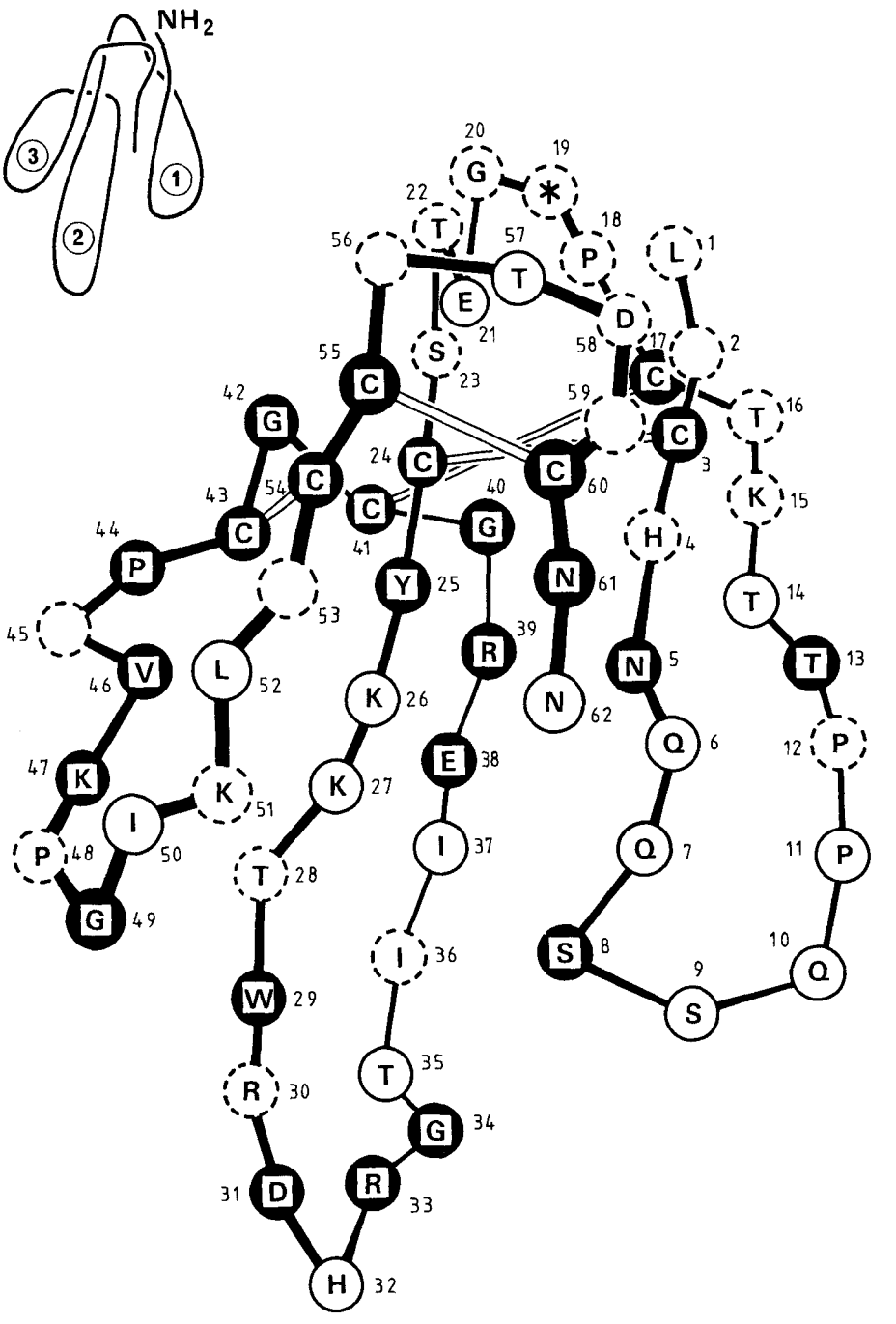

FIGURE 1. The backbone of a short neurotoxin showing the aggregate sequence of the group as a whole (Table 1). It is based on the crystal structure of the short neurotoxin erabutoxin b. The sequence shown may not correspond to an actual example. ● Residue shown is invariant; ○ most common residue (above 66% occurrence); ◌ most common residue (between 66 and 33% occurrence); where no residue is shown, none have a greater occurrence than 33%; * deletion in sequence; inset: loop nomenclature.

Figure 1 shows that the disulphide bridges are localized to one end of the molecule and generate three major polypeptide loops. The predominant secondary structuring evident from the crystallographic map is β-sheet, but there are several β-turns located at the chain reversals. Despite the sequence variation amongst the short neurotoxins, prediction of β-structure by theoretical methods consistently produces a particular pattern which matches that seen in the crystal structure.[15,16] Since the accuracy of these predictions is enhanced by the strongly nucleated conformations of these toxins (as shown by denaturation/refolding studies[38-40]) it is valid to regard all of them as approximating closely to the configuration of erabutoxin b.

It should be noted in Figure 1 that most of the invariant residues are either localized in the immediate vicinity of the disulphide bridges (i.e., the main core of the molecule) or towards the extremities of the three major loops. In contrast, those positions whose side chain nature is the least conserved are clustered towards the 'top' of the molecule in the sense of Figure 1.

The short neurotoxin homologues are a group whose sequences show features only seen in the true short neurotoxins, and in particular, the sequence CGC (41 to 43). Their distinction lies in a lack of toxicity and this can be associated with the absence of certain key residues. For example, whereas all short neurotoxins possess a tryptophan at position 29, many of the homologues have a different residue (Table 1). In terms of predicted secondary structure, however, these homologues do not differ substantially from the active neurotoxins.[41]

B. Long Neurotoxins

The properties of this group are very similar to those of the short neurotoxins, but an important difference lies in the rates of association and dissociation with the cholinergic receptors — long neurotoxins generally associate and dissociate more slowly.[36,37]

Regarding amino acid sequence, there are major differences to short neurotoxins. The characteristic eight half-cystine residues are present, but there are an additional two (forming a fifth disulphide bridge) in the central loop of the molecule. Besides other residue insertions and deletions within the main chain, the remaining outstanding feature is a C-terminal extension, or "tail", that brings the total length to between 65 and 74 residues.

A distinct subgroup can be identified in the sequences (LN 25-29) in which glycine-54 is replaced by tyrosine, but there are not sufficient other differences to warrant a separate classification.

The folding pattern of a long neurotoxin (α-cobratoxin, LN 03) has also been determined by X-ray crystallography and the overall arrangement is very similar to the short neurotoxin, erabutoxin b.[42] Where the differences in sequence and chain length occur, they do not disrupt the clustering of the disulphide bridges or the generation of the three major loops. The predominant secondary structuring is again of the β-type with theoretical predictions in the other members of the group indicating that this structure is quite appropriate for all.[15,16] Indeed, such predictions and their comparison with those for short neurotoxins enabled a close approximation to be made of the long neurotoxin configuration in advance of the crystallography.[13,15]

Figure 2 shows the essential conformation of the long neurotoxins together with aggregated sequence information. The diagram is based on the short neurotoxin backbone in order to facilitate a direct comparison with Figure 1, with certain small sections distorted for purposes of clarity. This representation is not to be considered definitive, but it is nevertheless quite in keeping with the experimentally determined shape of the long neurotoxin (LN 03).[42]

In comparison with Figure 1, Figure 2 shows that there are proportionately fewer

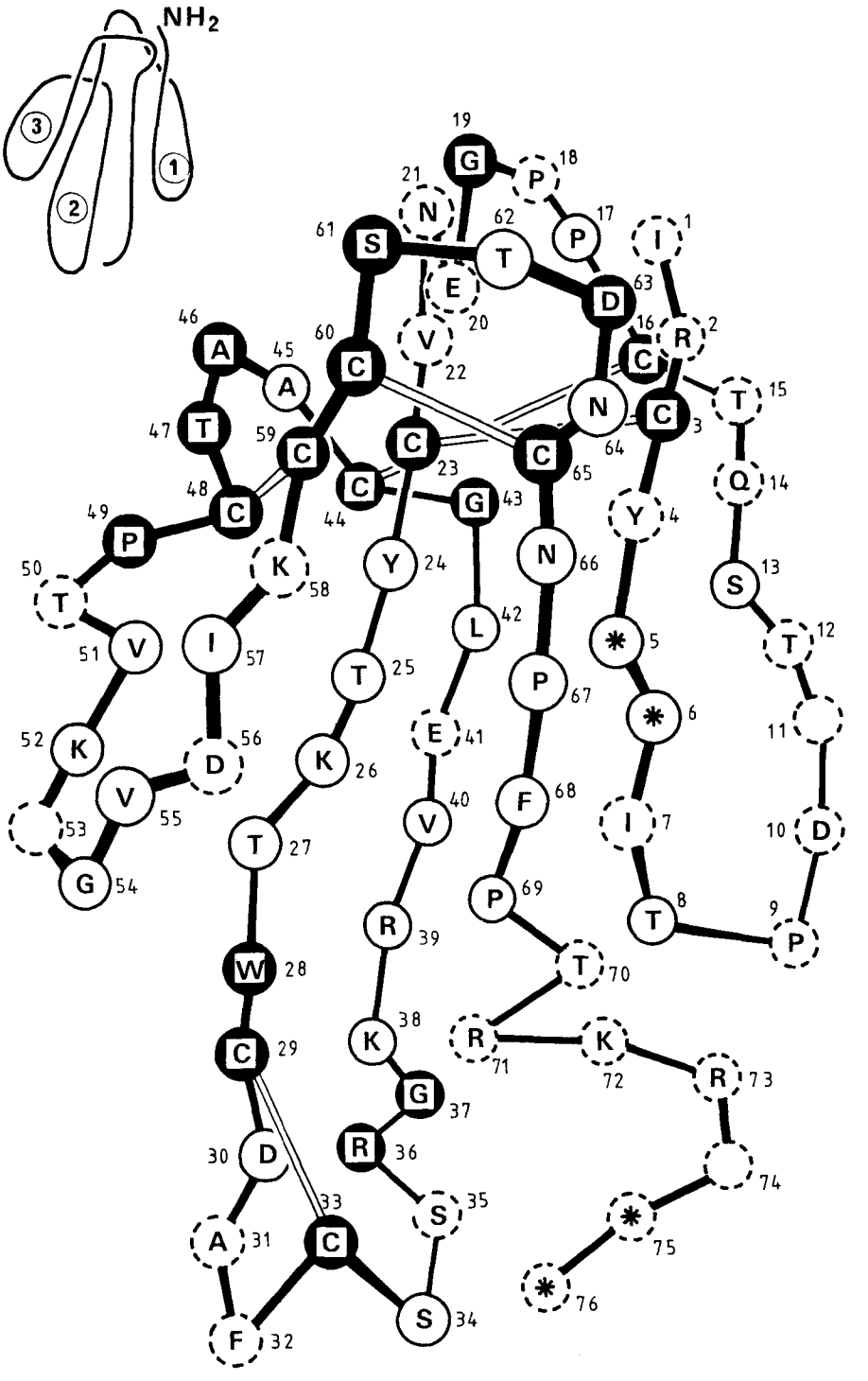

FIGURE 2. The backbone of a long neurotoxin showing the aggregate sequence of the group as a whole (Table 2). It is extrapolated from the crystal structure of the short neurotoxin, erabutoxin b. The sequence shown may not correspond to an actual example. ● residue shown is invariant; ○ most common residue (above 66% occurrence); ◌ most common residue (Between 66 and 33% occurrence), where no residue is shown, none have a, greater occurrence than 33%. * Deletion in sequence; inset: loop nomenclature.

highly conserved positions in the long neurotoxins. Despite this, there are still marked similarities in sequence in the vicinity of the disulphide core and loops 2 and 3. Most of the variable positions are located in the first loop and in the C-terminal "tail".

The long neurotoxin homologues lack the fifth disulphide bridge (29 to 33) of true long neurotoxins, but in other respects they are mostly similar, and they still possess neurotoxicity.[26]

C. Cytotoxins

In contrast to the neurotoxins, the pharmacological properties and sites of action of these toxins are obscure. Many effects, such as hemolysis, cytolysis, and muscle depolarisation have been attributed to them, but these have not yet been reconciled in terms of a common target.[10,35,43] They tend to be less toxic than the neurotoxins with LD_{50} levels falling in the 0.75 to 2.0 mg kg^{-1} range.

No detailed crystallographic data is available for cytotoxins, but sequence comparisons reveal that in overall length (60 residues) and cystine content, they are very similar to short neurotoxins. The major distinction is the higher content of hydrophobic and basic residues, but secondary structure prediction has again suggested that one can expect a molecular configuration similar to the short neurotoxin archetype.[15,16]

Figure 3 depicts the aggregated cytotoxin sequence information on a modified short neurotoxin backbone that is anticipated to be largely appropriate. Amongst the three toxin groups, the cytotoxins show the greatest conservation of residues. Some invariant residues lie, as in the neurotoxins, around the disulphide core and in loops 2 and 3. An interesting difference to the short neurotoxins concerns the distribution of the variable positions. Whereas loop 1 and the extremity of loop 2 are conserved in the short neurotoxins, they are variable in the cytotoxins. Similarly, the region of the core remote from the major loops is variable in the short neurotoxins, but conserved in the cytotoxins. Thus, the variable and conserved areas in these two groups are largely reversed.

The cytotoxin homologues are a separate group, which, although extremely similar to cytotoxins, have certain distinguishing features. These are an extra residue in the first loop and a lack of the sequence IDV or INV between positions 38 and 42. Toxicities are not invariably less in these homologues, but they do tend to be quite low, and as in the short neurotoxin homologues, this can be associated with the absence of some key residues, notably methionine-26 (Table 3). Secondary structure predictions show much similarity to the true cytotoxins, but the additional residue at position 4 is responsible in part for lowering the β-sheet potential of the first loop.[41]

D. Miscellaneous Types

Amongst the sequence data are 12 examples that have no obvious affinity to any of the previously described groups, but they do seem to be related to both short neurotoxins and cytotoxins on the basis of chain length, loop size, and the presence of certain residues (Table 4). There is one subgroup (MT 5 to 9) which is unique in having a fifth disulphide bridge spanning the extremity of the first major loop. These varied types have no pronounced toxicity according to the usual assays, but it is still possible to accommodate their sequences on the basic framework found appropriate for the other toxins.

E. Deductions from Sequence Information

Turning attention first to the similarities rather than the differences between the toxin groups, Figure 4 shows the residues that are invariant throughout, or commonly found. The highly conserved residues are clearly important from the structural point of view because they are mostly half-cystine, proline, and glycine. They lie towards the top of the

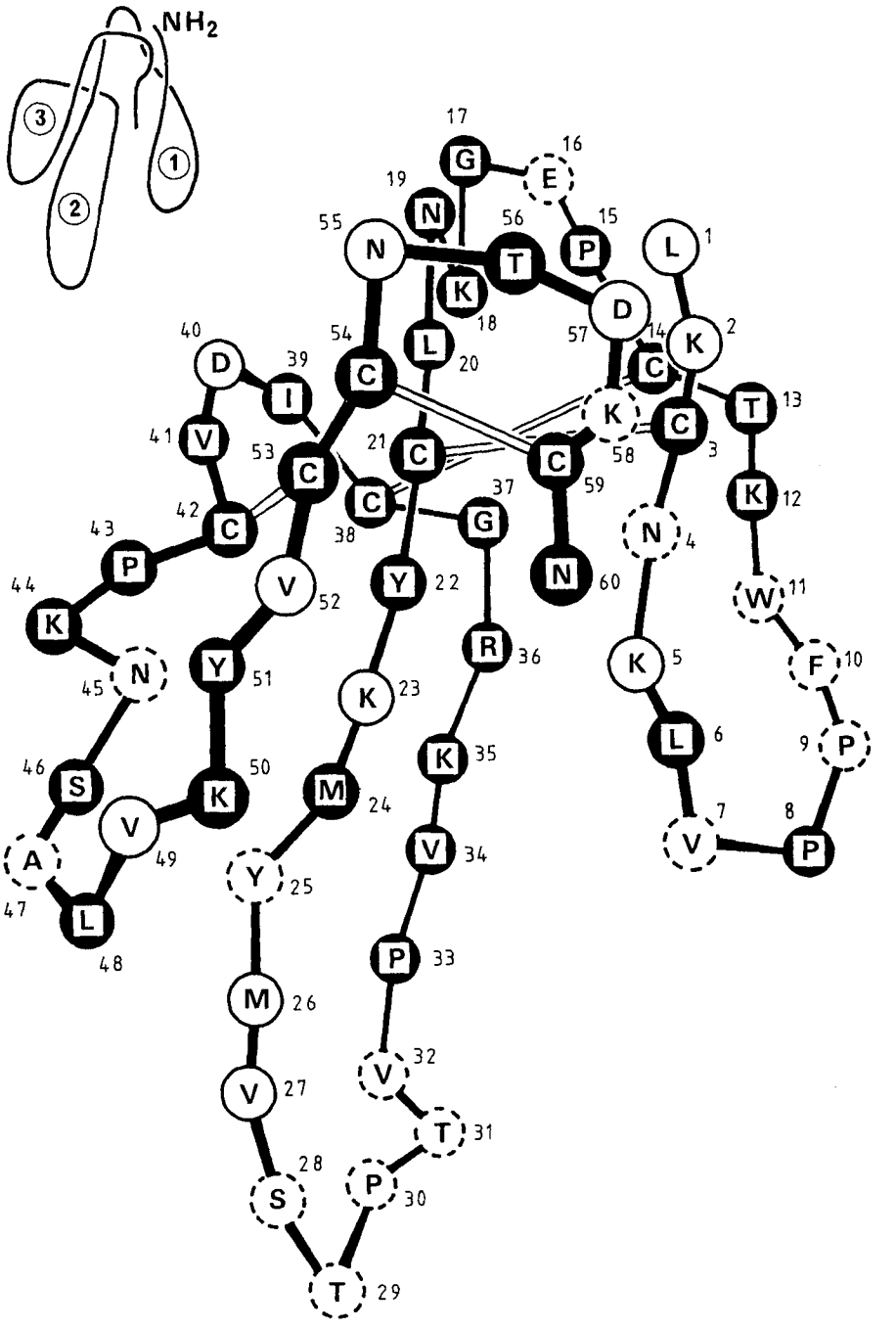

FIGURE 3. The backbone of a cytotoxin showing the aggregate sequence of the group as a whole (Table 3). It is extrapolated from the crystal structure of the short neurotoxin, erabutoxin b. The sequence shown may not correspond to an actual example. ● residue shown is invariant, ○ most common residue (above 66% occurrence), ◌ most common residue (Between 66 and 33% occurrence). Inset: loop nomenclature.

