Articles

Role of Tyrosine and Tryptophan Residues in the Structure–Activity Relationships of a Cardiotoxin from *Naja nigricollis* Venom†

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ABSTRACT: This paper is an attempt to localize the critical area determining toxicity in a snake cardiotoxin. Toxin γ is a single-chain polypeptide of 60 amino acids, which has been isolated from the venom of the African spitting cobra, *Naja nigricollis*. Three aromatic residues, namely, Trp-11, Tyr-22, and Tyr-51, have been individually modified by chemical means. The structure of the native toxin and of each derivative has been carefully investigated by circular dichroism, fluorescence, proton magnetic resonance spectroscopy, and two specific monoclonal antibodies. None of the chemical modifications alters the overall structure of the toxin, which in all cases remains folded into three adjacent loops (I, II, and III) rich in β-pleated sheet emerging from a small globular region containing four disulfide bridges. A number of subtle changes, however, have been detected in the structure of each derivative compared with that of the native toxin. In particular, nitration of Tyr-51 provoked a structural perturbation in the globular region. Nitration of Tyr-22 induces a more substantial change in the β-sheet area of the molecule. Thus, the strong inter-ring NOE that is observed in the native toxin between Tyr-22 and Tyr-51 vanishes in the Tyr-22 derivative, and significant changes are observed in the globular region. In contrast, no alteration of the β-sheet structure of loops II and III has been detected after modification of Trp-11. All changes observed for this derivative remain located in the vicinity of the indole side chain of Trp-11 in loop I. The biological consequences of the modifications were measured: the lethal potency in vivo in mice and the cytotoxic activities in vitro on FL-cells. Lethal activities correlate with cytotoxicity: Tyr-51 modified toxin is equally potent as native toxin, whereas Tyr-22 and Trp-11 derivatized toxins are characterized by substantially lesser activities, the Trp-11 derivatized toxin being the least potent. We conclude that (1) Tyr-51 is not involved in the functional site of the toxin, although it is in interaction with the core of the molecule, (2) Tyr-22 may play a dual structural and functional role, and (3) Trp-11 is in, or in close proximity to, the functional site of the toxin. These data indicate the importance of loop I in determining toxicity of the cardiotoxin.

Cardiotoxins constitute the major toxic components of a number of Elapid snakes. They are characterized by various biological activities (Harvey, 1985) including blockade of the heart in systole (Sarkar, 1947), depolarization of a number of excitable cells (Chang et al., 1972; Condrea, 1974; Harvey et al., 1982), and lysis of various cells including erythrocytes (Louw & Visser, 1977, 1978). Cardiotoxins are small and highly basic single-chain polypeptides comprising 60 amino acid residues (Karlsson, 1979). Their chain is folded like that of neurotoxins (Dufton & Hider, 1983), with three adjacent loops rich in β-pleated sheets emerging from a small globular region with four disulfide bonds (Rees et al., 1983, 1987). The high toxicity of cardiotoxin to mammals (Lee et al., 1968) has suggested both the existence of a specific physiological target (Dufton & Hider, 1983) and the presence on cardiotoxin surface of a critical area determining toxicity. At present, these complementary sites have not been identified, as illustrated by the numerous hypotheses proposed in the literature (Lauterwein & Wüthrich, 1978; Dufourcq et al., 1982; Hider & Khader, 1982; Batenburg et al., 1985; Hodges et al., 1987). These proposals suggest commonly, however, that the functional site of cardiotoxins involves one or more of the three loops. There is also evidence based on biological investigations (Hider & Khader, 1982; Harvey, 1985), amino acid sequence examination (Botes & Viljoen, 1976), secondary structure analysis (Grognet, 1984), and evolution (Dufton and Breckenridge, unpublished results), which suggests that there are subclasses of cardiotoxins. This might explain the forementioned heterogeneity as to identification of both the target and the functional site of cardiotoxins.

The purpose of the present paper is to localize the functional site of a well-characterized cardiotoxin, toxin γ from *Naja nigricollis* venom (Fryklund & Eaker, 1975). The principal effect of toxin γ is on cell membranes. In particular, it kills animals by ventricular fibrillation, produces contracture of striated muscles (Tazieff-Depierre et al., 1969, 1972), depolarizes skeletal muscle fibers in culture (Gatineau and Harvey, unpublished data), and possesses a strong cytotoxic activity toward FL-cells (see below). One approach to locating a functional site in a protein consists of modifying a number of individual amino acid residues in order to pinpoint the modification(s) that alter(s) the biological activity of the protein. Then the conclusion that the residue(s) in question is (are)

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effectively incorporated in the functional site can be drawn, provided appropriate structural analyses of the modified protein are made concomitantly. In order to localize the functional site of toxin γ, we have selectively modified three individual aromatic amino acids—one in each of the three loops. The single tryptophan at position 11 (loop I) has been modified with nitrophenylsulfenyl chloride and the two tyrosines at positions 22 (loop II) and 51 (loop III) have been nitrated individually with tetranitromethane. The consequences of these modifications on the global and local structures of toxin γ were analyzed by circular dichroism, by emission fluorescence, and by two monoclonal antibodies. Furthermore, some effects of the chemical modifications on the local structures in the globular part of the toxin γ have been examined by proton nuclear magnetic resonance spectroscopy. We studied the biological activities of the toxin derivatives in two different ways. First, we determined in vivo their toxicities in mice. Second, we measured their cytotoxic activities on FL-cells (Takechi et al., 1986) to estimate their capacities to perturb cell membranes. The results indicate a critical role of loop I and possibly of a part of loop II in toxicity of toxin γ.

Materials and Methods

Materials. Toxin γ from *Naja nigricollis* venom (Pasteur Institute, Paris, France) was isolated as previously described (Fryklund & Eaker, 1975). ^{3}H-Propionylated toxin γ was prepared according to Grognet et al. (1986). Tetranitromethane was obtained from Aldrich (Strasbourg, France), formic acid (99% pure) was from Merck (Darmstadt, Germany), Bio-Rex 70 and Bio-Gel P2 were from Bio-Rad (Richmond, CA), Sephadex G-25 coarse and G-50 medium were from Pharmacia (Uppsala, Sweden), and the high-performance liquid chromatography (HPLC) C_{18} μBondapak column was from Waters Associates (Milford, MA). 2-Nitrophenylsulfenyl chloride and α-chymotrypsin were purchased from Sigma (St. Louis, MO). Balb/C mice were from IFFA-CREDO (Lyon, France). FL-cells (cultured human amnion cells) were obtained from Gibco-BRL (Cergy-Pontoise, France) and cultivated as previously described (Fogh & Lund, 1957). Monoclonal antibodies, called $M_{\gamma 1}$ and $M_{\gamma 2/3}$, specific for *Naja nigricollis* toxin γ were produced by hybridoma cells obtained by cell fusion (Grognet et al., 1986). Amino acid compositions were analyzed by using an LKB 4400 automatic analyzer. Circular dichroism spectra were recorded with a Jobin-Yvon dichrograph III. Fluorescence spectra were recorded with a Fluorolog 2-FIT 11 SPEX. ^{1}H NMR spectra were recorded at 500 MHz on a WM 500 Bruker spectrometer equipped with an Aspect 3000 computer, an array processor, and a CDC 32 disk drive. The spectrophotometric titration of pK_a was performed with a Minisis 8000 pH meter from Tacussel and a Beckman DU 7 spectrophotometer.

