Structure and Chemical Modifications of Neurotoxin from *Naja nigricollis* Studied by Raman Spectroscopy

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ABSTRACT: Raman spectroscopy was used to determine structural features of the native toxin α from *Naja nigricollis*, which contains only one Trp and one Tyr, and of chemically modified toxins having chromophores added to these two conserved aromatic amino acids. The percentages of secondary structure were determined by using amide I polypeptidic vibration analysis and are in agreement with X-ray structure [Low et al. (1976) *Proc. Natl. Acad Sci. U.S.A. 73*, 2991-2994] as well as with the geometry of the disulfide bridges estimated by using the ν(S-S) vibrations. In the native toxin α, the single invariant tyrosine 25 appears to be buried in the structure and involved in a strong hydrogen bond. We have chemically modified these two invariant aromatic side chains by addition of chromophores. The presence of a (nitrophenyl)sulfenyl (NPS) chromophore bound to the Trp does not perturb the secondary structure of the toxin as shown by the analysis of the polypeptidic amide I vibrations; however, the environment of this Trp and the geometry of a disulfide bridge seem to be modified. The secondary structure is not affected by the presence of the NPS chromophore; therefore, the decrease in binding affinity observed after modification of Trp-29 by the reagent NPS-Cl [Faure et al. (1983) *Biochemistry 22*, 2068-2076] is due to an alteration of the environment of this aromatic amino acid and/or a steric hindrance and not to an overall modification of the toxin structure. The binding assays of [nitrotyrosyl]toxin show that after nitration the affinity toward the monoclonal antibody $M_{\alpha 1}$ is unchanged and that the affinity toward the cholinergic receptor (AcChR) from *Torpedo marmorata* remains high. We concluded that the structure of toxin α after adding the NO_2 chromophore to Tyr-25 is the same as it is in native toxin.

It is well documented that snake venom neurotoxins are potent blockers of the nicotinic acetylcholine receptor [reviewed by Changeux (1981)]. In the past decade, the primary structures of more than 50 of them were elucidated, revealing the existence of two toxin classes. The short-chain toxins are single-chain proteins with 60-62 amino acid residues and four disulfide bridges, and the long-chain ones have 66-74 residues and four or five disulfide bonds (Mênez et al., 1978). Toxin α from *Naja nigricollis* (Figure 1) belongs to the former class (61 amino acids and MW = 6.8 kDa). Examination of the sequences disclosed that several positions are highly conserved. Comparison with structurally homologous cardiotoxins led to the proposal that some residues of the neurotoxins are conserved in relation with their neurotoxic function, whereas other are invariant because they maintain the tertiary folding of the molecule (Ryden et al., 1973; Mênez et al., 1984). As judged from X-ray crystallographic studies (Low et al., 1976; Tsernoglou & Petsko, 1976; Kimball et al., 1979) on several short- and long-chain toxins, the overall folding pattern is highly conserved, consisting of three adjacent loops of β pleated sheets protruding from a core where the invariant disulfides are cross-linked. Neurotoxins are flat molecules, the plane of which is defined by the sheet of the three loops. Interestingly, most functionally invariant residues are located on one face of the molecule, all pointing toward the same direction. These residues essentially belong to the second and third loops, forming a homogeneous surface by which, presumably, they recognize the nicotinic acetylcholine receptor.

A variety of chemical studies have been reported with the aim of investigating the functional and/or structural role of the conserved residues [for reviews, see Dufton and Hider (1983) and Endo and Tamiya (1987)]. Among these experiments, chemical modifications of particular side chains have been made and the remaining affinity toward AcChR[1] or monoclonal antibodies was compared to that of native toxin (Chicheportiche et al., 1972; Mênez et al., 1982; Boulain et al., 1982; Faure et al., 1983). These studies have shown that the chemical modifications of Lys-27 and -47 and Trp-29 give rise to a decrease in affinity toward AcChR, and it was concluded that these amino acids are involved in the site of binding to AcChR. EPR (Tsetlin et al., 1979; Rousselet et al., 1984) and fluorescence (Tsetlin et al., 1982) investigations have also identified lysines 27 and 47 in the active site of toxin α.

In this Raman spectroscopic investigation, we were interested in studying the consequences of chemical modifications of the two conserved aromatic side chains of Tyr-25 and Trp-29 of toxin α *N. nigricollis*. These modifications are the addition of a NO_2 group to Tyr-25 and of a nitrophenyl thioether (NPS) group to Trp-29. We describe the spectral features of the NPS chromophore, and we compare the native and modified toxins in order to detect possible structural modifications induced by the addition of the chromophores.

Materials and Methods

Chemical Modifications of the Toxin. Toxin α from *N. nigricollis* was isolated as described by Fryklund and Eaker

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[1] Abbreviations: AcChR, acetylcholine receptor; EPR, electron paramagnetic resonance; TNM, tetranitromethane; NPS Trp-29 toxin α [C_2-[(2-nitrophenyl)sulfenyl]-Trp^{29}]toxin α; NO_2 Tyr-25 toxin α, [C_3-nitro-Tyr^{25}]toxin α; NPS-CL, 2-nitrobenzenesulfenyl chloride.

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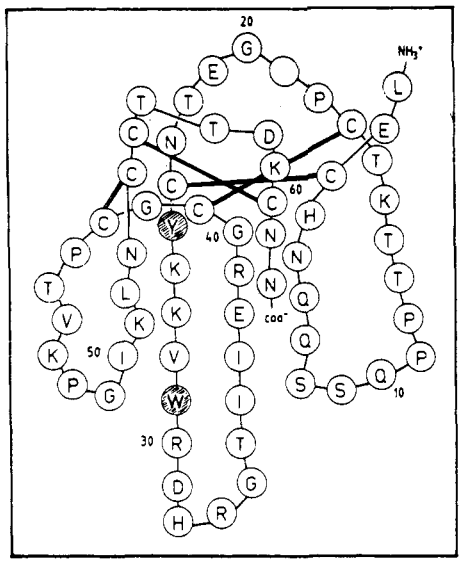

FIGURE 1: Primary structure of toxin α from *N. nigricollis*. Tyr-25 and Trp-29, on which are added preresonance chromophores, are hatched.