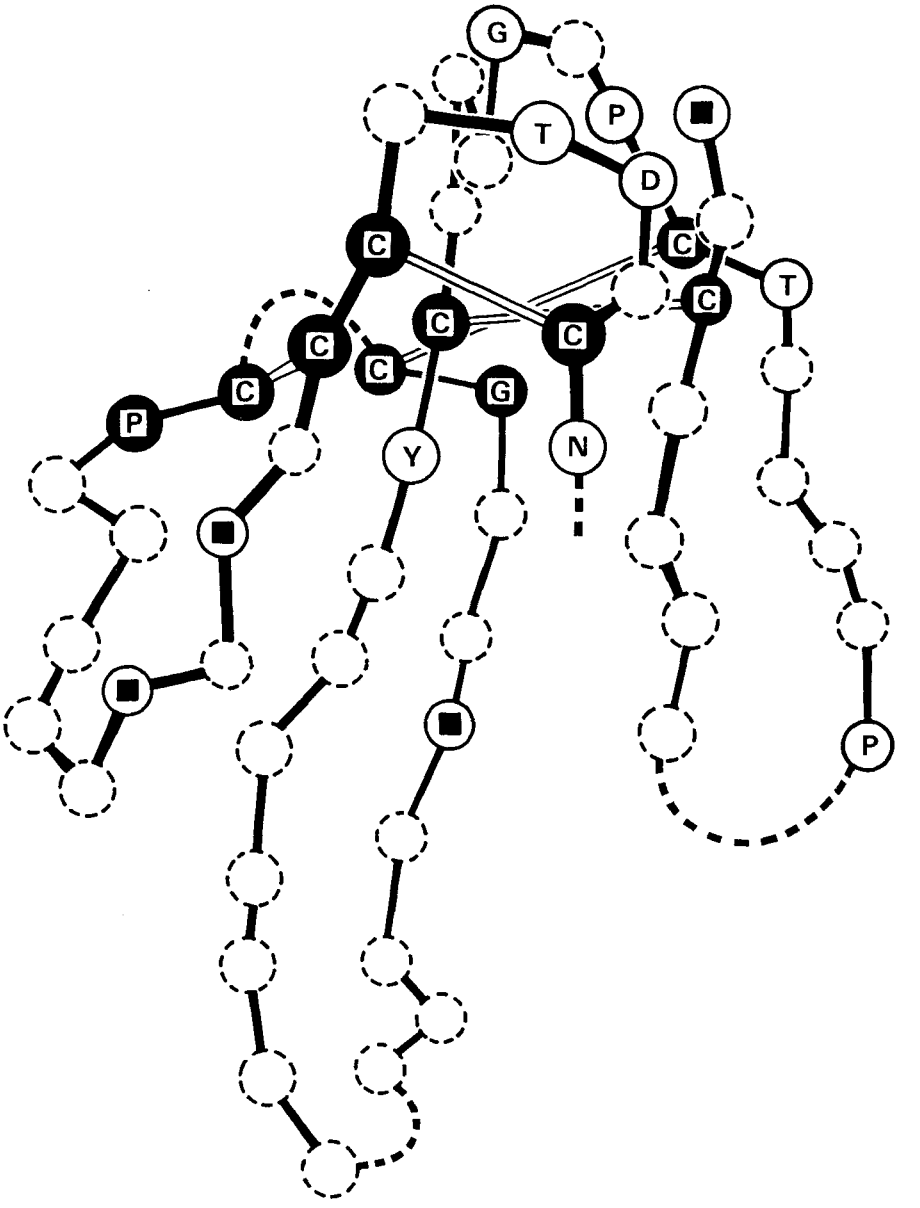

FIGURE 4. Residues common to short neurotoxins, long neurotoxins and cytotoxins. The generalized structure is based on the crystal structure of the short neurotoxin erabutoxin b. ● residue shown is invariant, ○ residue shown is predominant in each group, ◌ no one residue predominant in all groups, ◍ hydrophobic residue.

generalized toxin molecule as drawn and are responsible for determining and retaining the fundamental tertiary structure. The minimum requirement for a toxin of this kind, therefore, is a clustering of the four disulphide bridges at one end of the molecule with three polypeptide loops emerging from this core.

The determinants of neurotoxicity and cytotoxicity can be found by treating the aggregated sequences (Figures 1, 2, 3) rather like simultaneous equations. Thus Figures 5 and 6 display those residues that are uniquely associated with each mode of action. These residues need not be the sole basis of the toxic mechanism and might be better understood

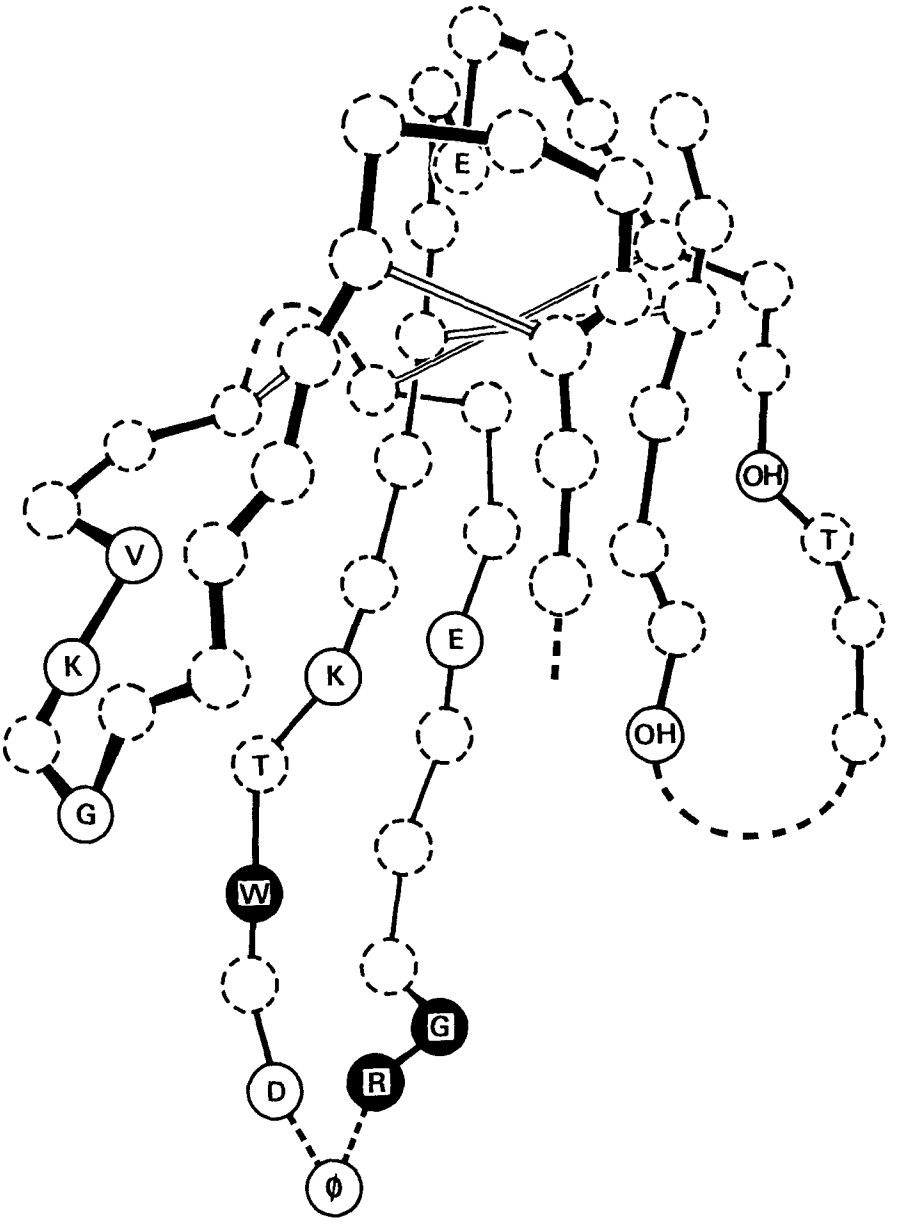

FIGURE 5. Residues found commonly in short and long neurotoxins, but rarely in cytotoxins. The generalized structure is based on the crystal structure of the short neurotoxin, erabutoxin b. ● common in neurotoxins only (invariant); ○ common in neurotoxins only (Above 66% occurrence in both types of neurotoxin); ◌ common in neurotoxins only (in at least one of the neurotoxin types, occurrence falls to between 33 and 66%) where no residue is shown, there are none unique and common; OH serine or threonine; Ø aromatic residue.

as "targeting" some common property of the toxins. According to the figures, specificity seems to be dictated by residues in the three major loops, and particularly by loops 2 and 3. Amongst neurotoxins, there are three invariant residues in the extremity of loop 2, but there are also seven other residues in the vicinity whose chemical character is conserved in over 66% of the sequences. Some of these residues have been subject to specific chemical

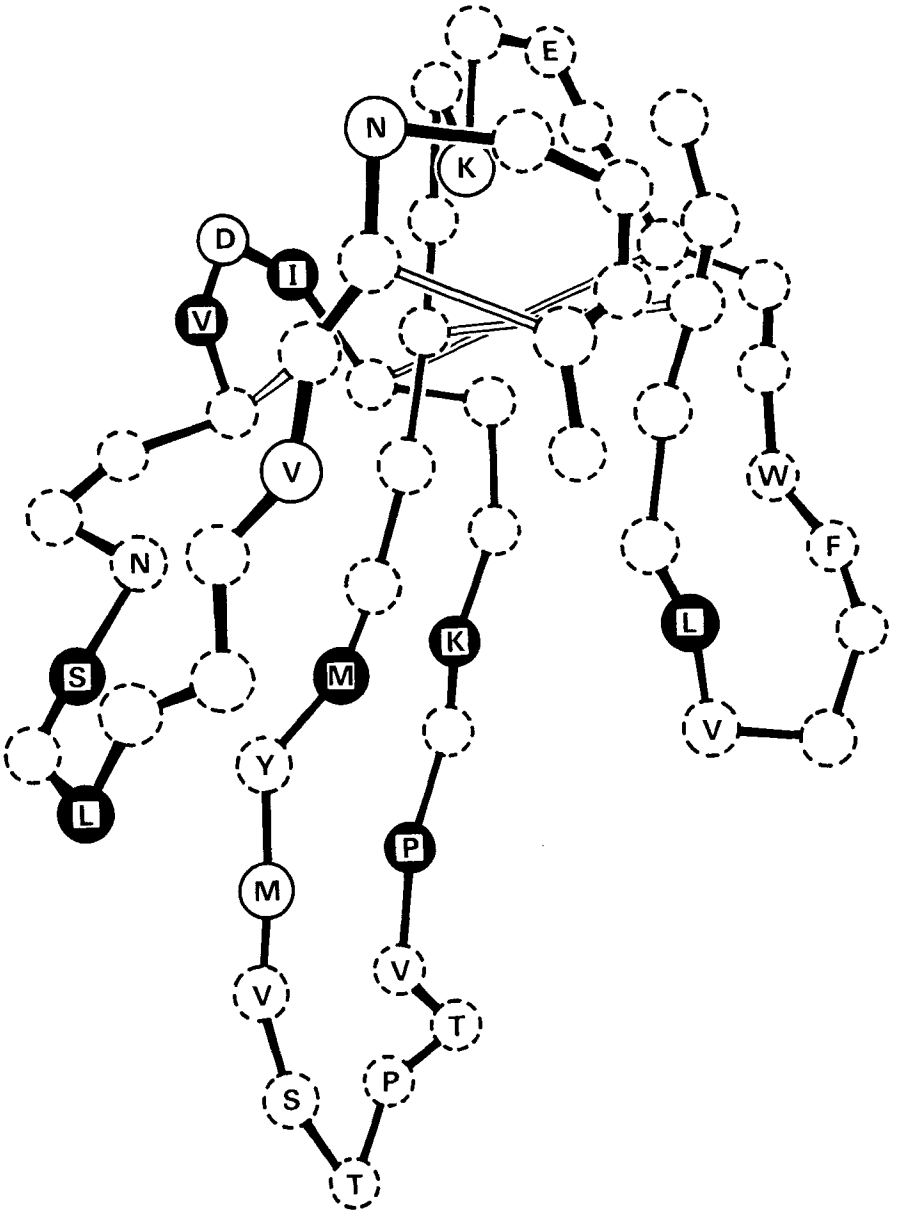

FIGURE 6. Residues found commonly in cytotoxins, but rarely in the neurotoxins. The structure is based on the crystal structure of the short neurotoxin, erabutoxin b. ● common in cytotoxins only (invariant); ○ common in cytotoxins only (Above 66% occurrence); ◌ common in cytotoxins only (Between 66 and 33% occurrence); where no residue is shown, there are none unique and common.

modification and often clear decreases in toxicity can be observed. Indeed, a considerable effort has been made in this respect which has been fully reviewed elsewhere.[10,13,15,44,45] Suffice it to say that most of the residues found to be functionally important are highlighted in Figure 5. No one residue appears absolutely vital for toxicity as would befit a recognition process dependent on many points of interaction. The permitted evolutionary variation of each site may provide some clue to the relative dispensability of each of these residues. In cytotoxins, the unique residues are similarly distributed over

the three major loops (Figure 6), but the evolutionary dispensability of each position is clearly different to the neurotoxins. The association of this area with specificity has been confirmed by the chemical modification of methionine which results in a reduction of toxicity.[131]

III. TOXIN CONFORMATION IN THE NATIVE STATE

A. Short Neurotoxins

1. Erabutoxin b

X-ray crystallography is presently unrivalled in its ability to discern the precise tertiary structure of proteins, but with the recently improved resolution of nuclear magnetic resonance (NMR) techniques, it is now possible to confirm whether conformations are similar in both the crystal and solution states. Elapidae neurotoxins are ideal for this type of comparison because their relatively low molecular weights generate many sharp resonances.

Two X-ray maps of erabutoxin b, a short neurotoxin (SN 18), have been reported and the overall tertiary structure is very similar in both.[46,47] It consists of a globular core, extensively cross-linked by cystine, with three large emergent loops. These loops are so arranged that the neighboring sections of chain run antiparallel to one another (Figure 7).

The globular region is defined by chain segments 1 to 4, 16 to 27, 38 to 44, and 52 to 62 and in addition to the disulphide bridges it is stabilized by an extensive network of hydrogen bonds with electrostatic and hydrophobic interactions. The C-terminal section (52 to 62) is held by two salt bridges, one between arginine-39 and the α-carboxyl of asparagine-62, and the other between glutamate-58 and the N-terminal α-amino (pK = 9.0).[48] Important hydrogen bonds also occur between isoleucine-2 (carbonyl oxygen) and valine-59 (NH), and between tyrosine-25 (O) and asparagine-61 (γ-amide NH).[13] These are shown in Figure 8. Tyrosine-25 is sited at the origin of loop 2 and participates in an extensive hydrophobic interaction* with the adjacent residues lysine-27, glutamate-38, proline-44, and leucine-52. Together with the nearby chain section 40 to 43 (GCGC), these residues effectively enclose the tyrosine (Figure 7). This is confirmed in solution by the tyrosine's broad NMR signal at room temperature which originates from hindered rotation about its C_α-C_β bond.[49] The extremely high phenolic pK_a value of tyrosine-25 (12 compared to 10 under normal conditions) is attributable to its hydroxyl group forming a hydrogen bond, and according to both the X-ray maps, glutamate-38 is the acceptor. Since the tyrosine is much restricted by the tertiary structure, this provides a ready explanation for the difficulties associated with its chemical derivatisation and the collapse of the global structure should this be achieved.[10]

The three loops which emerge from the globular area are hydrogen bonded in the crystal state (Figure 8), but the length and strength of these bonds varies according to the proximity of the core. Thus the shorter and hence stronger hydrogen bonds are to be found amongst half-cystine-24, tyrosine-25, lysine-27, glutamate-38, and glycine-40.[46]

In loop 2, several van der Waals** contacts occur between tryptophan-29 and isoleucine-36, an interaction also confirmed in solution.[13,48] The crystal conformation also shows a salt link between arginine-33 and aspartate-31, and many hydrophobic attractions between the two antiparallel chains of the 43 to 54 loop.[13,46,47] The orientation of loops one and two with respect to each other is apparently determined by a hydrogen bond between serine-8 (NH) and isoleucine-37 (carbonyl oxygen) (Figure 8) together with a hydrophobic interaction that includes the side chain methylenes of glutamine-6 and arginine-39 (Figure 7). Similarly, loop 3 is held alongside loop 2 via a number of van

* Carbon-carbon distances < 5.5 Å.

** Carbon-carbon van der Waal contact distance < 3.5 Å.

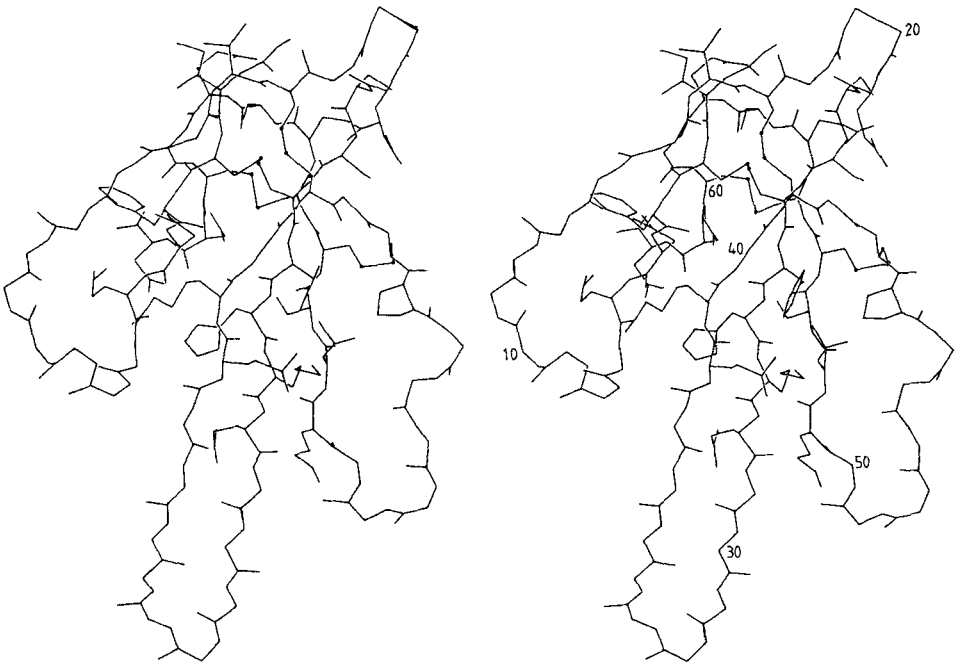

FIGURE 7. Stereo diagram of the crystal structure of erabutoxin b at 1.35 Å resolution.[46,91] Only those side chains involved in the globular core are shown. The side chains of residues 18 and 19 have been omitted because the sequence is reported to be prolylseryl and not serylprolyl, as recorded in the X-ray data.[175,177] The coordinates were obtained from the Protein Data Bank.[118]

der Waals contacts between tryptophan-29 and isoleucine-50 (confirmed by NMR)[48] and between glutamine-28 and lysine-51 (Figure 7).

