Perutz, M. F., Pulsinelli, P. D., & Raney, H. M. (1972) *Nature (London), New Biol. 237*, 259.
Pisciotta, A. V., Ebbe, S. N., & Hinz, J. E. (1959) *J. Lab. Clin. Med. 54*, 73.
Pulsinelli, P. D., Perutz, M. F., & Nagel, R. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3870.
Shimizu, A., Hayashi, A., & Yamamura, Y. (1965) *Biochim. Biophys. Acta 97*, 472.
Viggiano, G., & Ho, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3673.
Viggiano, G., Ho, N. T., & Ho, C. (1979) *Biochemistry 18*, 5238.
Wiechelman, K. J., Charache, S., & Ho, C. (1974) *Biochemistry 13*, 4772.

Conformation of Two Homologous Neurotoxins. Fluorescence and Circular Dichroism Studies†

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ABSTRACT: Two homologous short neurotoxins isolated from snake venoms (*Laticauda semifasciata* erabutoxin b and *Naja nigricollis* toxin α) have been studied by means of fluorescence spectroscopy and in aqueous solution at various pH values. In parallel experiments, the stability of toxin conformations was analyzed on the basis of ultraviolet circular dichroism. Total luminescence spectra (77 K) were recorded for both toxins in neutral and alkaline solutions. The data obtained indicate that, at neutral pH, the fluorescence emission is only due to the single and invariant tryptophan (29). From a comparative study with erabutoxin a, which differs from erabutoxin b by a single residue, it is unambiguously shown that the protonation of His-26 of erabutoxin b is responsible for a decrease of Trp-29 fluorescence. Also, on the basis of available X-ray data it is proposed that the protonation or deprotonation of the following titrable groups is responsible for an alteration of Trp-29 fluorescence. These are Asp-31 ($pK \simeq 4$) and Lys-27 ($pK = 9.6$) for both toxins and Lys-26 ($pK \simeq 9.6$) for toxin α. No tyrosinate emission can be observed at neutral pH and 77 K. Excitation spectra of toxin α revealed that 50% of the light absorbed by Tyr-25 in water is transferred to Trp-29. From the energy transfer measurements, the distance separating these two aromatic chromophores in the native toxin was estimated to be 13 Å. A similar experiment was made for toxin α dissolved in trifluoroethanol. The data indicate that the distance separating the two aromatic side chains does not depend greatly on the nature of the solvent.

Elapidae and *Hydrophiidae* venoms possess neurotoxins which block the nicotinic acetylcholine receptor specifically and with a high affinity (Lee, 1972; Bourgeois et al., 1972). These compounds are single chain polypeptides of either 60–62 amino acids and 4 disulfide bonds (short neurotoxins) or 66–74 amino acids and 5 disulfide bonds (long neurotoxins) (Maeda & Tamiya, 1974). A chart alignment of 52 neurotoxin sequences shows that 22 residues or groups remain invariant (Menez et al., 1978).

On the basis of circular dichroism analysis and secondary structure predictions, it has been shown that short neurotoxins share a homologous conformation (Hseu et al., 1977; Dufton & Hider, 1977; Menez et al., 1978), essentially organized into β-pleated sheets and resembling the X-ray structure solved from erabutoxin b crystals (Tsernoglou & Petsko, 1976; Low et al., 1976). This structure reveals that a short toxin molecule largely consists of three β-sheet loops which are joined side by side. It also shows that most of the "functionally" invariant residues (or groups) are concentrated on the central loop or in its spatial proximity. This loop seems therefore of importance for the neurotoxic activity of the whole molecule and it is noteworthy that the sole invariant aromatic amino acids, Tyr-25 and Trp-29, are precisely located in the core of this region. Taking advantage of the fact that most of the short neurotoxins have no additional tyrosine or tryptophan residues, we used Tyr-25 and Trp-29 as fluorescent probes to investigate local and/or environmental perturbations occurring in the toxin molecules. Erabutoxin b[1] from *Laticauda semifasciata* and toxin α from *Naja nigricollis* were used for this work.

In the present study (i) ionizable groups responsible for changes of Trp-29 fluorescence emission are tentatively assigned, (ii) an average distance between Tyr-25 and Trp-29 is estimated from the determination of excitation energy transfer efficiency, (iii) conformational modifications induced by pH changes are investigated by circular dichroism (CD), and (iv) total luminescence spectra at 77 K are examined for both toxins.

Materials and Methods

Toxin α from *N. nigricollis* was prepared as described (Fryklund & Eaker, 1975). Erabutoxins a and b from *L. semifasciata* were kindly supplied by Professor N. Tamiya (Sendai, Japan). *N*-Ac-Trp-NH_2 and the dipeptide L-Tyr-L-Trp were purchased from Sigma Chemical Co. Trifluoroethanol was from Merck and was used without further purification. Methanol was from Sigma.

Fluorescence and energy transfer experiments were performed at 8 °C with dilute protein solutions [$(2–5) \times 10^{-6}$ or $(2–5) \times 10^{-5}$ M] by using a FICA 55000 differential absolute spectrofluorometer. The appropriate buffer was placed in the reference compartment. Excitation and emission slits were adjusted to 7.5 and 2.5 nm, respectively, for emission spectra

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[1] Abbreviations used: toxin α, toxin α from *Naja nigricollis*; erabutoxins a and b, erabutoxins a and b from *Laticauda semifasciata*; TFE, trifluoroethanol.

