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Lipid-induced Changes in the Secondary Structure of Snake Venom Cardiotoxins*

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The secondary structures of three snake venom cardiotoxins (from *Hemachatus hemachatus*, *Naja naja atra*, and *Naja naja naja*), in aqueous solution and in a lipid-bound form, were investigated by Fourier-transform infrared spectroscopy. The conformation-sensitive protein infrared bands in the amide I region were analyzed using deconvolution and band-fitting procedures. The spectra of the three cardiotoxins in aqueous buffer are very similar; they indicate a high content of both antiparallel β -sheet structure and unordered conformation. Moreover, component bands characteristic of turns can also be identified. The binding of cardiotoxins to bilayers of dimyristoylphosphatidylglycerol results in an increased content of a β -structure at the expense of the nonordered conformation. It is suggested that lipid-induced conformational transitions to a β -structure, similar to that observed with cardiotoxins, may be operative also in membrane interaction of other proteins and peptides, particularly with those which have a small tendency to form α -helices.

Snake venom cardiotoxins constitute a group of basic proteins consisting of a single polypeptide chain of 60–61 amino acids. The family of cardiotoxins is highly homologous, particularly in respect to the location of four disulfide bridges and the presence of numerous invariant residues (Louw, 1974; Dufton and Hider, 1983). A certain degree of homology also exists between cardiotoxins and short neurotoxins (Louw, 1974; Dufton and Hider, 1983), although these two classes of snake venom toxins have pharmacologically different effects. Whereas the neurotoxins specifically block nicotinic acetylcholine receptors, cardiotoxins act nonspecifically by perturbing the structure of cell membranes and are also referred to as cytotoxins (for a review see Dufton and Hider, 1983; Harvey, 1985).

The crucial role of phospholipids has been implicated in the action of cardiotoxins (Patel *et al.*, 1969; Vincent *et al.*, 1976). Since the finding of a strong cardiotoxin interaction with acidic phospholipids (Vincent *et al.*, 1978; Dufourcq and Faucon, 1978), numerous model studies have been performed revealing details of the effect of the protein on the molecular organization of lipid bilayer (Bougis *et al.*, 1981; Gulik-Krzywicki *et al.*, 1981; Bougis *et al.*, 1983; Faucon *et al.*, 1983; Batenburg *et al.*, 1985). Models of the location of the toxin molecule in the membrane have also been proposed (Lauter-

wein and Wüthrich, 1978; Dufourcq *et al.*, 1982; Batenburg *et al.*, 1985). One important aspect of cardiotoxin-phospholipid interaction that still remains largely unexplored is the conformation of the protein in a lipid environment. Whereas folding of cardiotoxins in water has been extensively studied by a variety of techniques (Hung and Chen, 1977; Visser and Louw, 1978; Lauterwein *et al.*, 1978; Takamatsu *et al.*, 1980; Drake *et al.*, 1980; Galat *et al.*, 1985), we are aware of only one report pertaining to the structure of a lipid-associated protein (Pézolet *et al.*, 1982). More details in this respect seem crucial for the better understanding of the mechanism of pharmacological action of cardiotoxins. Studies along this line are also important from the more general point of view of the basic mechanisms of protein-lipid interactions. Although the association of many polypeptides or proteins with phospholipids have been reported to be connected with an increase in α -helical conformation (Wu *et al.*, 1979; Epand *et al.*, 1983; Lau *et al.*, 1983; Hanssens *et al.*, 1985; Surewicz *et al.*, 1986), the family of cardiotoxins provides an example of proteins which, while interacting strongly with phospholipids, have only a very limited potential to form α -helical structures (Dufton and Hider, 1977; Visser and Louw, 1978; Menez *et al.*, 1978).