Although the core region in the two independently determined X-ray maps is virtually identical, the peripheral regions show appreciable differences in both the main chain (Figure 9) and side chain orientations. In particular, the orientation of isoleucine-50 with respect to the central loop is different. According to the map by Tsernoglou and Petsko, this residue's side chain is positioned on the opposite face of the β-sheet to tryptophan-29 (Section V.B.2), but in the map by Low et al. it is on the same side (Figure 2 of Reference 47). Tsernoglou and Petsko have drawn attention to the extremity of loop 2 because difficulty was encountered in attempting to fit a particular conformation to the observed data.[46]

It is possible that this and similar areas may possess an appreciable degree of flexibility, thereby rendering them susceptible to unequal perturbation by different crystallizing media. A marked perturbance that can be associated with both the media used concerns histidine-7. Whereas both crystal structures depict the imidazole function exposed to the solvent,[46,47] this is incompatible with its high resistance to chemical modification,[50] low pK_a value (<3 compared to ~ 6.5) and very low exchange rate with D_2O when in solution.[49] Indeed, access to this moiety only becomes possible with the denaturation of the global conformation. The implication that histidine-7 is normally buried within the tertiary structure has support from Nuclear Overhauser Effect (NOE) experiments which demonstrate that it must lie within 10 Å of phenylalanine-4 (crystal distance 15 Å).[51] Inagaki et al. have shown that ethyl mercury phosphate, a heavy atom derivative used for one of the crystal structure determinations,[12] binds to the histidine-7 imidazole and thereby draws it out on to the molecular surface where it becomes proximate to

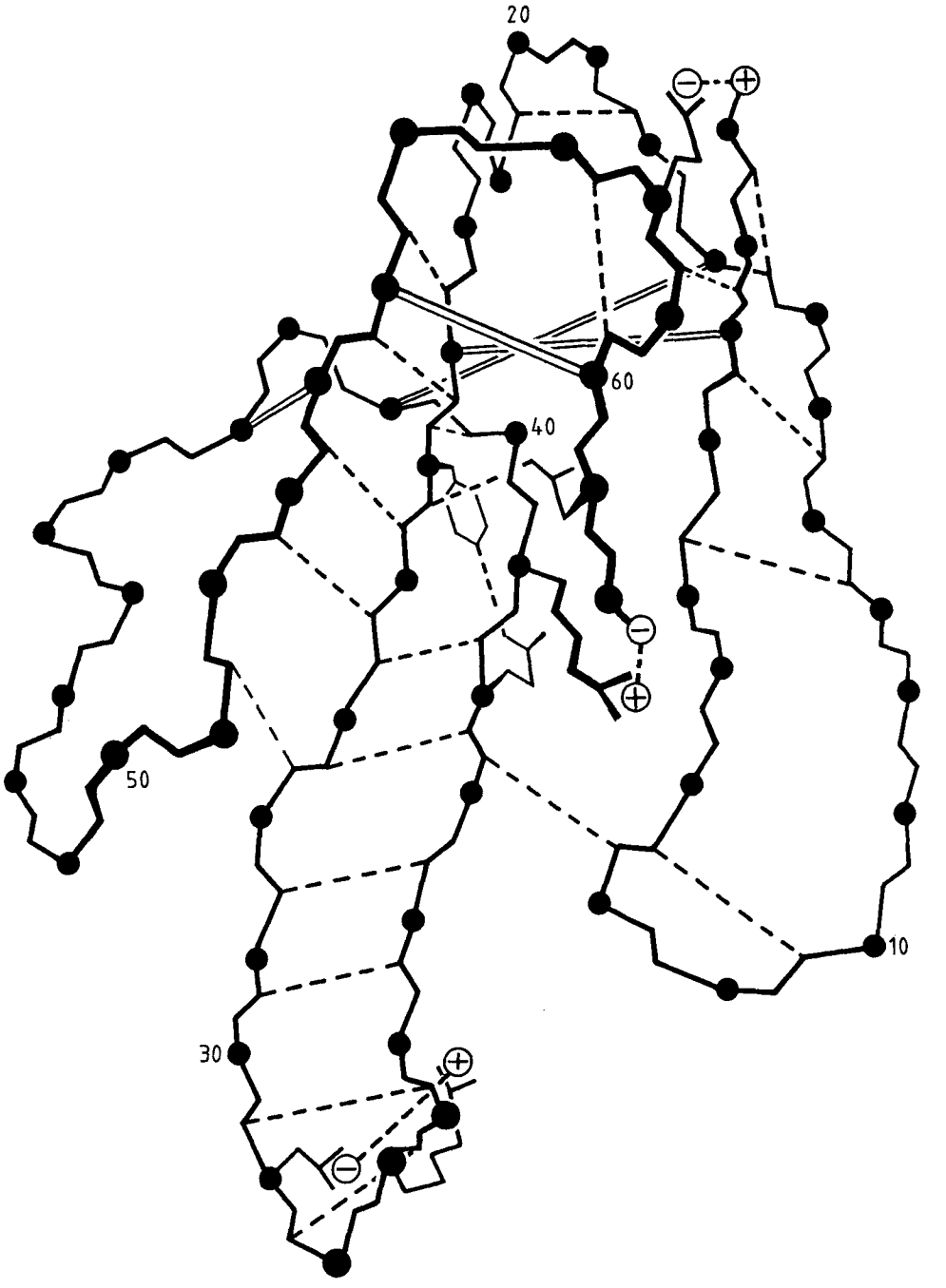

FIGURE 8. Schematic representation of the hydrogen bonding in erabutoxin b. ----- hydrogen bond (N/O distance ≃3.2Å), ⊕ ⊖ salt link. The hydrogen bonds were placed according to X-ray coordinates obtained by Tsernoglou and Petsko.[46,91]

isoleucine-36 and tryptophan-29 (Figure 10). This conformational change has also been seen spectroscopically by changes in the methyl region of the NMR spectrum.[51] Mercury compounds were also used in the other crystallographic analysis by Kimball et al. and it is possible that histidine-7 was again similarly perturbed.[47] Therefore, the exposed position

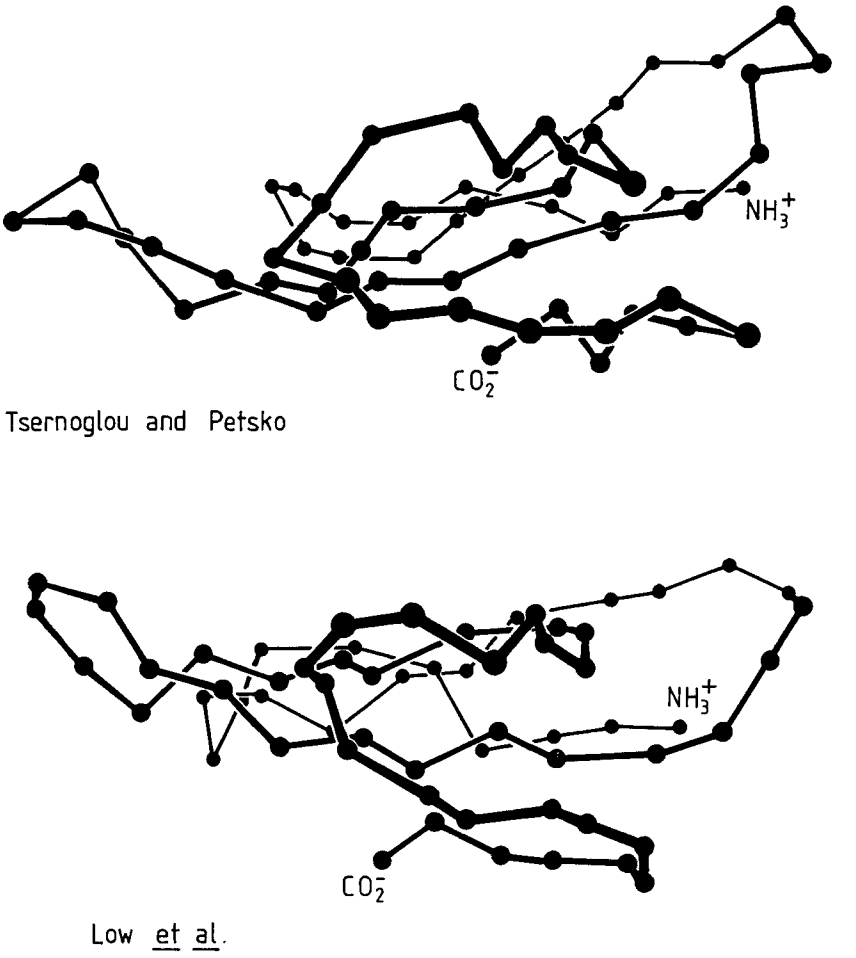

FIGURE 9. A comparison of the two independently determined crystal structures of erabutoxin b.[12,13]

of histidine-7 with isoleucine-36 and tryptophan-29 nearby as depicted in both X-ray maps of erabutoxin b does not represent the native solution conformation. It is difficult to envisage such a major reorientation of histidine-7 taking place without some other concerted changes in loop one, so ultimately the positioning of this loop with respect to the remainder of the molecule in physiological conditions may have to be reconsidered.

2. Other Short Neurotoxins

The homology between erabutoxin b and other members of the short neurotoxin group (Table 1) gives good reason to suppose that their tertiary structures must all be very similar. The use of secondary structure prediction to confirm this has already been mentioned but further means of comparison also exist in circular dichroism and laser Raman spectroscopy. Both these methods are capable of demonstrating the predominant secondary structural forms in these toxins, but they also have the advantage that measurements can be made in solution and under physiological conditions. NMR complements such information with more specific detail on intramolecular interactions.

The CD spectra of short neurotoxins show that a common feature of both the crystal and solution structures is a preponderance of β-structure. Indeed, the CD bands at

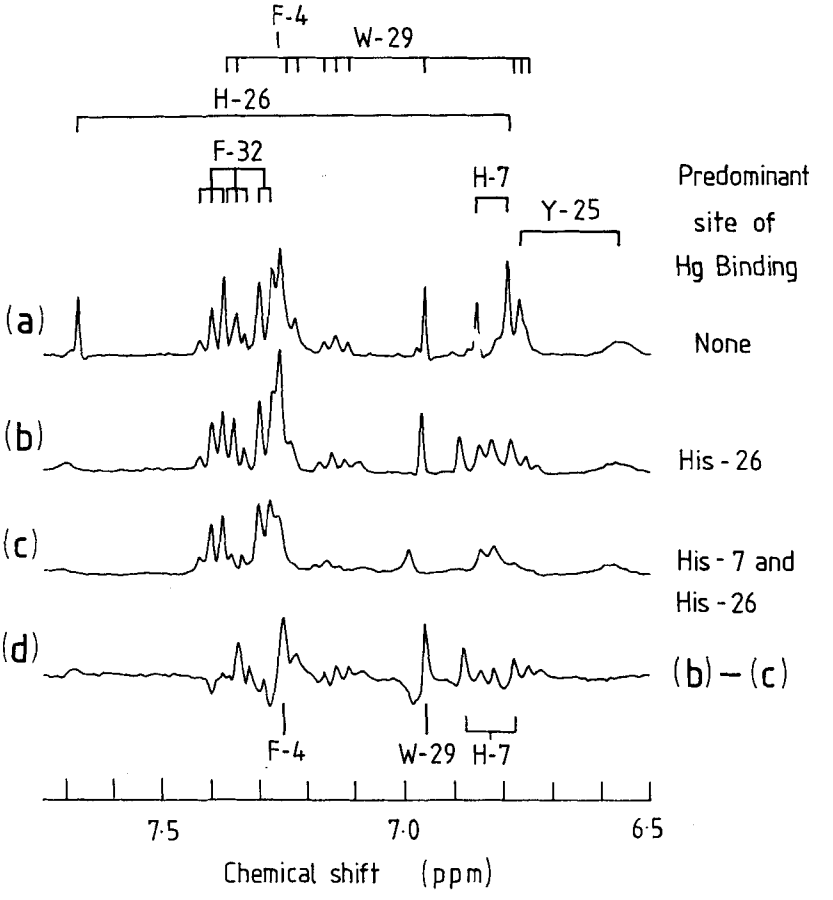

FIGURE 10. The effect of binding ethyl mercury phosphate to erabutoxin b. ^{1}H 300-MHz NMR spectrum in the aromatic region at 37°C and pH 7.0.[51] Molar ratio of ethyl mercury phosphate to erabutoxin: (a) 0, (b) 5.0, (c) 20.0.

195 nm and 215 nm resemble quite closely the "classic" β-sheet spectrum.[52] Figure 11 shows the average of the known spectra together with an indication of the possible variation. Whereas the peptide CD bands below 220 nm are consistent, large differences are possible in the magnitude of the 230 nm band. Laser Raman studies with short neurotoxins from *Enhydrina,*[59] *Pelamis,*[60] *Lapemis.*[61] *Laticauda,*[62] and *Naja*[63] species confirm that β-structure is dominant, but they also show some random coil and the occasional minor helical element.

The residues considered to be important for maintaining the conformation of erabutoxin b are highly conserved in the short neurotoxin group (Figure 1). An exception is the pair of residues at positions 28 and 51 (glutamine and lysine in erabutoxin b) which are threonine and lysine, respectively, in many of the sequences. In principle, however, it is possible to preserve the essential feature of the interaction by a hydrophobic attraction between the β, γ carbons of residue 28 and the γ, δ carbons of residue 51.

According to NMR studies, the conserved structural residues are performing similar roles to those in erabutoxin b. The rotation of tyrosine-25 about its β, γ bond is severely restricted in at least 9 short neurotoxins (SN2,[64] 3,[65] 9,[66] 10,[67] 11,[57], 14,[68] 17, 18, and 19[48]) and a hydrogen bond with the adjacent glutamate-38 has been inferred in 6 (SN 2,[64] 3,[65]

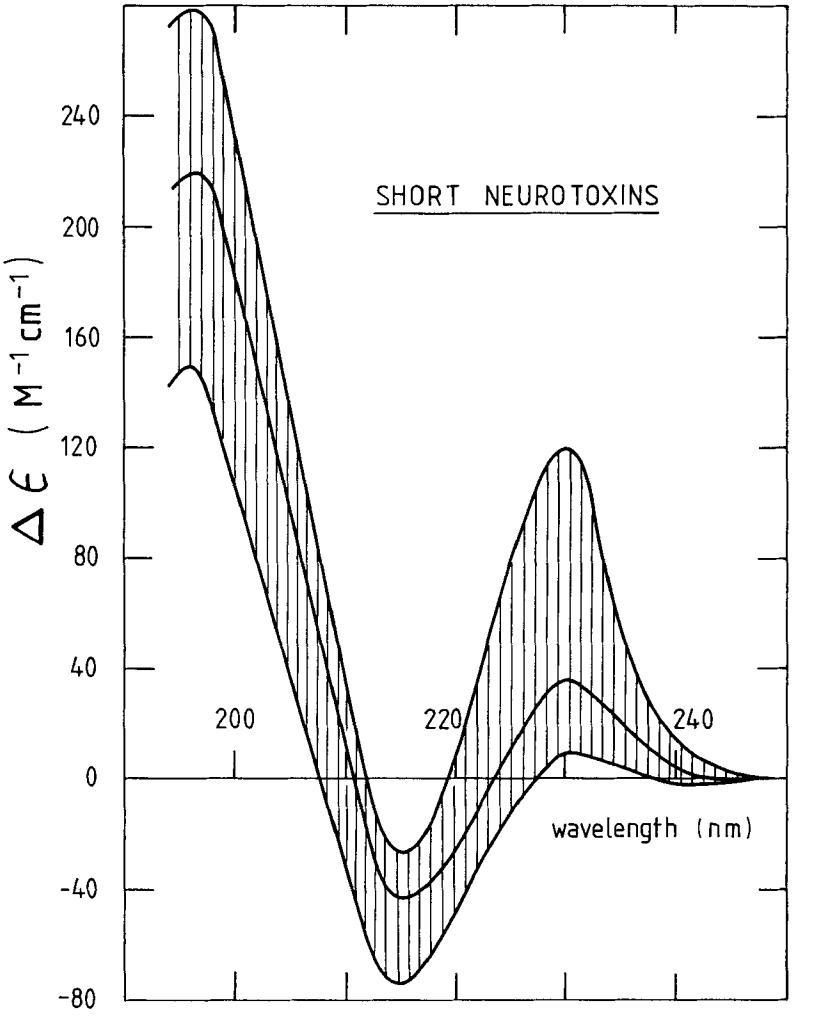

FIGURE 11. The average of the known short neurotoxin CD spectra together with the range in which individual spectra may fall. The toxins considered were SN 01,[58] SN 08,[38] SN 09,[55] SN 10,[54] SN 11,[57] SN 12,[58] SN 13,[56] SN 14,[53] SN 17,[16] SN 18,[55] SN 24,[55] SN 26[38] and SN 31.[38]

6,[69] 10,[70] 11,[57] 18[48]) by the extremely high pKa of the phenolic hydroxyl (≃12). In the case of cobrotoxin (SN 10) this hydrogen bond has proved to be stable over the pH range 1.3 to 11.5.[70] Laser Raman spectroscopy has also identified the bond in SN 11 and SN 24.[62] In addition, tyrosine-25 is shown by NMR to be involved in a hydrophobic interaction with leucine-52 in erabutoxin b, and a similar association has been noted with the isoleucine-52 of cobrotoxin (SN 10).[70] It is to be expected, therefore, that tyrosine-25 occupies the same restricted environment in all short neurotoxins and that the difficulty connected with its chemical derivatisation in those tested is typical.[10]

The proximity of lysine-27 and glutamate-38 seen in erabutoxin b (Figure 7) has been confirmed for SN 11,[71] and apart from SN 26, these residues are invariant. In this exceptional case, position 27 is methionine, but by virtue of its unbranched nature, it is still possible for it to enter into non-bonded interactions with glutamate-38. The salt-link between the N-terminal α-amino group and residue 58 reported for erabutoxin b is also detectable in SN 10, SN 11, and SN 3, where aspartate-58 replaces the glutamate.[48,65,70,71]

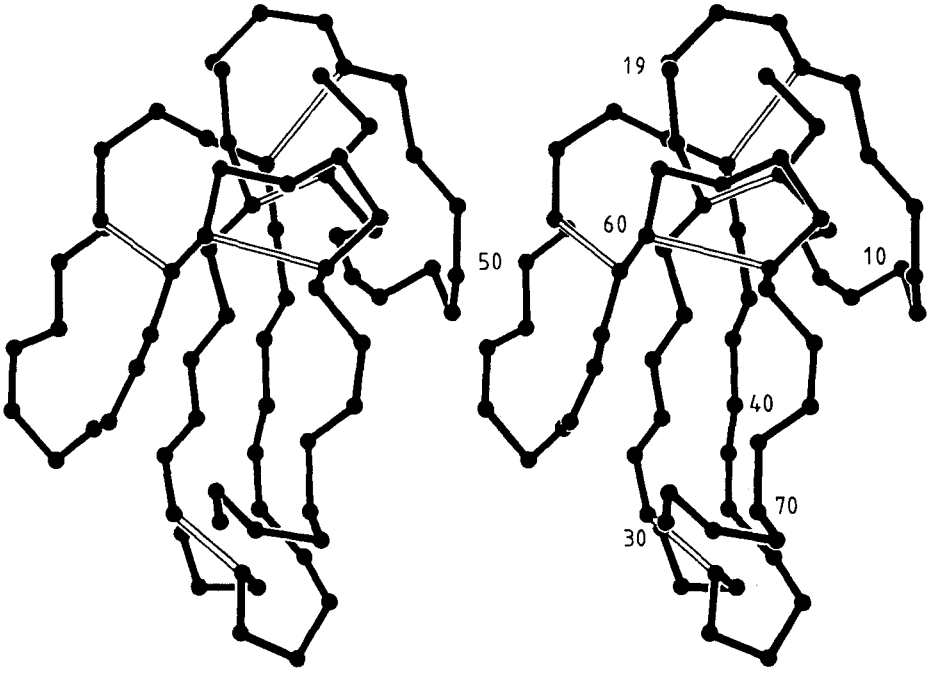

FIGURE 12. Stereo diagram of the crystal structure of α-cobratoxin (a long neurotoxin) at 2.8 Å resolution.[42]

Intricate pH titrations of native, acetylated, and spin labeled short neurotoxins have shown that the three major loops in SN 3,[65] 10[70], and 11[72,73] are compatible in arrangement with the crystal structure of erabutoxin b. However, the lack of good NMR probes in the first loop of many short neurotoxins prevents a detailed comparison of this area throughout the group. This is of special interest because of the discrepancies seen here between the solution and crystal structures of erabutoxin b. Nevertheless, since histidine or phenylalanine-4 is similarly positioned near residues 39 and 59 in erabutoxin b,[46,48] SN 2,[64] SN 3,[65] SN 9,[66] SN 10[70] and SN 11,[72] at least the section of the first loop nearest the core is structurally conserved. On the basis of the loop sequences and the secondary structure predictions, overall uniformity in each short neurotoxin is probable.

B. Long Neurotoxins

1. α-Cobratoxin and α-Bungarotoxin

The crystal structures of two long neurotoxins, α-cobratoxin (LN 3)[42] and α-bungarotoxin (LN 27),[74] have been determined and their folding patterns are very similar to erabutoxin b (SN 18) (Figures 12 and 13). They have a compact globular region containing the four disulphide bridges, with two polypeptide loops and the C-terminal "tail" emerging from this core. The protruding loops (2 and 3) in both form a triple stranded β-sheet as verified by ^{1}H NMR, but its extent is less in α-bungarotoxin. The "tail" is directed alongside one of the strands of loop 2.

The major contrast to the organization of erabutoxin b (SN 18) is the first of the three major polypeptide loops which in both long neurotoxins is more properly considered as part of the globular core than an emergent structure.

