^{1}H n.m.r. STUDIES OF A NEUROTOXIN AND A CARDIOTOXIN FROM Naja mossambica mossambica: AMIDE PROTON RESONANCES

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SUMMARY: Proton n.m.r. spectra at 360 MHz of neurotoxin II and cardiotoxin V^{II}_4 from the venom of Naja mossambica mossambica are reported. From the n.m.r. spectra the solution conformations of the two proteins seem to be quite closely related. However, the exchange rates of the n.m.r. observable labile protons with deuterium of the solvent were markedly different, showing that the molecular structure of the cardiotoxin must be more flexible than that of the neurotoxin and suggesting that the different functional properties of the two toxins might be related to the different molecular dynamics.

Snake neurotoxins and cardiotoxins are parent proteins with extensive structural homologies (1-5). Short neurotoxins and cardiotoxins consist of one polypeptide chain with 60 - 62 amino acids, including four disulfide bridges. The eight half-cystine residues in cardiotoxins and neurotoxins are placed at the same positions, and in addition seven other amino acid residues are found to be common to both groups of toxins. In spite of these homologies there are pronounced differences in the mode of action of the two types of toxins. Neurotoxin binds to a protein receptor at the post-synaptic level and blocks acetylcholine transmission (6). Cardiotoxin apparently binds to a lipid-type receptor structure, to trigger a structural rearrangement in the membrane that inactivates the Na^+, K^+-ATPase (5). The present paper reports a ^{1}H nuclear magnetic resonance (n.m.r.) study of the neurotoxin II and cardiotoxin V^{II}_4 from the snake Naja mossambica mossambica, which was undertaken to obtain additional insights into the structural basis for the different functional properties of the two proteins.

MATERIALS AND METHODS: Neurotoxin II and cardiotoxin V^{II}_4 from the venom of Naja mossambica mossambica were prepared as described previously (5,7). For the n.m.r. measurements, 0.002-M solutions of the proteins in D_2O at pD $\sim$ 4.5 and pD $\sim$7.0, which contained also 0.1-M NaCl, were prepared. pD values are the pH meter readings without correction for isotope effects.

High resolution proton n.m.r. spectra were obtained on a Bruker HX-360 spectrometer. Chemical shifts are in parts per million (ppm) relative to internal sodium 3-trimethylsilyl propionate (TSP).

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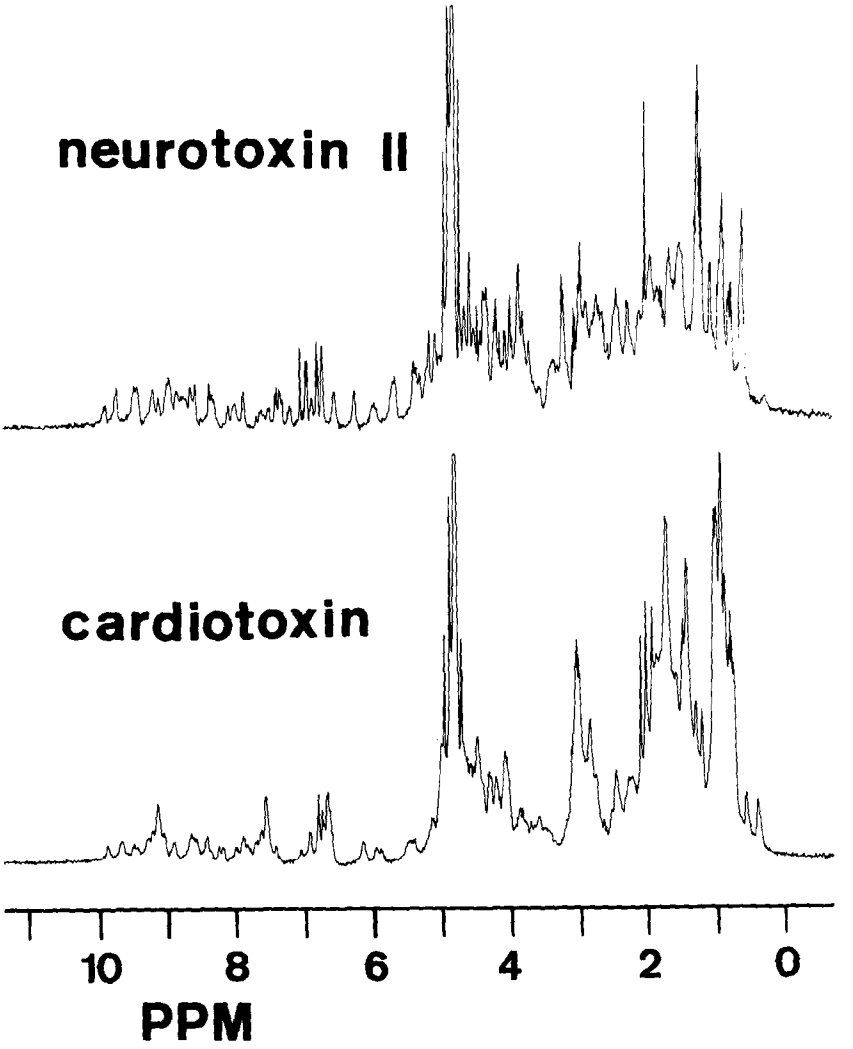