In the present communication, we have used infrared spectroscopy to establish the effect of membrane binding on the secondary structure of cardiotoxins isolated from the venoms of *Hemachatus hemachatus*, *Naja naja atra*, and *Naja naja naja*. Recent progress in Fourier-transform infrared spectroscopy and, particularly, the development of spectral resolution enhancement methods now allow for a much more penetrating insight into the secondary structure of polypeptide backbones in different environments. The technique has proven particularly useful in structural studies of membrane-associated proteins and peptides (Lee *et al.*, 1985; Arrondo *et al.*, 1987; Surewicz *et al.*, 1987a, 1987b, 1987c).

EXPERIMENTAL PROCEDURES

Materials—*H. hemachatus* venom was from Miami Serpentarium, *N. naja atra* venom (lot 125C-0215) from Sigma, and *N. naja naja* (India) venom from Bioactives Inc. (Arlington, VA). Dimyristoylphosphatidylglycerol was from Avanti Polar Lipids (Birmingham, AL).

Purification of Cytotoxins from Crude Venoms—*H. hemachatus* venom was fractionated by gel permeation followed by cation exchange chromatography as described by Fryklund and Eaker (1973). Toxin 11A (Joubert, 1977) was separated from its homologue, toxin 11, by chromatography on CM-52 cellulose using a linear gradient (2400 ml total volume) from 200 to 1000 mM ammonium acetate/acetic acid, pH 5.2. Chromatography on CM-52 cellulose was repeated twice to ensure complete separation of the toxins.

Cobramine B was isolated from *N. naja naja* venom by the ion-exchange chromatography as described by Larsen and Wolff (1968). A major contaminating protein was subsequently removed by chromatography on CM-52 cellulose (linear gradient of 50–600 mM am-

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monium bicarbonate, 4000 ml total volume).

Cardiotoxin analogue I from the venom of *N. naja atra* was isolated by gel permeation and ion-exchange chromatography as described by Kaneda *et al.* (1976). Minor contaminants were separated by chromatography on CM-52 cellulose (linear gradient of 170–600 mM ammonium acetate/acetic acid, pH 6.6, 4000 ml total volume).

Homogeneity of all three toxin preparations was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by amino acid analysis.

Sample Preparation and Spectroscopic Measurements—Cardiotoxins were dissolved at a concentration of 50 mg/ml in 50 mM Hepes¹ buffer prepared in ²H₂O and adjusted to pH 7.4. For preparation of cardiotoxin-phospholipid complexes, protein solutions were added to the powdered lipid (1,2-dimyristoylphosphatidylglycerol) so that the lipid:protein weight ratio of 3:1 was reached. The mixture was vortexed for approximately 5 min during which time the sample was warmed and cooled repeatedly through the transition temperature.

Fourier-transform infrared spectra were measured at 30 °C with a Digilab FTS-15 instrument equipped with a high sensitivity mercury cadmium telluride detector. Samples were examined in a demountable infrared cell that was fitted with two calcium fluoride windows separated by a 12- μ m teflon spacer. For each spectrum, 500 interferograms were co-added, apodized with a triangular function, and Fourier-transformed to give a resolution of 2 cm⁻¹ and an encoding interval of 1 cm⁻¹. To eliminate spectral contributions of atmospheric water vapor, the instrument was continuously purged with dry nitrogen. Overlapping infrared bands were resolved using Fourier self-deconvolution procedures (Mantsch *et al.*, 1986).

RESULTS

The correspondence between infrared vibrational modes of proteins and the secondary structure of the polypeptide backbone is best understood for the so-called amide I band in the spectral region between approximately 1600 and 1700 cm⁻¹. The conformation-sensitive amide I mode represents the stretching vibrations of the C=O groups of the protein backbone. The frequency of this vibration is determined by the nature of hydrogen bonding of the C=O groups; this, in turn, depends on the particular secondary structure adopted by the polypeptide chains (Susi, 1969).

