

# Identification of Two Antiparallel-sheet Structure of Cobrotoxin in Aqueous Solution by $^1\text{H}$ NMR

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## 1. INTRODUCTION

Cobrotoxin is a neurotoxic protein isolated from the venom of Formosan cobra (*Naja naja atra*). This protein, which blocks the neuromuscular transmission at post-synaptic membrane by the specific binding to acetylcholine receptors, contains 62 amino acid residues (Mr 6949) with four disulfide bridges (1,2). The concept of a structurally and functionally invariant residue of cobrotoxin has been proposed which is essential for both conformational stability and neurotoxicity (3). To elucidate the chain conformation and roles of amino acid residues of cobrotoxin in solution, nuclear magnetic resonance spectra have to be analyzed.

High field  $^1\text{H}$ NMR spectroscopy is one of the most powerful techniques available for studying the solution structure and dynamics of cobrotoxin. Recently, Wüthrich et al. (4) showed that 2D correlated spectroscopy could be used to identify protons that are scalar coupled, while 2D NOE spectroscopy could give through space connectivity and provide sequential assignments as well as conformational information. We demonstrated the secondary structure of cobrotoxin was found by NOESY spectrum after sequential assignment has been made.

## 2. EXPERIMENTAL

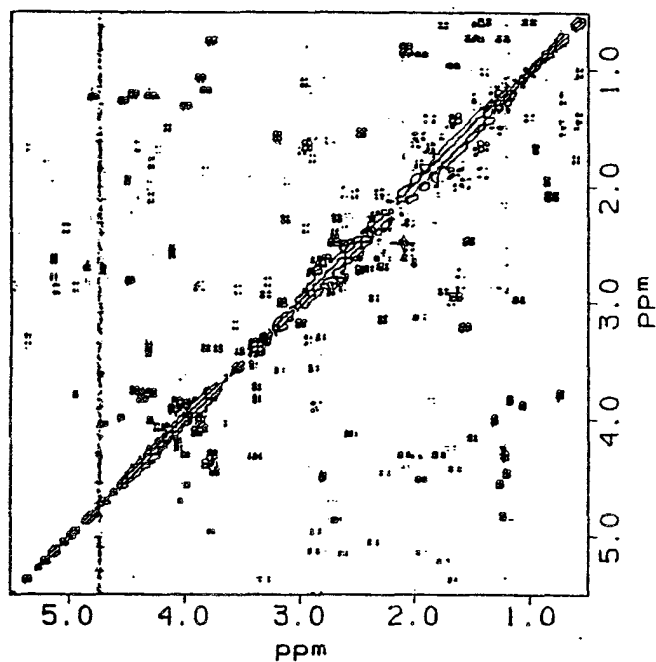
Formosan cobra venom was obtained from commercial source in lyophilized powder. Crude venom of Formosan cobra was first fractionated on a SP-Sephadex C-25 column preequilibrated with 0.025 M sodium phosphate buffer, pH 7.5. The column was then washed with 200 ml of the equilibrium buffer and elution was carried out with sodium chloride in a linear gradient from 0 to 0.5M. The cobrotoxin was further rechromatographed on a SP-Sephadex C-25 column with a linear gradient of sodium chloride with concentration varied depending on the chromatographic behavior of toxin fraction being applied. Each toxin fraction was then desalted by filtration on a Sephadex G-25 column and lyophilized.

Samples were prepared from lyophilized protein as follows: weighted amounts of solid 70 mg were dissolved in either 100%  $\text{D}_2\text{O}$  or 90%  $\text{H}_2\text{O}/10\% \text{D}_2\text{O}$  solution. pH were adjusted to 3.0 with 0.2 M of DCl. DSS was added as an internal standard. All the NMR spectra were acquired at 20°C on a Bruker AM400 spectrometer in the Fourier transform mode. All two-dimensional NMR spectra were recorded in pure phase absorption mode using the time proportional phase incrementation approach (5,6). All the 2D data were processed on a micro Vax III computer (Digital Equipment Cooperation), using a version of

software provided by Dr. Dennis Hare (7).