Nitration of Tyrosine Residues. The nitration of tyrosine groups in toxin γ was performed with tetranitromethane (Sokolovsky et al., 1966; Riordan et al., 1967) under denaturing conditions (Karlsson & Sundelin, 1976; Hung et al., 1978; Carlsson, 1980). Sixteen micromoles of *Naja nigricollis* toxin γ was dissolved in 8.5 mL of 6 M guanidinium chloride in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8. Tetranitromethane was diluted in 95% ethanol (10 mg/mL), and 235 μL of this solution (2-fold molar excess) was added to the toxin solution. The reaction mixture was stirred in the dark at room temperature for 30 min and then filtered through a Sephadex G-50 column (2 × 90 cm) in 0.1 M ammonium acetate, pH 7. The elution was monitored by measurement of UV absorption at 278 nm. The elution rate was slow (0.2 mL/min). This permitted a good separation of the toxin from the denaturing agent and therefore allowed refolding of the protein. The toxin monomer fraction was rechromatographed on a Bio-Rex 70 column (2 × 55 cm) equilibrated in 0.05 M ammonium acetate, pH 8 (Carlsson, 1980). The elution was done over a linear gradient from 0.05 to 0.7 M ammonium acetate, pH 8, for 24 h. The number of moles of 3-nitrotyrosine per mole of nitrated derivatives was determined according to the method of Sokolovsky et al. (1966). Acid hydrolysis of samples of each derivative was carried out under vacuum in sealed tubes with 6 M HCl at 120 °C for 24 h and amino acid analysis (Spackman et al., 1958) was performed with an automatic analyzer. Nitrotyrosine was eluted after phenylalanine (Sokolovsky et al., 1966). Tryptophan was determined spectrophotometrically (Raymond et al., 1972). Finally, control experiments showed that treatment of toxin γ with guanidine hydrochloride under the same conditions as above, followed by removal of the denaturing agent, led to a protein with the same biological and structural properties as the native toxin γ.

Identification of the 3-Nitrotyrosine Residue in the Two Mononitrated Derivatives. Native toxin γ and each mononitrated derivative (36 nmol) were dissolved separately in 0.2 mL of 0.1 M Tris-HCl buffer, pH 8, containing 6 M guanidinium hydrochloride, and dithiothreitol (3.8 μmol) was added. Reaction mixtures were allowed to stand for 75 min in the dark at room temperature. Free sulfhydryl groups were blocked with 31.5 μmol of iodoacetic acid (4-fold molar excess in terms of sulfhydryl groups) in 0.1 M Tris-HCl buffer, pH 8, for 15 min. The reduced and S-carboxymethylated products were desalted by gel filtration through a Bio-Gel P2 column (1 × 20 cm) in 10% acetic acid and lyophilized. These products were dissolved in 0.1 M Tris-HCl buffer, pH 7.5 (final concentration of RCm-toxin 1 mg/mL) and hydrolyzed with α-chymotrypsin at a substrate–enzyme ratio of 100/1 (w/w) for 4 h at 37 °C (Fryklund & Eaker, 1975). Chymotryptic digests were chromatographed on a Sephadex G-25 coarse column (1 × 80 cm) in 0.02 M HCl. Peaks containing nitrotyrosine fragments were rechromatogaphed on a C_{18} μBondapak column (3.9 mm × 30 cm) using a system of 0.1 M triethylamine–phosphoric acid buffer, pH 3.2, in acetonitrile. Eluted peptides were detected by monitoring UV absorption at 220 nm. Peptides containing 3-nitrotyrosine were hydrolyzed in 6 M HCl at 120 °C for 24 h, and their amino acid compositions were determined.

Modification of Tryptophan 11. Introduction of a nitrophenyl moiety at C_2 of the indole side chain by a thioether linkage was done according to the method of Fontana et al. (1966). Toxin γ (1.5 μmol, 10 mg) was dissolved in 2 mL of pure acetic acid, and 0.3 mg of 2-nitrophenylsulfenyl (NPS) chloride was added in 2 mL of the same solvent. After 17 h at room temperature, the reaction mixture was filtered through a Bio-Gel P2 column (1 × 20 cm) equilibrated in 10% acetic acid. The fraction eluting in the void volume and containing the native or modified toxin was freeze-dried. It was then chromatographed on a C_{18} μBondapak column (9.4 mm × 25 cm) equilibrated in 0.1 M triethylamine buffer adjusted to pH 4.2 with formic acid and containing 25% acetonitrile. Elution was done with a linear gradient of 25–40% acetonitrile in 0.1 M triethylamine, pH 4.2. The UV spectrum in the region 220–470 nm and the amino acid composition were determined for each peak.

Circular Dichroism (CD). CD measurements were performed with a Jobin-Yvon dichrograph III at room temperature with a peptide in water solution at a concentration close

to 10^{-5} M. The path length of the quartz cell was 0.05 cm. Each spectrum was the average of 16 repetitions and was expressed as molar ellipticities (θ) in deg·cm^2·dmol^{-1}.

Monoclonal Antibody Binding Assay. A monoclonal antibody ($M_{\gamma 1}$ or $M_{\gamma 2/3}$, ≈ 1 μM) was incubated overnight at 4 °C with ^{3}H-propionylated toxin γ (2.4 nM or 35 000 total cpm) and various amounts of modified toxins (0.4–50 nM) in 0.3 mL of 0.05 M sodium phosphate buffer, pH 7.2, containing 0.45% NaCl plus 1% (w/v) bovine serum albumin, and 20 μL of normal horse serum. Toxin–antibody complexes were precipitated by poly(ethylene glycol) 6000 (0.5 mL) at a final concentration of 12.5% (w/v) and were centrifuged down at 2000g in a Jouan K110 SX centrifuge at 4 °C for 20 min. The supernatants were removed, and the pellets were dissolved in 0.01 M NaOH (0.75 mL), placed in 10 mL of scintillator (Aqualuma Plus), and counted in a liquid scintillation counter (LKB-Rackbeta 1211). The background was about 16% of the added radioactivity. The affinity constants for each derivative were deduced from competition curves according to a method derived from that of Ishikawa et al. (1977).

Spectrophotometric Titration. Aliquots of native and derivatized toxin γ were dissolved separately in 0.1 M KCl at a concentration close to 8×10^{-5} M (native toxin or NPS derivative) or in 0.05 M acetic acid plus 0.1 M NaCl at a concentration close to 5.5×10^{-5} M (nitrated derivatives). The pH of the solutions was adjusted with small amounts of NaOH (0.1–5 M) during bubbling with argon at room temperature. Optical densities were measured in a 1-cm quartz cell. The pH and the absorbance change at 430 nm for nitrated tyrosine and at 295 nm for unmodified tyrosine were measured immediately after each addition of NaOH. The differential molar absorbances were calculated for each pH.

Emission Fluorescence. The emission-corrected spectra were recorded at 20 °C. The excitation and emission slits were set at 3.6 and 1.8 nm, respectively. The wavelength increment was 0.5 nm and the integration time 1 s.

^{1}H Nuclear Magnetic Resonance Spectroscopy. All of the NMR measurements were carried out in D_2O solution. In order to allow exchange of labile protons with ^{2}H, 2–4 mg of protein, depending on the amount of material available, was dissolved in D_2O (99.96% ^{2}H), and after 3 h of incubation at room temperature, the solutions were freeze-dried. This step was repeated twice and, finally, the resulting material was dissolved in 0.3 mL of D_2O. Sodium 3-(methylsilyl)-[2,2,3,3-$^2H_4$]propionate was added as internal standard for the chemical shift determination. Scalar connectivities were obtained by the standard two-dimensional correlated spectroscopy (COSY) technique (Nagayama et al., 1980). Two-dimensional nuclear Overhauser effect (NOESY) spectra were run in the phase-sensitive mode by the TPPI method (Bodenhausen et al., 1984) in order to achieve better peak resolution and a higher signal to noise ratio. The spectra were acquired in quadrature detection with the carrier frequency placed at the center of the spectral width. Preliminary low-resolution NOESY spectra (ca. 10 Hz) were carried out with different values of the mixing time τ_M (80–400 ms), which was randomly modulated (±20 ms). Higher resolution NOESY spectra (ca. 4 Hz) were run at 25 °C for all sample with τ_M = 100 ms in order to minimize spin diffusion effects (Kumar et al., 1981). The NOE's mentioned further in this work refer to these conditions. The residual water signal was suppressed in all the two-dimensional experiments by selective preirradiation.

