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Photochemically Induced Nuclear Polarization Study of Exposed Tyrosines, Tryptophans, and Histidines in Postsynaptic Neurotoxins and in Membranotoxins of Elapid and Hydrophid Snake Venoms†

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ABSTRACT: The accessibility of surface tyrosines, histidines, and tryptophans in snake venom neurotoxins (short and long) and in membranotoxins to excited triplet 10-(carboxyethyl)-flavin was studied by photochemically induced dynamic nuclear polarization at 270 MHz. Trp-29 is accessible in the short neurotoxins—erabutoxins a, b, and c and cobrotoxin—and also in the long neurotoxins—α-cobratoxin and α-bungarotoxin. Tyr-25 is practically inaccessible in all neurotoxins. Tyr-39 in cobrotoxin and Tyr-55 in α-bungarotoxin are accessible. His-6 (revised sequence) is inaccessible in the erabutoxins while His-26 is only very weakly accessible. His-22 of α-cobratoxin is inaccessible as are His-4 and -68 in α-bungarotoxin and His-4 of cobrotoxin. His-33 of cobrotoxin is accessible. The rigidity order α-bungarotoxin ≥ α-cobratoxin ≥ erabutoxins, with respect to the unfolding effect of 7 M urea, was deduced in this study. In the membranotoxins studied (cardiotoxin and its analogues I, II, and IV as well as cytotoxin I and II), the two tyrosines Tyr-25 and Tyr-58 are only weakly accessible. Tyr-14 is completely accessible and so is in all probability Tyr-29. These studies allow deductions to be made about the accessibilities in analogous systems. Thus, the accessibility of His-33 and the inaccessibility of His-4 in cobrotoxin can be used to deduce the conformations of these residues in a large group of neurotoxins including the α-toxin of *Naja nigricollis*, neurotoxin II of *Naja naja oxiana*, and neurotoxins I and III of *Naja mossambica mossambica*. As a generalization of this study, we suggest that in snake venom toxins residues distant only one or two peptide units from a disulfide bond are inaccessible, while more distant residues have accessible side chains. Significant differences were observed between solution and crystal conformations in α-bungarotoxin and α-cobratoxin.

The solution conformations of snake venom toxins (Tu, 1977; Yang, 1978; Karlsson, 1979; Low, 1979; Strydom, 1979) are of obvious importance in determining their physiological effects, their binding at the target organs, and their inactivation by specific immunoglobulins (Boquet, 1979). Very recently, much detailed information on many aspects of solution conformations of the snake venom toxin proteins has been provided by high-resolution nuclear magnetic resonance studies [see, e.g., Arseniev et al. (1981), Endo et al. (1982), Miyazawa et al. (1983), Steinmetz et al. (1981), Fung et al. (1979), and Hider et al. (1982)]. These studies considered parameters such as pH, nuclear proximity, and inter peptide residue interactions, temperature, and group mobility, as well as effects of chemical modifications and their influence on chemical shifts, signal structure, and exchange rates.

In this paper we describe a different approach to the study of solution conformations of these molecules. This approach is based on the application of the triplet-dye-induced nuclear polarization method (protein photo-CIDNP)[1] [see, e.g., Muszkat et al. (1981, 1983) and Berliner & Kaptein (1981) and references cited therein] to the study of surface conformations and surface residue exposure of the snake venom toxins.

Briefly speaking, the protein photo-CIDNP method consists of a normal proton high-resolution NMR experiment modified by a simultaneous process of chemically reversible radical pair formation. The availability of suitable radical pair forming processes allows the application of this method to three peptide residues, tyrosine, histidine, and tryptophan. The radical pair formation process in question involves the reversible transfer of a hydrogen atom H from the aromatic hydroxyl of a tyrosine residue (AOH) or an imidazolyl NH of a histidine residue

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[1] Abbreviations: photo-CIDNP, photochemically induced dynamic nuclear polarization; SDS, sodium dodecyl sulfate.

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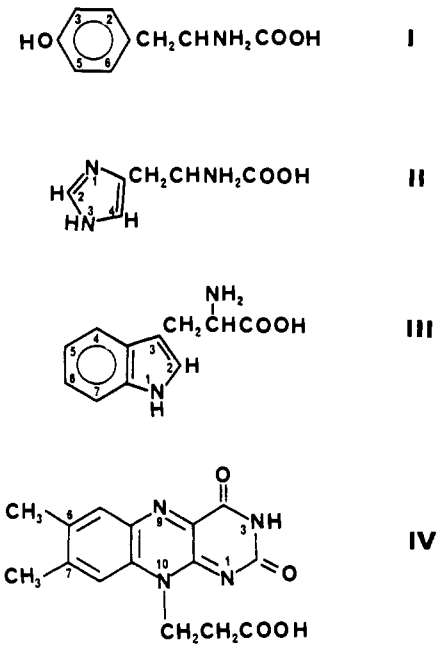

FIGURE 1: Numbering of protons in I–IV.

(BH) to a dye molecule D photoexcited to its triplet state (processes 1 and 2, respectively):[2]

$$AOH + {}^3D \rightarrow {}^3\overline{AO\cdot + DH\cdot} \rightarrow A^*OH + D \quad (1)$$

$$BH + {}^3D \rightarrow {}^3\overline{B\cdot + DH\cdot} \rightarrow B^*H + D \quad (2)$$

For tryptophan (C), there is a possibility of a parallel reversible radical pair formation process 3, involving electron transfer:

$$C + {}^3D \rightarrow {}^3\overline{C^{+}\cdot + D^{-}\cdot} \rightarrow C^* + D \quad (3)$$

Nuclear polarization (CIDNP) results from the operation of a magnetic nuclear spin selection mechanism [for reviews, see Muus et al. (1977)] in the radical pairs in processes 1–3. It is observable as either positive or negative enhancement of signals (often very substantial). Tyrosine C-3 and C-5 aromatic protons show large negative (emission) effects; histidine C-2 and C-4 imidazolic protons, as well as tryptophan C-2, C-4, and C-6 protons (Figure 1, I–III), show large positive enhancements. Substantial CIDNP effects are also observed in the methylene protons.