(1975). The sulfenylation of the toxin was performed according to Fontana and Scoffone (1972) and was described by Faure et al. (1983), who have measured a 30-fold decrease in affinity for the NPS Trp-29 toxin toward AcChR.

The nitration of the toxin was carried out according to Karlsson and Sundelin (1976) by reaction of tetranitromethane (TNM) and tyrosine with a TNM/tyrosine molar ratio of 20. Before this reaction, the denaturation of toxin (1 μmol) without breaking the disulfide bridges was performed in 6 M guanidinium chloride and 0.1 M Tris buffer at pH 8. TNM (20 μmol) in 50 μL of ethanol was added to the solution of denatured toxin, which was gently stirred at 20 °C in the dark for 2 h. Nitrated toxin was then purified: byproducts have been eliminated by two successive filtrations through columns. A first purification was realized with a Sephadex G50 column equilibrated with 0.1 M ammonium acetate buffer, pH 7.1; the optical density of 1-mL fractions was measured at 352 nm. Higher OD fractions were freeze-dried. A second purification was done with a Bio-Rex column through which a 0.05–0.5 M linear ammonium acetate gradient at pH 8 was passed for 2 h. The fraction corresponding to higher optical density was assayed for activity toward AcChR and a monoclonal antibody, $M_{\alpha 1}$. This fraction was freeze-dried after preparation. In order to record spectra, the native and modified toxins are dissolved in 10^{-2} M Tris buffer, pH 7.4, to obtain concentrations of 50 and 20 mg/mL, respectively.

TNM was purchased from Sigma; absorption spectra were recorded on a Beckman spectrophotometer with a 2-mm path length quartz cell.

Binding Assays. Binding activity of [nitrotyrosyl]toxin was determined from competition assays between the modified and tritiated toxins and compared with competition assays done with native and tritiated toxins. The method was described by Faure et al. (1983), who have used this technique for several derivatives, including NPS Trp-29 toxin α. These competition measurements were performed with varying amounts of modified toxin (from 1×10^{-7} to 0.3×10^{-9} M) and constant amounts of [^{3}H]toxin (5×10^{-9} M) and of receptor (2×10^{-9} M), which was added after the toxins were mixed. The incubation time was 2 h. The solutions were filtered through Millipore filters, and the radioactivity of [^{3}H]toxin retained was counted. Cholinergic receptor rich membranes were prepared according to Sobel et al. (1977) and Saitoh et al. (1979) from *Torpedo marmorata* electric organ in the presence of proteinase inhibitors.

Raman Spectroscopy. Samples (5–10 μL) in Tris-HCl buffer at pH 7.4 were placed in capillary glass tubes held in a thermostated cell at 10 °C. Excitation was provided by an argon ion laser (Coherent Radiation Innova 90-3) tuned at 488 or 458 nm, with interference filters placed between the laser and the sample. The excitation power was maintained as low as possible to avoid thermal denaturation. The power used depended on the wavelength and on the sample, as indicated in the figure captions. The double-monochromator Raman spectrometer was a Coderg PH 1. It was calibrated with Rayleigh scattering of argon laser lines by water and by comparing known spectra of dissolved compounds or solvents such as acetylcholine or ethanol. The two halographic gratings were blazed at 1800 grooves/mm. An ITT 130 photomultiplier was used for detection and cooled at −30 °C to reduce noise. The slits were adjusted to provide a resolution of 6 or 4 cm^{-1}. An Apple IIe computer controlled a stepper motor driving the gratings and recorded data from a Racal-Dana timer–counter. Several scans were collected (12–25) by measuring the Raman of scatter in 1-cm^{-1} steps with a dwell time of 1 s. Each scan was examined for the presence of spikes before averaging.

Digital recording was needed to subtract the buffer spectrum and the fluorescence baseline from original spectra. Smoothing was performed by Fourier transform methods. To analyze the secondary structure content of toxin α and NPS Trp-29 toxin α, we have used all the steps of the method developed by Williams (1983, 1986), which is based on the sensitivity of polypeptide chain vibrations (amide I band) and the relationship between known structures and frequencies (Miyazawa, 1960). Before analysis, the spectrum of the buffer, recorded under the same conditions, was subtracted from the original spectrum of the toxin. The 2500–3200-cm^{-1} region of each spectrum was recorded just following the measurement in the 400–1800-cm^{-1} region, and the linearity of the baseline from 2500 to 3200 cm^{-1} was used as a criterion of well-performed subtraction. Then, the vibration bands from aromatic side chains in the range 1582–1620 cm^{-1}, which overlap the amide I band, were fitted by a least-squares method. The resulting fitted curve was subtracted. In this way we subtracted the peak from NPS, close to the amide I band, instead of subtracting the spectrum of a NPS-derivatized amino acid because that one can be shifted with respect to the protein-incorporated NPS amino acid. To perform the structural analysis, the amide I band was assumed to be a linear sum of vibrations from the six possible structures (ordered and disordered helices, parallel and antiparallel β sheets, β turns, and undefined structure). The normalized intensity was compared to a set of reference Raman spectra from proteins, the structure of which have been determined by X-ray diffraction. We have used the data set from Williams' method (1986), and we have already applied this technique to another protein in solution, the enzyme acetylcholinesterase (Aslanian et al., 1987).