Toxicity Assays. Various doses of native and derivatized toxin γ in 0.9% NaCl were injected intravenously into the tail vein of mice (10 mice per dose) of the Balb/C strain weighing 20–22 g. The administration volume was about 0.2 mL. The number of mice that died during 3 days was recorded, and the percentage of dead mice was calculated for each dose. The median lethal dose (LD_{50}) was determined according to the method of Miller and Tainter (1944).

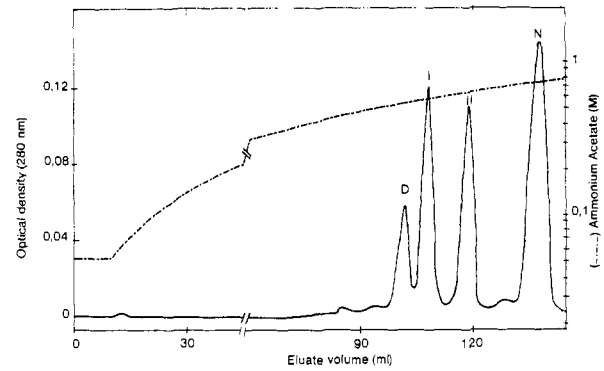

FIGURE 1: Elution on a Bio-Rex 70 column (2 × 55 cm) of the toxin monomer fraction obtained after nitration of 16 μmol of toxin γ by 32 μmol of tetranitromethane and after filtration of the reaction mixture through a Sephadex G-50 column. Fractions I and II correspond to the toxins modified at tyrosine 22 and at tyrosine 51, respectively. D and N represent dimodified and unreacted toxins, respectively.

Cytotoxicity Assay. Three- or four-day-old FL-cells were dispersed with 0.1% trypsin and suspended in phosphate-buffered saline (PBS) (140 mM NaCl + 8 mM Na_2HPO_4 + 1.5 mM KH_2PO_4 + 2.7 mM KCl, pH 7.2) at a concentration close to 3.3×10^6 cells/mL. Three different concentrations (1.3, 1.9, and 2.5 μM) of toxin γ or its derivatives in PBS buffer were added to 0.2-mL aliquots of cell suspensions. The cytotoxic activities of native or derivatized toxin γ after 30 min of incubation at 37 °C were measured by the trypan blue exclusion test (Braganca et al., 1967). The dead cells were counted, and the percentage of dead cells was calculated for each dose. The median effective dose (EC_{50}) was determined for each compound.

RESULTS

Chemical Modification of Toxin γ

(1) Isolation of Nitrated Derivatives. Nitration of toxin γ using tetranitromethane yielded a complex mixture that was separated by gel filtration on a Sephadex G-50 column. In a control experiment, toxin γ was exposed to reaction buffer without tetranitromethane for 90 min. A subsequent gel filtration gave rise to two peaks at 15 and 23 min. The latter peak contained 8% of the protein and consisted of denatured toxin. The first fraction corresponded to native toxin γ. After nitration, two additional peaks were observed. One of them consisted of polymers of the toxin, and the other corresponded to low molecular weight reagents and buffer constituents. Pilot experiments were carried out in the presence of a 4-fold molar excess of tetranitromethane for 2 or 5 h. In both cases, a high level of dimerization was observed. In contrast, when the experiment was performed in the presence of a 2-fold molar excess of tetranitromethane for 30 min, the monomeric form of the toxin predominated ($\approx$60%). The monomeric fraction was freeze-dried and then purified by ion-exchange chromatography on a Bio-Rex 70 column with a linear gradient of ammonium acetate at pH 8. As shown in Figure 1, four fractions were well resolved. As judged by UV determination (Sokolovsky et al., 1966), amino acid analysis (Karlsson & Sundelin, 1976) (not shown), and NMR spectroscopy (see below), fractions I and II are mononitrated derivatives, whereas

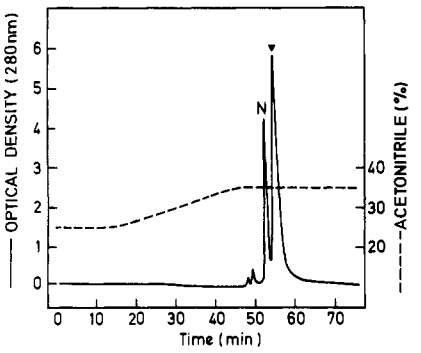

FIGURE 2: Elution on a C_{18} μBondapak column (9.4 mm × 25 cm) of the desalted (Bio-Gel P2 column) reaction mixture obtained after reaction of toxin γ with 2-nitrophenylsulfenyl chloride. A binary gradient of 0.1 M triethylamine buffer, pH 4.2/acetonitrile was applied (---). The arrow shows the position of the pure monomodified toxin, and N represents unreacted toxin.

fraction D corresponds to the dinitrated derivative. Fraction N corresponds to the unmodified toxin γ. Side reactions have been reported in the literature [see review in Riordan and Vallee (1972)]. In the present case, however, no such undesirable effect has been detected. Thus, the lack of oxidation of methionine is indicated by NMR spectroscopy since the four signals assigned to S-methyl protons of methionine residues remain unchanged after nitration. Tryptophan 11 is certainly not nitrated since the nitroderivatives of toxin γ are colorless at pH 3, in contrast to a derivative with nitrotryptophan that becomes yellow at this pH (Cuatrecasas et al., 1968).

(*2*) *Identification of the 3-Nitrotyrosine Residue in the Two Mononitrated Toxin γ Derivatives.* The elution profiles of the chymotryptic digests of reduced and S-carboxymethylated native toxin and of fractions I and II (see above) on a Sephadex G-25 column revealed three well-resolved fractions having characteristic tyrosine UV spectra. The second fraction, which is the major one, was rechromatographed on a C_{18} μBondapak reverse-phase column. In the case of the native toxin, two main tyrosine-containing peaks were resolved. An amino acid analysis (not shown) revealed that they correspond to the peptide sequences 49–51 (Ile-Lys-Tyr) and 49–52 (Ile-Lys-Tyr-Met). These peptides eluted at acetonitrile concentrations equal to 5% and 7.5%, respectively. No fragments containing unambiguously the tyrosine residue at position 22 have been recovered. We focused our attention, therefore, on tyrosine residue 51 and in particular on the major peptide 49–51, which can be readily and clearly detected even with very small amounts of toxin. This peptide was indeed recovered intact in the chromatogram of the digest of (250 μg) RCm-mononitrated derivative I (not shown). We conclude therefore that tyrosine 51 is not modified in derivative I. Since one tyrosine is nitrated in this derivative (see above), it has to be tyrosine 22. In contrast, we observed that the peak corresponding to peptide 49–51 disappeared from the chromatogram of derivative II. A new peak eluted at 11% acetonitrile. It corresponds to the tripeptide Ile_{49}-Lys_{50}-(3-nitroTyr_{51}). We conclude, therefore, that the monoderivatives I and II correspond to (3-nitrotyrosine 22)–toxin γ and (3-nitrotyrosine 51)–toxin γ, respectively.