Nuclear polarization[3] is limited to those substrate molecules that participate in processes 1–3 and, as such, can serve as a nuclear spin-label for these molecules. Because of the requirement of close approach of ^3D to its substrate for the transfer processes to take place,[4] nuclear polarization is an indicator for accessibility of residue to dye.

Thus for conformational studies involving these three residues photo-CIDNP offers very substantial advantages: higher sensitivity than NMR, chemical and labeling specificity, and surface exposure dependence. Considering the special roles of the three susceptible residues, these advantages can often outweigh the limitation imposed by the small number of residues showing photo-CIDNP effects. As in our other recent studies (Muszkat et al., 1982a,b), the triplet dye accessibility probe used in this work was 10-(carboxyethyl)flavin, introduced for protein photo-CIDNP work by Lerman & Cohen (1980). This flavin derivative was chosen because of its significant long-term light stability.

Three groups of venom toxins of Asian Elapid and Hydrophid snake species are included in our study.

(*a*) *Short (Group I) Neurotoxins.* We have examined the three erabutoxins a and b (Sato & Tamiya, 1971) and c (Tamiya & Abe, 1972), all isolated from the venom of the sea snake *Laticauda semifasciata*,[5] and cobrotoxin (Yang et al., 1969), the neurotoxin principle from the venom of the Formosan cobra (*Naja naja atra*). These toxins have 62 residues and four disulfide-bridged loops.

(*b*) *Long (Group II) Neurotoxins.* We have examined two members of this group, α-cobratoxin (*Naja naja siamensis* neurotoxin; Arnberg et al., 1973) isolated from the venom of the Thailand cobra and α-bungarotoxin (Mebs et al., 1972) obtained from the venom of the Formosan banded krait *Bungarus multicinctus*. These toxins have five disulfide-bridge loops and contain respectively 71 and 74 peptide residues. In addition to the *N. naja siamensis* α-cobratoxin, we have also examined the α-cobratoxin isolated from the venom of the Indian cobra *Naja naja kaouthia* (Karlsson & Eaker, 1972; K. Hayashi, unpublished results). This neurotoxin and *N. naja siamensis* α-cobratoxin have identical amino acid compositions, differing from Indian *Naja naja* toxin C by substitution of Asn for Asp in residues 65 and 67.

(*c*) *Cardiotoxins and Cytotoxins (Tu, 1977; Yang, 1978; Karlsson, 1979).* Four cardiotoxins isolated from the venom of the Formosan cobra *N. naja atra*, cardiotoxin (Narita & Lee, 1970) and cardiotoxin analogues I, II, and IV (Hayashi et al., 1975; Kaneda et al., 1976a,b), were investigated. The *N. naja atra* cardiotoxin analogue III sample (Hayashi et al., 1972), which is identical with cardiotoxin, was also included.

In addition to these four toxins, we have also studied two toxins from the cytotoxin group, the India cobra *Naja naja* cytotoxin I and cytotoxin II (Hayashi et al., 1972; Takaechi & Hayashi, 1972). The six toxins in this group are all basic single-chain proteins consisting of 60 peptide residues cross-linked by four disulfide bridges to form four loops, as in the short neurotoxins.

Despite the overall structural similarity suggested by the alignment of Table I, the snake venom categories included in this study differ in important details of their covalent structure as well as in their physiological activities (Tu, 1977; Yang, 1978; Karlsson, 1979). While the short and long neurotoxins have potent postsynaptic acetylcholine receptor blocking (curaremimetic) activity, this activity is very much less pronounced in the cardiotoxins and cytotoxins series. On the other hand, the cardiotoxins and cytotoxins show strong membranotoxic activities absent in the neurotoxin series: the cardiotoxins have a cardiotoxic activity (causing systolic arrest) and the cytotoxins exert strong cytolytic activity (assayed, e.g., toward Yoshida sarcoma cells or ascite hepatoma cells) (Tu, 1977; Karlsson, 1979).

Finally, it is important to stress at this stage the considerable value of studying series of closely related toxin molecules. This

[2] The horizontal bar denotes an electron spin correlated radical pair. [For reviews, see Muus et al. (1977).] The asterisk (*) denotes nuclear polarization.

[3] We refer here to primary nuclear polarization as opposed to secondary [transfer, or cross-polarization; cf., e.g., Berliner & Kaptein (1981)]. These two effects are easily distinguishable because of their different time evolutions.

[4] Close approach of dye to substrate is also required by electron transfer as indicated by the absence of tryptophan nuclear polarization of Trp-86 in *Streptomyces subtilisin* inhibitor (Akasaka et al., 1981). Viscosity-dependence studies of ^3D quenching rates point to a similar conclusion (K. A. Muszkat and T. Wismontski-Knittel, unpublished results).

[5] The previous amino acid sequence of the three erabutoxins at positions 6, 7, 18, and 19 (Gln^6-His^7 and Pro^{18}-Ser^{19}) has been revised after the completion of this study to His^6-Gln^7 and Ser^{18}-Pro^{19} (S. Nishida, Y. Kokobun, and N. Tamiya, unpublished results).

approach was adopted in this study and proved invaluable for establishing spectral assignments and analyzing accessibility results.