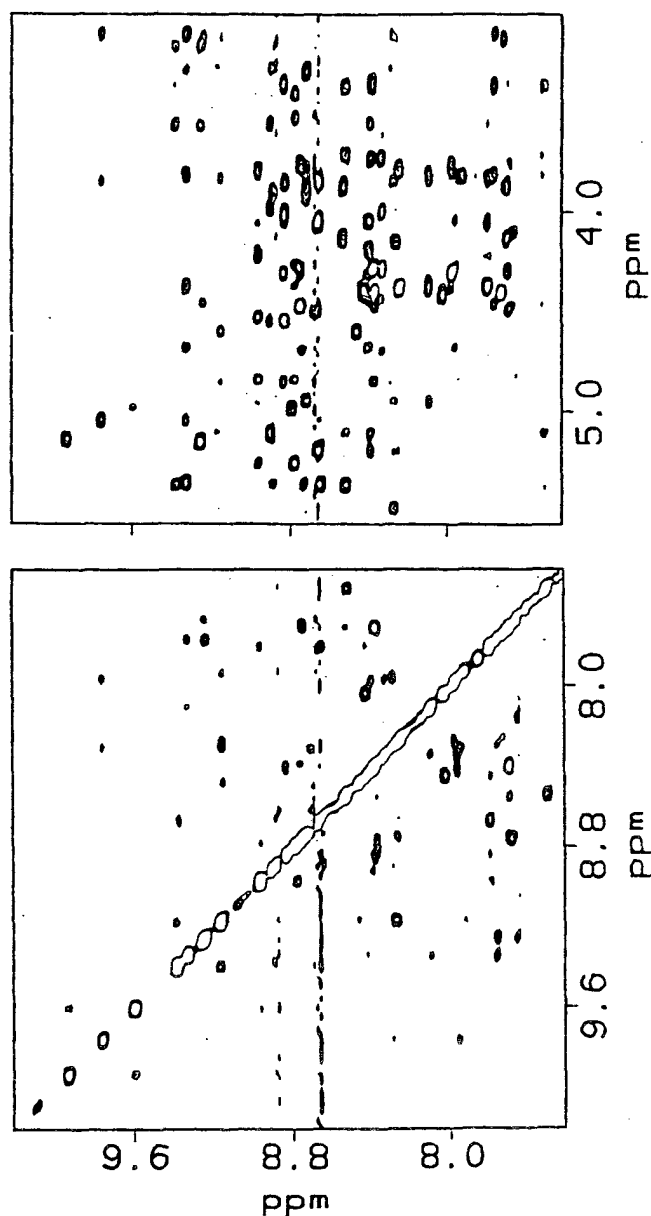
### 3.RESULTS AND DISCUSSION

The  $^1\text{H}$ NMR resonance assignments are obtained by following two steps. First, by using 2QF and RELAYED COSY spectrum (5,9,10), all the spin systems of amino acid residues could be identified. Each spin system could be classified as unique (Gly, Ala, Thr, Val, Ile, Leu, Arg, Lys and Pro), 3-Spin (Ser, His, Tyr, Phe, Asn) three and 5-Spin (Gln) five spins system according to the pattern of their scalar coupling (8). Figure 1 shows the 2QF-COSY spectrum of cobrotoxin in  $\text{D}_2\text{O}$  solution.