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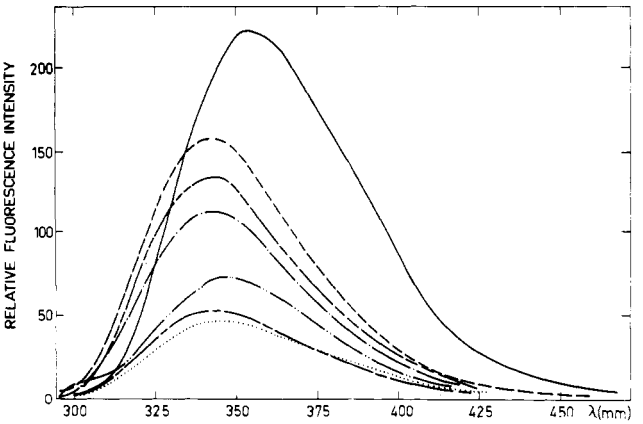

FIGURE 1: Emission fluorescence spectra of *N*-Ac-Trp-NH_2, toxin α from *N. nigricollis*, and erabutoxin b from *L. semifasciata* in aqueous solution. (—), (- - -), and (-·-) correspond to spectra of *N*-Ac-Trp-NH_2, toxin α, and erabutoxin b, respectively, in a 10^{-3} M cacodylate buffer, 10^{-3} M NaCl, and 2×10^{-4} M EDTA, pH 7. (-·-·-) and (-··-) are for toxin α and erabutoxin b, respectively, at pH 1.2. This pH was obtained by adding minute amounts of concentrated HCl to a neutral toxin solution. (- - -) corresponds to the fluorescence spectrum of toxin α immediately after its dissolution in 0.1 N NaOH and (···) after overnight incubation. Peptide concentrations are 6.8×10^{-5}, 4.1×10^{-5}, and 4.4×10^{-5} M for *N*-Ac-Trp-NH_2, toxin α, and erabutoxin b, respectively. Excitation wavelength is 280 nm.

and inversely for excitation spectra. Spectra recorded with this apparatus were automatically corrected for the wavelength dependence of lamp intensity, monochromator transmission, and photomultiplier response.

pH titrations were performed by adding minute amounts of concentrated HCl or NaOH to a neutral toxin solution. The pH was monitored before and after recording the fluorescence intensity. Mixtures of solvents are expressed in terms of volume percentage. Calculations of energy transfer efficiency were made according to the method described by Eisinger (1969) by using the averaged excitation spectrum obtained from three independent experiments.

Ultraviolet absorption spectra were recorded with a Cary 15 spectrophotometer. Circular dichroism measurements were performed at 20 °C by using a Jouan dichrograph, Model II. The peptide concentration was $(5–10) \times 10^{-5}$ M with cells of 0.2–0.5-cm path length. The circular dichroism data were expressed as molar ellipticities (θ) in deg·cm^2·dmol^{-1}.

Luminescence and phosphorescence lifetime measurements at 77 K were made with a Jobin Yvon spectrofluorometer modified to correct for lamp fluctuations as described elsewhere (Brun et al., 1975). Proteins were dissolved in a mixture of propylene glycol and aqueous buffer (1:1) and frozen at 77 K. The pH of the solution was measured before adding the organic solvent.

Results

(1) Fluorescence Emission. Figure 1 shows the fluorescence emission spectra of toxin α, erabutoxin b, and the model compound *N*-Ac-Trp-NH_2 in aqueous solution. At neutral pH, the toxin spectra display a broad peak centered at 342 nm due to Trp-29 emission as proved by excitation spectra (see below). No tyrosine emission could be detected in the 305-nm range when the excitation wavelength was varied between 270 and 290 nm. At the same pH, *N*-Ac-Trp-NH_2 exhibits a wide peak centered at 352 nm. The difference in maximum fluorescence wavelength between both toxins and the model compound is a feature commonly observed in fluorescence spectra of proteins (Longworth, 1971). At acidic pH, the erabutoxin b emission spectrum is modified. Thus, at pH 1.2

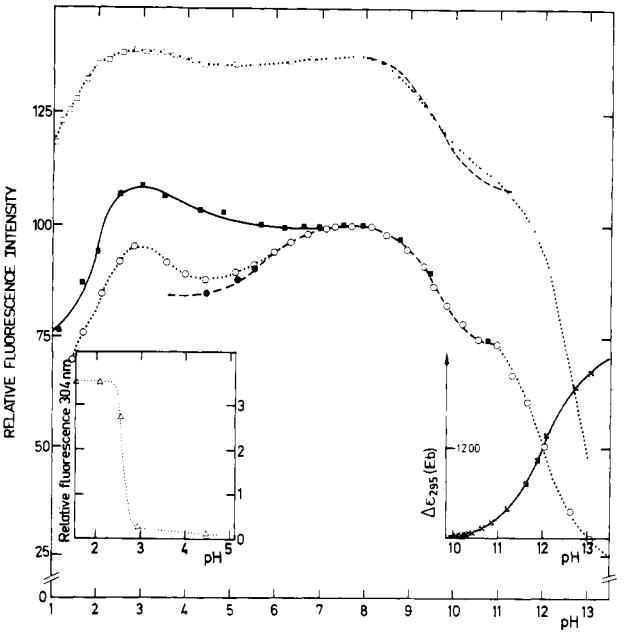

FIGURE 2: The pH dependence of relative fluorescence intensity for toxin α (□), erabutoxin b (○), and erabutoxin a (■). Dashed lines represent semiempirical curves calculated for pK values of 9.6 for toxin α and erabutoxin b and 5.8 for erabutoxin b (see the text). In the latter case, the points (●) are deduced by subtracting experimental intensity values (○) obtained for erabutoxin b from the corresponding increment observed for erabutoxin a. This increment is calculated relative to the fluorescence intensity values of both erabutoxins after normalization at pH 8. Excitation wavelength is 278 nm. The left side inset represents the dependence of erabutoxin b relative fluorescence at 304 nm vs. pH. The right side inset shows the ultraviolet differential absorption at 295 nm as a function of pH for erabutoxin b.