Results

(1) Native Toxin α. (a) Secondary Structure. The Raman spectrum of native toxin α in aqueous solution and the assigned vibrations are shown in Figure 2a and in Table I, respectively. Very little fluorescence was observed with 488-nm excitation and subtracted from the spectrum. The spectrum gives clear indications about the secondary structure of the polypeptide chain of toxin α. The main amide I and III vibration bands from the polypeptide backbone have frequencies of 1668 and 1243 cm^{-1}, respectively, indicating that the chain contains a

Table I: Frequencies and Assignments of the Vibrations of Raman Spectra of Native Toxin α and NPS Trp-29 Modified Toxin α (Figure 2)

frequencies (cm^{-1})			
native toxin α	NPS Trp-29 toxin α	assignments: vibrations	assignments: groups
509–544	509–519	ν(S–S)	Cys
604	602	δ(C=O), amide VI	polypep backbone
645	640	ν(C–C) ring	Tyr
686–730		ν(C–S)	Cys
759	745–760	indole	Trp
838	827–838	ν(C–C) ring	Tyr
880	886	ν(C–C) indole	Trp
933	932	ν(C–C)	polypep backbone
1000		ν(C–C) ring	Tyr
1014	1009	ν(C–C) indole	Trp
1076	1086	ν(C–N)	side chains
1129		ν(C–C) + ν(C–N)	Tyr + backbone
1171	1168		Tyr
1198	1201	$\nu(C-C_6H_4)$	Tyr
1243–1250	1252	amide III	polypep backbone
1317		$\delta(CH_2)$	aliphatic side chains
1350		indole	Trp
1015–1415		$\nu_s(COO^-)$	Asp + Glu
1447	1452	$\delta(CH_2) + V(CH_3)$	aliphatic side chains
1552	1551	indole	Trp
1617	1613	ν(C–C) ring	Tyr + Trp
1668	1660–1670	amide I	polypep backbone

large amount of β structure. The shoulder at 1250 cm^{-1} reveals the presence of a small component due to disordered structure. Quantitative estimation of secondary structure content was obtained by analysis of the amide I vibrations after subtracting aromatic side-chain contributions (Figure 3 and Table II). As indicated in Table II, β structure is predominant: 54% β sheets and 22% β turns. No helical structure was observed, but some random coil structure is present (14%). The value obtained for disordered helix involves 5 ± 2 amino acids; such a small number does not give rise to a helical segment and may correspond to a constrained turn between adjacent sheets. The total structure, indicated in Table II, is not the total obtained by summing up the results of different structures but is calculated by least-squares methods, as done for each structure.

Clearly, the secondary structure of toxin α is similar to those of other homologous toxins for which the three-dimensional structures have been determined by X-ray diffraction (Low et al., 1976; Tsernoglou & Petsko, 1976; Kimball et al., 1979).

(*b*) *Side Chains.* The Raman spectrum also gives valuable information about the side-chain organization in toxin α. The geometry of the disulfide bridges can be determined by the frequencies of the ν(S–S) stretching vibration. In the spectrum of native toxin α (Figure 2a), the ν(S–S) vibrations are well-defined and show two components located at 509 and 544 cm^{-1}.

In order to describe the geometry of disulfide bridges from the vibrational frequencies provided by Raman spectroscopy, two models were proposed. Sugeta et al. (1972, 1973) have assigned frequencies of the ν(S–S) stretching vibrations to different "gauche–trans" conformations. According to this model, the intense peak at 509 cm^{-1} corresponds to disulfide bridges with the C–S–S–C bonds each in a gauche conformation (GGG), and the less intense component at 544 cm^{-1} more likely indicates a disulfide bridge with a trans–gauche–trans conformation (TGT). Van Wart et al. (Van Wart & Scheraga, 1976; Van Wart et al., 1976) have proposed another model, based on the study of model compounds, in which ν(S–S) frequencies are related to the values of χ(SS–CC) and φ(CS–SC) dihedral angles. According to this general model, the 509-cm^{-1} frequency that we observed corresponds to χ(SS–CC) angles greater than 50° and a φ(CS–SC) unconstrained angle greater than 65°. The other component located at 544 cm^{-1} (Figure 2) would correspond to the ν(S–S) vi-

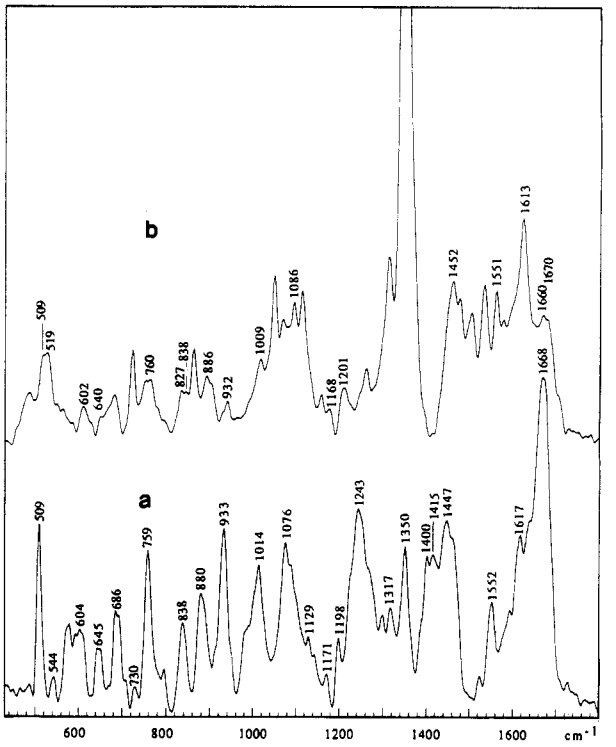

FIGURE 2: Raman spectra of (a) native toxin α and (b) NPS Trp-29 modified toxin α. λ_0 = 488 nm; T = 10 °C; P = 150 mW; spectral slits, 4 cm^{-1}. These spectra are water subtracted, as described under Materials and Methods.

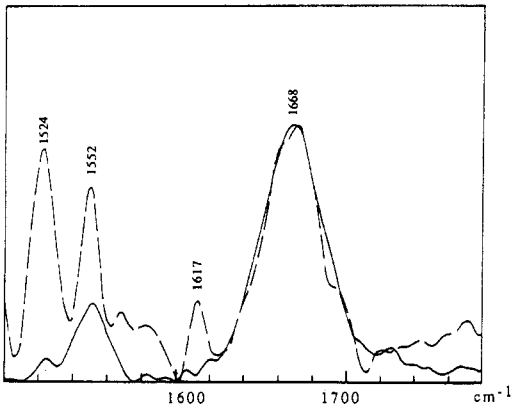

FIGURE 3: Amide I polypeptidic vibration bands after the subtraction of aromatic amino acid vibrations in order to analyze secondary structure. (—) Native toxin α; (- - -) NPS Trp-29 modified toxin α.