Experimental Procedures

The three erabutoxins were obtained in the laboratory of N. Tamiya, while all the other snake venom toxins used in this study originate from the laboratory of K. Hayashi (see the introduction for references to original literature). Prior to the photo-CIDNP measurements, a check on the purity of these toxins was carried out by disc SDS–polyacrylamide gel electrophoresis. The sample of 10-(carboxyethyl)flavin (CE) used was kindly provided by Dr. D. Porter, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA. The photo-CIDNP experiments were carried out at 270 Mhz on the previously described set up (Muszkat et al., 1982, 1983a,b), which consists of a WH270 Bruker spectrometer and a mercury–xenon 5000-W compact arc lamp used as a light source. The parallel light beam emerging from the lamp is filtered by a 23 cm long water filter to remove any IR component, reflected by 90°, and focussed into a vertical light guide (quartz) mounted rigidly inside the normally spinning 5-mm sample tubes. For a more detailed discussion of the optical irradiation method, see Muszkat et al. (1984). The dye is largely stable under the present irradiation conditions. The lamp shutter in these experiments is controlled by the spectrometer's computer. Light (e.g., photo-CIDNP) spectra are obtained by a Fourier-transform procedure modified so that each free-induction decay acquisition is preceded by a 15-s cooling delay followed by a 0.3–0.4-s optical irradiation, followed in turn by a magnetic relaxation delay D_3, usually 0.05 s but increased to 0.3–0.5 s when searching for polarization-transfer effects. The samples of the snake venom neurotoxins (3×10^{-4} M in ^2H_2O) contained 4,4-dimethyl-4-silapentane-1-sulfonate (DSS, 3×10^{-4} M) used as a chemical shift ($\delta = 0$) and intensity standard. The concentration of the flavin dye (CE) was 3×10^{-4} M. [$^2H_4$]Urea (98 atom %, Aldrich Chemical Co., Milwaukee, WI) at 7 M concentrations was added for exploring unfolding effects.

Results and Discussion

The snake venom toxins were studied under three sets of conditions: in neutral solutions, at pH 7 or slightly above; in acid solutions, slightly above pH 4; and in 7 M urea solutions at pH 7. In general, all exposed (and CIDNP-susceptible) residues show polarization at pH 7 while at pH 4 in histidines no polarization is observed, because of protonation of the imidazolic ring at N-1. Thus, measurements at the acid pH should be indicative of histidine signals appearing in the neutral solutions. The measurements in the presence of 7 M urea were intended to provide qualitative data on the ease of unfolding of the peptide chain by breaking of the hydrogen-bond network. Figures 2 and 3 show the light-minus-dark difference (^1H, 270-MHz) spectra of the aromatic protons under the indicated conditions.

(*I*) *Neurotoxins.* (*1*) *Tryptophan-29.* We find that these invariant residues of neurotoxins are decidedly accessible to our triplet dye probe in all short and long neurotoxins examined. These residues are responsible for the prominent and characteristic tryptophan polarization pattern observed in Figures 2 and 3, e.g., signals b–d in Figure 2 (spectra 1A, 3B, 4A, 4B, 6B, and 7B). These signals are all strongly enhanced and are due to a primary CIPNP effect. Signal d is always a singlet, and b is a doublet (cf. spectrum 2B), while c is a multiplet (triplet). The weaker signals a and e (e.g., spectra

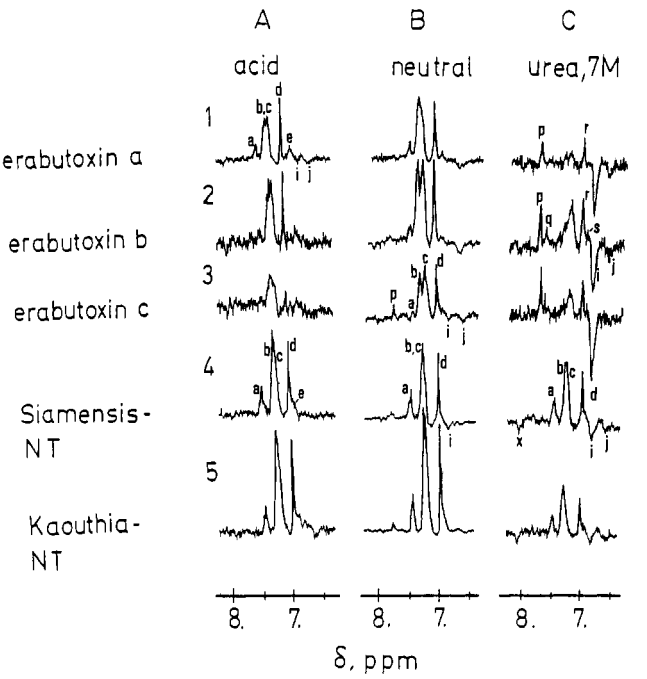

FIGURE 2: The 270-MHz proton photo-CIDNP difference spectra (light minus dark) of snake venom neurotoxins (aromatic proton region) ^2H_2O solutions in the presence of 0.4 mM 10-(carboxyethyl)flavin and of 0.4 mM DSS as intensity and $\delta = 0$ standard. Concentrations of neurotoxins in 1, 2, and 3 are 0.6 mM and in 4 and 5 are 1.2 mM. pH values (uncorrected) are as follows: 1A, 2A, and 3A, 4.9; 4A, 4.6; 5A, 4.8; 1B and 3B, 7.9; 2B and 4B, 7.5; 5B, 7.6; 1C and 2C, 8.0; 3C, 8.2; 4C, 7.3; 5C, 7.4. Light and dark spectra obtained from 60 accumulated free-induction decay acquisitions. Optical irradiation was 0.4 s. Assignments are as follows. Trp-29 signals: a, C-7; b, C-4; c, C-6; d, C-2; e, C-5. Tyr-25 signals in 3B: i, C-2 and C-6 protons; j, C-3 and C-5 protons. Tyr-25 signals in the presence of 7 M urea: i, C-3 and C-5 protons; j, C-2 and C-6 protons; (histidines) p and r, C-2 and C-4 protons, respectively, of His-6; q and s, C-2 and C-4 protons, respectively, of His-26.