Infrared spectra, between 1600 and 1700 cm⁻¹, of the aqueous (²H₂O buffer) solutions of cardiotoxins from *H. hemachatus*, *N. naja atra*, and *N. naja naja* are shown as solid lines in Fig. 1, A–C. The overall contours of the amide I bands of these three toxins are, at least qualitatively, very similar. They display maxima at around 1645 cm⁻¹ and have two distinct shoulders, one at wave numbers below 1635 cm⁻¹ and one above 1665 cm⁻¹. The position of the band maximum, at 1645 cm⁻¹, is typical for deuterium-exchanged polypeptide fragments in nonordered conformation (Susi, 1969; Byler and Susi, 1986), whereas the two shoulder bands occur at wave numbers characteristic of amide groups in a β -sheet structure (Susi, 1969; Krimm and Bandekar, 1986; Byler and Susi, 1986). Although similar general features, *i.e.* peak maxima around 1645 cm⁻¹ with two shoulder bands on either side, can be distinguished in the infrared spectra of cardiotoxins reconstituted with dimyristoylphosphatidylglycerol (dashed lines in Fig. 1, A–C), the amide I band contours of cardiotoxins in these two environments are not identical. The most prominent difference is the higher relative intensity of the low wave number amide I shoulder band in the spectra of lipid-associated proteins. Whereas this type of spectral change is compatible with an increased proportion of β -structure in the lipid-bound cardiotoxins, a more detailed interpretation of the spectra in Fig. 1 is obscured by the overlapping of the amide I component bands which contribute to the complex band contour.

Deeper insight into the secondary structure of cardiotoxins

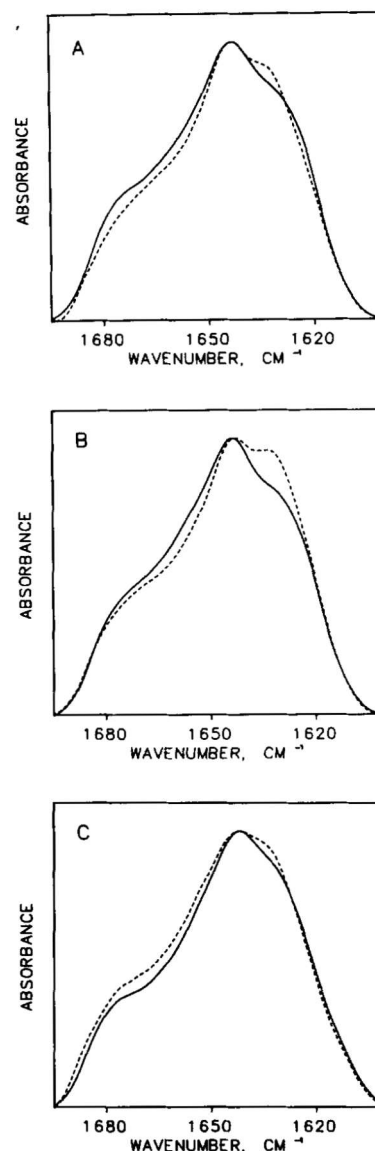


FIG. 1. Infrared spectra in the amide I region of cardiotoxins from the venoms of *H. hemachatus* (A), *N. naja atra* (B), and *N. naja naja* (C). Solid lines represent spectra of the cardiotoxins in aqueous solution, and dashed lines represent spectra of the cardiotoxins reconstituted with dimyristoylphosphatidylglycerol.

in solution and in a lipid environment may be obtained from Fourier self-deconvolution analysis of the infrared spectra (Lee *et al.*, 1985; Byler and Susi, 1986; Arrondo *et al.*, 1987; Surewicz *et al.*, 1987a, 1987b, 1987c). This computational procedure reduces the widths of infrared bands and thus results in an apparent increase in the resolution of the spectra. As a representative example, the Fourier self-deconvolved spectrum of *H. hemachatus* cardiotoxin in solution is shown in Fig. 2A. It reveals the presence of seven component bands in the amide I region. The same number of component bands was identified in the resolution-enhanced spectra of two other cardiotoxins studied (spectra not shown for brevity); the positions of these bands are summarized in Table I. These positions can be used subsequently as input parameters for curve fitting of the original amide I band contours (Byler and Susi, 1986; Surewicz *et al.*, 1987a, 1987b, 1987c). As an example, the result of such curve-fitting analysis of the spectrum of *H. hemachatus* cardiotoxin is shown in Fig. 3A. The fractional areas of the component bands in the spectra of various

¹ The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

cardiotoxins (as percentages of the total integrated intensity of the original amide I band contour) are also included in Table I.

