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Optical Activity and Conformation of Cobra Neurotoxin†

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ABSTRACT: Cobra neurotoxin from Formosan cobra (*Naja naja atra*) venom is a compact globular protein having an intrinsic viscosity of 4.5 mL/g. The protein is stable in 7.5 M urea but can be denatured in 4.1 M guanidine hydrochloride or at elevated temperature (above 70 °C). Its conformation remains virtually the same in solvents of lower polarity than water such as 1,2-ethanediol or a mixed solvent of 1-propanol–1,2-ethanediol–water (5:1:1 by volume). The circular dichroism spectrum is "atypical" in water in that the peptide chromophores show a small negative circular dichroic (CD) band at 215 nm, a large positive one at 199 nm, and another large negative one below 190 nm. The CD pattern resembles to some extent that of a β form but differs in both positions and magnitudes from the latter. It agrees qualitatively with the theoretical calculations of the reverse β bends, suggesting that cobra toxin contains a considerable amount of β turns and possibly a mixture of β form and β turns.

The neurotoxin of Formosan cobra (*Naja naja atra*) venom is a postsynaptic membrane binding protein (Chang and Lee, 1963, 1966). Its toxicity based on the 50% lethal dose (LD_{50}) in mice is most lethal in the venom of the snake *Naja naja atra* (Lo et al., 1966). Like α-bungarotoxin, it has also been used to characterize the biochemical preparations of acetylcholine receptor molecules (Changeux et al., 1970; Miledi and Potter, 1971; Raftery et al., 1972; Brockes and Hall, 1975). This toxin is an important subject of molecular neurobiology, the knowledge of which will help us understand other snake neurotoxins (see, for instance, Lee, 1972; Tu, 1973).

The cobra toxin molecule consists of a single polypeptide chain of 62 amino acid residues (Yang et al., 1969). Its 4 disulfide linkages divide this small molecule into 4 loops: (a) residues 3 to 24 (with no charged side groups except Glu-21); (b) residues 17 to 41 (having most of the charged groups); (c) residues 43 to 54; and (d) residues 55 to 60(Yang et al., 1970). Two loops share residues 17 to 24. The molecule contains 6 acidic residues and 11 basic residues (including 6 arginine), which accounts for its high isoelectric point (our preliminary study indicates a value of 9.1 to 9.2).

In this work we report the conformation of cobra toxin based on CD[1] and ORD. Unlike other globular proteins, this protein has no detectable CD bands that are characteristic of a helix or are associated with a β form. Instead, the CD spectrum resembles the calculated curves by Woody (1974) for Venkatachalam's β turns (1968), suggesting that this protein contains a considerable amount of reverse β turns and possibly a mixture of antiparallel β form and β turns, which is consistent with its compactness as inferred from viscosity measurements.

Experimental Section

Materials. Crude cobra neurotoxin of snake venom was prepared and repeatedly purified on CM-Sephadex C-50 or

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[1] Abbreviations used: CD, circular dichroism; ORD, optical rotatory dispersion; CM, carboxymethyl; UV, ultraviolet.

TABLE I: The Extrema of CD Bands (I–V) of Cobra Neurotoxin.[a]

Solvent	pH	T (°C)	I λ	I [θ]	II λ	II [θ]	III λ	III [θ]	IV λ	IV [θ]	V λ	V [θ]
Water	7.0	25	288	−210	282	−220	228	3530	215	−1290	199	14 000
	7.0	88	285	−70	281	−70		[b]		[b]	201	−13 200
	3.2	25	288	−210	282	−230	228	3300	215	−1390	199	14 600
	10.0	25	288	−210	282	−200	228	3330	215	−1550	199	14 000
	11.8	25	288	−170	282	−170	229	2190	215–216	−1810	199–200	11 500
	12.7[c]	25	289	−109	281	−107	232	561	215–216	−2870		
1,2-Ethanediol		25	289	−160	284	−170	230	3790	217	−1210	202	12 900
		88	285	−120	281	−130		[b]		[b]		
1-Propanol–1,2-ethanediol–water (5:1:1)		−10	288	−180	283	−280	229	4770	216	−530	201	14 730
		25	288	−170	283	−190	230	3060	217	−1500	202	7 470
		61	287	−100	282	−110		[b]		[b]		