fluorescence intensity decreases by 37% and its maximum is shifted to 347 nm. Such a red shift suggests that the erabutoxin b conformation has been altered. Confirmation of the structural change is obtained by the appearance of the Tyr-25 emission at 305 nm and by the CD study (see below). In contrast, the toxin α emission spectrum is essentially unmodified at acidic pH, except for a slight decrease in fluorescence intensity. Toxin α seems therefore to be more resistant than erabutoxin b toward acidic denaturing conditions. At alkaline pH, the fluorescence spectra of both toxins are modified in a similar fashion. Figure 1 shows the toxin α emission in 0.1 N NaOH both after immediate dissolution of the compound and after overnight incubation. A significant quenching of fluorescence intensity is observed in the two cases (~75%) whereas a red shift ($\Delta\lambda$ = 4 nm) of the maximum is observed in the latter only. Excitation energy transfer from indole to tyrosinate anion may account for such a quenching effect (Edelhoch et al., 1967).

The pH dependence of the neurotoxin maximal fluorescence intensities is reported in Figure 2. The excitation wavelength is 278 nm. Between pH 8 and 4.5, erabutoxin b fluorescence intensity decreases by 12%. Such a decrease is usually characteristic of the quenching effect of a charge-transfer complex occurring between a protonated side chain of a histidine residue and the indole side chain of a tryptophan residue (Shinitsky & Goldman, 1967; Shinitsky & Fridkin, 1969). It may also reflect a change in Trp-29 environment brought about by His protonation. It is of interest that erabutoxin a, which differs from erabutoxin b by a single residue at position 26 (Asn-26 in the first toxin is replaced by His-26 in the latter) and which displays the same X-ray structure as erabutoxin b (Tsernoglou & Petsko, 1977), does not exhibit this decrease.

Consequently, the fluorescence emission quenching can be unambiguously attributed to the protonation of His-26 and not to that of His-7 which is present in both erabutoxins a and b. Assuming that the increment of erabutoxin a fluorescence observed between pH 8 and 4.5 would be similar in erabutoxin b if His-26 was absent, the experimental values recorded for erabutoxin b were corrected and fitted with a semiempirical curve calculated for a single titrating group. A pK value of 5.8 was obtained. This result is in a good agreement with the NMR data previously reported by Inagaki et al. (1978). Thus protonation of His-26 alters the environment of Trp-29, even though both residues are separated by two amino acids. In contrast, in the same pH range the fluorescence intensity of toxin α from *N. nigricollis* remains unchanged despite the presence of two histidines (4 and 32). Between pH 4.5 and 3, a 7–10% increase in fluorescence intensity is observed for both erabutoxins, whereas a much weaker effect is detected for toxin α. Such an increase is probably due to the protonation of one (or more) carboxyl group(s). Below pH 3, quenching occurs in all three cases. However, fluorescence emission from Tyr-25 becomes apparent at 305 nm for erabutoxin b, whereas no tyrosine emission can be detected for toxin α. This observation suggests that a conformational change occurs in erabutoxin b leading to an unmasking of its single tyrosine chromophore. This is confirmed by CD measurements (see below). This change is probably controlled by the protonation of one or more carboxyl groups which, in view of the low pH value of the transition midpoint (pH 2.6, Figure 2), must be either buried or hydrogen bonded to another group in the native state of the toxin. For toxin α, the observed quenching can only be explained by a nonspecific proton quenching effect (Longworth, 1971) since no conformational change (CD pattern or tyrosine emission detection) is observed for this protein in the pH range 1–3. Similarly, below pH 1.5 the quenching of erabutoxin b emission can be attributed to a nonspecific effect of H^+ ions.

Above pH 8, titration curves are rather similar for the three toxins. They exhibit a 25–30% fluorescence intensity decrease between pH 8 and 10.5–10.8 with a transition midpoint value of 9.6. This emission quenching cannot be attributed to tryptophan energy transfer to a tyrosinate anion since the latter shows an abnormally high pK value (see Figure 2, right inset). More likely it is due to a proton transfer from the indole residue to a deprotonated amino group during the lifetime of the excited state (Edelhoch et al., 1967). A semiempirical titration curve with a pK value of 9.6 has been calculated and tentatively adjusted to the experimental curve. A good fit is obtained for the erabutoxins, indicating that the deprotonation of a single amino group is involved. In contrast, the calculated and experimental curves obtained for toxin α do not show such a good agreement (Figure 2). This discrepancy can be explained by the proximity of an additional lysine, the uncharged form of which may also participate in the observed quenching. Quenching of Trp fluorescence by amino groups was reported for other homologous toxins (Bukolova-Orlova et al., 1974). Figure 2 also shows that a 70% decrease of the Trp emission occurs when Tyr is deprotonated. Such a quenching is usually explained by an excitation energy transfer from Trp to Tyr^- (Longworth, 1971). In view of the abnormal pK value of the phenolic ring of Tyr-25 (p$K \simeq 12$, see Figure 2) the presence of a large concentration of OH^- ions in the medium has an additional quenching effect which prevents any accurate evaluation of energy transfer occurring from Trp to Tyr^-.

Addition of methanol to toxin solutions (not shown) induces an increase of the fluorescence quantum yield similar to that

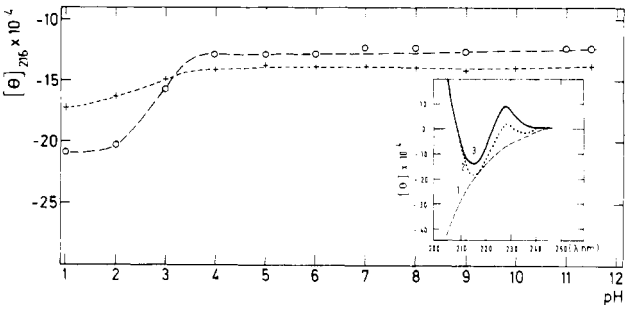

FIGURE 3: Variations of dichroic intensity at 216 nm as a function of pH for toxin α (+) and erabutoxin b (O). Inset shows CD spectra at pH 7 for toxin α (curve 3) and erabutoxin b (curve 3) and at pH 1.2 for toxin α (curve 2) and erabutoxin b (curve 1).

obtained with the model compound *N*-Ac-Trp-NH_2 (40% increase in the presence of 40% methanol). This result indicates a good accessibility of Trp-29 to methanol in agreement with the results of Seto et al. (1970) who used ethylene glycol as a perturbating agent. No change in CD spectra is observed in the presence of methanol.