Fig. 1 ^{1}H n.m.r. spectra at 360 MHz of $2 \cdot 10^{-3}$-M solutions of two toxins from Naja mossambica mossambica in D_2O, which also contained 0.1 M NaCl, T = 25°. The neurotoxin solution had pD = 4.8, the cardiotoxin solution pD = 4.1. Both spectra were recorded within 15 min after the proteins had been dissolved in D_2O.

RESULTS: Fig. 1 shows the 360 MHz ^{1}H n.m.r. spectra of the neurotoxin and the cardiotoxin recorded immediately after dissolving the proteins in D_2O. In addition to the resonances of the aliphatic protons between 0 and 6 ppm and the aromatic protons between 6 and 8 ppm (8), these spectra contain, in the range from 7 to 10 ppm, numerous quite well resolved lines which correspond to slowly exchanging labile protons in the protein. The present investigation concen-

trated on the spectral features of these labile protons. N.m.r. was used to determine the number of slowly exchanging protons in the two proteins, to measure their chemical shifts and to study the proton exchange rates with deuterium of the solvent.

Inspection of the ^1H n.m.r. spectra (Fig. 1) had shown that the molecular conformations of the neurotoxins and the cardiotoxin were maintained over the pH range from at least 4.0 to 8.0 and at temperatures up to at least 45°. Preliminary observations had also shown that in the pH region 4.0 to 8.0 the exchange rates of the labile protons observed between 7 and 10 ppm decrease at lower pH. This behavior is typical for amide protons in general (9), and in the absence of pH dependent changes of the molecular flexibility also for slowly exchanging amide protons in globular proteins (10-12). As a general strategy we decided on the basis of these qualitative observations, first to prepare protein solutions at pD ≈ 4.5 and 25°, where proton exchange would overall be rather slow, and then to pursue the kinetic studies at higher pD and higher temperature, where even the most slowly exchanging protons could be studied within a reasonable period of time.

The proteins were dissolved in 0.1-M NaCl solution in H_2O and the pH adjusted to 4.0 by the addition of HCl. These solutions were then lyophilized. The lyophilized proteins were redissolved in 0.4 ml of D_2O at 25°, and n.m.r. spectra (Fig. 1) of these 0.002-M D_2O solutions were recorded during several hours with time intervals of 15 minutes. pH measurements in the n.m.r. tubes showed that the actual pD values were 4.8 for the neurotoxin and 4.1 for the cardiotoxin. From comparison with spectra obtained after complete exchange of the labile protons it was found that under these conditions the spectra of both proteins contained resonances of approximately 30 labile protons. The chemical shifts of these resonances range from 7.55 to 9.84 ppm in neurotoxin and from 7.02 to 9.89 in cardiotoxin (Table I). Overall, the proton exchange rates derived from the time course of the resonance intensities were considerably faster in cardiotoxin than in neurotoxin (Table I). In the neurotoxin solution at pD = 4.8 and T = 25°, eight among the ca. 30 observable labile protons were found to exchange with a half-time of less than 1 hr. In cardiotoxin solutions at pD = 4.1 and T = 25°, thirteen of the ca. 30 protons had a half time of less than 1 hr (Table I).