[a] λ in nanometers denotes the position of the extremum. The dimension of [θ] is deg cm^2 dmol^{-1}. [b] The band disappears. [c] A broad shoulder appeared around 243 nm with [θ] ≅ 560.

C-25 columns (Lo et al., 1966). Its homogeneity was tested with acetylcellulose strip and acrylamide gel electrophoresis, both showing a single band. The purified protein in solution was ultrafiltrated with Amicon UM-3 membrane in a diaflow cell to remove traces of ammonium acetate. The solution was further clarified by passing it through Millipore filters of 5-μm pore size. The protein concentration was determined spectrophotometrically, using $E_{1\text{-cm}}^{1\%} = 13.7$ at 280 nm. The latter was based on micro-Kjeldahl nitrogen analysis, using 19.54% as the nitrogen content.

All chemicals used were of reagent grade. Water was double distilled.

Methods. CD was measured with a Jasco J-10 spectropolarimeter and ORD with a Cary 60 spectropolarimeter under constant nitrogen flush. In each instrument a specially designed aluminum block served as the cell holder and jacket; the latter was attached to a Haake constant-temperature regulator. Fused silica cylindrical cells of various path lengths were used so that the absorbance of the solution was kept below two. The data were expressed in terms of mean residue ellipticity, [θ], and mean residue rotation, [m], with a mean residue weight of 112.1.

Viscosities were measured in a Ubbelohde-type capillary viscometer. The flow time of water was about 1300 s at 20 ± 0.1 °C.

A Radiometer 25 pH meter with a scale expander was used for all pH measurements. The meter had been standardized against Beckman buffers.

Results

Conformation in Aqueous Solution. Figure 1 shows the CD spectra of cobra neurotoxin in water, urea, and guanidine hydrochloride at neutral pH. Like most proteins, the magnitudes of the CD bands in the near-ultraviolet region, such as bands I and II for nonpeptide chromophores, are small as compared with those due mostly to peptide backbones such as bands IV, V, and VI (band III probably belongs to the aromatic groups). Table I summarizes the results of the CD extrema under various conditions.

The most striking feature in Figure 1 is that the CD profile of the native protein is atypical. In the region below 250 nm it does not have the double minimum at 222 and 208–210 nm that is characteristic of a helical conformation (Greenfield and Fasman, 1969; Chen and Yang, 1971; Chen et al., 1972, 1974).

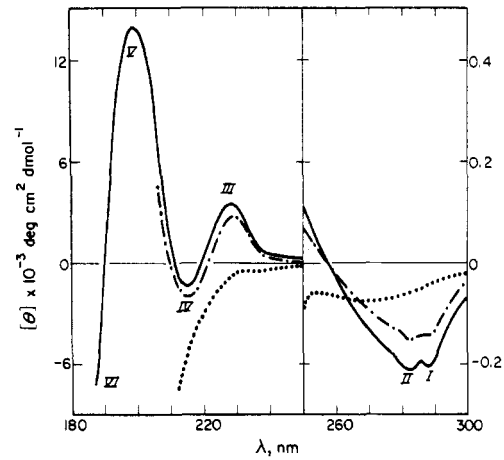

FIGURE 1: CD of cobra neurotoxin. (—) In water; (- - -) in 7.5 M urea; (···) in 4.1 M guanidine hydrochloride.

The pattern resembles to some extent that of the β form which has a minimum near 217 nm and a maximum at about 195 nm. But the ratio of the magnitudes at the extrema, in the present case $[\theta]_{199}/[\theta]_{215}$, is much larger than a similar ratio for the β form (~2). The spectrum also differs from that of an unordered form, which displays a large negative band near 190 nm and no large positive band around 200 nm.