In α-cobratoxin, the regular secondary structure most in evidence is the β-type (Figure 14), but there is proportionately less in comparison to erabutoxin b. Although the triple stranded β-sheet section between loops 2 and 3 is similar in both types of neurotoxin,

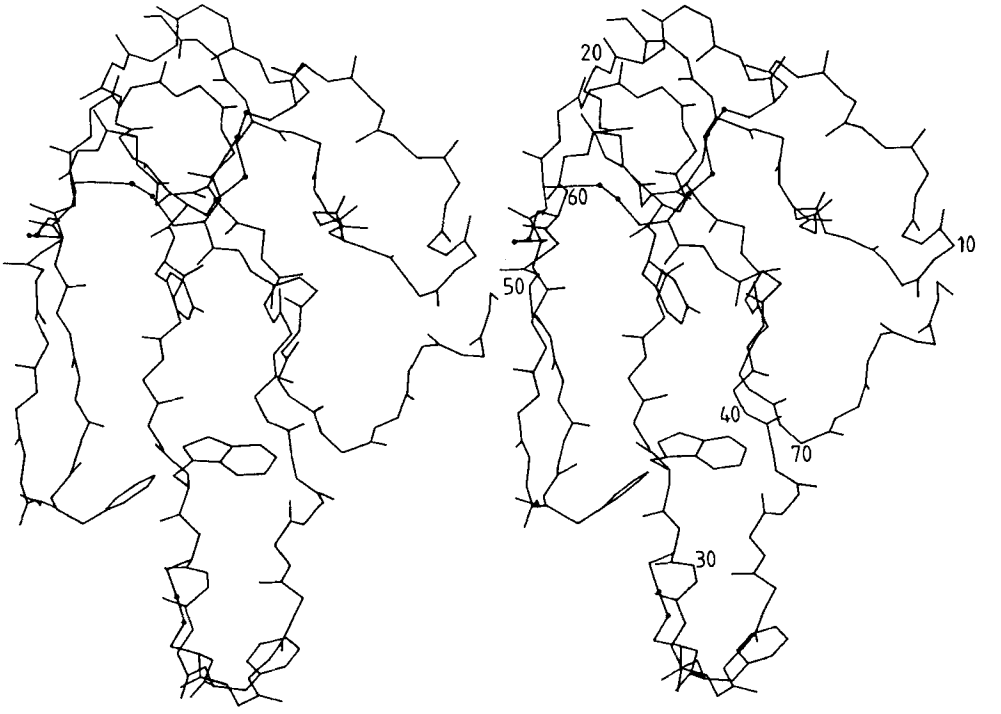

FIGURE 13. Stereo diagram of the crystal structure of α-Bungarotoxin (a long neurotoxin) at 3.5 Å resolution.[74] The position of the tryptophanyl residue should be noted.

loop I in α-cobratoxin and α-bungarotoxin differs by not having any defined β-structure (Figures 12 and 13). This difference in β-content is also evident from the respective CD spectra, for the two long neurotoxins do not have "typical" β-sheet spectra as do the short neurotoxins.[75,76] Instead, they have a type of spectrum that suggests some β-sheet together with an appreciable content of "random coil".[77] The CD spectra also confirm that relatively less β-sheet is to be found in α-bungarotoxin as compared to α-cobratoxin.

As shown in Table 2, α-cobratoxin and α-bungarotoxin are considered to represent two distinct subclasses of the long neurotoxins and so they warrant separate descriptions.[15] In addition, there are certain provisos concerning the individual crystallographic analyses.

a. α-Cobratoxin

According to its crystal structure, α-cobratoxin has two major clusters of closely packed hydrophobic side chains, one of which contains phenylalanine-4, threonine-12, valine-40, leucine-42, phenylalanine-68, and proline-69, while the other surrounds tyrosine-24.[78] The composition of this globular core and the associated triple-stranded β-sheet has been confirmed by NMR,[76] but this technique has also drawn attention to a perturbance of the native conformation produced by the crystallizing medium. This was an acid solution which has subsequently been shown to produce a pH-dependent conformational change in the toxin with a pKa of 5.5.[76,79] As judged by CD and NMR, this change does not markedly influence the secondary structure content (Figure 15), but it does have a profound effect on the microenvironment of histidine-21. When this residue becomes protonated, it reorients itself with respect to tyrosine-24, phenylalanine-4, alanine-45, leucine-42, and the half-cystines-23 and 44[76] (Figure 16). Since many of

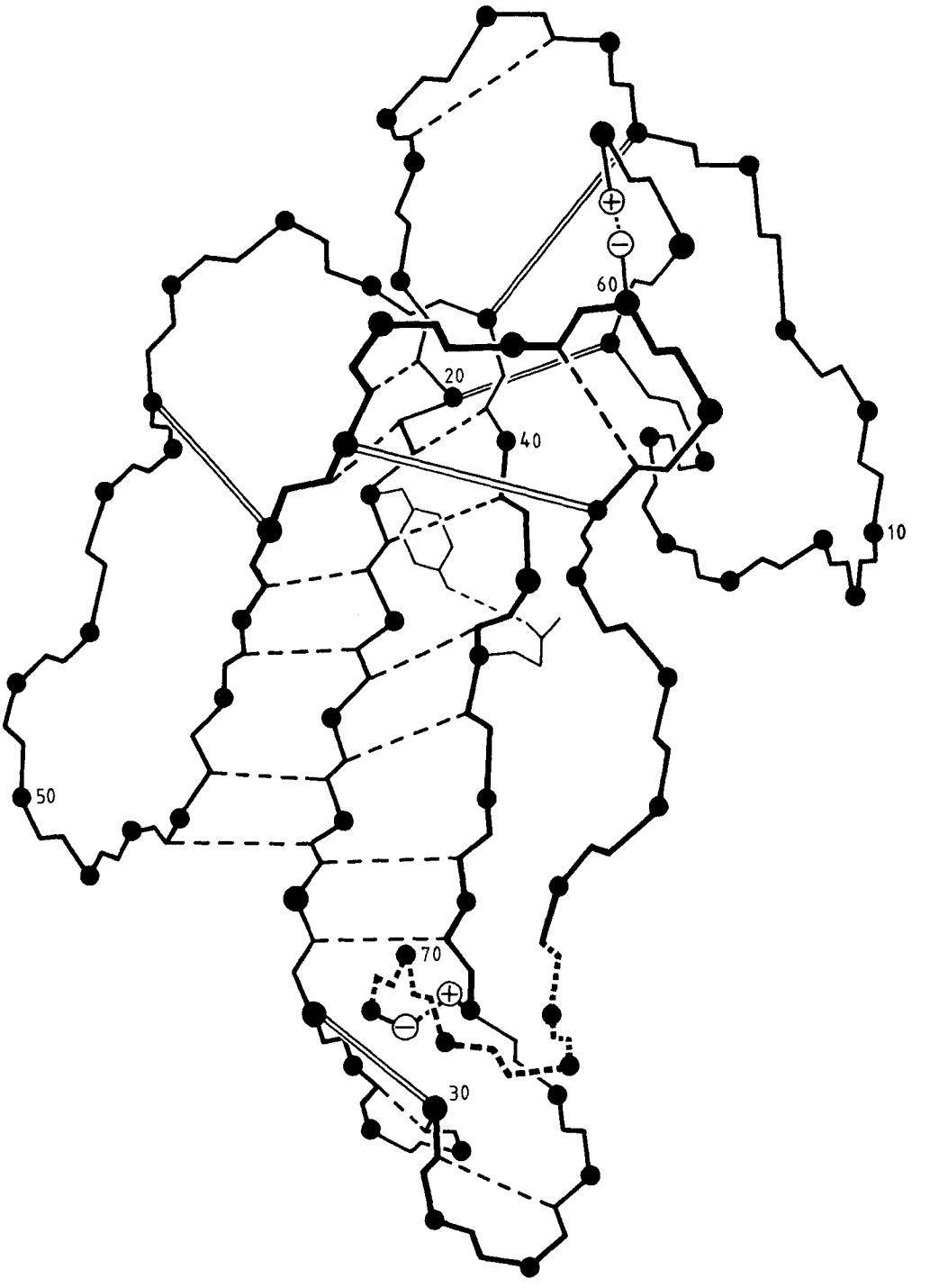

FIGURE 14. Schematic representation of the hydrogen bonding in the long neurotoxin α-cobratoxin.[42,78] Key: ----- hydrogen bond (N/O distance ≃3.2 Å), ⊕⊖ salt link. Doubt surrounds the precise location of the four C-terminal residues (see text).

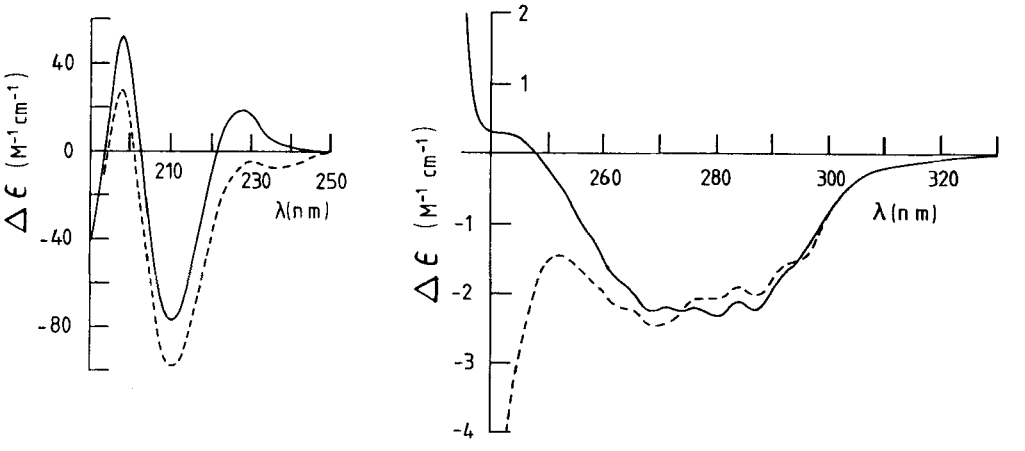

FIGURE 15. The conformational response of the long neurotoxin α-cobratoxin to the crystallizing media used by Walkinshaw et al.[42] Key: ——— CD spectrum in water, pH 8.0, ----- CD spectrum in 75% 2-methyl-2,4-pentanediol and 25% glycine-HCl (0.05M) at pH 2.85.

these residues form a hydrophobic cluster in the globular region, it is possible that in the native configuration, this histidine is also part of this cluster. Notably, for LN 07, a hydrogen deuterium exchange study does imply that a homologous histidine residue is not freely accessible.[80] Tsetlin et al. have also shown by NMR titration of α-cobratoxin that monotrifluoroacetylated lysines-14 and 26 can sense the protonation state of histidine-21.[79] These workers have interpreted these findings as indicating proximity of the three residues, but given their spatial separation in the crystal structure, it is more likely that the conformational change causes their unified responses.

The locality of histidine-21 in α-cobratoxin is subject to further perturbation in its crystalline form because it is the site of an intermolecular contact and HgI_2 binding.[42] Therefore, the crystal structure is expected to be at variance with the native solution structure by depicting an exposed histidine-21 with a distorted neighborhood.

The remaining area of doubt as regards the solution structure concerns the precise location of the C-terminus. The orientation shown in Figure 14 is uncertain because of a lack of continuity in the electron density map. However, only the last four residues are so affected.

In comparison with erabutoxin b, the major similarity in α-cobratoxin is the conformation of loops 2 and 3 and the associated amino acid sequence. The residues therein which have already been associated with neurotoxicity (Figure 5) possess similar orientations and juxtapositions as would befit their importance.

b. α-Bungarotoxin

Only four β-sheet hydrogen bonds are implied in the crystal structure of α-bungarotoxin, of which three occur between loops 2 and 3 (Figure 13).[74] However, Endo et al. have detected at least 16 slowly exchanging amide protons in aqueous solution,[81] a number which suggests that more than four such bonds must normally be present in the sheet superstructure after allowance has been made for β-turns and side chain amide interactions. Indeed, NOE experiments have identified six β-sheet hydrogen bonds in the

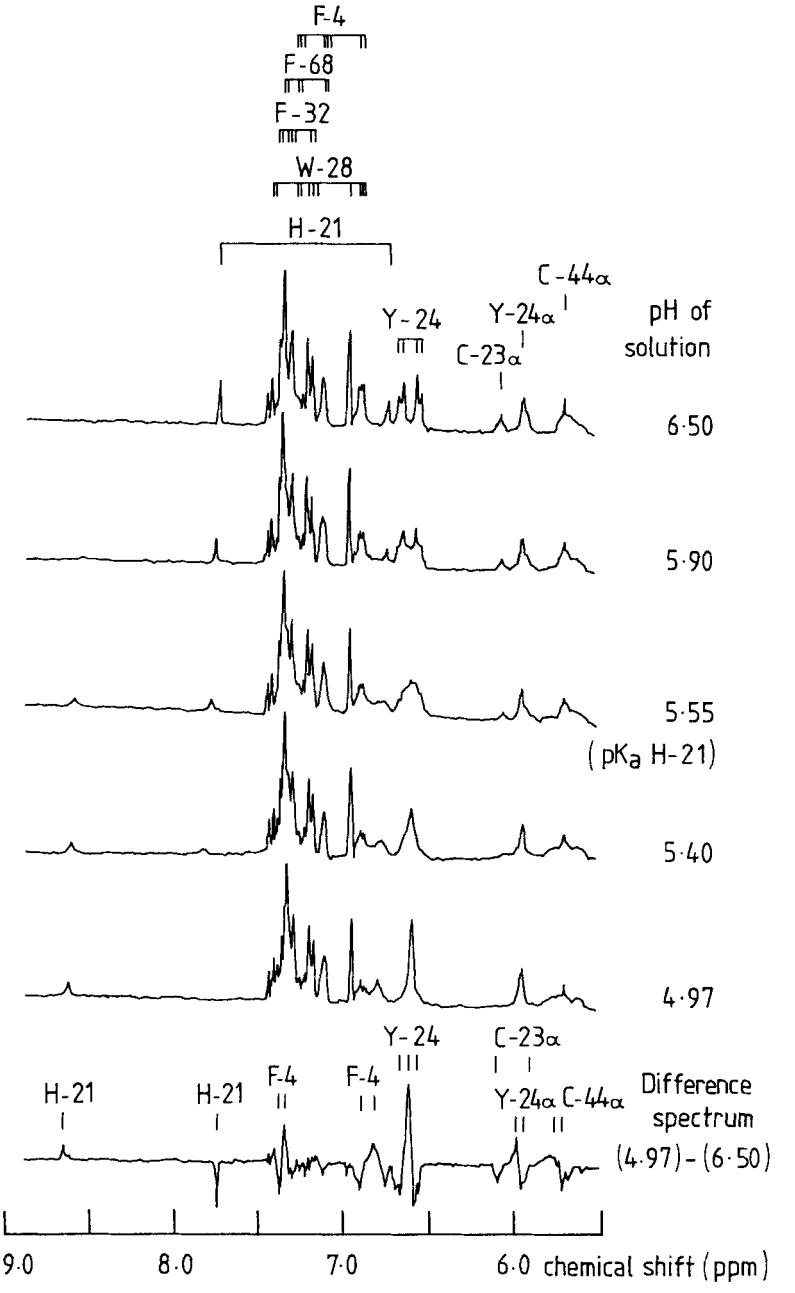

FIGURE 16. The pH dependence of the α-cobratoxin ^{1}H 300 MHz NMR spectrum at 27°C.[76]

vicinity of, and including, tyrosine-24.[81] Thus, under physiological conditions, the resemblance between the triple stranded β-sheet structures of erabutoxin b, α-cobratoxin and α-bungarotoxin is greater than is apparent from the X-ray map of the latter.

Similarly, in solution, tyrosine-24 has a pKa of about 12.0, which suggests that it could be hydrogen bonded to glutamate-41 as observed in other short and long neurotoxins.[81] No such bond is implied in the crystal structure, however. (Figure 13).[74]

A particularly unusual feature of the α-bungarotoxin crystal structure (Figure 13) is

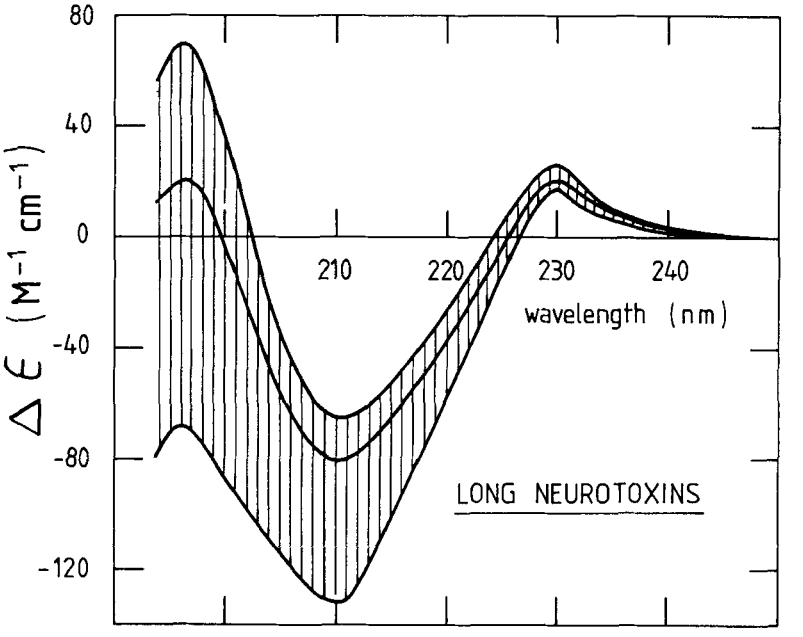

FIGURE 17. The average of the known long neurotoxin CD spectra together with the range in which individual spectra may fall. The toxins considered were LN 03,[76] LN 19,[77] LN 25,[77] LN 26[82] and LN 27[75].

the orientation of the "essential" tryptophan-28, for it projects from the opposite side of the β-sheet when compared to the homologous residues in α-cobratoxin and erabutoxin b. The usual position of this tryptophan is occupied instead by tyrosine-54,[46,47,78] a residue that along with the lack of valine at position 55, classifies the bungarotoxin-like subgroup LN 25-29. With the one exception of LN 22, which has the sequence GT at 54/55, all the remaining long neurotoxins have GV. In the light of this sequence difference, the crystal structure of α-bungarotoxin could give a representive view of the environment of tryptophan-28 in solution, and by implication, LN 25, 26, 28, and 29 could be similar.

2. Other Long Neurotoxins

The CD (Figure 17), laser Raman, and secondary structure prediction data for long neurotoxins shows that the group must be largely homogeneous in terms of tertiary structure.[15,16,62,83,84] The contrast to short neurotoxins of a proportionately lower content of β-structure is a group characteristic, though the variability of the CD spectra suggests a range of possible balances between β-structure and "random coil" (Figure 17). ^{1}H NMR studies of LS III (LN 22) have confirmed the existence of the triple stranded β-sheet in solution.[85]

Many of the residues identified as forming the hydrophobic core in the crystal structures (Residues 4, 12, 40, 42, 68, and 69) are conserved in the long neurotoxins, so it must be considered a common feature. In the case of LS III (LN 22) NMR has confirmed its presence.[86] Tyrosine-24, a highly conserved residue which lies close to lysine-26 and acidic residue-41, is homologous to the tyrosine-25 of short neurotoxins, but it differs in having a less restricted microenvironment.[86] In toxins LN 03,[76] 07,[81] 22,[86] and 27[81] the room temperature NMR signals from this aromatic residue show a rapid rotation about its $\beta\gamma$ bond. The high pKa value of the phenolic function in LS III (LN 22), as in

α-bungarotoxin (LN 27), suggests participation in a hydrogen bond, possibly with residue 41, but this bond is shown to be weaker or absent in long neurotoxin 03, and in 07 by laser Raman spectroscopy.[84] In both the latter toxins, aspartate-41 replaces glutamate-41, so presumably the shorter side chain of the aspartate precludes an optimum interaction. The greater freedom of tyrosine-24 in the long neurotoxins is probably associated with the CAATC sequence (44 to 48) which replaces the more confining sequence CGC (41 to 43) of the short neurotoxins. In the case of LS III (LN 22), NOE experiments show that alanine-45 is indeed close to the tyrosine.[86] Toxins b and c of *Astrotia stokesii* (LN 23 and LN 24) have phenylalanine-24 in place of the tyrosine, and significantly, residue 41 is isoleucine and valine, respectively. It seems that in this instance, phenylalanine-24 lies in a hydrophobic pocket surrounded by alanine-45 and residue 41.[87]

It can be concluded, therefore, that aromatic residue 24 of the long neurotoxins is accommodated in a hydrophobic pocket that is enlarged and less restrictive than that for the tyrosine-25 of the short neurotoxins. This explains why chemical modification of this residue causes less loss of activity in the long neurotoxins,[10] for there is less potential for conformational disturbance.