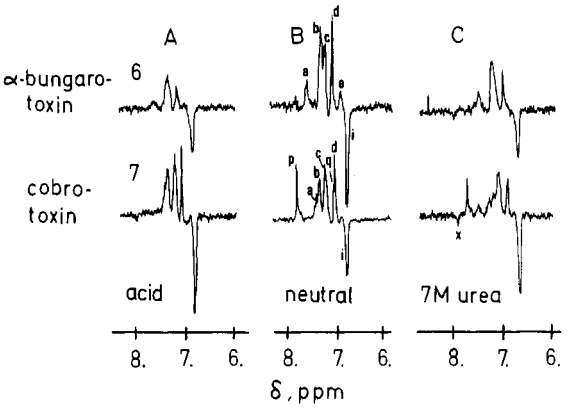

FIGURE 3: Aromatic proton region of 270-MHz photo-CIDNP difference spectra of two-tyrosine snake venom neurotoxins. For details, cf. caption to Figure 2. Concentrations were as follows: in 6, 1.2 mM; in 7 1.5 mM. pH values were as follows: 6A, 4.9; 6B, 7.5; 6C, 7.3; 7A, 4.8; 7B, 7.6; 7C, 7.2. Assignments are as follows. Trp-29 signals: a, C-7; b, C-4; c, C-6; d, C-2; e, C-5. i: C-3 and C-5 protons of Tyr-55 in 6B and of Tyr-39 in 7B. p and q in 7B: His-33 C-2 and C-4 protons, respectively.

1A, 3B, 4A, 4B, and 6B) are also due to the same Trp residues. These signals are positively enhanced but are due to a polarization-transfer (e.g., secondary) effect as shown by the results of Figure 4, obtained with Siamensis neurotoxin (NT) in 7 M urea. In this case, a post optical irradiation delay D_3 of 0.3 s (see Experimental Procedures) results in the growing-in of the a and e components of spectra 4A and 4B. The exact assignment of signals a–d is based on the previous NMR

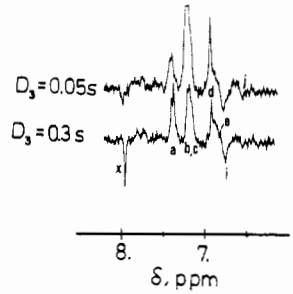

FIGURE 4: Trp-29 polarization-transfer effects in Siamensis NT in the presence of 7 M urea. Top spectrum identical with 4C of Figure 2. Bottom spectrum obtained under the same conditions, but post-optical irradiation delay D_3 is 0.3 s.

studies, making use of the information available from the CIDNP pattern mentioned above [see, e.g., McCord et al. (1981)] for discerning between the C-4 and the C-7 doublets and between the C-5 and C-7 triplets. We assign d to the C-2 proton, c (triplet in spectrum 3B) to the C-6 proton, and b (doublet in 3B and 7B) to the C-4 proton. These protons are expected to show a direct CIDNP effect. Protons expected not to show direct effects are C-7 and C-5 [see, e.g., McCord et al. (1981)]. Thus, the a doublet (spectra 1A and 4B) is unambigously assigned to the C-7 proton, and the e multiplet is assigned to the C-5 proton. These results differ somewhat from previous assignments and resolve usual ambiguities in the assignment of the components of the C-4/C-7 pair (and of the C-5/C-6 pair) in the erabutoxins (Inagaki et al., 1978), in Siamensis NT (Hider et al., 1982), in cobrotoxin (Fung et al., 1979; Endo et al., 1979), and in α-bungarotoxin (Endo et al., 1981) and could probably apply to other analogous cases as well, e.g., to the *Laticauda semifaciata* long neurotoxin III (Inagaki et al., 1981), to the *Naja naja oxiana* neurotoxins II and III (Arseniev et al., 1976, 1981; Bystrov et al., 1978), etc. Surprisingly enough, the present assignment of Trp-29 proton NMR signals is the same as the assignment deduced for Trp-14 protons in *Naja mossambica mossambica* cardiotoxin V^{II} 2 (Steinmetz et al., 1981). The present definitive conclusion about the accessibility of Trp-29 in snake venom neurotoxins [even to such a large probe as 10-(carboxyethyl)flavin] fits nicely previous deductions by other techniques, such as NMR titration [e.g., Inagaki et al. (1978)] and chemical modification [e.g., Tu (1977) and Yang (1978)]. The chemical shift of the C-6 proton and the pattern of the b–c–d signal system (at pH 7) allows us to divide the neurotoxins of Figures 2 and 3 into two groups. Group A contains neurotoxins in which signals b and c are clearly resolved at pH 7: erabutoxins b and c (spectra 2B and 3B) and α-bungarotoxin and cobrotoxin (spectra 6B and 7B). Group B contains neurotoxins having signal c merged with signal b, i.e., erabutoxin a and Siamensis (and Kaouthia) NT. This different pattern is clearly due to a relative shielding (upfield)–deshielding (downfield) effect on the C-6 proton (signal c) in group A. The sequence data of Table I indicate that the origin of the difference is traceable to residue 26. In group A, this residue has always a positively charged (basic) group (His-26 in erabutoxins b and c, Lys-26 in cobrotoxin, and Arg-26 in α-bugarotoxin) while in group B residue 26 has a neutral side chain (Asn-26 in erabutoxin a and Thr-26 in Siamensis NT). The crystal structure of erabutoxin b (Kimball et al., 1979) (if retained in solution by this part of the molecule) would suggest that any interaction between Trp-29 and the side chain of residue 26 should take place through intermediary residues such as Glu-42, Asp-42, Lys-27, etc. The merging of signal c with b going from pH 7 to 4 (2B and 2A, 3B and 3A, 6B

Table I: Peptide Residue Sequences of Snake Venom Toxins[a] Included in This Study, Aligned according to Karlsson (1973)