Recent theoretical calculations of Woody (1974) for Venkatachalam's β turns (1968) seem to provide a plausible explanation for the observed spectrum. Out of numerous variants of the β turns, three fundamental types, I, II, and III, are most prevalent. Their spectra are characterized by a negative CD band at about 225 nm, a positive one at about 205 nm, and another negative one below 190 nm, the ratio of $|[\theta]_{205}/[\theta]_{225}|$ varying between about 3 and 10. It is tempting to suggest that, in addition to the β form, native cobra toxin contains a considerable amount of β turns (the band positions differ by 5 to 10 nm from the theoretical calculations).

The CD of cobra toxin is the same in 1 M KF at pH 8 (not shown) as in water. Urea (7.5 M) as a denaturant has only a moderate effect on the conformation of toxin (Figure 1). Only in concentrated guanidine hydrochloride (4.1 M) does this toxin undergo a marked change in conformation. All six CD bands disappear and the remaining ellipticities are negative in the UV region. A new, large negative band appears below 230 nm, which indicates the presence of an unordered form.

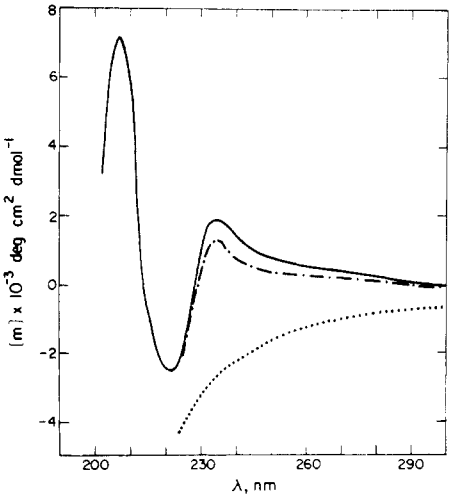

FIGURE 2: ORD of cobra neurotoxin. Symbols are the same as in Figure 1.

That cobra toxin is a compact globular protein is supported by the study of its hydrodynamic property. Its intrinsic viscosity, $[\eta]$, in water or 0.1 M NaCl was found to be 4.5 mL/g (after density correction according to Tanford's formula (1955)). In 4.1 M guanidine hydrochloride, a 0.8% toxin solution had a reduced viscosity of 7.8 mL/g, suggesting some degree of unfolding of this small molecule. However, the presence of four disulfide bonds in the molecule restricts its expansion, thus causing only a small increase in viscosity upon denaturation.

The ORD of cobra toxin in water shows two maxima at 234 and 207 nm, one minimum at 221 nm, and probably another minimum below 200 nm (Figure 2). Here again the pattern differs from that of a right-handed helix (see Discussion), β form or unordered form. The rotatory contributions of the CD bands III and V overshadow those of bands I and II, thus resulting in a plain curve between 250 and 320 nm. However, above 290 nm the rotations become levorotatory. The effect of 7.5 M urea on the ORD spectrum is again insignificant. But the denatured protein in 4.1 M guanidine hydrochloride makes the ORD negative throughout the visible and UV region studied in a manner similar to the "coiled form" (Greenfield et al., 1967).

Cobra toxin is stable over a wide range of pHs at room temperature (Table I). In acidic solution, where the protein molecule is protonated (with 11 positive charges), its CD spectrum remains virtually the same as that in neutral solution. On the alkaline side, the potentiometric titration indicated that Tyr-35 behaved normally, but Tyr-25 only began to ionize at pH 11.3 (Chang et al., 1971). The magnitudes of the CD bands are slightly modified at pH 10, where Tyr-35 is known to be ionized. At pH 11.8 further minor reduction in the intensity of the CD bands is observed, except that of band IV is increased slightly. Raising the pH of the solution to 12.7 (Table I) causes significant changes in magnitude, although the CD profile is still retained. The secondary and tertiary structures of toxin seem rather insensitive toward the local conformation around the two tyrosine residues as a result of ionization of the phenolic groups.