The "tail" of the long neurotoxins lies alongside the protruding polypeptide loops and so may contribute to the general stability of the area. In comparison with short neurotoxins, there are some highly conserved residues in loop 2 of the long neurotoxins which can be uniquely associated with the presence of this tail.[15] Arginine-39 in the short neurotoxins forms a salt bridge with the C-terminal α-carboxylate function, but it is replaced by leucine-42 in the long neurotoxins in order to extend the hydrophobic core and anchor the "tail". An alternative salt bridge with the C-terminus of the tail could be provided by lysine-38 which lies further toward the extremity of loop 2 (Figure 14).[15,87] Significantly, toxins LN 23 and LN 24 possess glutamate-38 instead of lysine-38, and in both cases, the C-terminus is amidated.[87] This anchorage for the C-terminus is not confirmed in the X-ray structures, but there are complications that affect this area. Apart from the difficulty in interpreting the electron density map of α-cobratoxin already mentioned, there are intermolecular contacts in both the α-cobratoxin and α-bungarotoxin crystals that could influence the orientation of the tail.[42,74] In addition, luminescence spectra of *Naja naja oxiana* α (LN 26) do suggest the close proximity of tryptophan-28 and/or tryptophan-32 with the C-terminal residues.[88]

C. Cytotoxins

Since no crystallographic studies have been completed, the information on cytotoxin conformation comes from secondary structure prediction,[15,16] circular dichroism (Figure 18) and laser Raman spectroscopy.[90] All these methods suggest that cytotoxins are similar to the neurotoxins, both in terms of chain folding and content of β-structure. However, the CD spectra show that the balance of β-sheet and "random coil" is variable. In some cases, the peptide CD bands resemble those of long neurotoxins, but in the majority, they approximate closely to the β-sheet bands of short neurotoxins (Figure 18). This greater resemblance to the short neurotoxins is appropriate considering overall chain length and the number of disulphide bridges, but two of the cytotoxin features, namely the small hydrophobic first loop and the presence of three residues between half-cystines-38 and 42, are only mirrored in the long neurotoxins. When using the crystal structure of erabutoxin b (SN 18) as a basis for cytotoxin conformation, therefore, it is prudent to regard loop 1 as forming part of the globular core of the molecule and not an emergent structure. Further, the presence of residues 39 to 41 should ensure that tyrosine-22 has a greater freedom than tyrosine-25 of the short neurotoxins. An indication that cytotoxins do have conformational features in common with the long neurotoxins is

given by comparing asymmetric units in their crystals. Whereas erabutoxin b (SN 18) only has one molecule per unit cell,[11,91] the two cytotoxins (CT 18 and CT 11)[92,93] and the two long neurotoxins (LN 3 and LN 27) have two.[42,94]

A detailed NMR comparison has been made between a short neurotoxin (SN 2) and a cytotoxin (CT 18), both of which were isolated from *Naja mossambica mossambica*. This confirmed that the backbone conformations were essentially similar,[64] with β-sheet as the dominant secondary structuring. The tyrosine-22 in the cytotoxin generated a broad ^{1}H-NMR signal below 20° C which indicated restricted rotation about its $\beta - \gamma$ bond, but this restriction was not so severe as that seen for the homologous residue in the short neurotoxin. The moieties that surround this tyrosine in the cytotoxin are the unbranched side chains of methionine-24 and lysine-35, together with tyrosine-51 and proline-43. These are constant features in the cytotoxin sequences and probably provide a similar microenvironment to that of tyrosine-24 in the long neurotoxins. The phenolic pKa of tyrosine-22 is above 12,[64] which suggests either inaccessibility to the solvent and/or participation in a hydrogen bond. If the latter is the case, then it cannot be equivalent to the tyrosine-25 hydrogen bond in short neurotoxins because there is no suitable acceptor at position 35 (Figure 3). Cytotoxins are highly basic molecules and contain less acidic residues than neurotoxins. In *Naja mossambica mossambica* cytotoxin (CT 18), there are only three such groups, of which two are likely to be involved in salt-links (i.e., N-terminal amino and aspartate-57, and C-terminal carboxyl and arginine-36) while the third (glutamate-16) is too remote to hydrogen bond with the tyrosine-22. An alternative acceptor that needs no reorientation of the aromatic ring is methionine-24, a residue that is absent when phenylalanine-23 replaces the tyrosine in some of the cytotoxin homologues (Table 3).

The above cytotoxin (CT 18) has another 2 tyrosyl residues at positions 11 and 51. One of these again has a phenolic pKa above 12, and in conjunction with chemical modification data for another cytotoxin (CT 11), it can be identified as the constant tyrosine-51.[95] If it is hydrogen bonded to another residue, the proximate asparagine-40 (normally conserved as aspartate, Figure 3) could be invoked as a suitable acceptor. Both tyrosines-22 and 51 are in close proximity to the same isoleucine residue (probably isoleucine-39[64]) and together they form the nucleus of an extensive hydrophobic core. The existence of this core has recently been confirmed for CT16.[178] Whatever the actual microenvironment of tyrosine-51, it is clearly important for stabilizing the overall conformation. This is shown by the nitration of tyrosine-51 in CT 11 which brings about a major structural disruption.[95] Presumably, the triple stranded β-sheet structure which lies at the heart of short and long neurotoxin conformations is also a basic feature of the cytotoxins, so modification of any residue therein (i.e., tyrosine-51) could be expected to have this drastic effect. From these preliminary NMR studies and the comparisons with the homologous neurotoxins, it appears that cytotoxins, like long neurotoxins, possess two hydrophobic clusters, one centered on tyrosine-22 and the other on the aromatic residues in the first loop.

D. The Dynamics of Toxin Structure in Solution

There is mounting experimental evidence to show that there can be considerable internal motions within protein structures.[96–98] Relatively large amplitudes of motion in the 1 to 100 psec range have also been predicted by theoretical analyses of protein dynamics.[99,100] In principle, NMR relaxation time measurements will reveal details of these dynamics, but in practice, complications can be caused by cross-relaxation effects. Nevertheless, Inagaki et al. have recently obtained interesting data on erabutoxin b (SN 18) through the use of ^{1}H and ^{13}C NMR.[101–103] Their work shows a wide range of mobilities for different parts of the toxin molecule. The aromatic ring of tyrosine-25, for

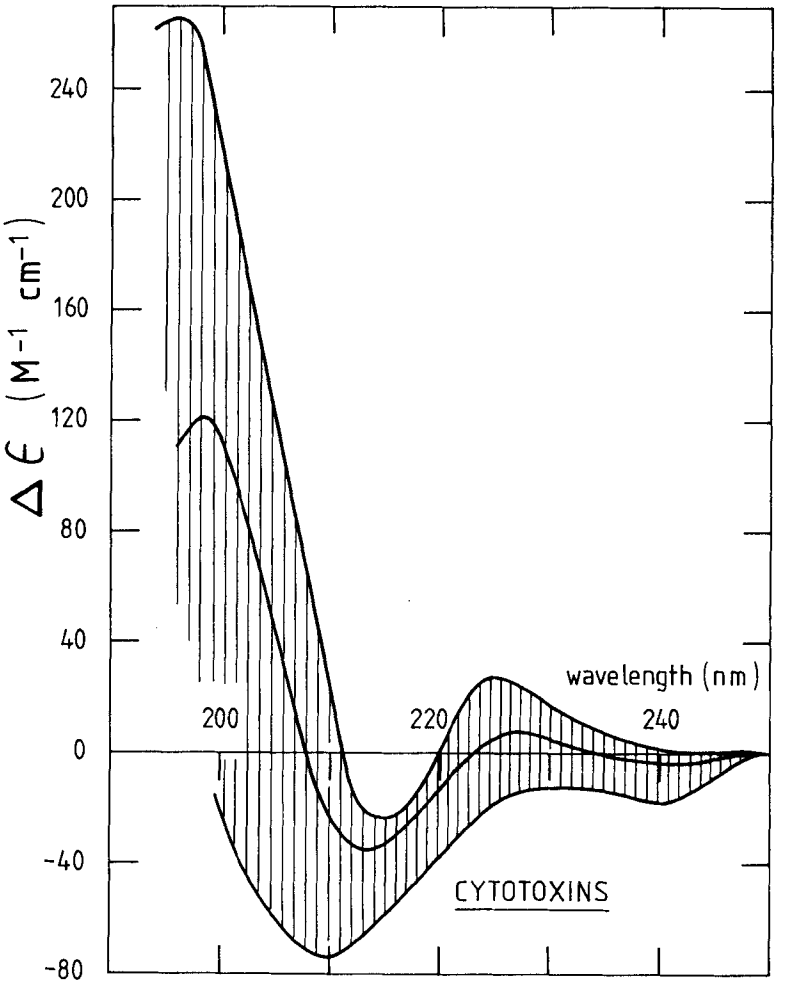

FIGURE 18. The average of the known cytotoxin CD spectra together with the range in which individual spectra may fall. The toxins considered were CT 01,[56] CT 05,[89] CT 06,[89] CT 08,[82] CT 13,[58] CT 16,[58] CT 18,[77] CT 21[58] and CT 27.[58] Some spectra have not been reported below 200 nm, so at these wavelengths, the average spectrum is less representative. (A) The superimposed average CD spectra of short neurotoxins, long neurotoxins and cytotoxins. The resemblance between the short neurotoxins and cytotoxins should be noted.

instance, has a low rate of "ring-flip" that befits its buried position, while phenylalanine-33 has a high rate. For tryptophan-29, its narrow resonances imply some mobility. Information on a homologous tryptophan in *Naja naja naja* long neurotoxin (LN 07) has been obtained by fluorescence techniques, and it is again apparent that the residue can be mobile.[104] Moreover, it is more mobile compared to tryptophan residues in other types of protein and it can sense a change in environment within a time scale of 5 μsecs. Returning to erabutoxin b, ^{13}C NMR relaxation studies of the methyl groups have shown some to possess considerable segmental freedom at 27°C[101] i.e., isoleucine-36δ (±50°), isoleucine-37δ (±70°), valine-46 (±50°), and isoleucine-50γ,δ (±50°). Large amplitude motions (±50 to ±70°) also require cooperative motion in adjacent side chains to account for the lack of steric hindrance. In contrast, for the methyl groups isoleucine, 2γ, leucine-52γ, and valine-59γ, the calculated segmental motions indicate that they are

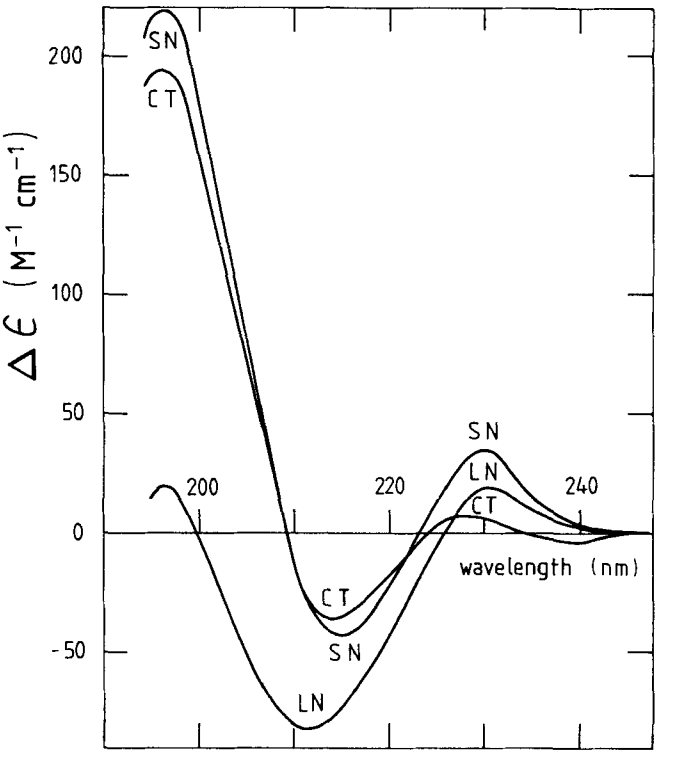

FIGURE 18A

relatively immobile. From the distribution of these aliphatic and aromatic residues, the most mobile areas of erabutoxin b are those sections of the second and third loops that are distant from the central core.[103]

The dynamics of the secondary structures within toxin molecules also vary, and these are measurable by the H-D exchange rates of interchain amide hydrogen bonds, a high rate of exchange being commensurate with a high lability. Appropriately, the four slowly exchanging hydrogen bonds ($T\frac{1}{2} > 30$ hr) in α-bungarotoxin (LN 27) are found towards the core of the molecule where mobility generally would be expected to be at a minimum.[74,81] Given the similarity between long and short neurotoxins, the slowly exchanging hydrogen bonds detected in the latter may also be located near the core area (Table 6).

The greater mobility of the loop extremities implied by the various NMR techniques may account for other NMR observations that are not entirely compatible with the crystal structures. For instance, in four short neurotoxins (SN 2,[64] SN 3,[65] SN 11,[57] SN 09[66]), the indole nucleus of tryptophan-29 can sense the protonation states of both histidine-32 and aspartate-31, and in erabutoxin b (SN 18) it senses the protonation state of aspartate-31. Fluorescence studies and the indole NH exchange rate suggest that the tryptophan and aspartate are hydrogen bonded together,[105] a finding incompatible with the crystal structure of erabutoxin b where aspartate-31 appears to be salt-linked with arginine-33 (Figure 8). Fluorescence spectroscopy has also demonstrated that tryptophen-29 in both SN09 and SN18 senses the protonation states of residue 26 (lysine in SN09 and histidine in SN 18), lysine-27, and aspartate-31.[130] Furthermore, spatial proximity of histidine-32 and the tryptophan indole function in solution has been implied by ^{1}H NMR and fluorescence.[105] This could be sustained by a stacking of the aromatic

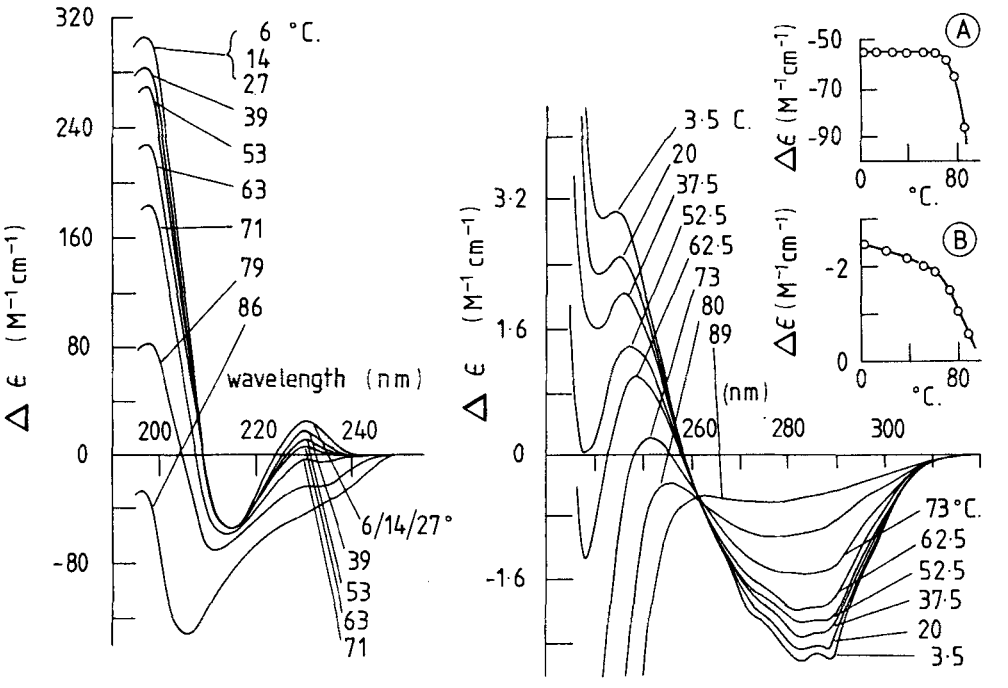

FIGURE 19. Variable temperature CD spectra of erabutoxin b (SN 18).[120] The changes at the fixed wavelengths of 215 nm (A) and 290 nm (B) are also shown.

rings, but the 10.5 Å separation between the centers of homologous residues in the crystal structure of erabutoxin b is too great to permit such an interaction. In addition, the tyrosine-35 residues of SN 10 and SN 03 have been reported to be near the side chains of aspartate-31 and histidine-32,[65,70] despite the implication from the crystal structure of erabutoxin b that they should be positioned on the opposite side of the β-sheet.

Considering the exposed nature of the hydrogen bonds in the central loop extremity of short neurotoxins, a tentative explanation of these apparently anomalous interactions can be made. In essence, this area and its associated hydrogen bonding can be compared to a "Schwyzer-form" cyclic hexapeptide, many examples of which are known to favor two transannular bonds in the solid state.[106,107] In aqueous solution, however, NMR has shown that the structure can become randomized due to the competing tendency of water molecules to form intermolecular hydrogen bonds with the peptide acceptor and donator moieties.[108,109] Therefore, given that the extremity of the central toxin loop will be extensively solvated, the same phenomenon could bestow sufficient conformational freedom on the associated side chains to allow the interactions shown by NMR.[110]

E. Conclusions on Crystal and Solution Structure

The clear sequential homology between the three major toxin groups is evidently reflected as homology of conformation. Where crystal structures have been determined, it appears that they are largely representative of the corresponding solution structures, but differences do exist in the orientations and environments of certain residues. Under normal conditions, it is possible for a toxin to have structural elements that are inherently flexible, and it is to be expected that the distribution and nature of such flexibilities is a common feature of these molecules.