The peak wave number positions of the amide I component bands identified in the spectra of the three cardiotoxins studied are very close, indicating that these bands represent similar elements of protein secondary structure. Thus, bands at 1621–1623 cm^{-1} and at 1631–1632 cm^{-1} are highly characteristic of the amide groups in a β -sheet conformation (Byler and Susi, 1986; Krimm and Bandekar, 1986). The presence of two low wave number " β -bands" suggests that two types of β -strands with a somewhat different pattern of hydrogen bonding are present in these cardiotoxins, although a detailed discrimination between these β -structures (e.g. parallel and antiparallel sheets) is at present not feasible. The prominent band in the spectra of cardiotoxins around 1644 cm^{-1} can be assigned to nonordered polypeptide fragments in which the amide groups are hydrogen-bonded mainly to solvent ($^2\text{H}_2\text{O}$) and not to other backbone C=O or N-H moieties (Byler and Susi, 1986). The relatively weak band around 1655 cm^{-1} may be associated with α -helices (Byler and Susi, 1986; Krimm and Bandekar, 1986), although its assignment in the spectra of cardiotoxins is not completely unambiguous (see "Discussion"). Turns are represented by a band at 1663–1665 cm^{-1} (Byler and Susi, 1986) and by one of the two bands at wave numbers above 1670 cm^{-1} . The second of these high-frequency

bands most likely represents "in phase" vibrations of neighboring peptide C=O groups in an antiparallel β -sheet conformation. Previously, bands around 1675 cm^{-1} have been assigned to the β -structure and those between 1680 and 1985 cm^{-1} to turns (Byler and Susi, 1986). Although such an assignment may be correct also in the present case, the discrimination between the "high frequency β -band" and the "turn bands" in the spectra of cardiotoxins remains to be clarified once there are more data available from model peptides/proteins. Fourier self-deconvolved spectra of cardiotoxins reconstituted with dimyristoylphosphatidylglycerol reveal amide I component bands at positions very close to those observed for the aqueous solution of the proteins (Fig. 2 and Table I). The curve-fitting analysis of the spectra clearly indicates that while the fractional integrated intensities of bands around 1655, 1675, and 1682 cm^{-1} are very similar for cardiotoxins in the two different environments, membrane binding of all three proteins results in a significant increase in the total fractional areas of the β -bands around 1622 and 1632 cm^{-1} , concomitant with the decrease in the fractional

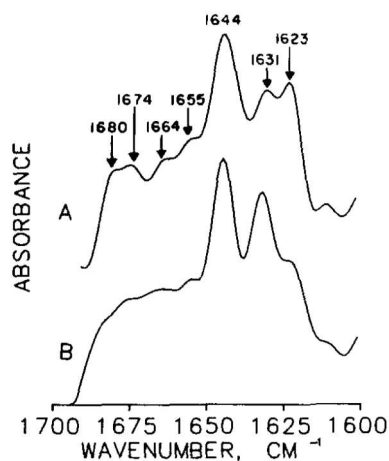


FIG. 2. Infrared spectra of the cardiotoxin from the venom of *H. hemachatus* after band narrowing by Fourier self-deconvolution using a 15 cm^{-1} half-width Lorentzian line and a resolution enhancement factor (k value) of 2.1 (Mantsch *et al.*, 1986). A, cardiotoxin in buffer; B, cardiotoxin reconstituted with dimyristoylphosphatidylglycerol.