(*3*) *Modification of Tryptophan 11.* A preliminary description of the NPS derivative has been previously reported (Grognet et al., 1986). Figure 2 shows an HPLC profile of the desalted mixture of toxin γ after treatment with 2-nitrophenylsulfenyl chloride. Two peaks were observed, which elute at about 52.5 and 54.5 min and which correspond to the unmodified toxin γ and NPS-modified derivative, respectively. The amino acid composition of the derivative is identical with

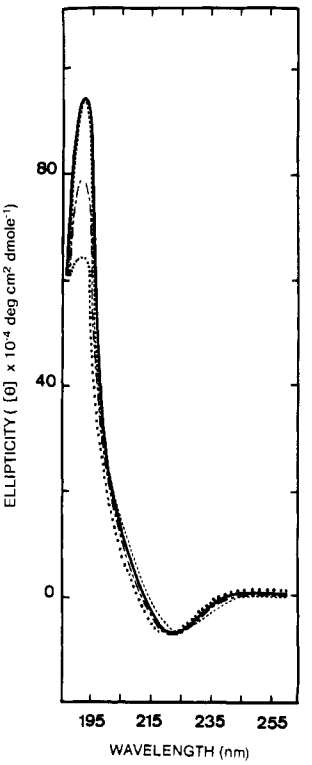

FIGURE 3: Far-UV circular dichroism spectra of native (—) and monomodified toxins at positions 11 (···), 22 (---), and 51 (–·–). In all cases, the compound was dissolved in water, and the protein concentration was approximately 10^{-5} M. The cell path length was 0.05 cm.

that of native toxin (not shown). The derivatized toxin is characterized by a UV spectrum that presents two maxima centered at 282 nm (molar absorbance 16 000) and 365 nm, indicating that a tryptophan has been modified. As deduced from NMR spectra (see below), no side reactions had occurred.

Structural Comparison of Native and Modified Toxin γ

(*1*) *Circular Dichroism.* The far-ultraviolet CD spectra of the native and modified toxins are shown in Figure 3. They all share very similar characteristics with a negative trough centered approximately at 215 nm and an intense positive peak at 195 nm. These spectra resemble those of homologous cardiotoxins (Hung & Chen, 1977) and neurotoxins (Drake et al., 1980; Dufton & Hider, 1983). The tertiary structures of some of these toxins have been demonstrated to involve a high content of β-sheet and β-turn structures (Rees et al., 1983, 1987). Presumably, therefore, the two CD bands observed with toxin γ correspond respectively to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the peptide chromophores, as previously suggested (Ménez et al., 1978). The chemical modifications of the aromatic residues do not substantially alter the two main dichroic signals. Conservation of these contributions in both native and modified toxins indicates that the *overall* structure of the polypeptide backbone of toxin γ is essentially unaffected by the chemical derivatizations.

(*2*) *Immunological Probes.* $M_{\gamma 1}$ and $M_{\gamma 2/3}$ are monoclonal antibodies that bind with high affinities on loop I and loops II/III of toxin γ, respectively (Grognet et al., 1986). The binding affinities of both antibodies for the two mononitrated toxins were determined by competition experiments. Both modified toxins possess a native-like affinity for each antibody, indicating that the tyrosines are not incorporated in the epitopes which recognize $M_{\gamma 1}$ and $M_{\gamma 2/3}$ and that the structures of these epitopes are not altered by nitration. Previously, it

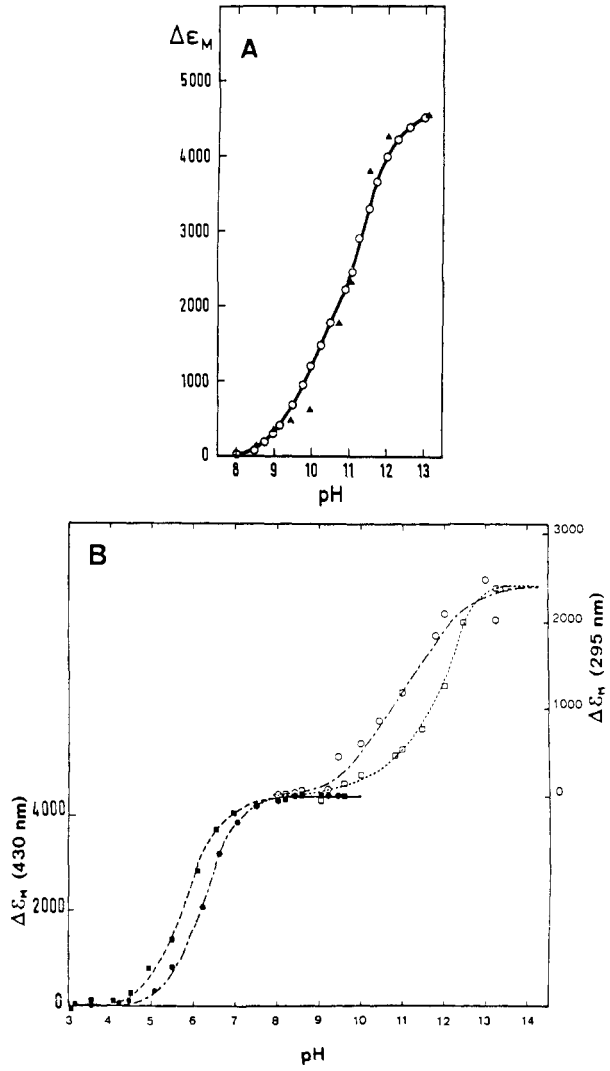

FIGURE 4: (A) Spectrometric titration of toxin γ (○) and its derivative at Trp-11 (▲). Toxins were dissolved in 0.1 M KCl. Titration of tyrosine was performed at 295 nm: the maximum ΔOD were 0.39 for native toxin γ and 0.37 for Trp-11 modified toxin. (B) Spectrometric titration of (3-nitrotyrosine 22)–toxin γ (---) and (3-nitrotyrosine 51)–toxin γ (– – –). Toxins were dissolved in 0.05 M acetic acid plus 0.1 M NaCl. Titration of 3-nitrotyrosine was performed at 430 nm: the maximum ΔOD were 0.25 (■) and 0.20 (●) for derivatives at positions 22 and 51, respectively. Titration of unmodified tyrosine was performed at 295 nm: the maximum ΔOD were 0.13 (□) and 0.09 (○) for derivatives at positions 22 and 51, respectively.

was shown that modification of Trp-11 selectively affects the binding affinity of the toxin for $M_{\gamma 1}$ but not for $M_{\gamma 2/3}$ (Grognet et al., 1986).

(*3*) *Spectrometric Titration.* The spectrometric titration of native toxin γ (Figure 4A) indicates that one (pK_a = 10) of the two tyrosines is exposed to the solvent, whereas the other (pK_a = 11.7) is buried in the molecule and/or involved in stabilizing interactions. Derivatization of tryptophan 11 in toxin γ does not modify the pK_a of the tyrosine residues (pK_a = 10.4 and 11.4), indicating that the environment of the tyrosines is not altered by the incorporation of a bulky nitrophenyl on Trp-11. In mononitrated derivative I, 3-nitrotyrosine 22 and unmodified tyrosine 51 are characterized by pK_a's of 5.8 and 11.9, respectively. Nitrated tyrosine 22 is therefore probably accessible (the pK_a of the model compound *N*-acetylnitrotyrosinamide is equal to 7, whereas tyrosine 51 is certainly not. This result suggests, in agreement with other findings concerning homologous cardiotoxins (Hung et al., 1978), that a similar situation exists in native toxin γ. Surprisingly, however, in the mononitrated derivative II, 3-nitrotyrosine 51 has a pK_a of 6.2, indicating that this nitrated phenolic ring is also accessible. The pK_a of unmodified tyrosine 22 could not be determined precisely but was found to be approximately equal to 11. Since the pK_a difference observed between the two tyrosines in the native state is not found between the two derivatized tyrosines, the tyrosine residue that is buried in the native toxin, presumably Tyr-51, has a different status after nitration.