Table II: Analysis of the Secondary Structure Using Polypeptide Backbone Vibrations: Comparison of Structures between Native Toxin α and NPS Trp-29 Modified Toxin α

	structures (%)						
	ord helix	disord helix	∥ β struct	anti∥ β struct	β turns	undef struct	total
native toxin α	0 ± 2	9 ± 2	4 ± 3	50 ± 5	22 ± 2	14 ± 2	99 ± 2
NPS Trp-29 toxin α	0 ± 4	9 ± 3	3 ± 3	52 ± 5	22 ± 3	14 ± 2	96 ± 5

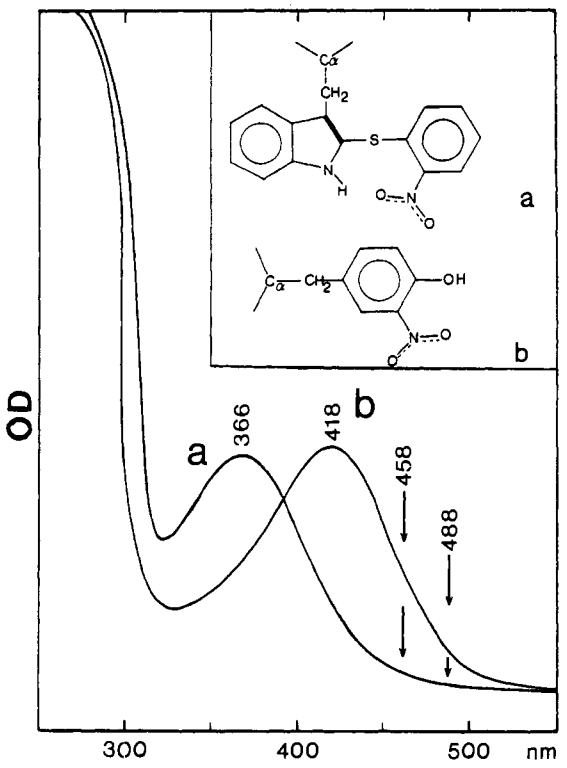

FIGURE 4: Absorption spectra of (a) NPS Trp-29 modified toxin and (b) [nitrotyrosyl]toxin at pH 8. The excitation wavelengths provided by the argon laser are indicated by the arrows. The inset shows the chromophores added to toxin α: (a) nitrophenyl thioether Trp-29 and (b) nitrotyrosine 25.

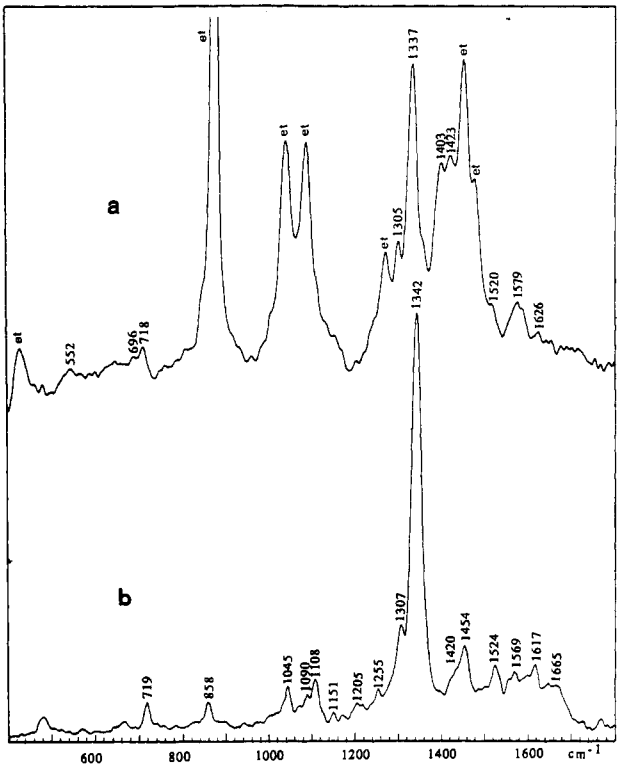

FIGURE 5: Raman spectra of NPS chromophore in ethanol solution (a) and bound to Trp-29 of toxin α (b). The peaks labeled "et" come from ethanol. λ_0 = 458 nm; T = 10 °C; P = 50 mW; spectral slits, 4 cm^{-1}.

bration frequency of two dihedral angles χ(SS–CC) smaller than 50°. This interpretation is in accord with values close to 90° for three angles φ given by X-ray diffraction for erabutoxin, which is homologous (Kimball et al., 1979).

The phenol vibrational doublet located near 830–850 cm^{-1} comes from a Fermi interaction between two vibrations. The intensity ratio of this doublet is indicative of the tyrosine environment (Siamwiza et al., 1975): a freely accessible phenolic ring is usually characterized by a vibrational component located at 850 cm^{-1} and a buried one by a component at 830 cm^{-1}. The single tyrosine of native toxin α gives rise to a singlet at 838-cm^{-1} frequencey (Figure 2a). The absence of the second component at 850 cm^{-1} indicates that the Tyr-25 residue is not freely accessible to solvent and that this hydroxyl group could be involved in a hydrogen bond with a charged polar residue.