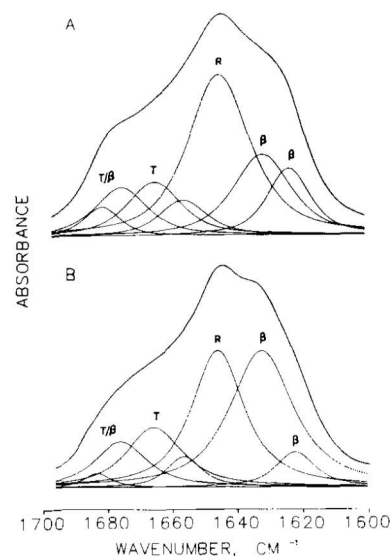


FIG. 3. The infrared amide I band contour with the best-fitted individual component bands for *H. hemachatus* cardiotoxin in aqueous solution (A) and in a lipid (dimyristoylphosphatidylglycerol) environment (B). Half-widths of the best-fitted component bands ranged between 16 and 22 cm^{-1} , except for the highest wave number component which was narrower ($\sim 11 \text{ cm}^{-1}$). The band shapes were 70–80% Lorentzian and 20–30% Gaussian. The symbols β , T, and R stand for β -structure, turns, and nonordered (random) structure.

TABLE I

Wave number positions (in cm^{-1}) and fractional areas (percentages) of the amide I component bands of cardiotoxins in buffer and in a lipid environment. Wave numbers and percentages were rounded off to the nearest integer.

<i>H. hemachatus</i> cardiotoxin				<i>N. naja atra</i> cardiotoxin				<i>N. naja naja</i> cardiotoxin			
Lipid-free protein		Protein complexed with DMPG ^a		Lipid-free protein		Protein complexed with DMPG		Lipid-free protein		Protein complexed with DMPG	
cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%
1623	(11)	1622	(6)	1623	(10)	1622	(10)	1621	(7)	1623	(11)
1631	(18)	1632	(35)	1631	(19)	1632	(28)	1632	(22)	1633	(25)
1644	(39)	1645	(29)	1644	(35)	1644	(24)	1643	(32)	1644	(24)
1655	(7)	1655	(5)	1655	(10)	1654	(14)	1655	(13)	1653	(12)
1664	(11)	1664	(14)	1665	(12)	1666	(12)	1663	(7)	1663	(11)
1674	(10)	1675	(10)	1674	(10)	1674	(10)	1674	(14)	1675	(14)
1680	(4)	1683	(2)	1681	(4)	1681	(2)	1683	(5)	1684	(3)

^a DMPG, dimyristoylphosphatidylglycerol.

area of the "unordered band" around 1644 cm^{-1} (Table I).

DISCUSSION

Analysis of the infrared amide I bands indicates that the secondary structure of the three cardiotoxins studied is very similar, consisting mainly of β -sheets. This is in accord with the general qualitative conclusions of previous circular dichroism and laser Raman spectroscopic studies of a number of homologous snake venom toxins (Hung and Chen, 1977; Visser and Louw, 1978; Takamatsu *et al.*, 1980; Drake *et al.*, 1980; Galat *et al.*, 1985). Our quantitative estimate of the β -structure content in cardiotoxins in aqueous solution, derived from the total fractional area of all β -bands, amounts to between 39 and 43% (more likely) or to 33–34% (less likely), depending on whether the band around 1675 cm^{-1} or the band around 1682 cm^{-1} is chosen as the high wave number " β -component" (see "Results"). The infrared spectroscopic estimate of nonordered structures (based on the intensity of the band at $1643\text{--}1644\text{ cm}^{-1}$) amounts to between 32 and 39%. Another abundant structural element of cardiotoxins are turns. Whereas turns could be anticipated from the analysis of amino acid sequences (Dufton and Hider, 1977), this study provides the first direct experimental evidence for such structures in aqueous solution of cardiotoxins. No separate features representing turns could be distinguished in circular dichroism or Raman spectra of proteins.