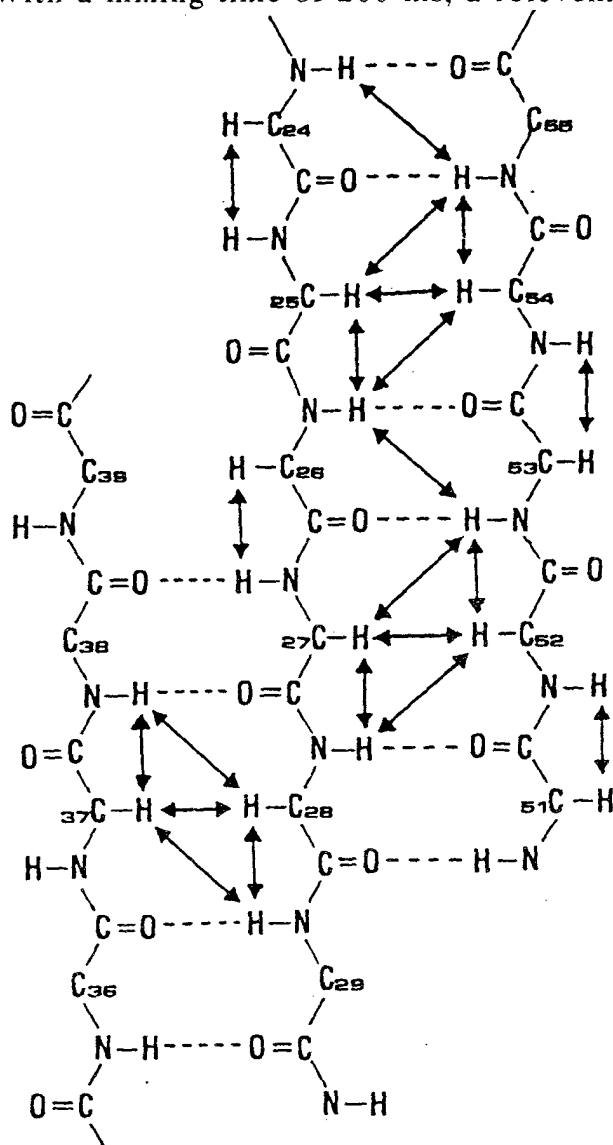
**Figure 1.** Contour plot of the aliphatic region ( $\omega_1 = \omega_2 = 0.5\text{-}5.5$  ppm) of a 400 MHz H 2QF-COSY spectrum of 20 mM cobrotoxin in 99.97%  $\text{D}_2\text{O}$  at pH=3.1 and 28°C .

In this figure, most of spin systems could be identified. Others could also be done with the supplement of RELAYED COSY experiment. The second step is using NOESY (11) spectrum to achieve sequential assignment. By combining



**Figure 2.** Contour plot of a 400-MHz H NOESY spectrum recorded with a mixing time of 200 ms of 20 mM cobrotoxin in 90%  $\text{H}_2\text{O}$  / 10%  $\text{D}_2\text{O}$  solution. The upper panel shows the region ( $\omega_1 = 3.1\text{-}5.4$  ppm,  $\omega_2 = 7.3\text{-}10.1$  ppm) containing the intraresidue NH- $\alpha$  H and sequential  $\alpha$  H-NH NOESY cross peaks. The lower panel shows the region ( $\omega_1 = \omega_2 = 7.3\text{-}10.1$  ppm) containing sequential NH-NH cross peaks.

information from 2QF/RELAYED COSY and NOESY spectrum of cobrotoxin in H<sub>2</sub>O solution, NOE cross peaks could be identified. With a mixing time of 200 ms, a relevant

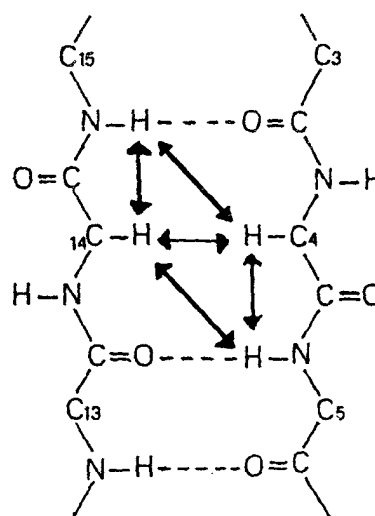


**Figure 3A.** Antiparallel triple-strand  $\beta$ -pleated. Schematic representation of the  $\beta$ -sheet secondary structure in cobrotoxin. The backbone atoms NH,  $\alpha$ -carbon and carbonyl group are drawn and sequence position is given. Solid arrows indicate NOEs observed in cobrotoxin. The inter-strand hydrogen bond between amide protons and carbonyl oxygens are indicated by dotted lines.

spectral region of such NOESY spectrum is shown in Figure 2.

The sequential distances  $d_{\alpha N}$ ,  $d_{NN}$  and  $d_{\beta N}$  between adjacent amino acid residues are most important evidence for the assignment.