	1	10	20	30	40	50	60	70
α-cobratoxin	IRCF--ITPDITSKDCPNG-HVCYTKTWCDAFCSIRGKRVDLGCAATCPTVKTGVDIQCCSTDNCNPFPTRKRP							
α-bungarotoxin	IVCH-TTATIPSSAVTCPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPSKKPYEEVTCCSTDKCNHPPKRQPG							
erabutoxin a	RICFNHQSSQPQTTKTCSPGSESCYNKQWSD-F---RGTIIERGC--GCPTVKPGIKLSCCESEVCNH							
erabutoxin b	RICFNHQSSQPQTTKTCSPGSESCYHKQWSD-F---RGTIIERGC--GCPTVKPGIKLSCCESEVCNH							
erabutoxin c	RICFNHQSSQPQTTKTCSPGSESCYHKQWSD-F---RGTIIERGC--GCPTVKPGIMLSCCESEVCNH							
cobrotoxin	LECHNQQSSQTPTTTGCSGGETNCYKKRWRD-H---RGYRTERGC--GCPSVKNGIEINCCTTDRCNN							
cardiotoxin	LKC-N--KLVPLFYKTCPAGKNLCY-KMFMVA---TPKVPVKRGCIDVCPKSSLLVKYVCCNTDRCN							
cardiotoxin-a.I	LKC-N--KLIPIASKTCPAGKNLCY-KMFMNS---ELTIPVKRGCIDVCPKSNLLVKYVCCNTDRCN							
cardiotoxin-a.II	LKC-N--KLVPLFYKTCPAGKNLCY-KMFMVS---NLTVPVKRGCIDVCPKNSALVKYVCCNTDRCN							
cardiotoxin-a.IV'	RKC-N--KLVPLFYKTCPAGKNLCY-KMFMVS---NLTVPVKRGCIDVCPKNSALVKYVCCNTDRCN							
cytotoxin I	LKC-N--KLIPLAYKTCPAGKNLCY-KMYMVS---NKTVPVKRGCIDVCPKNSLVLKYECCNTDRCN							
cytotoxin II	LKC-N--KLVPLFYKTCPAGKNLCY-KMYMVA---TPKVPVKRGCIDVCPKSSLVLKYVCCNTDRCN							

[a]One-letter code: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Origin: erabutoxins, *L. semifasciata*; cobrotoxin, cardiotoxin, and cardiotoxin analogues, *N. naja atra*; cytotoxins, *N. naja*; α-bungarotoxin, *B. multicinctus*; α-cobratoxin, *N. naja siamensis*.

and 6A, 7B and 7A) supports such possibilities.

(*2*) *Tyrosine-25 and Other Tyrosines.* The structurally essential residue Tyr-25 (invariant in all snake venom postsynaptic neurotoxins), as well as other tyrosines, is obviously amenable to investigation by the CIDNP method. The results of Figure 2 show quite conclusively that (on the time scale of the creation of nuclear polarization effects) Tyr-25 is practically inaccessible to a probe of the size of 10-(carboxyethyl)flavin. Very weak signals (e.g., i and j in spectrum 3B) are, however, observable, indicating very slight accessibility [for signal pattern of accessible tyrosines see, e.g., Muszkat et al. (1981)]. Signal i should be assigned to aromatic protons C-2 and C-6 and j to protons C-3 and C-5. In the presence of 7 M urea, the three erabutoxins undergo sufficient unfolding to render Tyr-25 accessible (spectra 1C, 2C, and 3C). In the unfolded neurotoxins, the assignment of i and j should be however reversed according to the CIDNP pattern: i (spectrum 2C) should be assigned to the C-3 and C-5 protons and j to the C-2 and C-6 protons, as i is more intense than j. Attempted unfolding of α-cobratoxin (Siamensis or Kaouthia NT) in the presence of 7 M urea (spectra 4C and 5C) reveals that this neurotoxin is resistant to such perturbation, at least in the microenvironment of Tyr-25. This clear lack of accessibility of Tyr-25 to flavin triplets observed in this study for α-cobratoxin and deduced in all certainty for α-bungarotoxin implies that previous conclusions to the opposite reached on the basis of results of nitration, iodination, and protonation reactions [see, e.g., Karlsson (1979)] should be attributed largely to the small size of the reactive species. On the basis of the present results for the exposure of Tyr-25 in the erabutoxins and in α-cobratoxin, we can interpret now the results obtained for the two-tyrosine neurotoxins α-bungarotoxin and cobrotoxin. We use the accessibility data obtained from the short neurotoxins (erabutoxins) to analyze the accessibility of Tyr-25 in cobrotoxin, and similarly, the accessibility of Tyr-25 in α-bungarotoxin is analyzed on the basis of the results for Tyr-25 in α-cobratoxin. We thus come to the conclusion that in solution the additional tyrosine residues, Tyr-55 in α-bungarotoxin and Tyr-39 in cobrotoxin, are clearly accessible to the present probe. The fact that the negative signal i (spectra 6B and 7B) is narrow and resolved is an indication that it is due to a single tyrosine, i.e., that Tyr-25 remains inaccessible in these two neurotoxins. These conclusions agree with the assignments of regular NMR spectroscopy (Fung et al., 1979; Endo et al., 1981). Tyr-25 is accessible in the crystal conformation of α-bungarotoxin (Stroud, 1981; Agard & Stroud, 1982). Thus, this toxin probably assumes different conformations in solution and in the crystal.

Cobrotoxin is closely related to the well-studied neurotoxin I of *N. mossambica mossambica* (Lauterwein et al., 1978). Thus, the conclusion about the accessibility of Tyr-39 in cobrotoxin should apply as well to neurotoxin I. Similarly, our conclusion about the accessibility of Tyr-55 in α-bungarotoxin should apply as well to Tyr-55 in toxin 3.9.4 of *Naja melanoleuca* and in neurotoxin I of *N. naja oxiana* [cf. Yang (1978); Table I].

(*3*) *Histidines.* (*A*) *Erabutoxins.* The three erabutoxins have a common His-6 residue (revised sequence; cf. footnote 5). In erabutoxin a, this is the only histidine. Erabutoxins b and c have, however, an additional histidine, His-26. The lack of histidine polarization in spectra 1B and 2B is thus an indication that His-6 is inaccessible in general. (See below for the case of erabutoxin c.) Lack of accessibility of both His-6 and His-26 is indicated by the absence of histidine polarization in 2B. Unfolding with 7 M urea renders His-6 accessible in erabutoxin a (signals p and r in spectrum 1C). In erabutoxins b and c unfolded with 7 M urea, signals q and s are thus due to His-26. These signals are weaker than the pair p and r due to His-6. Thus, we conclude that in unfolded erabutoxins b and c (but not in the natural neurotoxin) His-26 is more effectively covered and even less accessible than is His-6. Signal p in spectrum 3B of natural erabutoxin c should be assigned to a marginally accessible His-26, on the basis of the NMR data for erabutoxin b (Inagaki, 1978). The present results fully confirm previous conclusions of Inagaki et al. (1981) about the lack of accessibility of His-6 (previously designed as His-7) in aqueous solutions. In the crystal of erabutoxin a the residue at position 7 now known to be Gln-7 is a clearly accessible residue (Tsernoglou & Petsko, 1976; Kimball et al., 1979). Thus the previous discrepancy between the X-ray structure and the solution accessibility results is resolved for this histidine.