Conformation in Organic Solvents. In spite of the apparent lack of any secondary structure, cobra toxin appears to be a compact protein partly due to the restriction arising from the four -S-S-bridges and partly the long-range interactions among the side groups of the amino acid residues. To test further the hydrophobic effect on the conformational stability,

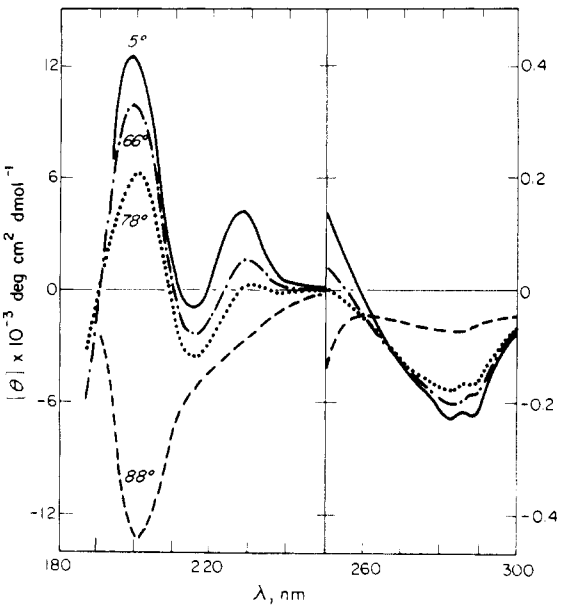

FIGURE 3: CD of cobra neurotoxin at several temperatures.

we studied the CD of this toxin in 1,2-ethanediol and also in a mixture of 1-propanol-1,2-ethanediol-water (5:1:1 by volume). The CD profiles in these two solvents resemble the spectrum in aqueous solution, but the positions and magnitudes of the extrema change to some extent (Table I). The red shift is in accord with similar change in absorption maximum from 278 nm in water to 279-280 nm in the other two solvents.

Thermal Denaturation. Cobra toxin in solution is sensitive to temperature. In aqueous solution the magnitudes of all six CD bands change gradually from 5 to 78 °C. This is accompanied by a small blue shift for bands I and II (the corresponding absorption maximum also blue-shifts 1 to 2 nm) and a red shift for bands III, IV, and V (Figure 3). At 88 °C all six bands disappear; instead, a large negative band at 201 nm emerges. This suggests the existence of an unordered form; that is, the protein is completely denatured.

Figure 4 shows the change of the ellipticities of toxin with temperature at three wavelengths, 200 nm (band V), 215 nm (band IV), and 228 nm (band III). In water (Figure 4A) and 1,2-ethanediol (Figure 4B), a drastic change occurs above 70 °C and is complete between 80 and 90 °C, whereas in the mixed solvent of 1-propanol-1,2-ethanediol-water (5:1:1) (Figure 4C) the conformation of toxin is more easily altered and the denaturation is completed at lower temperature (near 60 °C).

Thermal denaturation of toxin is reversible only if the protein in neutral solution is heated at 78 °C for less than 3 h. Prolonged heating of the toxin solution at 88 °C, for instance, for 1 h, would only partially restore the protein conformation when the solution is cooled back to room temperature. Cobra toxin has been reported to regain its biological activity after a heating-cooling cycle (Yang, 1965). However, the activity tests are carried out under physiological conditions, where the protein conformation may differ from that at elevated temperatures.