After the proton exchange had been followed for several hours at pD ≈ 4.5 and 25° (for details see figure captions 2 and 3), the temperature was raised to

Table I Chemical shifts, δ, and life times with respect to chemical exchange, $\tau_{\frac{1}{2}}$, of the slowly exchanging labile protons in neurotoxin II and cardiotoxin $V^{II}4$ of Naja mossambica mossambica.

$\tau_{\frac{1}{2}}$	Neurotoxin II δ (ppm)	Cardiotoxin δ (ppm)
0.1 hr - 1 hr at 25° and pD = 4.8 (neurotoxin) or pD = 4.1 (cardiotoxin)	7.55 7.58 7.62 7.98 8.05 8.28 8.60 (2)[a]	7.02 7.51 7.60 7.67 7.77 7.85 7.96 8.21 8.31 8.39 8.86 9.25 9.38
0.1 hr - 10 hr at 35° and pD = 6.2	7.44 7.89 8.65 8.72 8.87 8.95 9.05 9.09 9.33 9.53	7.21 7.46 7.51 8.58 (2-3)[a] 8.84 8.94 9.02 (2)[a] 9.16 9.24 9.44 9.60 (2)[a]
10 hr - 100 hr at 35° and pD = 6.2	7.64 7.94 8.17 8.52 9.09 9.43 (2-3)[a] 9.65	8.16 8.58 9.89
> 100 hr at 35° and pD = 6.2	7.96 8.87 9.41 9.84	

a For resonances with intensities corresponding to more than one proton, estimated resonance intensities are indicated in parentheses.

35° and the pD changed by the addition of 0.01 ml of 2-M deuterated neutral phosphate buffer. pH measurement in the n.m.r. tubes showed that pD = 6.2 was thus obtained in both protein solutions. The time course of the resonance in-