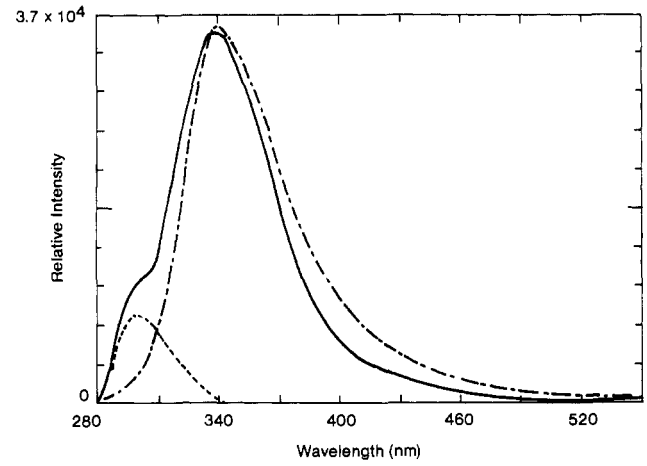

FIGURE 5: Emission fluorescence spectra of native toxin γ for excitation wavelengths equal to 275 (—) or 295 nm (– – –). The difference spectrum (---) shows the emission of tyrosines. Toxin γ was dissolved in 10^{-3} M cacodylate buffer, 10^{-3} M NaCl, and 2 × 10^{-4} M ethylenediaminetetraacetic acid (EDTA), pH 7; θ = 20 °C.

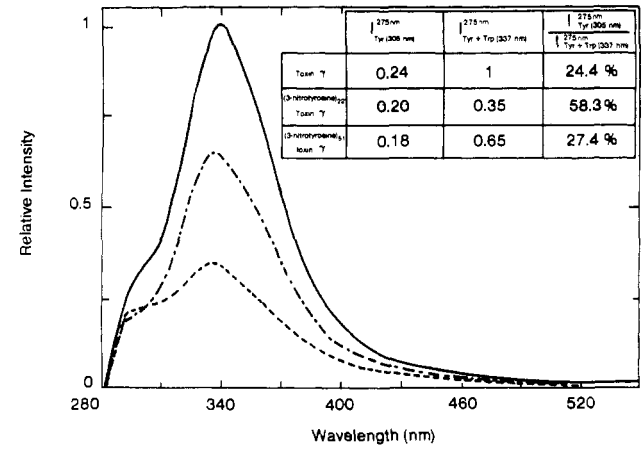

FIGURE 6: Emission fluorescence spectra of native toxin γ (—) and its derivatives mononitrated at positions 22 (---) and 51 (– – –). The excitation wavelength was 275 nm. The inset indicates the contribution of fluorescent residues in total fluorescence at this excitation wavelength. The compounds were dissolved in 10^{-3} M cacodylate buffer, 10^{-3} M NaCl, and 2 × 10^{-4} M EDTA, pH 7; θ = 20 °C.

(*4*) *Fluorescence.* The fluorescence emission spectra of native toxin γ are shown in Figure 5. The emission of tryptophan 11 is characterized by a broad band centered at 337 nm. Under similar conditions, the fluorescence emission of a model compound, *N*-acetyltryptophanamide, is centered at 352 nm (Ménez et al., 1980). The difference in the maximum wavelength suggests that tryptophan 11 is located in a hydrophobic environment. Tyrosine emission is clearly observed when the excitation wavelength is at 275 nm, and the difference spectrum reveals that the maximum emission of it is centered at 305 nm. The fluorescence emission spectra of native toxin γ, (3-nitrotyrosine 22)–toxin γ, and (3-nitrotyrosine 51)–toxin γ are compared in Figure 6 with an excitation wavelength at 275 nm. No screening effect due to

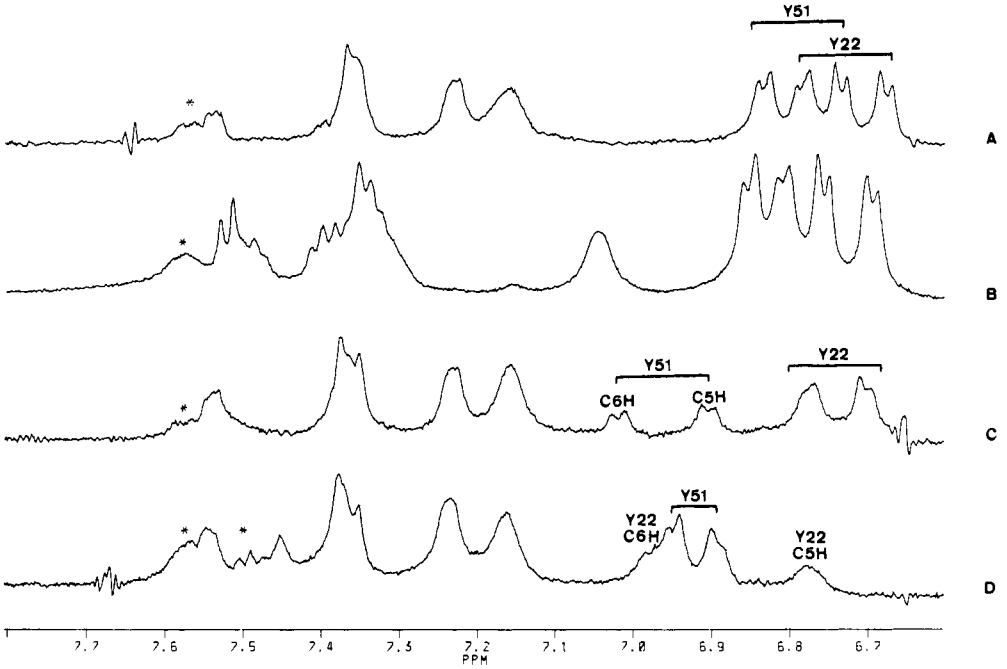

FIGURE 7: 500-MHz ^1H NMR spectra (25 °C) in the region of the aromatic protons (6.6–7.7 ppm): (A) native toxin γ, pH 5.6; (B) Trp-11 modified toxin γ, pH 4.5; (C) (3-nitrotyrosine 51)–toxin γ, pH 5.4; (D) (3-nitrotyrosine 22)–toxin γ, pH 5.5. The compounds were dissolved in D_2O. See assignment in Table I. The asterisks indicate exchangeable proton resonances.

nitrotyrosine absorption has been detected. In the three cases, the maximum fluorescence emissions due to tryptophan 11 and to tyrosine residue(s) remain centered at 337 and 305 nm. Pilot experiments have shown that the fluorescence emission of *N*-acetylnitrotyrosinamide is strongly quenched (not shown). The emission observed for the modified toxins at 305 nm is due, therefore, to the contribution of nonnitrated tyrosine residues only. As indicated in the inset of Figure 6, the contributions under excitation at 275 nm are equal to 0.20 and 0.18 for tyrosine 51 and tyrosine 22 in (3-nitrotyrosine 22)–toxin γ and (3-nitrotyrosine 51)–toxin γ, respectively. The sum of these contributions (0.38) is greater than that due to the two tyrosines in native toxin γ. Presumably, therefore, one or both of the nonnitrated tyrosines in derivatized toxins are localized in an environment which differs from that in native toxin. Figure 6 shows also that nitration of each tyrosine alters the fluorescence intensity due to tryptophan 11. However, it is the modification at residue 22 that induces the greatest decrease (65%). It is difficult to interpret the significance of such quenching effects, which might be due simply to some change in the electronic distribution in the vicinity of Trp residue. However, the maximum wavelength remains centered at 337 nm for both nitrated derivatives, suggesting that the environment of tryptophan has not been greatly changed. Indeed, complete unfolding of the toxin shifts this maximum to a longer wavelength (not shown). Fluorescence excitation spectra have been monitored for native toxin γ. No energy transfer between tyrosine(s) and tryptophan 11 has been detected in these excitation spectra.