(*B*) *α-Cobratoxin (Siamensis and Kaouthia NT) and α-Bungarotoxin.* According to the results of Figure 2, the single histidine-22 of both Siamensis NT and of Kaouthia NT α-cobratoxin is clearly inaccessible in the physiological-state (pH 7) conformation [cf. Hider et al. (1982)] and also in the presence of 7 M urea (spectra 4A–C and 5A–C). This last result implies (when taken together with the results for Tyr-25 accessibility) that the natural conformation is retained in the presence of 7 M urea in the region of both His-22 and Tyr-25. As in the previous case of the erabutoxins, the present results for α-cobratoxin allow one to establish a clear-cut difference between the crystal conformation (Walkinshaw et al., 1980) in which His-22 is completely exposed and the "physiological" state in aqueous solution in which His-22 is inaccessible. Thus, we can fully confirm the conclusion of Hider et al. (1982) to the same effect, reached on the basis of CD and NMR measurements. Our results indicate that the two histidines of α-bungarotoxin, His-4 and His-68, are inaccessible in aqueous solution (spectrum 6B) as well as in the presence of 7 M urea (spectrum 6C). Both His-4 and His-68 are exposed surface residues in the crystal of α-bungarotoxin (Stroud, 1981; Agard & Stroud, 1982). We thus have here a case of difference of crystal and solution conformations.

(*C*) *Cobrotoxin.* Of the two histidines of cobrotoxin, His-4 and His-33, only one is clearly accessible, as may be seen from spectrum 7B. In this spectrum, only one histidine shows the typical pair of strongly enhanced singlets (p and q). According to the chemical shifts of these protons (C-2 and C-4 imidazole protons; Endo et al., 1979), these are assigned unequivocally to His-33 at the center of the active segment Asp^{31}-Arg^{37}. His-4 is thus clearly inaccessible. This situation remains unchanged in 7 M urea solutions, reflecting the resistance of the hydrogen-bond network to unfolding. The same conclusion about the accessibility of His-33 and the lack of accessibility of His-4 can be also reached on the basis of structural analogy with α-bungarotoxin, where His-4 is inaccessible, as was seen above.

The simultaneous observation of nuclear polarization in the three residues tyrosine, histidine, and tryptophan (Tyr-39, His-33, and Trp-29) in cobrotoxin at neutral pH is of considerable experimental significance beyond that of the present context of neurotoxin conformations. The implication of this situation is that the reactivities of the three accessible residues (in the initial steps of processes 1–3) are sufficiently similar[6]

[6] This is also the conclusion of the flash photolysis study of the quenching of the 10-(carboxyethyl)flavin triplet by these and by other side chains (K. A. Muszkat and T. Wismontski-Knittel, unpublished results).

so that absence of nuclear polarization in an existing Tyr, His, or Trp residue has to be attributed to lack of accessibility and not to competition in the abstraction step of 1, 2, or 3.

(4) General Conclusions on the Conformation of Postsynaptic Neurotoxins. Histidine-33 of cobrotoxin is an invariant residue of the short neurotoxins, conservatively substituted with Phe in the erabutoxins and long neurotoxins. Position 4 in many short and long neurotoxins is also conservatively substituted in many of these neurotoxins with His, Tyr, or Phe. Thus, the accessibility data for positions 4 (inaccessible) and 33 (accessible) in cobrotoxin can be applied to deduce the conformation at one or both of these positions in some thirty neurotoxins (and to some extent in membrane toxins as well). Some closely related and well-studied neurotoxins for which these deductions should apply are thus neurotoxin II of *N. naja oxiana* (Arseniev et al., 1976), α-toxin of *Naja nigricollis*, and neutoroxins I and III of *N. mossambica mossambica* (Arseniev et al., 1981; Lauterwein et al., 1978). Residue 33, which occupies the tip of the reactive charge pair segment Arg^{37}–Asp^{31}, is a phenylalanine in the erabutoxins. We thus suggest that in analogy with His-33 in cobrotoxin this residue is exposed in the erabutoxins, to be expected of a residue on an active segment of a loop. The fact that His-33 is the only frankly exposed histidine in these series is to be attributed to that special position at the active tip. Comparing the effect of 7 M urea on the neurotoxins of Figures 2 and 3 reveals some interesting differences between the eratuboxins and the other neurotoxins. Thus, 7 M urea can unfold the three erabutoxins and expose their tyrosine and histidine residues. The effect of 7 M urea on the *N. naja siamensis* or *N. naja kaouthia* α-cobratoxin is almost imperceptible, being limited to a small increase in the intensity of the C-3 and C-5 proton signals of Tyr-25. In α-bungarotoxin and in cobrotoxin no effects of 7 M urea could be observed. These results seem to conform to the trends of the values of association and dissociation rate constants of the neurotoxins with the acetylcholine receptor, which are larger in the short neurotoxins than in the long neurotoxins (Chicheportiche et al., 1975). This difference is reflected in the more pronounced lack of reversibility of the neuromuscular blocking action of the long neurotoxins (Lee & Chen, 1976) and can be correlated with molecular rigidity as judged by the values of the rates of $^1H/^2H$ exchange (Endo et al. 1981). The differences detected in the present study, leading to the ridigity relationship α-bungarotoxin $\geq$ α-cobratoxins > erabutoxins, largely correlate with the order deduced by Chicheportiche et al. (1975).

Finally, the possibility of aggregation of toxins in these series as well as in the membranotoxins (see next section) under the present conditions seems to be largely ruled out by the limited effects of pH changes on the CIDNP pattern of tyrosines (and of tryptophans as well). In particular, no CIDNP signals disappear on going from acid to neutral or basic pH (cf. e.g., spectra 4A and 4B, 5A and 5B, 6A and 6B, and 7A and 7B).