The indole ring of tryptophan also is characterized by a vibrational doublet located at 1340–1360 cm^{-1} due to a Fermi interaction. Harada et al. (1986) have shown in a study of indole and tryptophan derivatives that changes of environment of the indole ring modify the intensity ratio of this doublet. Supporting these data, Thomas et al. (1983) have observed an increase of the 1360-cm^{-1} component correlated with hydrophobic environment in the case of virus proteins. For toxin α, the frequency of this vibration is located at 1350 cm^{-1} (Figure 2a). According to Harada's interpretation, this suggests that part of Trp-29 is in a hydrophobic environment, whereas the other part is positioned within a more polar one. The partially hydrophobic nature of its neighborhood may be further deduced from the 880-cm^{-1} peak, as suggested by Kitagawa et al. (1979), who have observed an intensity decrease of this vibration upon denaturation in spectra of Bence–Jones proteins.

(2) Toxin α Modified at Trp-29 Possessing a Nitrophenyl Thioether Group (NPS). NPS Trp-29 toxin α has an absorption spectrum that is pH-independent (Figure 4a). The two arrows indicate the wavelength available with the argon ion laser that we have used. The excitation at 458 nm, which is the nearest wavelength to the absorption maximum, gives rise to an enhancement of the chromophore vibrations that permits their identification. The second excitation wavelength (488 nm) has been used to study the structure of the modified NPS Trp-29 toxin.

Table III: Frequencies and Assignments of the Vibrations for the Raman Spectrum of the NPS Chromophore, in Ethanol Solution and Covalently Bound to Trp-29 of Toxin α (Figure 5)

frequencies (cm^{-1})		
NPS/ethanol	NPS/toxin	assignments
552		ν(C–S)
696		ν(C–S)
718	719	ν(C–C) nitrophenyl
	1045–1108	nitrophenyl ring vib
1305	1307	NPS ring vib
1337	1342	$\nu_s(NO_2)$
1403–1423	1405–1420 sh	NPS ring vib
1520	1524	$\nu_a(NO_2)$
1579–1626		ν(C–C) NPS ring

(a) The NPS Chromophore. 2-Nitrobenzenesulfenyl chloride is a specific reagent for tryptophan residues, used to incorporate a nitrophenyl group via a thioether bond to the C_2 carbon of the indole ring (Fontana & Scoffone, 1972). Trp-29 of toxin α was modified with this reagent, and the modified toxin was purified according to the procedure previously described (Faure et al., 1983). Figure 5 compares the spectra of the NPS free in ethanol solution and bound to Trp-29 (see Table III for the corresponding vibrations). The spectral features of the modified toxin spectrum (Figure 5b) obtained with an excitation wavelength of 458 nm are different from those recorded by using a 488-nm excitation (Figure 2b). With 458-nm excitation, the preresonance scattering due to

the NPS bound to Trp-29 is predominant, and the amide I vibrations from the polypeptide backbone of the toxin appear with very low intensity at 1665 and 1255 cm^{-1}. The side-chain vibrations are not visible under these conditions (Figure 5b). The spectrum of NPS in ethanol solution (Figure 5a) allows us to identify its vibrations when bound to toxin.

The prevailing peak at 1337 cm^{-1} (Figure 5a) comes from the $\nu_s(NO_2)$ symmetric stretching vibration. Its frequency is shifted to 1342 cm^{-1} when the chromophore is bound to Trp-29 (Figure 5b). The antisymmetric stretch $\nu_a(NO_2)$ appears at 1520 cm^{-1} as a shoulder near an ethanol peak when NPS is in solution and at 1524 cm^{-1} when it is covalently bound to the toxin.

The ν(C–S) stretching vibrations from monosulfide in NPS are also present and give rise to two weak peaks, which are located at 696 and 552 cm^{-1} for NPS in ethanol (Figure 5a). Storer et al. (1979) have observed near frequencies for ν_s(C–S) vibrations of several monosulfide compounds. The intensity of these vibrations is not sufficient to assign them with certainty after binding to Trp-29. The frequencies are likely shifted after the chemical reaction, but no study of the relationship between geometry and vibrational frequencies for monosulfides is available, as it is for dialkyl disulfides.

The vibration at 718 cm^{-1}, which is not disturbed by binding to Trp (719 cm^{-1}), is assigned to the ν(C–C) stretch of the nitrophenyl ring of NPS. Other ring vibrations are apparent at 1045 and 1090 cm^{-1} when NPS is bound to the toxin (Figure 5b) but are hidden by ethanol peaks when NPS is in solution (vibrations at 858 and 1108 cm^{-1} appear as shoulders; Figure 5a). The ν(C–N) stretching vibrations between phenyl and NO_2 groups are located at 1220 and 1151 cm^{-1} (Scheule et al., 1980).

(b) NPS Trp-29 Toxin α. We have used 458-nm excitation to assign the vibrations of the chromophore, but excitation at 488 nm, farther from the absorption maximum, provides preresonance conditions that permit the resolution of the polypeptide and side-chain vibrations. This allows the use of the amide I band to determine the secondary structure of the modified toxin by Williams' method. The Raman spectra of both native and modified toxins (Figure 2) were recorded under the same conditions, except that the laser power was lower for the modified toxin. Figure 2b presents the spectrum of NPS-modified toxin α, which can be directly compared to that of the native toxin; the vibrational assignments are listed in Table I.

Many vibrations of the aromatic side chains are masked by preresonance peaks and are difficult to analyze, for example, the tyrosine and tryptophan peaks located at 838 and 1350 cm^{-1}, respectively; the latter completely disappears because of the presence of the strong $\nu(NO_2)$ signal at 1340 cm^{-1}. However, an environment-sensitive vibration of the indole ring is apparent at 886 cm^{-1} (880 cm^{-1} for native toxin). The changes of this vibration must be compared with a vibration that is not environment-sensitive, such as $\delta(CH_2)$ from aliphatic side chains, located near 1450 cm^{-1}. The intensity ratio I_{886}/I_{1452} is lower for the modified toxin than for the native toxin and indicates a change in the environment of the indole ring (Kitagawa et al., 1979; Thomas et al., 1983). Thus the contact of Trp-29 with hydrophobic groups seems to be less tight. The indole vibration (759 cm^{-1} in the native toxin; Figure 2a) has two components at 754 and 757 cm^{-1} for the modified toxin (Figure 2b). The second component may arise from the chromophore ring or from the indole ring.