(*2*) *Circular Dichroism.* Far-ultraviolet circular dichroism spectra of erabutoxin b and toxin α have been previously reported (Ménez et al., 1976, 1978). They are very similar in shape and in intensity, displaying a positive band at 197 nm and a negative one at 216 nm. Both bands are characteristic of β-sheet structures (Greenfield & Fasman, 1969) and possibly of β-turn structures (Chen et al., 1977). The pH dependence of the ellipticity at 216 nm has been studied between pH 1 and 11.5 for both toxins (Figure 3). In both cases, it appears that changing the pH between 4 and 11.5 has no detectable effect on the dichroic intensity. This observation indicates that the overall conformation of both toxins remains stable throughout this pH range. Below pH 4, the ellipticity associated with erabutoxin b undergoes a reversible transition with a midpoint at pH 2.9. In agreement with the fluorescence data presented above, it is likely that such a transition corresponds to a conformational change of the polypeptide chain. As the transition proceeds, the CD spectrum of erabutoxin b changes, finally resulting at pH 1.2 in a pattern characteristic of denatured proteins (Tiffany & Krimm, 1969) (Figure 3, inset). Similar observations were recently reported by Inagaki et al. (1978). In contrast, toxin α does not display any conformational change in acidic medium (Figure 3, inset) although a slight increase of negative ellipticity is observed. This might correspond to the onset of a non cooperative structural destabilization of the polypeptide chain.

(*3*) *Determination of the Tyr → Trp Energy Transfer Efficiency.* Energy transfer measurements were performed for toxin α from *N. nigricollis* solubilized in water (pH 7) and in trifluoroethanol. The excitation spectrum of toxin α was recorded with the emission wavelength at 341 nm and compared to the spectra obtained for model compounds under the same experimental conditions (see Figure 4). A 100% energy transfer efficiency from Tyr to Trp was assumed in the case of the dipeptide L-Tyr-L-Trp.

The intermediate situation of the toxin excitation spectrum represented in Figure 4 demonstrates that a partial transfer from Tyr-25 to Trp-29 occurs. From these spectra, energy transfer efficiency (e) was estimated as follows. When toxin α is excited at wavelength λ, its fluorescence quantum yield (ϕ_{tox}) is given by eq 1, according to Eisinger (1969).

$$\phi_{tox}(\lambda)/\phi_{Trp}(\lambda) = f_{Trp}(\lambda) + e f_{Tyr}(\lambda) \qquad (1)$$

$\phi_{Trp}(\lambda)$ is the fluorescence quantum yield of Trp in toxin α in the absence of energy transfer ($\lambda_{ex} > 295$ nm). $f_{Trp}(\lambda)$ and

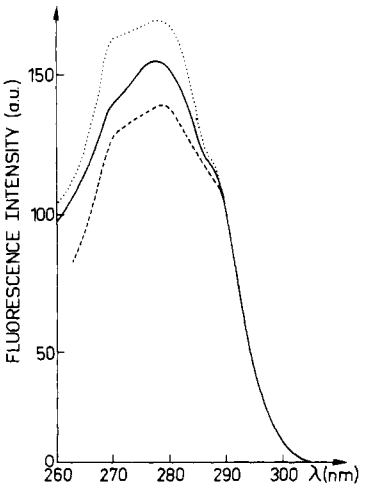

FIGURE 4: Excitation spectra in aqueous solutions of L-Tyr-L-Trp (···), *N*-Ac-Trp-NH_2 (- - -), and toxin α (—) at a 341 nm emission wavelength. Optical densities are 0.02 in all cases. θ = 8 °C.

$f_{Tyr}(\lambda)$ are the fractions of light absorbed by Trp and Tyr, respectively, in the toxin. In the present case, ratios of fluorescence excitation intensities were calculated, taking excitation intensities of both model compounds (L-Tyr-L-Trp and *N*-Ac-Trp-NH_2) as references. The data obtained were compared with theoretical curves calculated according to eq 2 and 3 (after all excitation spectra have been normalized at 295 nm where only Trp absorbs light)

$$I_{tox}(\lambda)/I_{L\text{-}Tyr\text{-}L\text{-}Trp}(\lambda) = f_{Trp}(\lambda) + ef_{Tyr}(\lambda) \qquad (2)$$

and

$$\frac{I_{N\text{-}Ac\text{-}Trp\text{-}NH_2}(\lambda)}{I_{tox}(\lambda)} = \frac{f_{Trp}(\lambda)}{f_{Trp}(\lambda) + ef_{Tyr}(\lambda)} \qquad (3)$$

On this basis, excitation transfer efficiencies of 0.45 and 0.5 fit theoretical curves obtained by using *N*-Ac-Trp-NH_2 and L-Tyr-L-Trp, respectively, as references (Figure 5). From X-ray data (Low, 1979; Tsernoglou & Petsko, 1976) as well as solution studies (Inagaki et al., 1978) it appears that Tyr-25 does not rotate freely. As a result, transfer efficiencies calculated above may be due to a particular orientation of this aromatic residue. However, in trifluoroethanol, a solvent which makes this chromophore much more accessible with no significant change of toxin α conformation (Ménez et al., 1976), a value of 0.6 was obtained for e by using recalculated fractions of light absorbed by Tyr and Trp in this solvent with *N*-Ac-Trp-NH_2 as reference (eq 3) (see Figure 5). Therefore, whatever the accessibility to the solvent of Tyr-25, 50–60% of its absorbed energy is transferred to Trp-29.