Discussion

The CD and ORD spectra of native toxin (Figures 1 and 2) are qualitatively similar to those found by other workers (Yang et al., 1967, 1968), but the magnitudes on the low wavelength

side in this work differ markedly from those in the literature. For instance, our $[\theta]_{199}$ of 14 000 is much larger than $[\theta]_{201}$ = 10 000 as reported by Yang et al. (1968). We found $[m]_{207}$ = 7230, whereas the other workers reported only 4470 (recalculated from [m']). At first, these authors (Yang et al., 1967, 1968) attributed the appearance of a maximum [m] at 233 nm and a minimum $[\theta]$ at 215 nm to the presence of a mixture of a left-handed helix and the β form. In a recent review, Yang (1974) still maintains this viewpoint. But the speculation of a left-handed helix in the toxin molecule is untenable. It is well known that the presence of a moderate amount of the helix in a protein molecule would dominate the CD and ORD spectra due to these peptide chromophores. The CD of a left-handed helix would have displayed a double maximum near 222 and 210 nm and a large negative band near 190 nm. This is not the case for toxin (Figure 1). The maximum [m] at 234 nm (Figure 2) most probably arises from the nonpeptide chromophore at 228 nm (Figure 1). Since the Cotton effect of an ORD spectrum is more difficult to resolve than the corresponding CD, the results in Figure 2 do not warrant a conclusion about secondary structure of toxin.

In view of Woody's theoretical calculation (1974) and the experimental results of polytetrapeptide, $(Val\text{-}Pro\text{-}Gly\text{-}Gly)_{40}$, which is believed to have repeating β bends (Urry et al., 1974), a mixture of β turns and β form in the toxin molecule seems to be a reasonable model. The helical conformation, if present, would have overshadowed other conformations as far as the CD and ORD spectra are concerned. The abundance of reverse β turns would bring distant portions of the polypeptide chain close to each other either through the formation of intramolecular β form or simply hydrophobic interactions or both. The viscosity of toxin also indicates a compact globular protein rather than an extended flexible one.

Recently, prediction of the secondary structure of proteins from their primary structure has become increasingly popular. These predictive methods are empirical in nature and involve many assumptions, but some of them have reported a 70–80% accuracy for native proteins (all of them do not apply to denatured proteins). Since the approach is so different from the optical measurements, it is helpful to compare such estimates with those inferred from our CD and ORD results. With known amino acid sequence of cobra toxin (Yang et al., 1969), Chen et al. (1975) used the Chou-Fasman method (1974) and reported that the predicted structure lacked any helix, contained two segments of the β form (residues 13–17 and 54–57), but was abundant with the β turns (residues 17–20, 22–28, 32–35, 39–46, 58–61), which was almost half of the protein molecule. In the absence of x-ray diffraction results, the agreement between our findings and the predictive method could be purely fortuitous. Nevertheless, it is a surprising coincidence that should not be completely overlooked.

The current CD analysis of protein conformation is most useful in detecting the helical conformation in the macromolecule. Interpretation of the β form and, more recently, the β turns is uncertain in at least three aspects. First, the CD of the β form depends on not only the length of each strand but also the number of neighboring strands; it is also different for the parallel and antiparallel β forms (Woody, 1969). This is further complicated by the fact that the β form in a protein molecule is by no means ideal; the strands may not precisely align themselves side by side, nor are they necessarily straight (they could have a gentle twist). Urry and Long (1976) have also proposed the β-spiral conformation for the polyhexapeptide of elastin and the cross-β form having regularly repeating β turns. Therefore, only a statistical average of various β-form

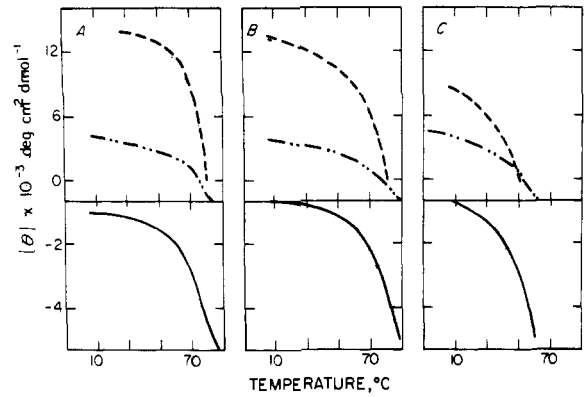

FIGURE 4: Thermally induced change in CD of cobra neurotoxin in different solvents. (- - -) $[\theta]_{200}$; (- · · -) $[\theta]_{228}$; (—) $[\theta]_{215}$. (A) In water; (B) in 1,2-ethanediol; (C) in mixed solvent of 1-propanol–1,2-ethanediol–water (5:1:1 by volume).