The side-chain vibrations of cysteine also provide structural information. The stretching vibrations of the disulfide bridges

Table IV: Frequencies and Assignments of the Vibrations for Raman Spectra of [Nitrotyrosyl]toxin in Solution (Figure 6)

frequencies (cm^{-1})	assignment
776	ring vib (*p*-disubst benzene)
830	ring vib
898	ring vib
1088	ν(C–N) + ν(C–C)
1120	ν(C–C)
1194	$\nu(C_6H_4–R)$
1270	$\nu(NO_2)$ + ν(C–OH)
1341	$\nu_s(NO_2)$
1451	$\delta(CH_2)$
1538	$\nu_a(NO_2)$
1622	ring vib

ν(S–S) are present for the modified toxin (Figure 2b). The two components at 509 and 519 cm^{-1}, which correspond to two different geometries of the S–S bridges, appear with similar intensities; this suggests the presence of two disulfide bridges in each conformation. Three bridges have the same conformation in the native toxin, as indicated by its Raman spectrum (Figure 2a). The frequencies 509 and 519 cm^{-1} correspond respectively to GGG and TGG conformations (Sugeta et al., 1972, 1973) or to dihedral angles χ(CC–SS) greater and smaller, respectively, than 50° (Van Wart & Scheraga, 1976, Van Wart et al., 1976). This change in the ν(S–S) frequency clearly indicates a geometrical modification—but not a drastic one—of one disulfide bridge after (nitrophenyl)sulfenylation of Trp-29. The ν(C–S) stretches of the cysteines are hidden by the peak at 715 cm^{-1} due to NPS.

In the spectrum of NPS Trp-29 toxin α (Figure 2b), amide I vibrations of the polypeptide chain give rise to a peak at 1660 cm^{-1} with a shoulder near 1670 cm^{-1}. The proximity of a preresonance peak from NPS-Trp vibrations at 1613 cm^{-1} modifies its apparent intensity, and moreover, there is a frequency shift of the amide III band (1252 cm^{-1}), which is close to the intense preresonance peaks at 1303 cm^{-1}. Thus, it was necessary to subtract the components due to the aromatic amino acids and to NPS from the amide I band in order to analyze these polypeptidic vibrations in the same way we did as for the native toxin (Figure 3 shows the amide I band after subtraction for both toxins). Components with higher intensity have been subtracted from the spectrum of the modified toxin, increasing the error in this case. The amide I band thus appears centered at 1668 cm^{-1}, with a maximum at 1672 cm^{-1}. The percentages of all structures are close to those of the native toxin (Table II). The differences between the values for the native and NPS-modified toxins are lower than the errors of the method, due essentially to possible differences in crystal and solution states of the reference proteins set (Williams, 1983, 1986) and to the subtraction of components due to aromatic side chains.

Our Raman experiments thus show that the secondary structure of the toxin α from *N. nigricollis* does not appear to be significantly affected by addition of the NPS chromophore to Trp-29.

(3) Toxin α Modified by Nitration of Tyrosine 25. The nitration of toxin α performed according to Karlsson and Sundelin (1976) modifies only Tyr-25. The denaturation before reaction avoids the use of a high concentration of TNM, which favors the formation of polymers. The absorption spectrum of nitrotyrosine is pH-dependent and shows a band due to nitrophenol with a maximum located at 418 nm at pH 8 (Figure 4). Excitation at 488 nm gives a resonance Raman spectrum of [nitrotyrosyl]toxin α similar to that obtained with excitation at 458 nm (Figure 6 and Table IV). The profile of the Raman resonance spectrum of [nitrotyrosyl]toxin α

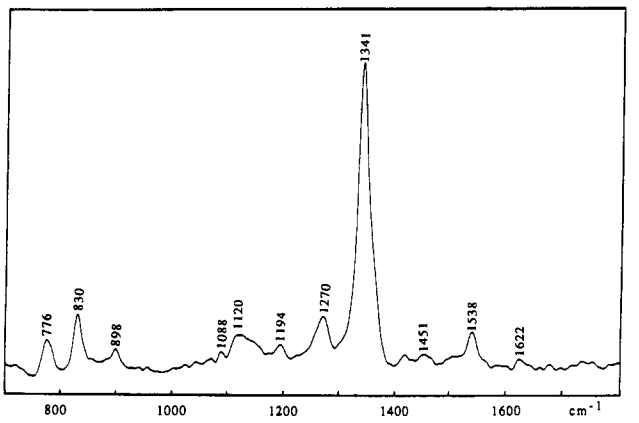

FIGURE 6: Resonance Raman spectra of [nitrotyrosyl]toxin in Tris buffer, pH 7.5; λ_0 = 458 nm; T = 10 °C; P = 50 mW; spectral slits, 4 cm^{-1}.

(Figure 6) is different from that of native toxin α (Figure 2a). The peak at 830 cm^{-1} comes from the ring of nitrotyrosine. This frequency depends on the presence of a phenol hydrogen bond, as shown by Siamwiza et al. (1975). Breaking of the hydrogen bond involving phenol as donor (the vibration of which is present in the Raman spectrum of native toxin at 838 cm^{-1}) would induce a shift to 850 cm^{-1} (corresponding to a moderate H bond with water). On the contrary, we have observed an 8-cm^{-1} downshift as a consequence of the H/NO_2 substitution. The absence of an 850-cm^{-1} component indicates a strong H bond for phenol. Since the amide I band from the polypeptide chain is not visible in the spectrum of the nitrated toxin, this spectral region cannot be used for structural analysis.