The question of conformational mobility seems particularly important for under-

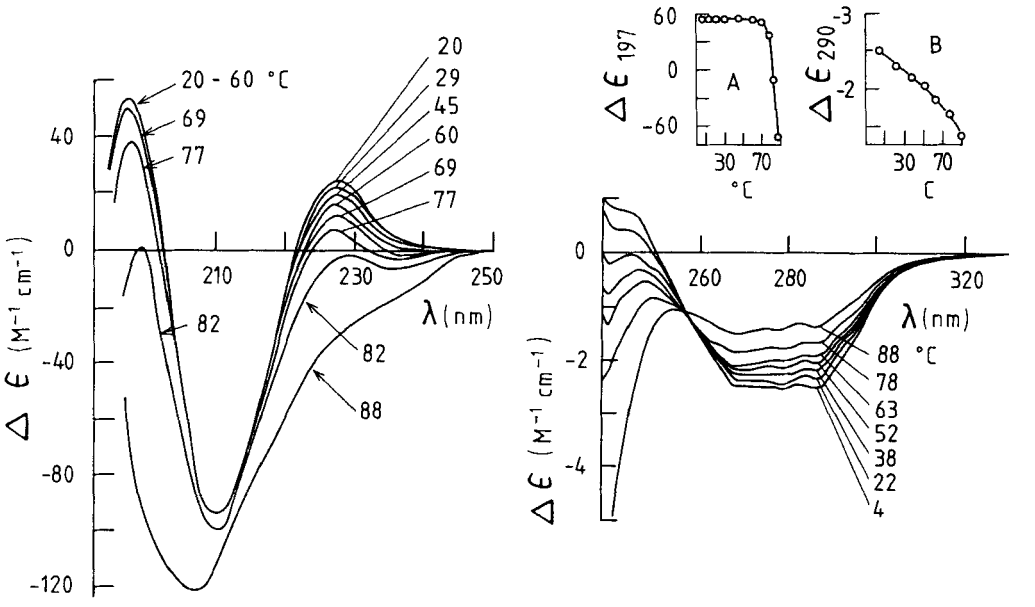

FIGURE 20. Variable temperature CD spectra of α-cobratoxin (LN 03). The changes at the fixed wavelengths of 197 nm (A) and 290 nm (B) are also shown.[76]

standing modes of action, for in neurotoxins at least, the molecular domain of high intrinsic mobility is also that which contains essential targeting residues. In these circumstances, methods which allow conformational changes to be detected and analyzed in solution assume a new relevance. The following section, therefore, is devoted to the information that circular dichroism, laser Raman and NMR has provided about the sensitivity of toxin structure to its environment.

IV. THE MODIFICATION OF TOXIN STRUCTURE

A. Heat Induced Modifications

The conformational responses of three representative toxins to heating, as seen by circular dichroism, are shown in Figures 19, 20, and 21. To complement the available X-ray data, erabutoxin b is the short neurotoxin chosen and α-cobratoxin, the long neurotoxin. The cytotoxin is *Naja mossambica mossambica* $V^{II}4$ (CT 18). Similar data has also been obtained for other toxins.[53,77,111,121]

The variable temperature spectra show that all three toxin types undergo structural changes, especially at higher temperatures, and that they differ in their individual resistance to denaturation by this treatment. Since each of these toxins has a unique spectrum and sensitivity to heat, it is necessary to make individual spectral assignments and interpretations as appropriate.

1. Erabutoxin b

The CD bands at 195 and 215 nm were mentioned earlier as being indicative of the dominant β-structure present in short neurotoxins generally and erabutoxin b in particular. In the variable temperature CD spectra of the latter, there is a steady decrease in the intensity of the 195 nm band up to 60° C, but there is no discernable change in the 215 nm absorption. Above 60° C, the changes in these bands are more dramatic. The 195 nm band undergoes a major collapse while the former 215 nm band increases in intensity,

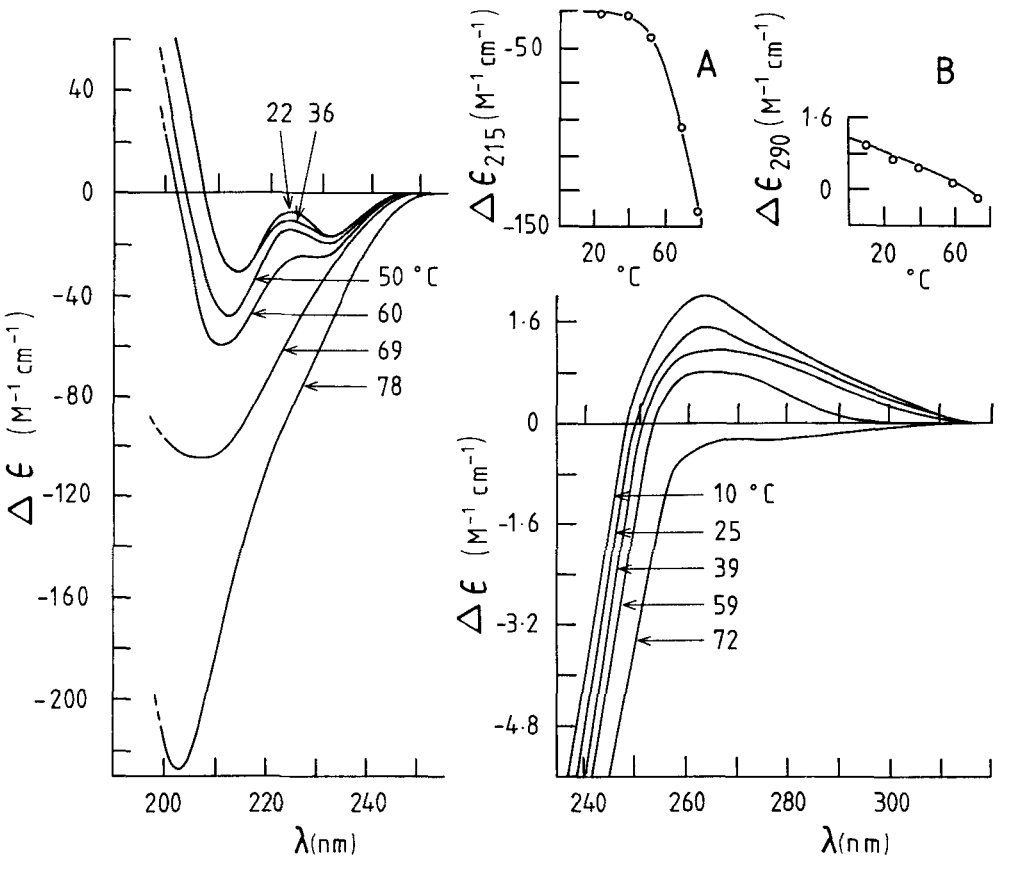

FIGURE 21. Variable temperature CD spectra of the cytotoxin *Naja mossambica mossambica* V[II]4 (CT 18).[77] The changes at the fixed wavelengths of 215 nm (A) and 290 nm (B) are also shown.

its λ_{max} moving to shorter wavelengths. This event can be described as the melting of the secondary structure, for it represents the disruption of the β-structure present in the molecule. In comparison, the changes seen in the peptide bands at temperatures up to 60° C are relatively minor, and so throughout this range, the secondary structure is little altered.

The positive band at 228 nm is less intense than that at 215 nm, but throughout the temperature range at which the latter is stable, the former shows a steady decrease. This 228 nm band is present in all the known neurotoxin CD spectra and in many cytotoxins (Figures 11, 17, and 18). In the case of erabutoxin b and the other short neurotoxins, it has been proposed that this band arises from an interaction between tyrosine-25 and tryptophan-29.[16,112] However, the absorption can also be seen in cytotoxins where the tryptophan is replaced by methionine, and in *Naja melanoleuca* 3.20 (CHO3), where phenylalanine replaces the tyrosine and leucine, the tryptophan. Suggestions that tyrosine-25 is itself a major contributor are unlikely because ionization of the homologous tyrosine-24 of α-cobratoxin barely changes the equivalent CD band.[76,117] Unless the band can originate from several juxtapositions of different aromatic residues, Figure 4 would imply that the constant disulphide bridges, or the peptide carbonyls contained within the conformational domain created by their presence, are the dominant source of the absorption. Choice in favor of the peptide carbonyls can be made by reference to other proteins and peptides in which a positive CD contribution near 228 nm has been noted. Thus for oxidised glutathione,[114] gramicidin S[115] and N-acetylcystine-

N,N-dimethylamide,[116] a similar CD absorption can be connected with the juxtaposition of carbonyls and cystine sulfur.[117] Although the ellipticity at 228 nm is rather weak for these molecules it is much higher for the seriously stereochemically confined gliotoxin antibiotic family.[179] In the X-ray structure of erabutoxin b, there are several instances where cystine sulfur atoms are in van der Waals contact with both amide carbonyl oxygen and carbon atoms. For example, half cystine-43(S) and half cystine-43 (carbonyl C), 33 Å; half cystine-54(S) and serine -53 (carbonyl C), 3.6 Å.[46,118] In erabutoxin b therefore, the 228 nm band probably arises from the vicinity of the disulphide core, and while the overall β-structure remains stable below 60° C, this particular part of the backbone appears to undergo some change.

At wavelengths above 250 nm, the CD absorptions are weaker and are usually attributed to aromatic side chains and disulphide moieties.[119] The fine structure seen in the broad negative band of erabutoxin b between 250 and 310 nm suggests that tryptophan-29 is a major contributor.[119] Upon heating, the band as a whole loses intensity consistently before the secondary structure collapses, and in this respect it resembles the 228 nm band. More significantly, there is an isocircular dichroitic point maintained in the variable temperature spectra at 263 nm up to 60° C which indicates that these two bands change in unison. This means that the conformational element which is changing below 60° C is sensed not only by parts of the polypeptide chain in the core (i.e., the 228 nm band) but also by certain aromatic residues like tryptophan-29 and possibly the disulphide bridges themselves.

2. α-Cobratoxin

When compared to erabutoxin b, the variable temperature CD spectra of α-cobratoxin have many similar features.[76] A major contrast, however, lies in the peptide CD bands at 195 nm and 210 nm. The former is much less intense than in erabutoxin b and the associated 210 nm band is more intense and at a shorter wavelength than the 215 nm equivalent in the short neurotoxin. Now although it is evident that these peptide bands reveal differences in secondary structure between short and long neurotoxins at normal temperatures, it is important to realize that the spectra are related. That is, the magnitudes and positions of the peptide CD bands in native α-cobratoxin (Figure 20) are mirrored in erabutoxin during its rapid structural collapse at about 80° C (Figure 19). This signifies that firstly, the secondary structural balance in erabutoxin b near 80° C is very similar to α-cobratoxin at physiological temperatures, and secondly, that proportionately less β-structure in relation to "random coil" is normal in the long neurotoxin.

Despite the different structural balance in α-cobratoxin, the peptide bands again show only relatively minor changes in intensity until high temperatures are attained. The major structural collapse occurs above 77° C, whereupon the 195 nm band starts to decrease dramatically while the former 210 nm band moves to shorter wavelengths becoming more intense. This trend closely follows that seen in erabutoxin b.

The CD bands above 220 nm are equivalent to those of erabutoxin b, even to the extent of showing a continuous decrease in intensity with heating insufficient to denature the secondary structure. Furthermore, an isocircular dichroitic point relating the change in the 228 nm band to that of the 250 to 310 nm band is to be found at 256 nm (Figure 20). It is also the case, therefore, that α-cobratoxin has a low temperature-sensitive conformational element that influences part of the polypeptide chain in unison with certain aromatics (e.g., tryptophan-28) and possibly the disulphide bridges at the core.

3. Naja mossambica mossambica $V^{II}4$

At physiological temperatures, this cytotoxin displays 195 nm and 215 nm CD bands similar to those of erabutoxin b which signify dominant β-structure in the molecule

(Figure 21).[77] The behavior of this β-structure during heat treatment is also the same as that in erabutoxin b, with a certain critical temperature initiating the familiar collapse of the peptide CD bands and the trend towards a typical denatured spectrum. However, a striking contrast lies in the lesser stability of the cytotoxin, for appreciable changes can be seen in the 215 nm band even before the major structural collapse occurs above 40° C. Evidently, the global conformation of the cytotoxin is very heat labile.[77]

As a consequence of the unstable secondary structure it is not possible to adequately discern whether there is any independent behavior of the bands above 220 nm. There is no 228 nm band as such, but its equivalent is less intense and has its λ_{max} at 225 nm. This too loses intensity rapidly with increasing temperature. Between 250 and 310 nm, there is normally a positive band which corresponds to the negative near UV CD absorption of the neurotoxins. The change in sign is probably due to the lack in the cytotoxin of a tryptophanyl residue homologous to the one conserved throughout the neurotoxins. For this reason, there is no suggestion of an isocircular dichroitic point in the overlapped spectra. The chromophores contributing in this region are again the various aromatics and disulphides.

4. Heat Stabililty of Toxins in General

In addition to the examples discussed, variable temperature CD data is available on the short neurotoxins cobrotoxin (SN 10),[111] *Naja naja oxiana* II (SN 11),[120] and *Dendroaspis viridis* 4.11.3 (SN 14);[53] the long neurotoxins α-bungarotoxin (LN 27),[120] *Notechis scutatus scutatus* III 4 (LN 28),[120] *Dendroaspis viridis* 4.9.3 (LN 19)[77] and *Naja melanoleuca* 3.9.4 (LN 25);[77] and the cytotoxins from *Naja naja atra* (CT 11)[121] and *Naja naja siamensis* (CT 06).[89]

The short neurotoxin *Dendroaspis viridis* 4.11.3 behaves in a very similar fashion to erabutoxin b on heating, but it requires a slightly higher temperature to bring about the major structural collapse.[53] The neurotoxins from *Naja naja oxiana* (SN 11) and *Naja naja atra* (SN 10) have the unusual feature of a 228 nm band that is about twice the usual intensity at physiological temperatures,[71,111] but the 228 nm and 260 to 310 nm bands are still related during heat treatment by the characteristic isocircular dichroitic points, so the underlying response of the conformation to heat appears to be quite similar to that shown for erabutoxin b and 4.11.3. The reason for the relatively high intensity of their 228 nm bands appears to be associated with the presence of tryptophan 28 in SN 11 and tyrosine 35 in SN 10.[16,58,180] Both these positions are on the opposite side of the β-sheet to the conserved tyrosine 25 and tryptophan 29. Presumably, the presence of these aromatics in place of the usual side chains (usually threonine at both positions, Figure 1) enhances the origin of the band through an effect on the side chain packing of the central loop. The relationship between the mobility of side chains in this area and the 228 nm intensity may be an important area of further investigation.

The variation in the short neurotoxin melting curves shows that individual examples can have different stabilities, and this has also been noted in an Infrared monitored H-D exchange study in which *Naja nigricollis* α (SN 09) proved to be more stable than erabutoxin b (SN 18).[122]

The other long neurotoxins and cytotoxins investigated conform very closely to α-cobratoxin and *Naja mossambica mossambica* $V^{II}4$, respectively, and only vary in the precise temperature at which the major denaturation occurs. Although *Naja naja siamensis* cytotoxin (CT 06)[89] has a 228 nm CD band much more like the versions seen in the neurotoxins than in *Naja mossambica mossambica* $V^{II}4$ (CT 18),[77] its response in conjunction with the peptide CD bands is very similar to the latter.

In general, it appears that short and long neurotoxins have a relatively stable secondary structure accompanied by some element that is labile at low temperatures. According to the suggested assignments of some of the CD bands, the sensitive area may

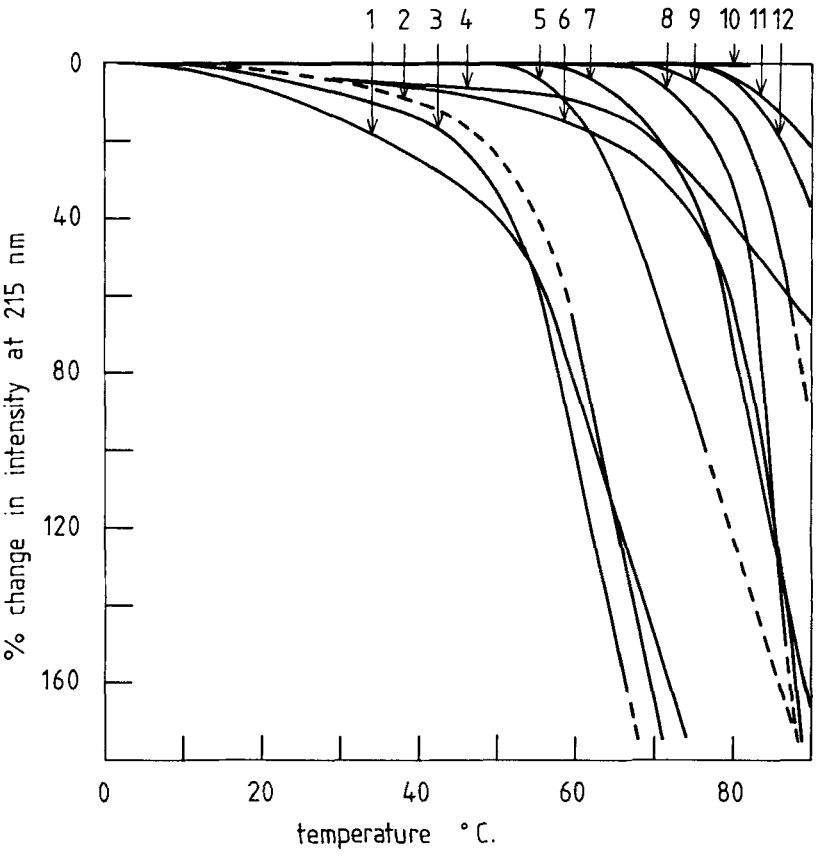

FIGURE 22. Comparative melting curves for short neurotoxins, long neurotoxins and cytotoxins. 1, CT 05;[89] 2, CT 11;[121] 3, CT 18;[77] 4, SN 11;[120] 5, LN 19;[77] 6, SN 10;[111] 7, LN 25;[77] 8, SN 18;[120] 9, LN 27;[120] 10, LN 28;[120] 11, SN 14[53] and 12, LN 03.[76] The response to heating is recorded as the percentage increase in the native 210 to 215 nm CD band. In the exceptional cases of SN 10 and SN 11, the 195 nm band was analyzed because of the anomalous behavior of the 215 nm band.

involve the vicinity of the 4 disulphide bridges and parts of loops 2 and 3. In contrast, the secondary structure of cytotoxins as a whole is quite heat labile and there is little distinction to be made between different parts of the backbone in terms of relative stability.

Although cytotoxins can be distinguished from neurotoxins on the basis of their heat stability, a similar separation cannot be made for short and long neurotoxins. As shown in Figure 22, both types have overlapping melting curves, so suggestions that one group is more stable than the other are not correct.[77,81] Complementary data from NMR on the hydrogen-deuterium exchange rates of the amide hydrogen bonds verifies that there is no clear distinction. Thus while *Naja naja* toxin B (LN 7) (which is very similar to α-cobratoxin (LN 3)) and α-bungarotoxin (LN 27) possess amide hydrogens that exchange slower than any in erabutoxin b (SN 18) and cobrotoxin (SN 10), the short neurotoxins *Naja naja oxiana* II (SN 11) and *Naja mossambica mossambica* II have the slowest rates of all (Table 6).

In seeking an explanation for the various stabilities, individual sequences can be examined for unusual features. Ultimately, stability is likely to be governed by many factors such as the amino acid composition and the placement of certain residues, but it is noteworthy that the two least stable long neurotoxins (*Naja melanoleuca* 3.9.4 (LN 25)

Table 6
HALF-TIMES OF AMIDE PROTON HYDROGEN-DEUTERIUM EXCHANGE REACTIONS FOR VARIOUS TOXINS[a]

Toxin	Conditions		Population of half-time ranges				Ref.
	pH	Temp. (°C)	>100 h.	100—10 h.	10 to 1 h.[b]	10 to 8.1 h.[b]	
Cobrotoxin (SN 10)	6.5	37	0	0	8	11	81
N. naja oxiana II (SN 11)	5.85	32	3	2	4	—	57
Erabutoxin b (SN 18)	4.82	30	1.5	4.5	3	—	123
	6.5	37	0	0	9	11	81
N. mossambica NT II	6.2	35	4	8	—	10	124
Naja naja b (LN 7)	6.5	37	0	3	12	14	81
α-Bungarotoxin (LN 27)	6.5	37	0	10	5	6	81
N. mossambica cardiotoxin IV (CT 18)	6.2	35	0	3	—	14	124
N. naja atra cardiotoxin (CT 11)	6.5	37	0	1	6	9	81

[a] Note that the exchange rate is dramatically dependent on pH as shown by the erabutoxin b data.
[b] Overlapping ranges.

and *Dendroaspis viridis* 4.9.3 (LN 19) [Figure 22]) probably have more than one glutamate residue incorporated into their triple stranded β-sheet.[15] This is a rare occurrence in β-sheet generally.[125,126]

Whatever toxins are involved, a similar type of CD spectrum is approached after the secondary structure has collapsed. It features an intense negative band at 200 nm with a slight shoulder near 210 nm. In some of the more stable neurotoxins, this final state is not completely attained in the available temperature range, but the trend towards it is clear. Presumably it reflects a predominantly random-chain conformation in which only the overall folding pattern is maintained by the disulphide bridges. As pointed out in the discussion of α-cobratoxin, consideration of the transitory intensities and wavelengths of the peptide CD bands as they denature from the β-sheet pattern to the totally randomized type gives insight into the different structural balances that are possible in the toxins. Thus, although all long neurotoxin and some cytotoxin peptide CD bands do not suggest extensive β-structure, they can nevertheless be reconciled as showing a mixture of β-sheet and random coil.