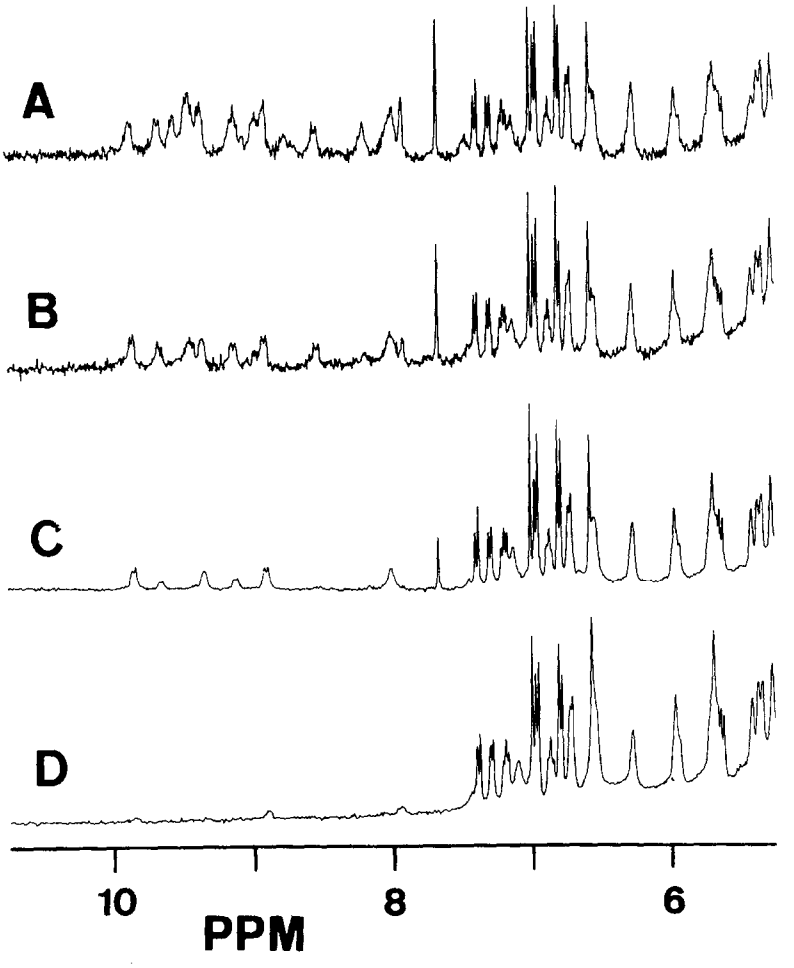

Fig. 2 Amide proton exchange in a D_2O solution of neurotoxin II. The sample of Fig. 1 had been kept at pD = 4.8 and T = 25° for 5.5 hrs. The temperature was then raised to 35° and the pH changed to 6.2 by the addition of a trace of deuterated 2-M neutral phosphate buffer. The spectra were recorded at the following time intervals after the addition of the phosphate buffer: A. 16 min, B. 17 hrs, C. 73 hrs, D. 454 hrs.

tensities at pD = 6.2 and T = 35° is shown in Fig. 2 for neurotoxin and in Fig. 3 for cardiotoxin. Again the two figures clearly show that the proton exchange is appreciably faster in cardiotoxin. Life times with respect to exchange with deuterium of D_2O estimated from experiments of the type of Figs. 2 and 3 are presented in Table I.

DISCUSSION: In all, the polypeptide backbone and the amino acid side chains of the neurotoxin and the cardiotoxin used in the present study contain of the order of 100 labile protons per molecule. The majority of these protons, i.e.