Although the NO_2 group is not a bulky substituent, it could be expected to modify the steric properties of tyrosine. In order to examine the effect of nitration of Tyr-25 on the biological properties of toxin α, we have performed binding assays of the [nitrotyrosyl]toxin to AcChR and to the $M_{\alpha 1}$ antibody using [^{3}H]toxin α competition (Figure 7). In the case of binding to the $M_{\alpha 1}$ antibody (Figure 7a), the two curves corresponding to native and nitrated toxins are superimposed. This shows without ambiguity that the nitration of Tyr-25 does not influence the binding site of this monoclonal antibody. In the case of binding the AcChR (Figure 7b), the K_D value for binding of modified toxin on AcChR is only 6 times greater than for the native toxin. The decrease of affinity induced by nitration of Tyr-25 is not high by comparison with the values obtained for other chemical modifications, especially those observed after monoacetylation of lysines 27 and 57, which are assumed to be in the binding site (Faure et al., 1983). Thus, the competition measurements show that the [nitrotyrosyl]toxin still possesses a high toxicity and its affinity toward the monoclonal antibody $M_{\alpha 1}$ is unchanged.

The affinity value and the spectral features of the modified toxin show that the secondary structure and the geometry of its binding site remain unaffected by the nitration of Tyr-25. The remaining toxicity after nitration, the buried position of this aromatic amino acid, and the presence of an internal hydrogen bond involving the phenol strongly indicate that Tyr-25 of toxin α from *N. nigricollis* is included neither in the binding site to AcChR nor that of $M_{\alpha 1}$.

Discussion

(1) Structure of Native Toxin α. The Raman spectrum of native toxin α from *N. nigricollis* shows several differences when compared to the spectrum of erabutoxin (Harada et al., 1976): spectral differences exist in side-chain vibrations, which can reflect the 29% nonidentical amino acids between these

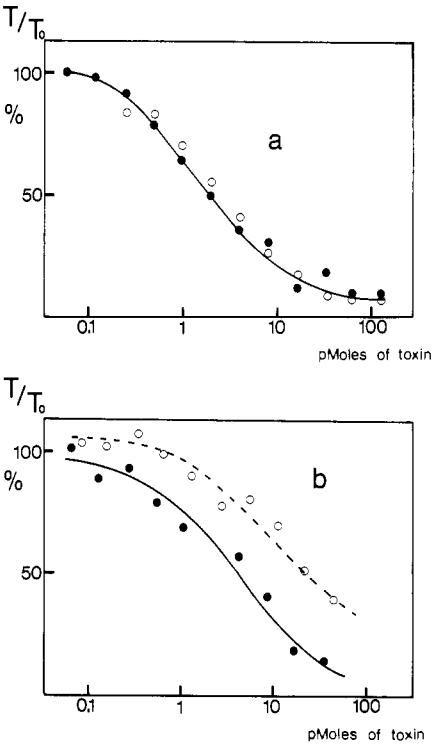

FIGURE 7: Biological activity of [nitrotyrosyl]toxin measured by comparative binding of [^{3}H]toxin α in the presence of varying amounts of modified toxin (○) and native toxin (●). (a) Binding to $M_{\alpha 1}$ antibody; (b) binding to AcChR. T and T_0 are respectively the radioactivities for bound [^{3}H]toxin in the presence and absence of tested toxin (see Materials and Methods).

two toxins. Harada et al. (1976) observed an amide I band located at a similar frequency for LS III toxin (from *Laticauda semifasciata*), but centered at 1674 cm^{-1} for erabutoxin. This value can be compared to the 1668 cm^{-1} that we obtained for toxin α from *N. nigricollis*. Both frequencies correspond to a predominant β structure. Nabedryk-Viala et al. (1980) have observed different amide I bands on infrared spectra of these two toxins. Using isotopic exchange ($^1H/^2H$), they observed a higher accessibility for the solvent in the case of erabutoxin than for toxin α. The latter shows a greater thermal stability. Thus, these two short neurotoxins, which have the same secondary structure, a high degree of sequence homology, and possess invariant residues, nonetheless exhibit differences in some of their physical properties and structure. Some of these differences can be reflected in their Raman spectra.

The shoulder at 1250 cm^{-1} in the Raman spectrum of the native toxin reveals a small amount of disordered structure. A frequency higher than 1240 cm^{-1} has already been observed for amide II vibrations from both β and disordered structures (Takamatsu et al., 1976; Thomas et al., 1986) in the case of long neurotoxins. The secondary structure content from analysis of amide I vibrations (Table II) and the geometry deduced for disulfide bridges are in agreement with values from X-ray experiments (Kimball et al., 1979). However, a difference is observed for one of the three disulfide bridges, which is seen in the TGG conformation by X-ray diffraction. Tsetlin et al. (1982) have noted that the conformation of disulfide bridges can change during crystallization without changing the secondary structure. Thomas et al. (1986) have observed differences in bungarotoxin conformation by comparing soluble and solid states of the molecule: the disulfide bonds are less constrained in solution than in the crystal state. Our spectral observations may be related to these hypotheses. If such a conformational difference between states exists for toxin α,

it is localized in only one C–S bond of one disulfide bridge.

Tyr-25 does not seem to be involved in the binding site of toxin α (Karlsson & Sundelin, 1976; Martin et al., 1983), but it is close to side chains that play a role in binding (Lys-27 and Trp-29), as shown by affinity measurements after chemical modifications (Faure et al., 1983). The IR study with isotopic $^1H/^2H$ exchange measurements (Thiéry et al., 1980) suggests that nine side chains, including Tyr-25, are located in the interior of toxin α. The X-ray study of a short neurotoxin (Tsernoglou & Petsko, 1976) indicated that Tyr-25 is completely buried and is involved in the hydrophobic interior and that the phenolic OH seems to be involved in internal hydrogen bonding. Our results are in agreement with these conclusions. Low et al. (1976) have estimated that Tyr-25 of erabutoxin is not buried but relatively inaccessible. The nitration not preceded by denaturation (Raymond & Tu, 1972) or with denaturation (Chicheportiche et al., 1972) suggests that this tyrosine is buried in case of short neurotoxins. After examining the structure of erabutoxin obtained by X-ray diffraction, Tamiya et al. (1980) have proposed that a hydrogen bond exists between Tyr-25 phenol and Glu-38. Thomas et al. (1986) have suggested that Tyr-24 of bungarotoxin binds to Glu-41 through a strong hydrogen bond. The Raman spectrum of toxin α clearly indicates that Tyr-25 is not exposed to solvent and participates in a strong hydrogen bond (likely with Glu-38). This residue, which is invariant in neurotoxins, is assumed to play a role by strongly maintaining the molecular structure and geometry of the site.