5. The Sensitive Conformational Element of Neurotoxins

The heat treatment of short and long neurotoxins has shown that the peptide chain in the vicinity of the central disulphide bridges is sensitive to temperature, along with certain aromatic residues, throughout the range in which the secondary structure remains essentially unchanged. It is to be expected that many proteins show a range of intramolecular stabilities that can be distinguished by their unequal response to heating, but the phenomenon seen in the neurotoxins seems to be of a definite and consistent character and not merely a random increase in local mobilities. This follows from its similar appearance and behavior (according to CD) in both short and long neurotoxins despite the extensive differences that exist between them in terms of sequence and composition. Further, any change that occurs in the 228 nm CD band is always related via an isocircular dichroitic point to changes in the 255 to 310 nm band, thereby indicating that all the contributing chromophores are responding in a concerted fashion.

While CD alone cannot give details of all the moieties involved in the low-temperature

conformational changes, NMR does have this potential and there is now specific information available for α-cobratoxin (LN 3).[76] Significant chemical shifts have been detected in the 0 to 60° C NMR signals of valine-22, lysine-26, threonine-27, tryptophan-28, leucine-42, valine-55, and isoleucine-57. Two of the half cystine residues, at positions 23 and 44, are also influenced. In contrast, there is relatively little change in the signals from histidine-21, tyrosine-24, isoleucine-7 and phenylalanines 4/32/68. These results suggest that the sensitive part of the structure is probably bounded by loops 2 and 3 and part of the disulphide core (Figure 23). This is quite appropriate when it is recalled that this region is the major resemblance between the two types of neurotoxin. The possible scope for reorientation in this area is limited, however, by the presence of the triple stranded β-sheet structure which, from the CD evidence, should remain virtually intact through the temperature range of interest. If part of the polypeptide chain is involved in the sensitive conformational element then it is probably located where loops 2 and 3 are rooted into the molecular core (as implied by the 228 nm CD band). The concerted behavior of a residue as far removed as the tryptophan-28/29 common to all neurotoxins suggests a transmitted allosteric effect across or around the β-sheet.

Certainly, the immediate environment of this tryptophan is prone to perturbation, for NMR has shown that the isoleucine-36 and isoleucine-50 residues which 'sandwich' the indole in erabutoxin b are very sensitive to temperature increases.[101] In addition, as pointed out earlier, the isoleucine-50 has quite different orientations in the two crystal structures of this toxin.

B. Solvent Modifications

When toxins are dissolved in nonphysiological solvents, various degrees of conformational disturbance are induced. CD is often used to record such changes and it transpires that the typical bands respond to denaturant solvents in much the same way as they do to an increase in temperature. For instance, when *Naja nigricollis* α-toxin (SN 09) is dissolved in increasing concentrations of trifluoroethanol (TFE), successive decreases are produced in the intensities of the 228 nm and 255 to 310 nm bands while the 195 nm and 215 nm peptide bands remain steady.[55] Even at 100% TFE the 215 nm band remains unperturbed. Erabutoxin b (SN 18) behaves similarly in this solvent, but above 85% TFE, there is a major change in the peptide CD bands that signifies alteration of the native secondary structure.[55] The modified far UV spectrum that results suggests that α-helix becomes the predominant secondary structure. Similar spectra are obtained when α-bungarotoxin (LN 27),[127] cobrotoxin (SN 10)[56] and cobramine B (CT 03)[56] are dissolved in 95% TFE, but like *Naja nigricollis* α (SN 09), *Hemachatus hemachates* IV (SN 13)[56] is resistant and shows only a loss of intensity in its 228 nm CD band. A short neurotoxin (SN 11), a long neurotoxin (LN 26) and a cytotoxin (CT 08), all from *Naja naja oxiana,* have been compared in their sensitivity to urea and TFE, and again it was the short neurotoxin that proved to be the most stable.[82] In studies of this kind, therefore, it appears that short neurotoxins are generally more resistant to denaturation, but notable exceptions are erabutoxin b and cobrotoxin. These two examples have also been highlighted in refolding studies because of their relatively slow rates of renaturation, and the rapid H-D exchange of their backbone hydrogen bonded structure (Table 6).[38]

In order to establish if the crystallizing media used for erabutoxin b (SN 18) could perturb its native conformation, a similar short neurotoxin (SN 14) was analyzed by CD in sodium sulphate (1 M) and ammonium sulphate (1 M).[53] Slight changes were noticed in the 228 nm and 255 to 310 nm CD band intensities which were reminiscent of those inducible by temperature increase, but there was no clear effect on the secondary structuring. Erabutoxin b has itself been compared in physiological media and ammonium sulphate solution by NMR, but no major perturbation was detected.[51]

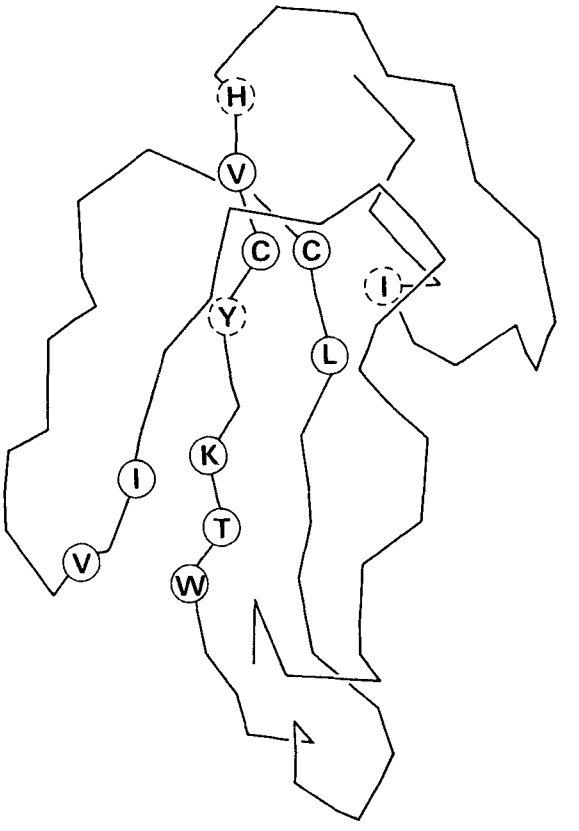

FIGURE 23. The crystal structure of α-cobratoxin showing those residues which show temperature induced chemical shifts between 0 and 60°C.[76] ○ Major shifts, ◌ Lesser shifts.

C. Chemical Modifications

The most informative chemical modification studies are accompanied by CD spectra of the native and derivatized toxins. A systematic study of *Naja naja oxiana* II (SN 11) has been undertaken in which its lysine residues were selectively acylated.[71] The five monoacetyl derivatives, corresponding to modification at a different lysine in each case, were purified and their CD spectra compared (Figure 24A). Depending on the location of each modified lysine, greater or lesser perturbations of the native CD bands were brought about. In comparison with the variable temperature spectra of this short neurotoxin (Figure 24B), it is clear that the conformation is responding in exactly the same way. All the lysyl derivatives cause a decrease in intensity of the 228 nm and 255 to 310 nm CD bands, but to different extents, and when their spectra are overlapped, an isocirculardichroitic point at 255 nm analogous to those seen in other neurotoxins during heat treatment is obtained.[71] The smallest change is produced by modifying lysines 45/47 and the largest by modifying lysine-26 (Figure 25). It is most significant that proximity to the disulphide core should produce the largest degree of disturbance because, as described earlier, it is expected that the core is the origin of the 228 nm band. When all the lysines and the N-terminus are acetylated, the native spectrum is perturbed more than with any of the single modifications. Notably, the hexa-acetylated form is the only derivative which shows a major change in secondary structure when dissolved in trifluoroethanol.[71]

The corresponding lysine residues in *Naja nigricollis* α (SN 09) have also been

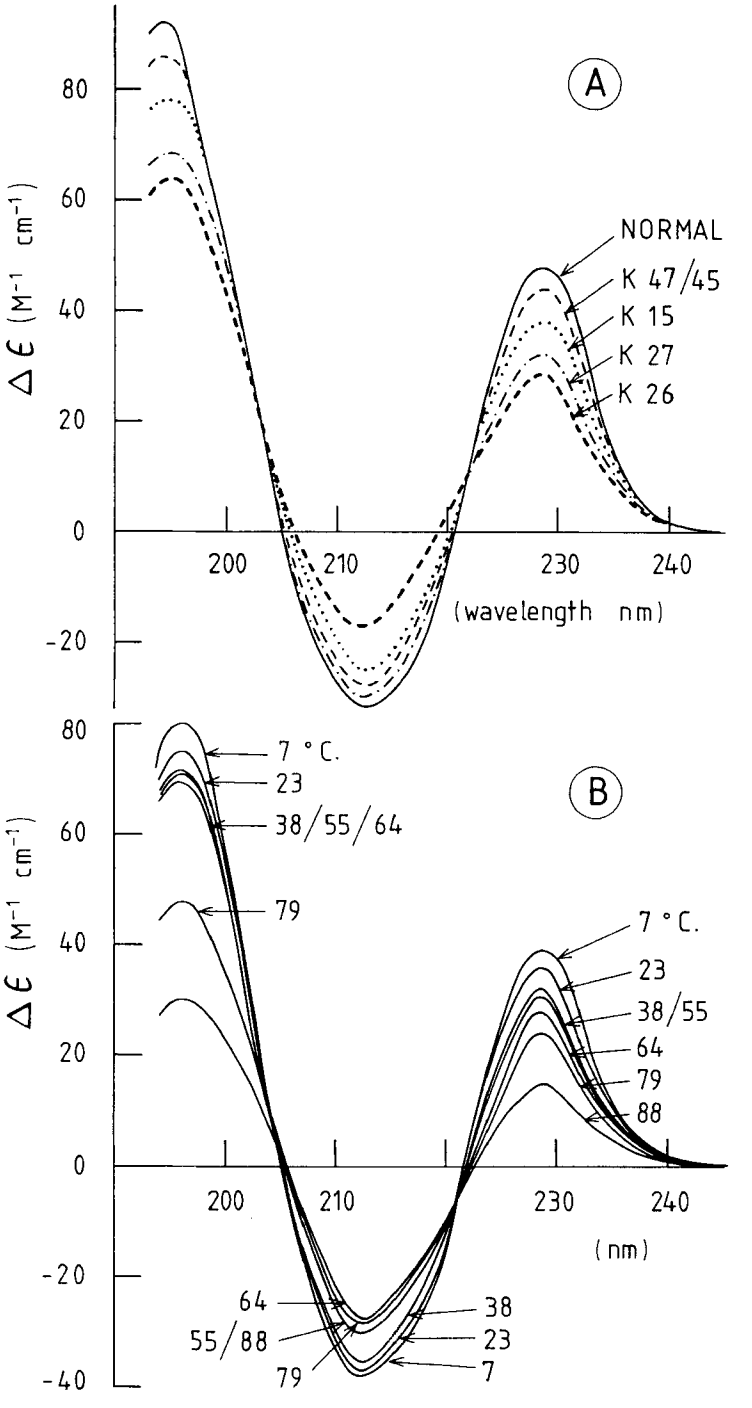

FIGURE 24. The effects of individual lysine acetylations on the CD spectrum of *Naja naja oxiana* II short neurotoxin (SN II),[71] (A) compared with the response of the same toxin to increasing temperature (B).[120]

acetylated individually, and again it can be observed by CD that proximity to the central area of the molecule causes the largest conformational disturbance.[128]

Aromatic residues are relatively easy to specifically derivatize, and the effect of

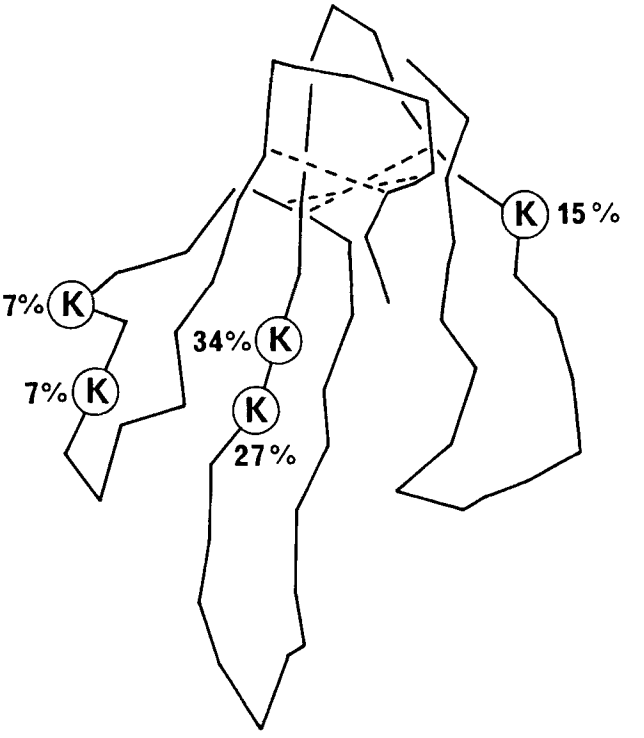

FIGURE 25. The backbone of *Naja naja oxiana* II (SN 11) (as extrapolated from erabutoxin b) showing how the perturbation of the native conformation by individual lysine modification is dependent on location.[71] The percentage figures relate to the loss in intensity of the native 228 nm CD band (Figure 24).

modifying the conserved tyrosine and tryptophanyl residues is of major interest. In α-bungarotoxin (LN 27) there are two tyrosines located at positions 24 and 54, of which the latter is the more accessible for iodination.[129] When tyrosine-54 is monoiodinated, the CD spectrum shows an increase in the intensities of the 228 and 255 to 310 nm bands. With diiodinated tyrosine-54, however, a larger change in the CD spectrum occurs, with a further increase in the intensity of the near UV bands, but a decrease in the magnitude of the 228 nm band.[75] In both the iodinated derivatives, the overall secondary structuring remains substantially the same.

The single tryptophan-29 of *Naja nigricollis* α (SN 09) has been modified by N-formylation and treatment with 2- hydroxy-5-nitrobenzyl bromide and the CD of both derivatives show abolition of the 228 nm band and lesser changes in the peptide CD bands.[128] This has been cited as evidence that the tryptophan makes a substantial contribution to the 228 nm CD band, but as has already been pointed out this residue is not essential for the presence of the same band in other toxins (e.g., many cytotoxins). If instead, the band arises from the disulphide core region, this modification suggests that the sensitive conformational element is being influenced in a similar fashion to that seen with the lysine modifications. When the tyrosine-35 of cobrotoxin (SN 10) is selectively nitrated, the major change in the native CD spectrum is again in the 228 nm band.[54]

Protonation can also be regarded as a chemical modification, and when histidine-26 of erabutoxin b (SN 18) is protonated, there is a decrease in the tryptophan-29 fluorescence.[130] Previously, this had been interpreted as a close spatial proximity between the two residues, even though their side chains project from different sides of the β-sheet.

However, since modification of lysine-26 in *Naja naja oxiana* II (SN 11) also influences tryptophan-29 of that toxin in association with the 228 nm CD band, the fluorescence data could indicate perturbation of the tryptophan via its conformational associations with the core region (i.e., via the defined element of flexibility).

The tryptophanyl residue characteristic of all neurotoxins is replaced by a homologous methionine in the cytotoxins, and so this latter residue has been implicated as being functionally important. Cytotoxin $V^{II}1$ from *Naja melanoleuca* (CT 13) has methionine residues at positions 24 and 26, and its native CD spectrum is similar to that of a typical short neurotoxin.[131] Upon oxidation of these methionines, the secondary structure remains intact, but decreases are seen in the 228 nm and near UV band intensities.[131] Apart from the positive nature of the near UV band, the observed perturbances resemble the deflections that can be caused in the CD spectra of neurotoxins over these wavelengths. As regards toxicity, this is also impaired by the methionine oxidation.

D. Conclusions on Stability

The evidence concerning the sensitivity of neurotoxin conformation to heat, solvent, and chemical modifications shows that while the perturbing influences may vary, the responses can be consistently similar. Thus, mildly denaturant conditions or residue modifications sited away from the triple stranded β-sheet will cause minor changes in the native conformation, but will not affect the overall secondary structuring.

If the denaturing influence is stronger, the perturbation of the conformation becomes even more pronounced, but it nevertheless continues to follow a defined pathway of change until the point is reached at which the predominant secondary structuring collapses. Then, similar reordered states are attained.

The particular point of interest to emerge from the perturbation studies on the neurotoxins concerns the nature of the readily induced changes that are independent of most of the secondary structure. In the light of the methionine modification study on the cytotoxin, similar conformational properties may be present in some, if not all, of these types of toxin, but they are not normally detectable because of the relative instability of cytotoxin secondary structure.

One of the diagnostic features of the sensitive element is the 228 nm CD band because it can show gradual changes while the bands typical of the β-structure remain stable. It follows from this that the magnitude of the 228 nm band could reflect the position of an equilibrium between two conformational extremes providing its origin is the same in all toxins. For example, although short neurotoxins usually have more intense 228 nm bands than long neurotoxins, heating of the former will eventually produce bands of the same lower intensity. If heating is continued, the 228 nm band will decrease even further until it resembles the very weak versions seen in some cytotoxins. Therefore, the variation in magnitude throughout the toxins could be indicative of an equivalent variation in conformational equilibria, in which case some groups of toxins are seen to be distinct from others (see Figures 11, 17, and 18). In addition, some toxins may have native conformational equilibria that are only achieved in other types under abnormal conditions or after specific modifications.

If it is accepted that the sensitive element does encompass the disulphide core and the "essential" tryptophan residue, then there are two conceivable ways in which the response of one moiety could be intimately linked with the other given that they are separated by a stable β-sheet. The first requires that there be side chain-side chain interactions along the triple-stranded β-sheet through which allosteric effects can be transmitted. The second involves a movement of the nonhydrogen bonded section of polypeptide chain in loop 3 which could in principle affect both its root in the core region and the packing of residues in the vicinity of the tryptophan. In view of the electrostatic network which has been demonstrated in the β-sheet,[65,71] and the perturbations caused by modifications to

residues within it, the hypothesis that invokes the side chain packing seems the most appropriate. If large movements of polypeptide chain were involved, major ^{1}H NMR shifts could be expected for many residues in loop 3 whenever the sensitive element alone is perturbed, but these have not been observed in erabutoxin b (SN 18)[48,49] or α-cobratoxin (LN 3).[76] In fact some of the most marked NMR shifts seen at nondenaturant temperatures are those of the Cα protons adjacent to the most stable hydrogen bonds in the β-sheet, a finding more compatible with the side chain packing model.