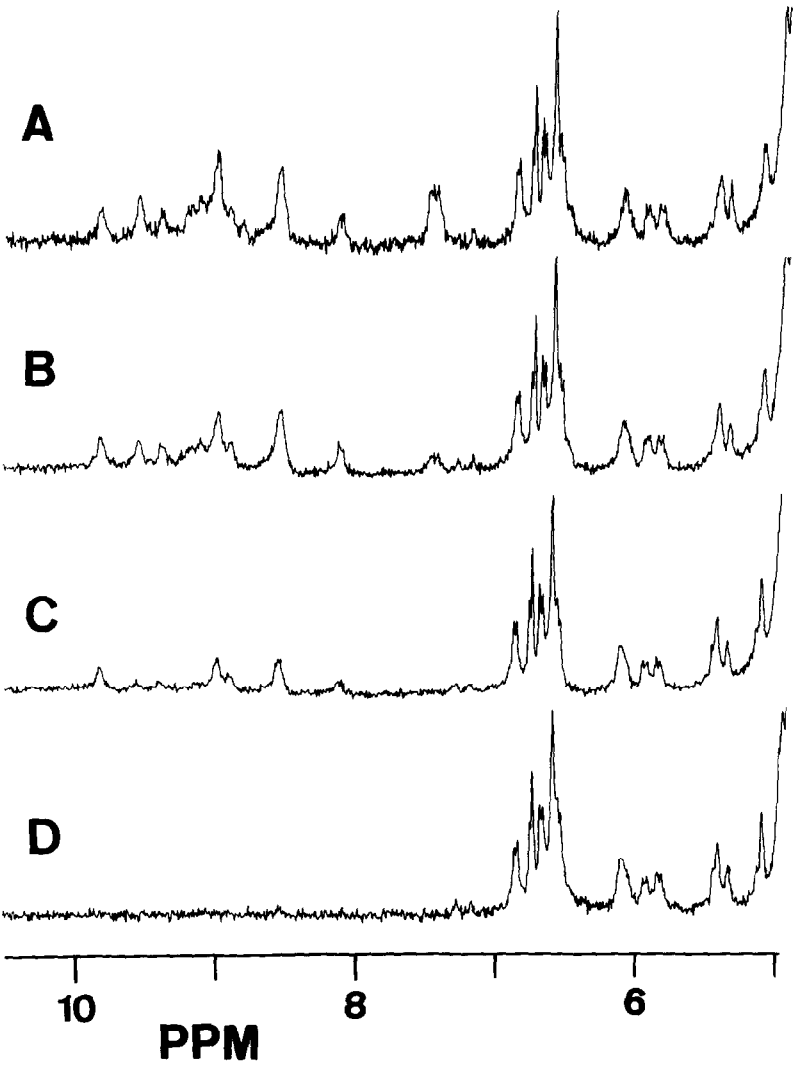

Fig. 3 Amide proton exchange in a D_2O solution of cardiotoxin. The sample of Fig. 1 had been kept at pD = 4.1 and T = 25° for 3 hrs. The temperature was then raised to 35° and the pH changed to 6.2 as described in Fig. 2. The spectra were then recorded at the following time intervals after the addition of the phosphate buffer: A. 10 min, B. 32 min, C. 3.6 hrs, D. 53 hrs.

those of the solvent accessible backbone amide groups and the bulk of the side chain protons, exchange too rapidly to be observed in the n.m.r. spectra of D_2O solutions of the proteins (8,9). General experience with peptides and proteins would therefore suggest that essentially all the labile protons observed in the spectra of Fig. 1 to 3 come from backbone amide groups located in inte-

rior parts of the molecule and hence not readily accessible to the solvent (8-12). This is further corroborated by the chemical shifts (Table I) and the multiplet structures of the resonance lines. As was previously extensively discussed (8-12), studies of the exchange kinetics of interior amide protons are a suitable method to investigate the rigidity of the protein conformation in solution.

More specific resonance assignments are indicated by the X-ray structures of homologous neurotoxins (13,14), which include extended regions of β-pleated sheets containing 25 to 30 hydrogen bonds. It is quite striking that the number of slowly exchanging protons listed in Table I coincides closely with the number of hydrogen bonds in the antiparallel β-pleated sheet structure of the neurotoxin crystal conformation. Since assignment of most of the resonances in Table I to backbone amide protons involved in intramolecular hydrogen bonds is also compatible with the observed chemical shifts (8), the n.m.r. data seem to imply that the neurotoxin polypeptide backbone conformation in solution is similar to the crystal structure. This conclusion is also supported by the results of an earlier n.m.r. investigation of a homologous neurotoxin (15), which was based mainly on spectral features of non-labile nuclei of the amino acid side chains and which appeared before the X-ray structure was known.

In view of the suggested different functional properties (5,6), it is of particular interest to compare structural features of neurotoxin and cardiotoxin. On the one hand, since in the two proteins essentially identical numbers of labile protons with similar chemical shifts exchange sufficiently slowly to be observable in D_2O solution (Table I), the present n.m.r. data indicate that the molecular conformation of cardiotoxin coincides with the structure type determined for neurotoxin (13-15). Considering the extensive homologies in the amino acid sequences of neurotoxin and cardiotoxin (1-5), it is not unexpected that the spatial structures should also be closely related. On the other hand, the amide proton exchange rates are considerably faster in cardiotoxin than in neurotoxin. In other words, there is a greater probability for cardiotoxin molecules to occur in "open forms" from which NH-exchange can take place (9-12). In conclusion these comparative studies would thus seem to indicate that the different modes of action on membranes are related to the different flexibility of the spatial structures of neurotoxin and cardiotoxin. Without pursuing possible mechanistic implications of this hypothesis, it may be pointed out that it is a priori more likely for a flexible molecule that conformational changes are induced by the different medium in or near receptor sites, which

might be amenable to experimental observation in future studies of structure-function relations in these toxins.

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