As stated above, at neutral pH Tyr-25 emission cannot be detected at any excitation wavelength. Since only ~50% of the energy absorbed by Tyr-25 is transferred to Trp-29, one must conclude that the remaining energy absorbed by Tyr-25 is quenched by another mechanism. The adjacent disulfide bond (3–24) might be responsible for such an effect (Longworth, 1971).

(*4*) *Luminescence Emission at 77 K.* The total luminescence and phosphorescence spectra of erabutoxin b, toxin α, and their corresponding 25–29 pentapeptide fragments have been recorded at 77 K in a 50% propylene glycol aqueous buffer. Since similar data were obtained for both toxins, an illustration of the results is given here for toxin α only. Figure 6 shows the total luminescence spectrum (uncorrected) of toxin α from *N. nigricollis* at pH 7. The spectrum is characteristic of tryptophan emission (Longworth, 1971), and no contribution

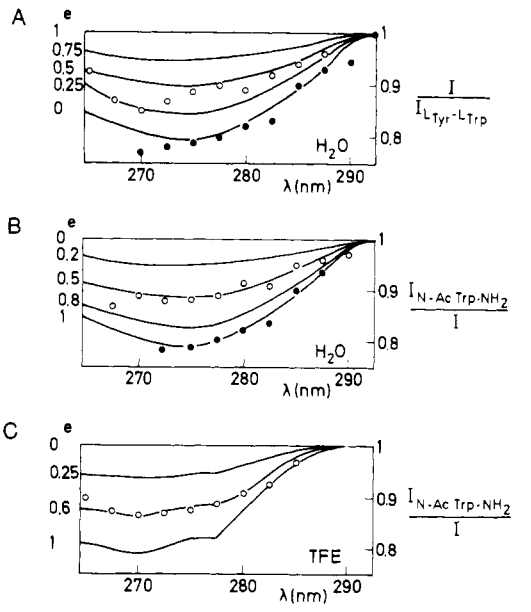

FIGURE 5: Determination of excitation transfer efficiencies for toxin α in water and in TFE. Solid curves are calculated as described in the text. (A) Open and closed symbols correspond respectively to fluorescence intensity ratios of toxin α or *N*-Ac-Trp-NH_2 and L-Tyr-L-Trp vs. excitation wavelength, in water. (B) Open and closed symbols correspond respectively to fluorescence intensity ratios of *N*-Ac-Trp-NH_2 or L-Tyr-L-Trp and toxin α vs. excitation wavelength, in water. (C) Fluorescence intensity ratios of *N*-Ac-Trp-NH_2 and toxin α in TFE (○).

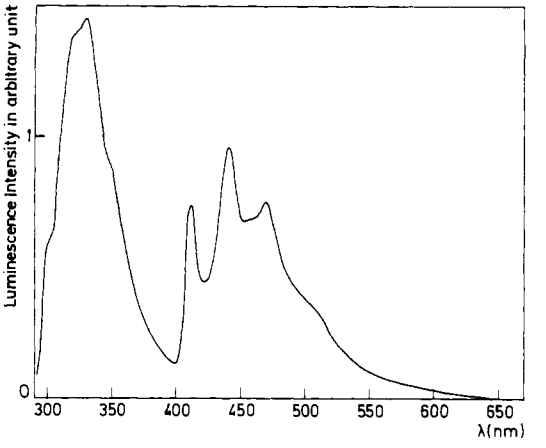

FIGURE 6: Total emission spectrum of toxin α (6.5×10^{-5} M) in a propylene glycol and aqueous buffer (1:1) at 77 K, for an excitation wavelength of 280 nm.

due to tyrosine emission is detected.

Figure 7 shows the phosphorescence spectra of toxin α at pH 7 and 13.5. At neutral pH, the spectrum is typical of tryptophan emission. The weak contribution observed between 350 and 400 nm could be attributed either to tyrosine or to tyrosinate emission resulting from an ionization of tyrosine in its excited state. Such an ionization could result from a strong interaction with a carboxylate anion as suggested by Szabo et al. (1978). However, under similar conditions, the pentapeptide *N*-Ac-Tyr-Lys-Lys-Val-Trp-NH_2 which corresponds to the 25–29 peptide fragment of toxin α displays the same total luminescence and phosphorescence spectra (not shown) and, in particular, the same weak contribution between 350 and 400 nm in spite of the absence of any carboxyl group in the sequence. Therefore the weak emission observed in the whole toxin seems unlikely to originate from tyrosinate species. In contrast, at pH 13.5 the contribution due to tyrosinate anion emission becomes observable in the 350–400-nm range.

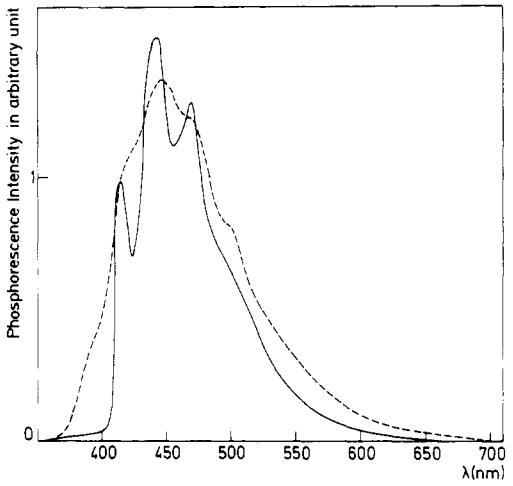

FIGURE 7: Phosphorescence emission spectra of toxin α (6.5×10^{-5} M) at pH 7 (—) and 13.5 (---) for an excitation wavelength of 270 nm, at 77 K.