V. DISCUSSION

A. The Possible Role of the Conformational Properties

The solution studies have revealed a facet of toxin structure that is not evident from the crystal structures alone, namely the existence of a conformational equilibrium under physiological conditions that can be readily perturbed. There are several circumstances which suggest a major significance of this property to the mode of action. These are as follows:

1. Despite the differences between long and short neurotoxins, the phenomenon appears and behaves in qualitatively the same fashion. Further, it may also be present in cytotoxins, and this would mean that it is a fundamental feature of the toxin family.
2. The normal conformational equilibrium of a toxin can be upset by the specific modification of residues implied as being functionally important. It is unlikely, therefore, that binding of these residues to a target could be achieved without a similar conformational response, especially if their individual influences on the overall structure were compounded. Indeed, if the disulphide core does sense the behavior of the relevant side chains, then a concerted influence from the simultaneous binding of many key residues could induce effects over a large area of the molecule via this molecular "fulcrum".
3. On theoretical grounds, maximum specific binding in the thermodynamic sense will be achieved by an exact stereochemical matching of the toxin and receptor surfaces. However, the rate at which the two surfaces are able to approach each other will inevitably be slow unless one or both has a degree of conformational mobility. As pointed out by Williams and co-workers,[103] a large number of available conformers increases the entropy of the free molecule and thus will reduce its binding strength. As an optimal compromise, the ligand should have a relatively rigid structure with a small number of defined conformers available. This would then allow fitting "as a hand fits a glove". A relatively stable, but partly articulated structure, as shown in the toxins, would appear to be highly suited.

Given this implied importance for the conformational properties, the themes that are thought to underlie neurotoxicity and cytotoxicity can now be considered.

B. Neurotoxicity

1. The Nature of the Receptor

The site of action of short and long neurotoxins is now clearly recognized as the nicotinic cholinergic receptor. This consists of four distinct subunits which are partly homologous in sequence and have a quarternary structure of $\alpha_2\beta\gamma\delta$.[132-134] Two of these complexes form a disulphide linked dimer joined through the two δ-chains.[135] Such receptor complexes adopt ring structures with the subunits arranged around a funnel-shaped ionophoretic channel.[74] They span the membrane and extend 5.5 nm on the synaptic side.[136-139] The functional significance of the subunit structure is largely

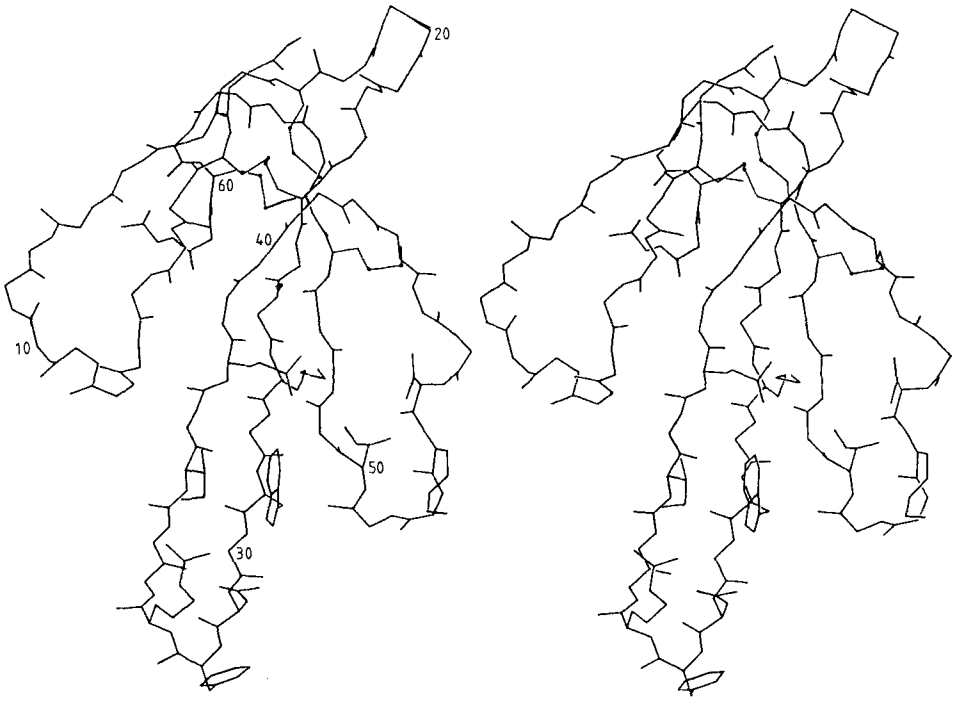

FIGURE 26. Stereo diagram of the crystal structure of erabutoxin b at 1.35 Å resolution.[46,91] Only those side chains thought to be involved in the interaction with the receptor are shown. The coordinates were obtained from the Protein Data Bank.[118]

unknown and only the α-chain has been unambiguously identified as a binding site for cholinergic ligands.[135,140,141] Thus there are at least two sites per receptor complex. Each site is located 5.5 nm from the most constricted part of the trans-membrane channel,[74,136] which suggests that some kind of allosteric change is transmitted through the oligomeric structure to the ion-channel. Indeed, X-ray diffraction studies and thermodynamic analyses have confirmed that a conformational change does occur in the receptor when snake neurotoxins bind.[142,143]

2. Specificity and Binding

The residues expected to be forefront in the toxin/receptor interaction have already been highlighted by sequence comparison and chemical modification (Figure 5). The X-ray maps of erabutoxin b (SN 18) (Figure 26), and α-cobratoxin (LN 3) show these residues as mostly oriented towards the same face of the molecule[12,13,78] so presumably they constitute the nucleus of the interaction. Further evidence that this face contacts the receptor is provided by antibody studies.[144–146] However, according to EPR spin label studies, the area of contact between a neurotoxin and its receptor is not confined to this region alone. This conclusion results from the modification of certain residues with EPR spin labels and the subsequent monitoring of their behavior on binding to receptor preparations. In *Naja naja oxiana* II (SN 11), lysine-27, lysine-47, and histidine-32 are implicated in the interaction, but so too are the leucine-1, glutamate-2, lysine-15, and lysine-26 residues which are not normally associated with neurotoxicity.[72,79,147] The lysine-26 of α-cobratoxin (LN 03) has similarly been shown to be involved.[79] Labeled residues that are not perturbed by binding are lysine-45 in *Naja naja oxiana* II (SN 11)[147] and histidine-68 in *Naja naja oxiana* α (LN 26).[79] It should be noted, however, that in these spin-labeled derivatives, the NO probe extends the side chain length by 4.5 Å and

converts the hydrophilic lysine side chain to one of hydrophobic character. Thus, the derivatised residues could possibly sense the receptor surface while being located close to the interface region.

For an effective binding to the protein receptor, one would expect a multipoint attachment in which electrostatic attractions, hydrophobic interactions, and hydrogen bonds feature. From amongst the varied agonists and antagonists of the cholinergic receptor, curare and acetylcholine have been chosen as those most likely to have clear mimics amongst the functional neurotoxin residues, but opinions differ as to the participating residues. The proposal by Dufton and Hider for a curare-like mimic cites lysine-47, arginine-33, tryptophan-29 and phenylalanine/histidine-32 as the essential components in short neurotoxins (Fig. 27A).[15] For long neurotoxins the equivalent residues are lysine/arginine-52, arginine-36, tryptophan-28 and phenylalanine/tryptophan-32. In contrast, Tamiya et al.[148] have suggested that tyrosine-25, lysine-27, tryptophan-29, aspartate-31, phenylalanine-32, arginine-33, isoleucine-36, and glutamate-38 could form a similar mimic in erabutoxin b (Figure 27B). The former model is not stereochemically compatible with the X-ray structures, but given that the neurotoxins have a degree of structural flexibility in the area, the mimic could be readily attained in solution.[149] The model put forward by Tamiya includes tyrosine-25 and phenylalanine-32, but the juxtaposition of these moieties that is required to produce the correct resemblance to the curare-like alkaloids cannot be achieved without disrupting the fundamental triple-stranded β-sheet structure. A more recent proposal by Menez removes these residues from the above model (Figure 27B).[145] In both the cases where the mimic is entirely located in the central loop, it is notable that lysine/arginine-47/52, a residue known to be one of the most functionally important,[71,128,150] is not incorporated. A difficulty associated with the curare mimic concept is the role played by the tryptophan-28 of α-bungarotoxin (LN 27), for it is positioned completely differently to its counterparts in erabutoxin b (SN 18) and α-cobratoxin (LN 3) by being on the opposite side of the molecule to the other functional residues (Figure 13). It is necessary, therefore, to speculate that in α-bungarotoxin, either tyrosine-54 substitutes for tryptophan-28 in forming the curare mimic or that the tryptophan is reoriented upon binding to bring it into the more usual position.

The crystallographers who determined the X-ray maps of erabutoxin b favor the hypothesis that a key mimic exists for acetylcholine.[13,14] The residues which have been highlighted in this respect are arginine-34 and aspartate-31 (Figure 27C), but the proposed mimic requires a salt-link to exist between them in solution, a situation not verified by NMR. Another possibility that can be put forward involves arginine-33 and glycine-34. Apart from tryptophan-29 these are the only two apparently functional residues that are invariant in all neurotoxins (Figure 5). In this instance, the lack of a side chain in the glycine would expose the peptide carbonyl, so along with the arginyl side chain, an even closer imitation of the characteristic moieties in acetylcholine could be produced (Figure 27C).

It should be noted that although there is good evidence for mutually exclusive toxin-cholinergic ligand competition for a common binding site,[37,151,152] there is also some evidence to the contrary.[143,153] Furthermore, the agonist and antagonist mimic sites need not be mutually exclusive as agonists and alkaloid antagonists bind at overlapping sites on the receptor and demonstrate competitive binding.[154]

While the mimic hypotheses may help to explain how specificity of binding is achieved, they do not in themselves suggest a reason for the unusual conformational properties of the neurotoxins. As suggested earlier, it is difficult to envisage binding without some conformational change taking place in the toxin as well as in the receptor. For instance, the lysine acetylation study on the short neurotoxin *Naja naja oxiana* II (SN 11) has shown that depending on the position of a lysine residue,[71] it has a greater or lesser

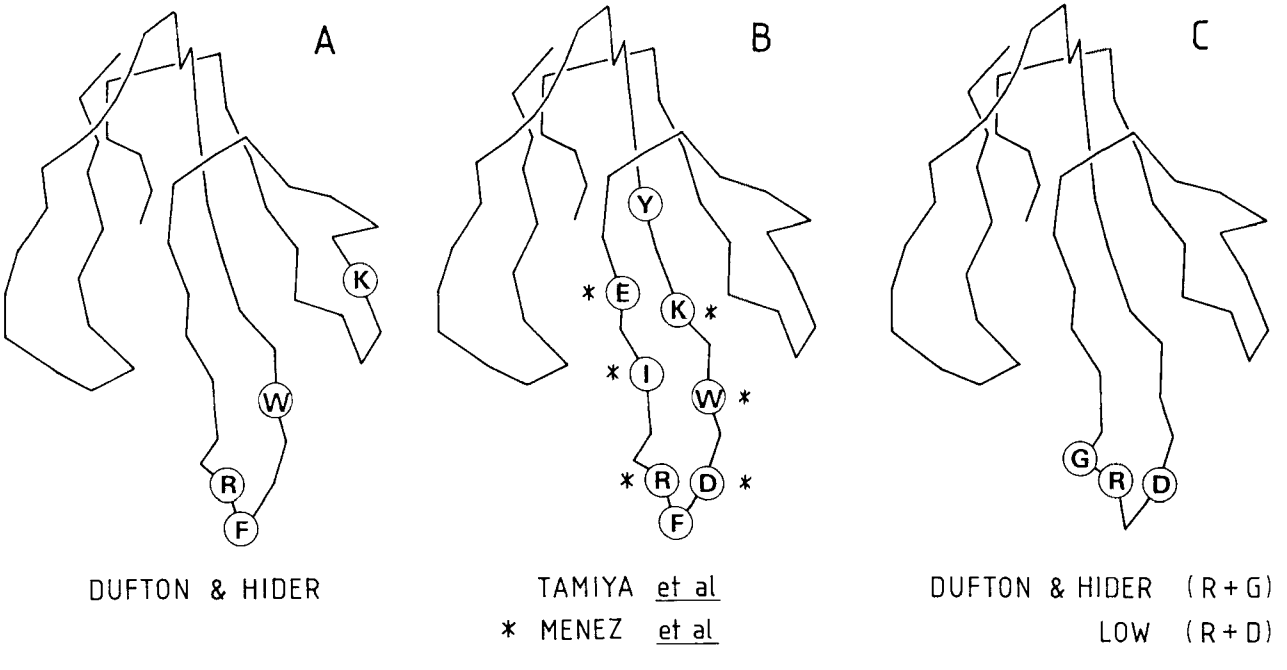

FIGURE 27. Hypothetical agonist/antagonist mimics amongst the functional residues of erabutoxin b. (A) Dufton and Hider.[15,110] Curare mimic. (B) Tamiya et al.[148] (* Menez et al.[145]). Curare mimic. (C) A. Dufton and Hider. Acetylcholine mimic B. Low et al.[13] and Tsernoglou et al.[14] Acetylcholine mimic.

involvement in the maintenance of the native configuration (Figure 25). In particular, lysines-26 and 27 are the most important, followed by lysine-15. It transpired that on binding to the receptor protein, EPR spin-labels on lysines-26 and -27 were perturbed the most, followed by lysine-15.[71,147] Only lysine-45 continued to behave as if it were in the free toxin. Therefore, the more influence a lysine residue exerts on the toxin configuration, the more central it appears to be to the association with the receptor, a correlation that is highly suggestive of an underlying importance for any conformational changes thereby induced. It is also significant that when all the lysines are acetylated in one molecule (a situation that could be considered equivalent to a simultaneous modification by receptor binding) the resistance of the global toxin conformation to denaturation is markedly lowered (Section IV.C).

The benefit of an induced conformational change in the toxin could be to enhance its affinity by allowing an optimum arrangement of its structure to be formed, and/or to allow the toxin to "follow through" with the conformational change in the receptor to produce a mutually locked complex. Alkaloid nicotinic antagonists, which can be effective as rigid molecules, possess a markedly lower affinity than toxins, typically 10^7 vs. $10^{10}-10^{11}M^{-1}$).[151] Thus, as a proteinase inhibitor depends for its action on the complementation of its own shape and that of its target enzymatic site,[176] then so could the conformation of a toxin complement that of its receptor. As the receptor seems to function by means of defined conformational responses, it would be appropriate if the toxin could complement it in this respect too.

3. The Differences Between Short and Long Neurotoxins

Although long and short neurotoxins bind to the acetylcholine receptor in a similar fashion there is a widely held view that long neurotoxins associate and dissociate to and from the receptor at slower rates. Whereas it is true for many toxins (i.e., SN 02,[36] SN 06,[36] SN 10,[155] SN 14,[156] SN 18,[155] LN 3,[155] LN 11,[36] LN 27[37]) it does not hold for all. For example, the long neurotoxin from *Dendroaspis viridis* (LN 19) dissociates rapidly while the short neurotoxin from *Naja nigricollis* (SN 09) has a dissociation rate like some long neurotoxins.[37,156] Therefore, the rate is not greatly influenced by the presence or absence of the features unique to the two neurotoxin groups. A related hypothesis that has been proposed by Inagaki is that the overall structural flexibility of a toxin influences its reversibility of binding,[103] the more flexible types being the more reversible.

A more marked distinction is that long and short neurotoxins have differential toxicities in a wide range of animals. For instance, short neurotoxins are generally more toxic in rodents and birds whereas long neurotoxins tend to be more effective against reptiles and amphibians.[155,157-159] It seems probable that both neurotoxin types are present in cobra and mamba venoms because of their varied diets, for King Cobras and Kraits, which feed almost totally on other reptiles, only have long neurotoxins. In view of this, the structural differences observed with neurotoxins may be connected with receptor variation amongst different prey species.

C. Cytotoxicity

Unlike neurotoxins, no common binding site has yet been identified for all the cytotoxins. This is due to the variety of toxic effects that can be induced by these proteins and the apparent lack of an underlying theme. It is often thought that they nonspecifically disrupt cell membranes and thereby cause the varied effects by disturbing associated membrane mechanisms.[10,43,160-162] While it can be shown that purified cytotoxins can induce lysis, contaminant phospholipase can sometimes be responsible.[163-165] The two proteins tend to copurify and show a significant degree of synergism, and so even slight traces of the enzyme cannot be tolerated in experiments. Nevertheless, some apparently phospholipase-free cytotoxins can still show lytic

properties,[165] but whereas they will form stable complexes with negatively charged phospholipids, they fail to do so with neutral lipids.[161,166] Indeed, some (CT 05 and CT 06) are only able to form mixed monolayers with phosphatidyl choline at low surface pressures, preferentially dissolving in the subphase at pressures higher than 20 dynes cm^{-1} [165] Since the concentration of negative lipids in the outer half of mammalian plasma membranes is quite low and typical surface pressures are 30 to 35 dynes cm^{-1},[167-171] cytotoxins in these circumstances have a poor penetrating power and are unlikely, therefore, to achieve depolarisation in this way.

On the basis of their highly conserved sequences and their fundamental conformational similarities to neurotoxins, it is strongly implied that cytotoxins could function via specific interactions with membrane receptors. Specificity is certainly shown in the cardiotoxic effects produced by these toxins, for when administered to mammals, they can precipitate a fatal ventricular fibrillation of the heart within 10 min (hence the alternative name of "cardiotoxins" for this toxin group).[35] They bring about depolarisation of the cardiac muscle, a phenomonon that can also be observed with smooth and skeletal muscle.[35,172] Since this requires an enhanced permeability of the membranes to calcium ions, the cytotoxins may be binding to some moiety associated with calcium movement or storage.

As has been shown in Figure 6, many of the residues uniquely associated with the cytotoxin group occupy positions in loops 2 and 3 and thus could form a binding site analogous to the one proposed for the neurotoxins.

In a similar manner to the neurotoxins, the residues associated with cytotoxicity have been examined for possible mimics of typical receptor antagonists. This is a speculative approach given that no common target is identifiable, but it has been suggested that if the muscarinic cholinergic receptor is involved,[173] a mimic of atropine might be identifiable.[15] Recent findings, however, have discounted involvement of this receptor in the action of cytotoxins CT 05 and CT 06.[172]

The information from solution studies, though lacking in comparison to neurotoxins, suggests that despite a relatively unstable superstructure, cytotoxins may have the familiar sensitive conformational equilibrium which can be perturbed by the modification of supposedly functional residues (i.e., the methionines). No other data is available to support this, but overall, the principles involved in cytotoxin action are expected to be like those of neurotoxin action, even to the extent of requiring an evolutionary relationship between the respective targets.

VI. CONCLUSION

This review of the conformational properties of snake neurotoxins and cytotoxins has drawn attention to several important principles, some of which do not apply solely to these proteins.

In general, it has shown that X-ray data alone, although highly informative, is not necessarily a sufficient basis for interpreting modes of action. In some cases, crystallization may require media capable of perturbing the native conformation, so parallel NMR and CD studies in such media, and in physiological media, should be performed for comparison.[174] Once the solution and crystal data have been matched, it can be relatively straightforward to ascertain the effects of chemical and physical modification by monitoring the CD and NMR spectra. The use of CD deserves special emphasis because it can provide a useful link between the overall conformation as deduced by X-ray crystallography and the detailed information on side chain environments from NMR.[76]

As regards the toxins, there is a wealth of detail that reflects the current interest. The solution studies have verified the overall accuracy of the crystal structures of erabutoxin

b and α-cobratoxin, but they have nevertheless shown important differences to the native configurations which have been induced by the crystallizing conditions. Most notably, they have detected a delicate conformational balance whose integrity is intimately linked with the functional residues.

The interaction of a toxin with its receptor has been compared to the interaction of a proteinase inhibitor with its enzyme,[36] but the conformational properties of the toxins suggest that there are important differences. Whereas the inhibitor has its disulphide bridges uniformly distributed throughout its structure, it is fundamental to the toxins that the four invariant bridges are clustered at one end of the molecule (Figures 8 and 14). Indeed, it is the only major feature to be found consistently in all the toxin types. Since it seems probable that this arrangement confers the potential for the defined conformational changes,[149] the latter are seen to be directly implicated as underlying the receptor binding mechanisms.

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