(II) Membranotoxins. The gross structure of the membranotoxins (see Table I) bears much similarity to that of the neurotoxins. Within the membranotoxin series there is much common covalent structure. However, within the context of this work, a number of important covalent structure elements distinguish membranotoxins from neurotoxins: Phe-29 in the cardiotoxins, Tyr-29 in the cytotoxins, and other substitution replace the invariant Trp-29 of neurotoxins. Tyr-25 is retained. Residue 58 is almost always a tyrosine. Residue 14 is often a tyrosine (in the membranotoxins of Table I) or a tryptophan. Histidines are absent in *N. naja atra*, *N. naja*, and *N. mossambica mossambica* membranotoxins. The other most salient

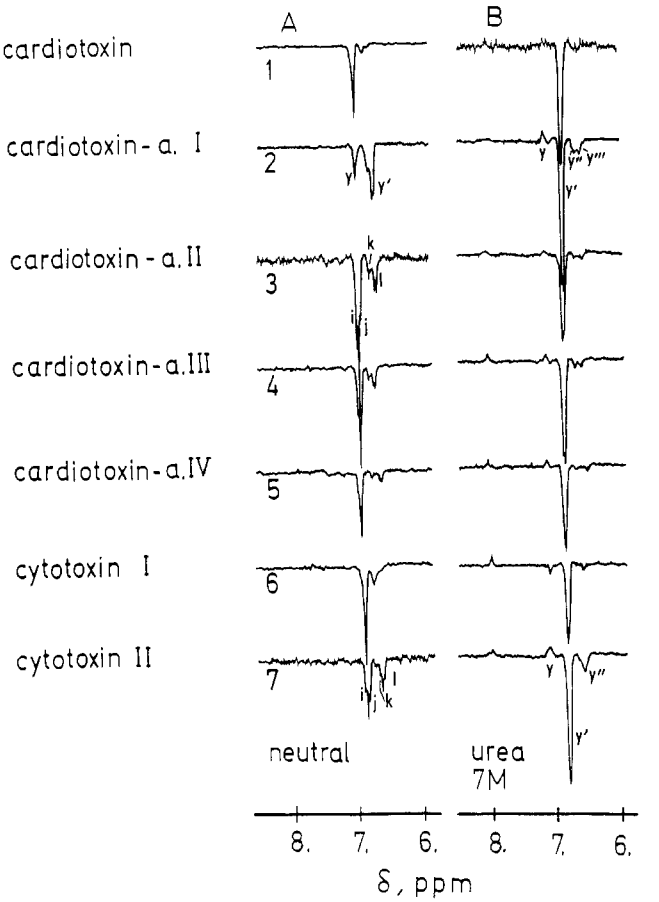

FIGURE 5: The 270-MHz photo-CIDNP difference spectra of aromatic protons of tyrosines of snake venom membranotoxins. Conditions are as in Figures 2 and 3. Concentration was 1.5 mM. pH values were as follows: 1A, 7.6; 2A–6A, 7.3; 7A, 7.1; 1B, 7.9; 4B, 7.5; 2B, 3B, and 5B–7B, 7.3. Sensitivity in 3A, 7A, and 1B is twice that in other spectra. Temperature in 2A, 3A, and 5A is 37 °C but otherwise is 25 °C. See text for assignment.

and persistent features of the structure of membranotoxins are the basic residues, e.g., lysines at positions 8, 15, 21, 27, 42, 51, and 57, arginines at positions of 43 and 65, and the absence of the Asp^{31}–Arg^{37} charge pair at the tip of the active loop. These features, however, are not directly treatable by the present experimental approach. We shall start our analysis of the photo-CIDNP spectra of membranotoxins by considering the photo-CIDNP effects in cardiotoxin analogue I (Figure 5, spectrum 2A). In this toxin as well as in the other members of this series, only tyrosine polarization can be expected. We select this toxin to start our analysis as it contains only two tyrosines, Tyr-25 and Tyr-58. The two negative signals y and y′ are only partially resolved. The single sharp negative signal y is due to the merging of two overlapping doublets, with centers separated by ca. 6–8 hz. A similar situation holds for y′. We thus conclude that both Tyr-25 and Tyr-58 are accessible. However, as these signals are weaker than those in spectra 1A or 4A (due to a completely accessible tyrosine), we conclude that Tyr-25 and Tyr-58 are only partially accessible. These two findings are quite general for all membranotoxins of Figure 5. In these, Tyr-25 is weakly accessible while it is almost inaccessible in neurotoxins. This is an important structural difference between the two groups of toxins. The exact assignment of the C-2/C-6 and C-3/C-5 doublets of Tyr-25 and Tyr-58 to the y and y′ signals can be arrived at in the following way. As y′ is more intense than y, it should be (tentatively) assigned to the two C-3/C-5 doublets of Tyr-25 and Tyr-58. Thus, y is to be assigned to

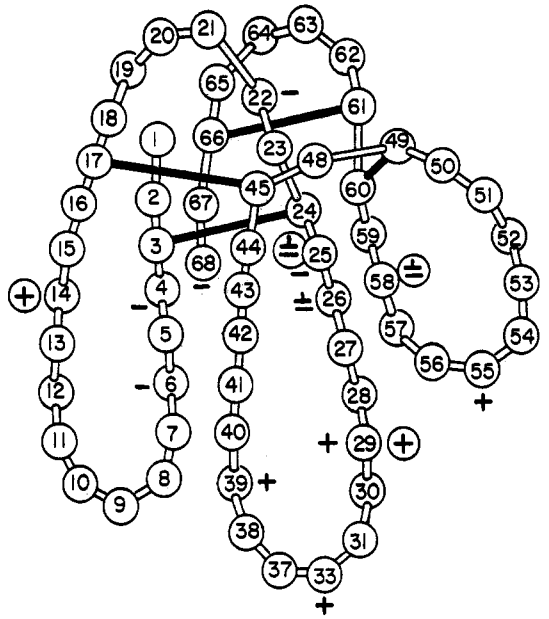

FIGURE 6: Schematic representation of conformation of snake venom toxins. Accessibility of side chains of tyrosines, tryptophan, and histidines is denoted by pluses (+). Partial accessibility is denoted by (±) and lack of accessibility by (−). Circles denote results in membranotoxins. Numbering is that of Karlsson (1979). Main-chain conformation is as in erabutoxin b crystal.