arrangements can be observed experimentally (Chen et al., 1972, 1974).

Second, the very resemblance in the CD spectra of the β form and β turns makes it difficult to distinguish these two types of structures. In addition, the β turns have numerous variants according to Woody's treatment and a statistical average of their CD has not yet been reported. At best the results in Figure 1 suggest the presence of β turns because of the large ratio of $[\theta]_{199}/[\theta]_{215}$, but they do not rule out a mixture of the β form and β turns.

Third, the CD spectra of both the β form and β turns are relatively small in magnitudes as compared with that of the helical conformation. Consequently, the rotatory contributions of nonpeptide chromophores in the ultraviolet region can no longer be overlooked. For instance, both tyrosine and tryptophan residues have absorption bands below 220 nm and their CD contributions may be significant with respect to those of the β form and β turns. Our present knowledge does not enable us to resolve these CD bands unequivocally. Thus, the interpretation of the CD spectrum must be viewed with some caution and reservation.

The CD contributions of the four disulfide bonds cannot be ascertained and are probably minor, noting that all CD bands except those of the unordered form disappear upon denaturation (disulfide linkages of opposite chirality would also cancel their optical activity). The assignment of CD bands in the near-ultraviolet region is also far from perfect. The tyrosine residues usually have three bands between 275 and 290 nm (the band position of a buried residue is located at higher wavelength than that of an exposed one by several nanometers). For instance, in ribonuclease A they are identified at 276, 282, and 288.5 nm (Horwitz et al., 1970). The tryptophan residues have two bands between 280 and 295 nm. For instance, in carboxypeptidase A they are found at 286 and 293 nm (Fretto and Strickland, 1971). Such overlappings make it difficult to identify various bands. Ionization of the tyrosine side chain would red-shift the band position to above 190 nm; thus, the relative changes in the CD magnitudes at various pHs might provide a clue to the CD assignment. Our data at pH 12.7 (Table I) seem to suggest a change in the overall structure of toxin rather than the change in tyrosyl CD alone. All one can say is that the CD bands I and II (and probably III) arise from the aromatic groups, Tyr-25 and Trp-29 (the ionization of the other Tyr-35 residue does not change the spectrum very much). Intact Trp-29 is essential for the toxicity of this protein (Yang, 1974) and therefore is very likely on the surface of the mole-

cule. The indole ring of Trp-29 probably contributes most to the first three bands, the intensities of which diminish at elevated temperatures because of increasing freedom of rotation of Trp-29. Bands IV, V, and VI in Figure 1 are mainly due to the peptide chromophores. The positive bands III and V overshadow the negative band IV, making the latter appear small. At elevated temperatures, the reduction in the intensity of bands III and V might be greater than the corresponding reduction of band IV. This could account for the apparent increase in magnitude of band IV with temperature.

After the submission of our paper, we learned of two x-ray diffraction studies on erabutoxin *b*, a structural analogue of cobra neurotoxin (Low et al., 1976; Tsernoglou and Petsko, 1976). According to Low and her co-workers, this toxin molecule contains about 40% twisted antiparallel β-pleated sheets and about 26% β turns. Its Tyr-25 is not "buried" but is relatively inaccessible, whereas Trp-29 is "half-buried." Qualitatively, our CD results are consistent with the x-ray diffraction findings of the cobra neurotoxin analogue.

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