(*5*) *^1H Nuclear Magnetic Resonance Spectroscopy.* (*a*) *Assignment of Singular Proton Resonances in Native Toxin* γ. Comparison of the NMR spectra of native toxin γ and those of the three derivatives modified at Trp-11, Tyr-22, and Tyr-51 enabled us to assign unambiguously the proton resonances of these aromatic groups (Figure 7 and Table III) using the standard one- and two-dimensional (COSY, NOESY) techniques. The proton chemical shifts observed for these three residues in toxin γ are identical to ±0.02 ppm with those found for Trp-11, Tyr-22, and Tyr-51 in cardiotoxin $V^{II}2$ (Steinmetz et al., 1981; Hosur et al., 1983). Cardiotoxin $V^{II}2$ from *Naja mossambica mossambica* venom is, like toxin γ, a 60-residue polypeptide with sequence differing from that of toxin γ by only three amino acid substitutions: Ala-28, Pro-30, and Met-31 in toxin γ are replaced in cardiotoxin $V^{II}2$ by Gly, Ser, and Lys, respectively (Louw, 1974). Individual sequential assignment of the α, β, β', and amide protons for 56 of the total 60 residues has been achieved for cardiotoxin $V^{II}2$ by a proton NMR study at 500 MHz (Hosur et al., 1983). The unassigned signals correspond to Leu-1, Lys-2, Pro-8, and Pro-15. In this work, it was suggested on the basis of the observed interresidue NOE connectivities and of the amide protons exchange data that cardiotoxin $V^{II}2$ contains a β-sheet structure with the 20–25 sequence as a nonperipheral strand flanked by two peripheral strands encompassing the 33–39 and 51–56 segments. In this preliminary investigation, we have further compared the results of the above study with the 500-MHz proton data observed for toxin γ in an attempt to obtain the sequential assignment of further singular resonances.

The 95% sequence homology between cardiotoxin $V^{II}2$ and toxin γ is reflected by the observation of a number of very close spectral parameters in the two toxins, including chemical shift values of individual proton signals, their scalar and dipolar connectivities, and the temperature dependence of chemical shifts. Thus, besides the identity of chemical shift values found for Trp-11, Tyr-22, and Tyr-51, the following additional close similarities are observed in the spectra of the two toxins: (i) the same number of slowly exchanging amide protons in the 7.8–10.0 ppm region; (ii) 3 well-separated methyl resonances in the 0.3–0.7 ppm region and 14 α-proton signals in the 6.5–5.0 ppm region (Table II); (iii) very low temperature coefficients (in the 25–60 °C range) for the chemical shifts of all of the above protons, except for those of the amide groups; (iv) dipolar H^{α}–H^{α} connectivities for the 6.23–5.84 ppm signals (6.20–5.82 ppm in cardiotoxin $V^{II}2$) and 6.10–5.98 ppm signals (6.06–5.94 ppm in cardiotoxin $V^{II}2$); (v) a strong NOE connectivity between the protons of the aromatic moieties of Tyr-22 and Tyr-51; and (vi) NOE between H2,6 of Tyr-22 and the signal at 0.50 ppm and between H3,5 of

Table I: Proton Chemical Shifts of the Aromatic Side Chains in Native and Derivatized Toxin γ (θ = 25 °C and pH ≈4)[a]

	toxin γ	toxin γ modified on		
		Trp-11	Tyr-22	Tyr-51
Trp	7.54	7.52	7.54	7.54
Trp, Phe		7.48		
		7.39		
	7.37	7.35	7.38	7.37
	7.36	7.34	7.36	7.36
	7.23	7.32	7.23	7.23
	7.16	7.04	7.16	7.16
Tyr-51, C2,6H	6.84	6.85	6.95	
Tyr-51, C3,5H	6.74	6.75	6.89	
Tyr-22, C2,6H	6.78	6.81		6.78
Tyr-22, C3,5H	6.68	6.69		6.70
NO_2-Tyr, C6H			6.98	7.01
NO_2-Tyr, C5H			6.77	6.90

[a] The values for protons C2H, C5H, and C6H of *N*-acetyl-3-nitrotyrosinamide are respectively 8.01, 7.14, and 7.55 ppm in the neutral form and 7.76, 6.74, and 7.24 ppm in the ionized form.

Tyr-22 and the methyl protons at 0.34 ppm. In the 500-MHz spectrum of cardiotoxin $V^{II}2$, 15 α-protons resonances are present in the 5.0–6.5 ppm region: the sequential assignment has been reported for all of them (Hosur et al., 1983) (Table II). Fourteen α-proton signals are found at 500 MHz for toxin γ in the same spectral region, two of them, centered at 5.52 and 5.42 ppm, are broad at room temperature and considerably sharpen at higher temperature. Among the above H^{α} resonances, those having simultaneously similar chemical shift values in the two toxins and scalar connectivities to β,β′ protons with close chemical shift values in both toxins have been assigned in toxin γ as in cardiotoxin $V^{II}2$ (Table II). This leaves the signals at 5.42 and 5.15 ppm unassigned in toxin γ. The signal assigned to H^{α} of Asn-4 in toxin γ presents in addition a NOE with H^{α} of Trp-11. This is consistent with the crystallographic data of cardiotoxin $V^{II}4$ from *Naja mossambica mossambica* venom (70% sequence homology with toxin γ), showing the proximity of the α-protons of Asn-4 and residue at position 11 in loop I (Rees et al., 1987).

(b) Consequences of the Chemical Modifications on the Proton NMR Spectral Parameters. The assignment of the proton resonances given for the three modified toxins (Tables I and II) were derived according to the procedure adopted for the parent compound. We have used the aromatic protons of Trp-11, Tyr-22, and Tyr-51, and the α-proton of Asn-4 as probes of possible structural effects on the three loops. We also investigated the α-protons of Tyr-22 and Cys-21, -38, and -53 and the protons of $C^{\gamma}H_2$ and the $C^{\delta}H_3$ in Ile-39 as probes of possible structural modifications in the globular region. The individual chemical modifications of Trp-11, Tyr-22, or Tyr-51 induce a number of local conformational changes that are reflected by variations of the chemical shifts (Figure 7 and Tables I and II) and NOE's of some protons. In the tryptophan modified toxin, the chemical shifts of most protons do not vary significantly with respect to the native toxin. At room temperature, two broader signals are observed at 5.68 and 5.62 ppm, respectively. One of these corresponds to the α-proton of Asn-4, although its formal assignment to either of these signals was precluded by the strong overlapping of the latter at the higher temperature chosen for he COSY experiments. No NOE is observed for the α-proton of Trp-11 with any of the other H^{α} signals, unlike that found in the native toxin. In contrast, the NOE's between the aromatic ring protons of the two tyrosine residues 22 and 51 are conserved, as well as the inter-α-proton NOE's of Tyr-22–Cys 53 and Cys-21–Cys-38 in the derivative. In (3-nitrotyrosine 22)–toxin γ, the chemical shifts of the unmodified aromatic protons undergo a downfield variation (+0.10 to +0.15 ppm). Concomitantly, the tyrosines 22–51 inter-ring NOE's vanish. Similar large variations of the chemical shift are observed for H^{α} of Cys-3 (+0.11 ppm), the $C^{\gamma}H_2$ signal of Ile-39 (−0.15 ppm), and the unidentified methyl group (*a*) (see Table II) (−0.18 ppm). In connection with these effects, the NOE's between the H3,5 and H2,6 of Tyr-22 and resonances (b) and (*c*) of Ile-39 (see Table II), which are observed in all the other compounds, are not present in the Tyr-22 modified toxin. In contrast, the inter-α-proton NOE's between Asn-4 and Trp-11, between Tyr-22 and Cys-53, and between Cys-21 and Cys-38 are conserved after nitration of tyrosine 22. Finally, although the aromatic proton chemical shifts of the unmodified tyrosine 22 in the (3-nitrotyrosine 51)–toxin γ are the same as in the native toxin, the interring NOE's are still present but much weaker than in the parent molecule. Simultaneously, the α-proton of Cys-53 is shifted downfeld (+0.07 ppm). The α-protons of tryptophan 11 and asparagine 4 show a NOE correlation as in the parent toxin.

Biological Activities of Native and Modified Toxin γ

(1) Lethal Activity. The LD_{50} of native toxin γ is equal to 1.06 ± 0.08 mg/kg of body weight (M ± SEM), intravenously, in mice. Nitration of tyrosine-51 does not significantly modify the lethal potency of the toxin. In contrast, monomodifications of tryptophan 11 and tyrosine 22 induce 51% and 30% loss of activity, respectively (Table III).