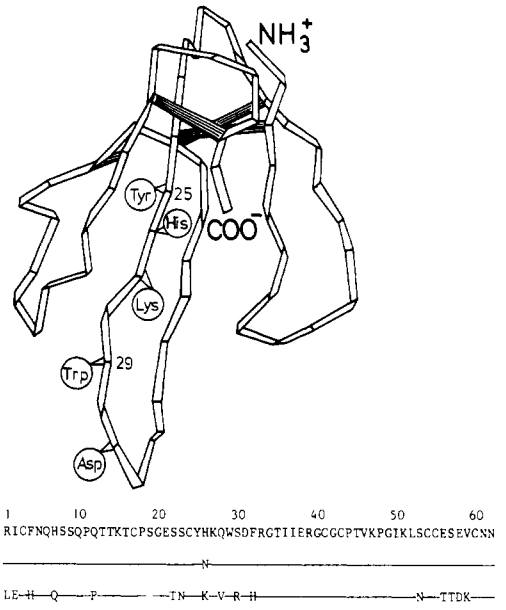

FIGURE 8: X-ray structure of erabutoxin b. [Copyright 1977 *Proc. Natl. Acad. Sci. U.S.A.*; reprinted with permission from Tsernoglou & Petsko (1977).] The lower part of the figure shows the difference in amino acids observed between the sequences of erabutoxin a, toxin α, and erabutoxin b. Amino acid residues are identified by the IUPAC one-letter symbol.

Further information on the nature of the species responsible for the phosphorescence emission of the toxins and of the 25–29 pentapeptides was obtained from phosphorescence lifetime measurements. These lifetimes are compared in the table to the values reported in the literature for model compounds. At neutral pH and for an emission wavelength of 440 nm, the phosphorescence lifetimes are comparable to those of tryptophan. For an emission wavelength of 375 nm, the values obtained for the 25–29 fragments are similar to those of tyrosine species, whereas they are significantly higher for the toxins. As shown from the experiments made at pH 13.5, the lifetimes measured at 375 nm are much smaller than at neutral pH and are characteristic of tyrosinate species. Under these conditions, there is no difference between the pentapeptides and the toxins. Phosphorescence lifetimes and intensity measurements exclude the possibility that the phosphorescence emitted by the toxins at neutral pH in the wavelength range 350–400 nm could be due to tyrosinate species. The comparison of data obtained from toxins and pentapeptides supports this finding. The longer lifetime for tyrosine phosphorescence in the toxins at neutral pH might be due to an unusual environment. This possibility is supported by the high pK value of Tyr-25.

Discussion

By use of the well-known fluorescence and luminescence characteristics of indole and phenolic chromophores, the single tryptophan (29) and, to a lesser extent, the single tyrosine (25) of snake neurotoxins have proved to be extremely useful structural probes. In particular, valuable information has been obtained concerning the nature of the interactions in which the two aromatic chromophores are involved. There is no doubt that at neutral pH fluorescence emission of neurotoxins is due to tryptophan only. First, the whole shape of the excitation spectrum is characteristic of this amino acid. Secondly, luminescence measurements at 293 and 77 K as well as phosphorescence lifetimes at 77 K demonstrate that no tyrosinate contribution exists at neutral pH. This eliminates the possibility that tyrosinate could be an emitting species at 340 nm as in the case of cytotoxins previously reported by Szabo et al. (1978). Therefore, alterations of Trp-29 fluorescence emission induced by pH changes should only reflect the two following alternatives: (i) a local modification of preexisting interactions involving the indole side chain (with either the solvent or an ionizable group), without conformational change in the molecule, or (ii) a local (or global) structural modification of the polypeptide chain. From circular dichroism data, it appears that no detectable conformational change occurs between pH 4 and 11.5 for both toxins (see Figure 3). Therefore the pH dependence of tryptophan fluorescence observed in this pH range can be analyzed in terms of the presence of titrating groups in proximity of the indole side chain 29 although this does not imply necessarily a direct contact since a slight localized conformational change undectable by CD might modify the preexisiting interactions of the tryptophyl residue. Then the results obtained may be discussed on the basis of the X-ray structure of erabutoxin b (Tsernoglou & Petsko, 1976; Low et al., 1976), which is considered as a reliable representation of all short-chain neurotoxins (Dufton & Hider, 1977; Mênez et al., 1978).

It has been shown that the fluorescence emission of erabutoxin b Trp-29 is altered by the protonation of (i) one or more carboxyl groups with pK values around 2 and 4 (examination of the X-ray structure and the sequence of erabutoxin b (Figure 8) suggests that Asp-31 is responsible for the latter titration), (ii) one histidine residue with a pK value of 5.8 (comparison of erabutoxins a and b enabled us to assign unambiguously this histidine to residue 26), (iii) one amino group with a pK of 9.6 belonging possibly to Lys-27, and (iv) the phenolic group of Tyr-25 with a pK of 12. Likewise, emission of Trp-29 of toxin α from *N. nigricollis* is modified by the protonation of (i) one or more carboxyl groups with pK values $\sim$3–4 (possibly Asp-31), (ii) at least two amino groups with pK values around 9.6 (possibly Lys-26 and -27), and (iii) the phenolic group of Tyr-25 (p$K \simeq 12$). It should be stressed that the amino acids which are quoted above are nearly invariant throughout the set of neurotoxin sequences (Menez et al., 1978) and that they are located on the same side of the central β-sheet loop of the toxin molecule (see Figure 8). The only exception is the residue at the 26th position. Such a situation is probably of particular importance for the toxic activity of the molecule.