As Trp-29 seems to be involved in binding function (Faure et al., 1983), it must be partially accessible and free to interact with the AcChR binding site. X-ray diffraction (Low et al., 1976) and fluorescence experiments (Chicheportiche et al., 1972) show that Trp-29 is half-buried in the molecule. Raman spectroscopy, which does not reveal a homogeneous environment for Trp-29 of toxin α, is in agreement with these investigations. In spite of the low molecular weight of this protein and a largely opened conformation of the adjacent β loops, the Trp side chain lies in hydrophobic surroundings.

(*2*) *Structure of Modified Toxins.* Modification of Trp-29 by nitrobenzenesulfenyl chloride is not accompanied by a change of the β sheet structure, as shown by analysis of the amide I vibrations (Table II). The decrease in binding affinity of the derivative toxin for the cholinergic receptor previously described (Faure et al., 1983) can therefore be interpreted as the consequence of changes in the orientation of local side chains and/or a steric constraint due to the presence of the added group, which prevents a tight contact between the two proteins. The latter proposal agrees with the observation that incorporation of the smaller formyl group on the C_2 carbon of the indole side chain has no effect on the binding affinity of the toxin (Faure et al., 1983). These conclusions are supported by our data: the decrease in intensity of the 886-cm^{-1} Trp ring vibration for NPS-modified toxin (Figure 2) is more likely due to a change in environment of the indole ring. The structural effects induced by the presence of NPS are restricted to the neighborhood of Trp-29.

The analysis of the amide I vibrations showed that the β sheet structure of the derivative is similar to that of the native toxin. However, the comparison between spectra of native and NPS-modified toxins clearly indicates the change of the conformation of one disulfide bridge, not yet identified. Is such a change compatible with the conservation of overall structure? From CD, UV, and fluorescence experiments, Ménez et al. (1976) have deduced that the β loop 25–40 is relatively independent of the other parts of the molecule. Furthermore, the length between the more distant amino acids of the loop can change (Tamiya et al., 1980). This loop could take up a more favorable geometry induced by the presence of NPS and thus slightly influence the geometry of a disulfide bridge (Cys-41–Cys-17 or Cys-3–Cys-24), the conformation of which is GGG in native toxin (Kimball et al., 1979). The four disulfide bridges of the toxin (see Figure 1) are located in the same globular core of the molecule (Low et al., 1976), and the amount of undefined structure and disordered helix is very likely located in that region of the molecule. The change in geometry of only one disulfide bridge does not affect this amount, since the analysis cannot distinguish between two different undefined conformations. That a slight change in the core of the molecule does not perturb the β loops reflects not only its stability but also its flexibility.

(*3*) *Role of Tyr-25.* The role of the invariant Tyr-25 has been much investigated [reviewed by Endo and Tamiya (1987)]. The importance of an amino acid for the biological activity may lie in the conservation of the exact geometry of the site (role in structure) or in direct binding with AcChR. The residues possibly involved in the interaction may be identified by using criteria such as changes in environment, reduction of mobility or accessibility after binding, or a decrease in affinity when chemically modified. The dissociation constant of toxin α–AcChR binding (2×10^{-11} M) indicates a high affinity and suggests numerous contacts between the two molecules (Weber & Changeux, 1974). Consequently, the toxicity is not totally abolished in the case of a single modification without structural change, reflecting the plurality of residues in close contact with AcChR in binding. Because chemical modifications of the toxin are usually followed by a decrease in affinity, the assays must be compared with other using toxins modified in different manners. Comparing our binding assays for modified Tyr-25 with those of Faure et al. (1983), which were performed in the same way for other residues, we conclude that the small decrease in toxicity of the nitrated toxin does not imply that Tyr-25 is part of the binding site. Our spectroscopic data confirm that in solution Tyr-25 is involved in a strong hydrogen bond, which persists after the phenol ring has been modified by the addition of a nitro group. This bond has previously been claimed to be important for maintaining the structure (Low et al., 1976). Perhaps it is because of the conservation of that bond that the nitro derivative still has a high affinity for the cholinergic receptor. The strong intensity of the nitro group vibrations unfortunately does not allow us to estimate possible changes in the secondary structure. Nevertheless, since the derivative binds with a high affinity to the receptor, we can conclude that its structure has not been substantially altered.

The strong hydrogen bond of this invariant tyrosine involves the side chain of an amino acid which thus appears important to maintain the structure and is most likely not exposed to the surface of the molecule. This position seems unfavorable for a residue that plays a role in binding, since the side chains involved in direct interaction with the receptor are expected to be exposed to the solvent. The presence of a strong H bond leads to the conclusion that this aromatic residue has structural importance in toxin α folding.

Conclusions

We have shown that the chemical modifications leading to addition of a nitrophenyl thioether on Trp-29 and of a nitro group on Tyr-25 of toxin α from *N. nigricollis* do not modify the secondary structure of this protein.

In resonance conditions (488-nm excitation), the spectrum of [nitrotyrosyl]toxin α reveals mainly the peaks of the ni-

trophenol chromophore, and in preresonance conditions other vibrations than those of the chromophore could be distinguished in the spectrum of the NPS derivative. The main feature of the spectrum of both derivative toxins is the $\nu(NO_2)$ peak. This intense peak can be used to estimate the contribution from other vibrations of the chromophore in spectra of the AcChR–toxin system.

The remaining toxicity and the presence of the resonance peaks from the chromophores bound to toxin α from *N. nigricollis* enable both the NO_2 Tyr-25 and the NPS Trp-29 modified toxins to be used in interaction with the nicotinic acetylcholine receptor. They thus constitute a powerful tool for studying the complex of AcChR and the toxin via the resonance vibrational spectra of the bound chromophores.

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