(2) Cytotoxicity. Table III indicates the median effective concentration (EC_{50}) of toxin γ and its derivatives. The EC_{50} of toxin γ, which contains less than 0.01% (w/w) phospholipase A2 contaminant as determined by the method of Dennis (1973), is 17.0 ± 1.03 mg/L (M ± SEM). This assay is specific for toxin γ since addition of 1% (w/w) phospholipase

Table II: Chemical Shifts (25 °C) of Some α-Protons and Other Singular Resonances in Cardiotoxin $V^{II}2$ from *Naja mossambica mossambica* [Data from Hosur et al. (1983)], Toxin γ, and Three Derivatives of Toxin γ

	Tyr-22	Cys-21	Cys-38	Cys-53	Trp-11	Asn-4	Cys-3	Asp-40	Cys-54	Asp-57	Cys-59	Met-26	*a*	Ile-39[b]	Ile-39[c]
N. mosambica mossambica $V^{II}2$[d]	6.20	6.06	5.94	5.82	5.57	5.44	5.21	5.16	5.08	5.06	5.00	5.06	0.60	0.49	0.31
toxin γ[e]	6.23	6.10	5.98	5.84	5.59	5.52	5.28	5.22	5.10	5.08	5.03	5.02	0.62	0.50	0.34
Trp-11 modified toxin γ[f]	6.23	6.10	5.97	5.84	5.56	[g]	5.28	5.22	5.11	5.08	5.05	5.02	0.63	0.50	0.33
Tyr-22 modified toxin γ[h]	6.26	6.11	5.94	5.80	5.62	5.52	5.39	5.24	5.10	NA[j]	5.05	NA[j]	0.44	0.35	0.31
Tyr-51 modified toxin γ[i]	6.25	6.14	6.01	5.91	5.60	5.51	NA[j]	5.21	NA[j]	5.08	5.04	5.02	0.61	0.55	0.34

[a] Unassigned methyl resonance. [b] $C^{\gamma}H_2$. [c] $C^{\delta}H_3$. [d] In cardiotoxin $V^{II}2$, three further H^{α} resonances are Lys-12 (5.21 ppm), Met-24 (5.27 ppm), and Met-24 (5.54 ppm); the signals corresponding to these residues could not be assigned in toxin γ. [e] In toxin γ, two resonances at 5.15 and 5.42 ppm remain unassigned. [f] Signals at 5.14 ppm and at 5.62 or 5.68 ppm are not assigned. [g] Can be assigned either at 5.62 ppm or at 5.68 ppm (see text). [h] Signal at 5.14 ppm not assigned. [i] signal at 5.15 ppm not assigned. [j] NA, not assigned.

Table III: Lethal Activity and Cytotoxicity of Native and Modified Toxin γ

injected compound	LD_{50}[a,c] (mg/kg)	EC_{50}[b,c] (mg/L)
native toxin γ	1.06 ± 0.08	17.0 ± 1.03
Trp-11 modified toxin γ	2.15 ± 0.10	32.0 ± 1.04
Tyr-22 modified toxin γ	1.52 ± 0.05	27.4 ± 1.04
Tyr-51 modified toxin γ	1.00 ± 0.06	14.1 ± 1.06

[a] For the toxicity measurements, the compounds in normal saline solution (0.2 mL) were injected intravenously into mice (20 g). Death occurs within 20 min. [b] For cytotoxicity measurements, the compounds in phosphate saline buffer were added to 6.6×10^5 cells from cultured human amnion. After a 30-min incubation, cytotoxicity was measured by trypan blue exclusion test. [c] Values are means ± standard errors of the means.

A_2 does not increase its cytotoxic effect. The cytotoxic potencies decrease in the same way as the lethalities.

Discussion

Recently, the three-dimensional structure of cardiotoxin $V^{II}4$ from *Naja mossambica mossambica* has been elucidated on the basis of X-ray crystallographic analysis at 3-Å resolution (Rees et al., 1987). The overall three-loop type structure of this toxin resembles closely that of the well-known snake neurotoxins (Low et al., 1976; Tsernoglou & Petsko, 1976; Walkinshaw et al., 1980; Love & Stroud, 1986). It involves a large triple-stranded antiparallel β-sheet formed by residues 20–27, 34–39, and 49–54, as well as two smaller β-sheet strands composed of residues 2–5 and 10–13. Cysteines 21, 38, 53, and 54 belong to the triple-stranded sheet (Rees et al., 1987). Previously, Hosur et al. (1983) reported a detailed NMR analysis of cardiotoxin $V^{II}2$ isolated from the venom of the same snake species. This toxin possesses 68% sequence homology with cardiotoxin $V^{II}4$. The authors proposed that the structure of cardiotoxin $V^{II}2$ involves a β-sheet structure composed of three strands 20–25, 33–39, and 51–56. There is an obvious excellent agreement between these NMR data and the X-ray structure of cardiotoxin $V^{II}4$. Very likely, therefore, cardiotoxins $V^{II}2$ and $V^{II}4$ possess similar folding patterns. Toxin γ, from *Naja nigricollis* venom, possesses 95% and 70% sequence homology with cardiotoxin $V^{II}2$ and $V^{II}4$, respectively. Furthermore, toxin γ and cardiotoxin $V^{II}2$ are characterized by nearly identical circular dichroism spectra (Grognet, 1984) that indicate the presence in both toxins of similar large contents of β-sheet structure, with no helicity (Woody, 1974). Moreover, the preliminary proton NMR analysis of toxin γ reported in the present paper indicates that this toxin possesses a number of common features with cardiotoxin $V^{II}2$ (Steinmetz et al., 1981; Hosur et al., 1983). These include close chemical shift values of the aromatic protons of residues Trp-11, Tyr-22, and Tyr-51, as well as the α-, β-, and β′-protons of 12 amino acid residues. Among these residues are found Cys-21, -38, and -53, Trp-11, and Tyr-22. We wish to suggest that toxin γ and cardiotoxin $V^{II}2$ have comparable folding patterns which presumably both resemble that of cardiotoxin $V^{II}4$ from *Naja mossambica mossambica* (Rees et al., 1987). Figure 8 represents the amino acid sequence of toxin γ folded according to the three-dimensional structure of cardiotoxin $V^{II}4$. Four additional results agree with this folding model for toxin γ. First, the inter-α-protons NOE's found for Tyr-22–Cys-53 and Cys-21–Cys-38 in toxin γ and cardiotoxin $V^{II}2$ are consistent with the presence of the above residues in a triple-stranded antiparallel β-sheet as observed in cardiotoxin $V^{II}4$ (Rees et al., 1987). Second, NOE's indicate that tyrosines 22 and 51 are characterized by a short interring distance, an observation that agrees with the localization of both residues in the model. Third, no NOE

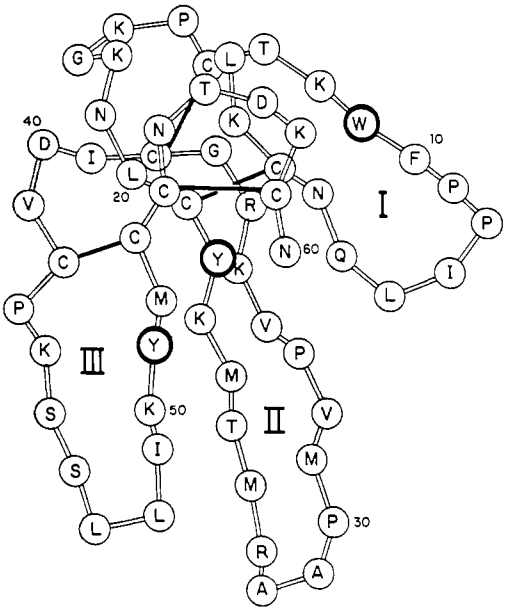

FIGURE 8: Amino acid sequence of toxin γ (Fryklund & Eaker, 1975). The backbone of the toxin has been schematically folded in a manner similar to that of *Naja mossambica mossambica* $V^{II}4$ as deduced from X-ray crystallographic data (Rees et al., 1983, 1987). The amino acid residues are represented by the one-letter code (IUPAC). Numbers I, II, and III indicate the three main loops of the toxin. The three residues that have been subjected to individual chemical modification are emphasized by a heavy ring.

connectivities have been detected between Trp-11 and either tyrosine, and no excitation energy transfer was observed from any tyrosine to Trp-11. This is compatible with the remoteness of Trp-11 from both tyrosine residues (Rees et al., 1987). Fourth, NOE connectivities indicate that Trp-11 is in proximity to Asn-4, as it is proposed in the model structure. The consequences of chemical modifications reported in the present paper will be now discussed on the assumption that toxin γ is folded as it is shown in Figure 8.