The second invariant probe, Tyr-25, does not display any fluorescence emission except under denaturing conditions. The

Table I: Phosphorescence Lifetime Values for Toxins and Model Compounds[a]

compound	τ (s) measured at 375 nm, pH 13.5	375 nm, neutral pH	440 nm, neutral pH
N. nigricollis toxin α	1.2	4.6	7.4
N-Ac-Tyr-Lys-Lys-Val-Trp-NH_2	1.4	2.4[b]	6.8
erabutoxin b	1.6	4	6.8
N-Ac-Tyr-His-Lys-Gln-Trp-NH_2	1.4	2.9[b]	6.8
tyrosine (Tyr)		3.0[c], 2.9[d]	
tyrosinate (Tyr^-)	1.3[c], 1.42[d]		
tryptophan (Trp)			6.4[c], 6.65[d]

[a] Measured at 77 K in a 50% propylene glycol-aqueous buffer. Excitation wavelength is 280 nm. [b] pH of the solution was adjusted at 4.5. [c] Hélène et al., 1968. [d] Longworth, 1971.

chemical unreactivity (Yang, 1974; Ménez et al., 1976) as well as the high pK value of the phenolic side chain confirms its masked situation. Recently, Harada et al. (1976) proposed that the hydroxyl group of Tyr-25 of erabutoxin b in solution is involved in hydrogen bonding. Subsequently, Inagaki et al. (1978) suggested that the carboxylate function of an aspartate or a glutamate residue could be possible acceptors. However, luminescence studies and phosphorescence lifetime measurements presented here (Figures 6 and 7 and Table I) do not provide any evidence in favor of the existence of a tyrosine–carboxylate complex at neutral pH which should lead to tyrosinate emission as reported by Szabo et al. (1978) in the case of other snake toxic polypeptides. From the energy transfer efficiency, an average distance separating the aromatic chromophores can be estimated (Förster, 1965). The critical Förster distance, i.e., the distance at which the probability of energy transfer is equal to that of all other modes of deactivation (e = 0.5), has been calculated to be 13 Å assuming a quantum yield of 0.1 for tyrosine. The indole ring is characterized by two transitions which have nearly equal energies but perpendicular polarization. This means that the efficiency of energy transfer depends much less on the orientation of the two chromophores than in the case where the acceptor molecule has only one transition moment in the overlap region (T. Montenay-Garestier and C. Hélène, unpublished results). Therefore a distance of 13 Å separating Tyr-25 and Trp-29 seems to be a reasonable approximation since the measured efficiency of energy transfer was ~0.5. This estimated value is compatible with the distance separating the γ carbons (12.2 Å) of the two residues as revealed by the crystalline structure (Kimball et al., 1979) (see Figure 8). At pH 13.5, Tyr-25 is in an ionized state. Dexter's theory (1953) indicates that, when two chromophores are in van der Waals contact, the chromophore with the higher triplet energy level (here Tyr^-) is able to transfer its excitation energy to the other (here Trp). As a consequence, phosphorescence emission (77 K) of the former should be quenched. That such a situation does not occur in the case of the toxins (Figure 7) indicates that at alkaline pH the two aromatic chromophores are still separated from each other by >5 Å.

As a conclusion, it appears that in solution the indole side chain of Trp-29 is in proximity of multiple titrating groups and most of the interactions can be explained on the basis of the crystalline structure of erabutoxin b (see Figure 8). However, the possible existence in water of an interaction between His-26 and Trp-29 of erabutoxin b might indicate that slight differences occur between solution and crystal structures. It has been reported that although Trp-29 is invariant throughout the entire series of neurotoxins, it has no "direct toxic" function (Karlsson, 1979). However, the numerous interactions in which this amino acid is involved, in particular, with the ε-amino group of Lys-27 and the carboxyl group of Asp-31, suggest that the indole side chain 29 plays a "supporting" role, generating the required orientation for vicinal interacting groups and thus allowing an adequate fit of the toxin to the receptor. Such a proposal would also explain why the activity of a toxin molecule is lost when the indole is modified by bulky reagents [see Karlsson (1979)] but is unaffected when indole bears a *N'*-formyl group (Chicheportiche et al., 1972).

Added in Proof

Since this paper was submitted, new experiments have demonstrated that Lys-27 is indeed responsible for the quenching of Trp-29 fluorescence intensity occurring in the alkaline pH range (pK = 9.6). Acylation of the amino group at position 27 abolishes specifically this quenching effect. Recent NMR results support also these findings (Inagaki et al., 1980).

Acknowledgments

We are very indebted to Professor N. Tamiya (Sendai, Japan) for his generous gift of erabutoxins. We thank Dr. O. Tremeau for kindly extracting toxin α from the *Naja nigricollis* venom. We are very grateful for Dr. R. C. Hider (Colchester, United Kingdom) for reading and correcting the manuscript and providing constructive criticism.