The presence of a minor infrared band around 1655 cm^{-1} is suggestive of a small amount (between 7 and 13%) of α -helix in cardiotoxins. Yet, no helical structures were detected in cardiotoxins by circular dichroism (Hung and Chen, 1977; Visser and Louw, 1978; Drake *et al.*, 1980) or by the predictive algorithms of protein secondary structure (Visser and Louw, 1978; Menez *et al.*, 1978). Furthermore, the recent x-ray crystallographic data for the cardiotoxin of *Naja mossambica mossambica* do not indicate the presence of α -helices in this protein (Rees *et al.*, 1987). The origin of the 1655 cm^{-1} band in the infrared spectra of cardiotoxins remains thus to be clarified. An alternative to the α -helix assignment of this band could be type II β -turns (Krimm and Bandekar, 1986) and/or nonordered polypeptide fragments that are inaccessible to the backbone hydrogen \rightarrow deuterium exchange.

Undoubtedly the most important finding of this study is that of protein conformational changes which accompany cardiotoxin binding to a phospholipid bilayer. Both the simple visual inspection of the original amide I band contours and the more detailed deconvolution and band-fitting analysis of the infrared spectra indicate a lipid-induced increase in the content of β -sheet structure, concomitant with a decrease in the number of amino acid residues in a random conformation. It should be emphasized that since only the β -bands around 1622 and 1632 cm^{-1} and a "random band" around 1644 cm^{-1} are influenced by the lipid binding, our conclusions regarding the conformational transitions in cardiotoxins are essentially unaffected by the uncertainty in the assignment of the component bands around 1655 cm^{-1} and those above 1670 cm^{-1} .

The nature and extent of lipid-induced changes in the secondary structure of the three proteins studied are very similar, suggesting that the effects observed in this investigation may be representative for the whole family of snake venom cardiotoxins. The three-dimensional structure of cardiotoxins is fairly rigid, mainly as a result of a stabilization by four disulfide bridges. However, certain segments of the polypeptide chain apparently possess enough conformational freedom to adopt a new conformation in a lipid environment. It may be postulated that the new β -structure encompasses the original (*i.e.* in an aqueous solution) unordered, water-

exposed polypeptide fragments which, as a result of electrostatic and hydrophobic interactions with the bilayer surface, are forced into a less polar membrane environment. In such an environment, they can no longer form hydrogen bonds with water and thus refold into a conformation in which the backbone amide groups are hydrogen-bonded to each other. Since cardiotoxins have a very low tendency to form α -helices (Visser and Louw, 1978), this new conformation is that of a β -type structure.

Previous laser Raman spectroscopic studies of cardiotoxins from the venoms of *N. mossambica mossambica* did not indicate differences in the secondary structure between the proteins in aqueous solution and in a lipid-bound form (Pézolet *et al.*, 1982). The factors that may contribute to the differences between the Raman and Fourier-transform infrared data are not fully clear. Whereas these discrepancies may reflect the different sources of cardiotoxins used in the Raman and infrared experiments, such an explanation is not very likely due to the similarity in the primary structure of the various snake venom cardiotoxins (Dufton and Hider, 1983). Another possibility is that relatively small conformational changes of the type observed in this study could remain undetected by Raman spectroscopy. This is particularly plausible in view of the fact that the positions of the Raman bands representing unordered structures and β -sheet conformations are similar; furthermore, the higher noise level in the Raman spectra may obscure subtle changes in the amide band profiles.

The most common conformation of polypeptide chains in a hydrophobic environment of a lipid bilayer is believed to be the helix (*e.g.* Engelman *et al.*, 1980; Engelman and Steitz, 1981). However, an increasing number of observations suggest that β -structures may also be present in some membrane-spanning proteins (Tobkes *et al.*, 1985; Lee *et al.*, 1985; Surewicz *et al.*, 1987a). Although the exact membrane location of cardiotoxins is not known, several lines of evidence indicate that the proteins can penetrate the hydrophobic portion of a bilayer (Faucon *et al.*, 1983; Batenburg *et al.*, 1985). The present data provide thus a direct experimental support to the notion (Tobkes *et al.*, 1985) that the membrane-embedded protein domains are not necessarily α -helical and may consist of intramolecularly hydrogen-bonded β -strands. Conformational changes of the type observed in this study are likely to be operative also in the interaction of other proteins and polypeptides with membranes, and they undoubtedly deserve further investigation.

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