Three positions have been individually modified. Tryptophan 11 (loop I) had a nitrophenyl group incorporated via a thioether bond at C_2 indole, whereas tyrosines 22 (loop II) and 51 (loop III) have been mononitrated. No overall structural change occurs in any of the derivatized toxins as compared to the native one, as judged by the conservation of their circular dichroic signals in the peptide region. Subtle changes have been detected, however, for each derivative. Nitrated tyrosine 51–toxin γ is characterized by an increase in the interring distance between Tyr-22 and Tyr-51. The nitrated tyrosine 22 derivative exhibits more substantial changes: the inter-ring NOE between the two tyrosines totally vanishes. This observation indicates that the phenolic groups are separated by more than 6 Å. Moreover, chemical shift changes appear for the α-proton of Cys-3, the $C^{\gamma}H_2$ of Ile-39, and the unassigned methyl resonance (*a*) (see Table II). Simultaneously, the NOE's between the side chain of the modified Tyr-22 and $C^{\gamma}H_2$ and $C^{\delta}H_3$ of Ile-39 disappear in the derivatized toxin. Clearly, therefore, the modification of tyrosine 22 induces structural changes that concern not only the close vicinity of this residue but also the core of the toxin, i.e., the region containing the disulfide bonds. In contrast, the structure at the periphery of the toxin does not seem to be altered after nitration, since two different toxin γ specific monoclonal antibodies recognized both nitrated derivatives with native-like binding affinities. One of these antibodies, $M_{\tilde{\gamma}2/3}$, recognizes an epitope localized at the tips of loops II and III, whereas the other antibody ($M_{\tilde{\gamma}1}$) binds to a site that involves Trp-11 (Grognet et al., 1986). It is noteworthy that a decrease in

Trp-11 fluorescence intensity is observed after either nitration. This effect certainly corresponds to quite a small change in the toxin structure, since the maximum wavelength of the fluorescence emission remains unchanged and since the monoclonal antibody $M_{\gamma 1}$, which recognizes an epitope involving Trp-11, does not perceive it.

Three observations indicate that incorporation of a bulky nitrophenyl moiety on Trp-11 does not alter the structure of loops II and III. First, the inter-ring distance between Tyr-22 and Tyr-51 remains unchanged as does the H^{α}–H^{α} distance for Tyr-22–Cys-53 and Cys-21–Cys-38. Second, the pK_a's of both tyrosines remain identical with those in the native toxin. Third, monoclonal antibody $M_{\gamma 2/3}$ that binds on the tips of the loops II and III binds to the Trp-11 modified toxin and the native toxin with identical binding affinities (Grognet et al., 1986). In contrast, a change is detected in loop I since the distance separating Asn-4 and Trp-11 increases, as revealed by the loss of NOE between the α-protons of these residues. Apparently, therefore, the structural changes that occur subsequent to Trp-11 modification mainly concern its close vicinity.

As emphasized by Harvey (1985), the mode of action of cardiotoxins remains obscure. A number of different actions have been described in the literature (Sakar, 1947; Chang et al., 1972; Condrea, 1974; Louw & Visser, 1977; Harvey et al., 1982), and so far, it has not been definitely established which of these actions is truly associated with the toxic effect of these molecules in mammals. There is general agreement, however, that cardiotoxins act primarily on cell membranes. Indeed, toxin γ, which is a potent toxin, is also characterized by a strong cytotoxic action toward FL-cells. It is striking that the lethal activities of derivatized toxins correlate perfectly with their cytotoxic abilities. Thus, the nitration of Tyr-51 does not alter both activities, whereas nitration of Tyr-22 or modification of Trp-11 induces a substantial decrease of both activities, the Trp-derivatized toxin being the less potent. Considering the present structural and biological investigations together, we draw the following conclusions: (1) the phenolic group 51, which is located on loop III, interacts with a number of residues located in the core of toxin γ but, nevertheless, is not directly involved in the "toxic" site of the molecule; (2) tyrosine-22, which is located on loop II, may play a dual role, ensuring the maintenance of the toxin structure and participating in some hydrophobic requirement for the toxic action; (3) Trp-11, which is localized on loop I, is incorporated in or in close proximity to the functional site of toxin γ, indicating, in agreement with previous proposals (Dufourcq et al., 1982), that loop I is involved the "toxic" site of a cardiotoxin. Further work is now in progress to delineate more precisely the functional site of toxin γ.

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Registry No. L-Tyr, 60-18-4; L-Trp, 73-22-3.

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Thermodynamics of Carbon Monoxide Binding to Monomeric Cytochrome *c'* †

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ABSTRACT: The thermodynamic parameters for carbon monoxide binding to monomeric *Rhodopseudomonas palustris* cytochrome *c'* are determined. An enthalpy change for CO(aq) binding to the cytochrome is measured directly by titration calorimetry as -6.7 ± 0.2 kcal/mol of heme, the CO binding equilibrium constant is measured at 35 °C as $(1.96 \pm 0.05) \times 10^5$ M^{-1}, and the binding equilibrium constant at 25 °C is calculated from the van't Hoff equation as $(2.8 \pm 0.1) \times 10^5$ M^{-1}. Comparison of the results to the known energetics of CO binding to dimeric cytochrome *c'*, where the CO binding site is buried in the protein interior, indicates that the heme binding site on the monomer form is, in contrast, more exposed.

The cytochromes *c'*, a class of high-spin *c*-type cytochromes, are widely distributed in the bacterial world. Although apparently functioning in bacterial electron transfer (Horio & Kamen, 1962a,b; McEwan et al., 1985), the nature of their physiological donors and acceptors are not well understood. Of particular functional interest is the fact that the cytochromes *c'* are usually isolated as dimers consisting of identical subunits, suggesting possible allosteric interactions. However, at least one example, from *Rhodopseudomonas palustris*, is isolated in monomeric form (Dus et al., 1967; Cusanovich, 1971). The high-resolution three-dimensional structure of *Rhodospirillum molischianum* cytochrome *c'* is known (Finzel et al., 1985), as well as the amino acid sequence of 12 examples (Ambler et al., 1981). On the basis of their spectral properties and their ability to bind CO, the cytochromes *c'* more closely resemble hemoglobin than the typical *c*-type cytochromes. A comprehensive literature review has been presented by Meyer and Kamen (1982).

This paper is a continuation of systematic structure–function studies on the reversible carbon monoxide binding reactions of the cytochromes *c'*. Cytochromes *c'* are especially attractive for rigorous thermodynamic analysis since their dimeric nature allows theoretical modeling of site–site interactions to be cast in the simplest possible terms. Additionally, the CO binding curves can be measured to high precision with a special thin-layer device (Dolman & Gill, 1978).

Previous work has demonstrated positive cooperativity in the CO binding curve of *Chromatium vinosum* cytochrome *c'*, the origin of which was found in a CO-linked dimer–monomer dissociation process (Doyle et al., 1986). A quan-

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