References

Bourgeois, J. P., Ryter, A., Ménez, A., Fromageot, P., Boquet, P., & Changeuse, J. P. (1972) *FEBS Lett. 25*, 127–130.
Brun, F., Toulme, J. J., & Hélène, C. (1975) *Biochemistry 14*, 558–563.
Bukolova-Orlova, T. G., Burstein, E. A., & Yukelson, L. Y. (1974) *Biochim. Biophys. Acta* 342, 275–280.
Chen, Y. H., Lo, T. B., & Yang, J. T. (1977) *Biochemistry 16*, 1826–1830.
Chicheportiche, R., Rochat, C., Sampieri, F., & Lazdunski, M. (1972) *Biochemistry 11*, 1681–1691.
Dexter, D. L. (1953) *J. Chem. Phys. 21*, 836–848.
Dufton, M. J., & Hider, R. C. (1977) *J. Mol. Biol. 115*, 177–193.
Edelhoch, H., Brand, L., & Wilcheck, M. (1967) *Biochemistry 6*, 547–559.
Eisinger, J. (1969) *Biochemistry 8*, 3902–3908.
Förster, T. (1965) *Mod. Quantum Chem., Lect. Istanbul Int. Summer Sch., 1964*, 93–137.
Fryklund, L., & Eaker, D. (1975) *Biochemistry 14*, 2865–2871.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.
Harada, I., Takamatsu, T., Shimanouchi, T., Miyazawa, T., & Tamiya, N. (1976) *J. Phys. Chem. 80*, 1153–1156.
Hélène, C., Prak, H., & Santus, R. (1968) *J. Chim. Phys. 65*, 160–166.
Hseu, T. H., Liu, Y. C., Wang, C., Chang, H., Hwang, D. M., & Yang, C. C. (1977) *Biochemistry 16*, 2999–3006.
Inagaki, F., Miyazawa, T., Hori, H., & Tamiya, N. (1978) *Eur. J. Biochem. 89*, 433–442.
Inagaki, F., Tamiya, N., & Miyazawa, T. (1980) *Eur. J. Biochem. 109*, 129–138.

Karlsson, E. (1979) *Handb. Exp. Pharmacol. 52*, 159–212.
Kimball, M. R., Sato, A., Richardson, J. S., Rosen, L. S., & Low, B. W. (1979) *Biochem. Biophys. Res. Commun. 88*, 950–959.
Lee, C. Y. (1972) *Annu. Rev. Pharmacol. 12*, 265–286.
Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 319–484, Macmillan, New York.
Low, B. W. (1979) *Handb. Exp. Pharmacol. 52*, 213–257.
Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D., & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2991–2994.
Maeda, N., & Tamiya, N. (1974) *Biochem. J. 141*, 389–400.
Mênez, A., Bouet, F., Tamiya, N., & Fromageot, P. (1976) *Biochim. Biophys. Acta 453*, 121–132.
Mênez, A., Langlet, G., Tamiya, N., & Fromageot, P. (1978) *Biochimie 60*, 505–516.
Seto, A., Sato, S., & Tamiya, N. (1970) *Biochim. Biophys. Acta 214*, 483–489.
Shinitsky, M., & Goldman, R. (1967) *Eur. J. Biochem. 3*, 139–144.
Shinitsky, M., & Fridkin, M. (1969) *Eur. J. Biochem. 9*, 176–181.
Szabo, A. G., Lynn, K. R., Krajcarski, D. T., & Rayner, D. M. (1978) *FEBS Lett. 94*, 249–252.
Tiffany, M. L., & Krimm, S. (1969) *Biopolymers 8*, 347–359.
Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68*, 1–4.
Tsernoglou, D., & Petsko, G. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 971–974.
Yang, C. C. (1974) *Toxicon 12*, 1–43.

Circular Dichroism and Fluorescence-Detected Circular Dichroism of Deoxyribonucleic Acid and Poly[d(A-C)·d(G-T)] in Ethanolic Solutions: A New Method for Estimating Circular Intensity Differential Scattering†

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ABSTRACT: A method is presented for determining the circular dichroism (CD) of systems whose CD spectra contain contributions from CD differential scattering. The technique is shown to detect light over 4π steradians, and thus, for the first time, a complete correction for scattering is possible. The method is applied to ethanol-condensed DNA and poly[d(A-C)·d(G-T)]. From the results obtained, the former are proposed to have an A-type secondary structure. The condensed polynucleotide particles are shown to exhibit behavior similar to that of cholesteric liquid crystals. CD difference spectra, obtained from the scattering corrections and showing the contributions to different sections of the scattering envelope, are displayed. It is asserted that these scattering patterns contain information about the tertiary structure of the condensed DNA particles studied.

Circular dichroism (CD) has proven to be a useful and sensitive tool in probing the structure of biological macromolecules. Many systems which have been studied with this technique, such as bacteriophage, membrane-incorporated proteins, DNA in chromosomes, etc., are intense light scatterers (Cantor & Hearst, 1969; Glaser & Singer, 1971; Ji & Urry, 1969; Schneider et al., 1970; Urry & Krivacic, 1970; Dorman & Maestre, 1973; Dorman et al., 1973). Since commercial CD spectrometers are ratio measuring devices (Velluz et al., 1965), structures which scatter both senses of circularly polarized light with equal efficiency will cause no change in the measured ellipticity. Many light-scattering suspensions, however, scatter left and right circularly polarized light to differing extents; i.e., they exhibit differential light scattering. This is measured by the CD spectrometer as if it were differential absorption and can lead to major distortions in the recorded spectra. The presence of such scattering can often be evidenced by observed CD signal in regions where the sample does not absorb light. Distorted spectra of this type can, of course, lead to great difficulties in interpretation of the measured signal.

The most common experimental method for correction of differential scattering is to increase the solid angle of detection in order to collect more scattered light. This has been accomplished by increasing the size of the detector, bringing it closer to the sample cell, or both (Dorman & Maestre, 1973; Dorman et al., 1973). Configurations of this type can collect light scattered by as much as 90° from the incident direction. Probably the most effective scatter correction method to date is the use of the fluorscat technique (Dorman & Maestre, 1973; Dorman et al., 1973). This method is effective even for scattering angles greater than 90° and has been successfully used in many applications (Dorman & Maestre, 1973; Dorman et al., 1973; Girod et al., 1973). Unfortunately, in none of the above experimental methods can the light detector be placed between the sample and the incident beam. Thus, one cannot correct for backscattering by the sample. In this paper we present a method, using fluorescence-detected circular dichroism (FDCD), in which a nonoptically active fluorescent substance can be used as a CD detector having a solid angle of detection of 4π steradians. Thus, backscattering by the particles can be detected. The CD spectra of DNA and

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0006-2960/80/0419-5208$01.00/0