the two overlapping C-2/C-6 doublets. These usually have either small positive polarization or no polarization at all. In the present case, the observed negative effect on y is due to intramolecular cross-polarization. However, this cross-polarization takes place during optical irradiation (0.3-s duration), e.g., is indetectable by prolonging the post optical irradiation delay to 0.3-s. This situation is observed for cardiotoxin analogues I, II, and III (≡cardiotoxin) at pH 7.3, 37 or 25 °C.

As far as the two tyrosines Tyr-25 and Tyr-58 are concerned, cardiotoxin analogue I bears a close relationship to the *N. mossambica mossambica* cardiotoxin V^{II} 2. The present assignment of y to the two C-2/C-6 pairs and of y′ to the two C-3/C-5 proton pairs of Tyr-25 and Tyr-58 gives a pattern similar to that observed in cardiotoxin V^{II} 2 (Steinmetz et al., 1981), thus indicating significant structural resemblance in these regions of the cardiotoxin series.

All the other *N. naja atra* cardiotoxins, e.g., cardiotoxin (and cardiotoxin analogue III), as well as cardiotoxin analogues II and IV, have a third additional tyrosine, Tyr-14, in the first loop (Cys^3–Cys^{24}). Examination of spectrum 1A (and 4A) as well as of spectra 3A and 5A reveals outright that this residue is completely accessible to our probe as a new high-intensity component contributes to the low-field signal y of these spectra, at ca. δ 6.8. This component is assigned to the C-3/C-5 pair, while the signals due to the C-2/C-6 pair are in all probability very weak, occurring at a still lower field as in a completely accessible tyrosine.

The three residues Tyr-14, -25, and -58 are also common to the *N. mossambica mossambica* cardiotoxin V^{II} 4, studied by proton NMR by Lauterwein et al. (1977). This analogy seems sufficient to suggest that the present conclusion about the accessibility of Tyr-14 applies in the latter case too.

In the two *N. naja* cytotoxins (I and II), a fourth tyrosine, Tyr-29, is substituted for the Trp-29 of the neurotoxins and for Phe-29 occurring in the cardiotoxins of *N. naja atra*. Because of lack of sufficient resolution in the spectra of Figure 5 (6A and 7A), these results do not allow by themselves to conclude at present about the accessibility of this residue. However, the analogy with the neurotoxins of Figures 2 and 3 in which Trp-29 is always accessible would suggest that Tyr-29 is also accessible.

Conclusions

Summary of Flavin Triplet Accessibility Data in Neurotoxins and Membranotoxins. The results obtained in this study are summarized in Figure 6. Accessibility is denoted by (+), lack of accessibility is denoted by (−), and partial accessibility is denoted by (±). Circles indicate results obtained in the membranotoxins. The sequence is that of a short neurotoxin drawn schematically on the basis of the crystal structure of erabutoxin b according to Inagaki et al. (1978) and numbered according to Karlsson (1979). Full lines indicate disulfide bonds. The accessibility data shown in Figure 6 lead us to two new, though not entirely unexpected generalizations.

(a) Side chains situated more than two residues away from disulfide bonds and in particular at the tip of a loop are usually accessible. Into this category fall Tyr-14 in loop 1, Trp-29, His-33, and Tyr-39 in loop 2, and Tyr-55 in loop 3. The inaccessible His-6 (revised erabutoxin sequence) in loop 1 seems to be the only exception to this rule (cf. discussion above).

(b) Side chains of residues near cysteines of disulfide bonds are either inaccessible or at most partially accessible. To this group of residue belong (i) His-4 in loop 1 and Tyr-25 in loop 2, which are next to Cys-3 and Cys-24, respectively, (ii) His-26 on loop 2 and Tyr-58 on loop 3, which are one residue away from Cys-24 and Cys-60, respectively, and (iii) His-22 and His-68, close in space to Cys^{61}–Cys^{66} and to Cys^{24}–Cys^3 disulfide bonds, respectively. One of the reasons for the lack of accessibility in i–iii is the crowding around the disulfide bonds.

Implications of CIDNP Results to Studies of Binding Phenomena of Snake Venom Toxins. Taken as a whole (cf. Figure 6), the neurotoxins and membranotoxins are thus seen to possess a significant number of accessible residues amenable to detailed CIDNP–NMR studies, which can be carried out under very advantageous conditions of much higher sensitivity and specificity than ordinary NMR studies. One particular area that we would like to single out as likely to profit very much from these new experimental possibilities is the study of various binding and association processes of fundamental importance in understanding the toxicity of these toxins, their antigenic activity, their interaction with the specific antitoxin immunoglobulins, and the mechanisms responsible for phenomea of snake autoresistance to own toxins. At present, binding to the postsynaptic acetylcholine receptor protein is believed to take place at the concave surface of the toxin (i.e., inner surface of chalice) while the antigenic activity and the neutralization by specific globulin involve in all probability the convex surface and the carboxyl terminal [outer surface and foot of chalice structure: see, e.g., Stroud (1981), Boquet (1979), and Smythies (1978)]. Previous CIDNP studies of binding of small proteins such as the binding of proteinase inhibitors with serine proteinases (Muszkat et al., 1982, 1983) provide ample illustration of the possibilities offered by this approch.

Acknowledgments

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Registry No. Tyr, 60-18-4; Trp, 73-22-3; His, 71-00-1; α-cobratoxin, 69344-74-7; α-bungarotoxin, 11032-79-4; erabutoxin a, 11094-61-4;

erabutoxin b, 9083-23-2; erabutoxin c, 37357-76-9; cobrotoxin, 12584-83-7.

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