In cobrotoxin, there are an antiparallel triple-stranded  $\beta$ -pleated, including the residues 24-29, 36-39 and 52-55, and a double-stranded sheet of residues 3-5 and 13-15 as showed in Figure 3A and 3B. The chemical shifts of these residues involved in antiparallel  $\beta$ -pleated are listed in Table I. A distinction feature of  $\beta$ -sheet structure is that the constituent strands are stabilized by hydrogen bonds between the amide and carbonyl groups of adjacent strands. The amide protons that participate in these hydrogen bond will exchange slowly with solvent, and could be observed in D<sub>2</sub>O solution. The NOESY spectra (data not shown) taken immediately after dissolving in D<sub>2</sub>O at 15°C, showed that all of the amide protons of central strand of triple-stranded sheet remained while the amide protons of two peripheral strands remained alternatively for being hydrogen-bonded. The antiparallel  $\beta$ -sheet structure gives a



**Figure 3B.** Antiparallel double stranded  $\beta$ -pleated.



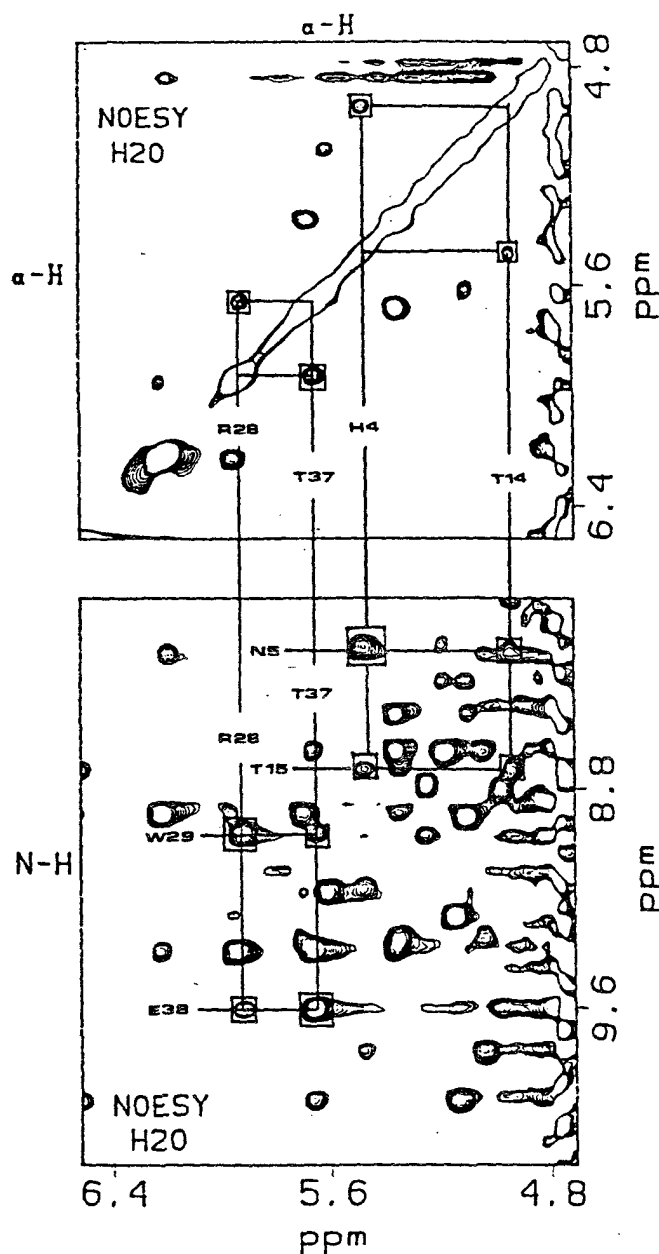
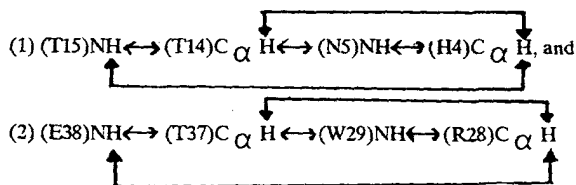


Figure 5. The NOEs for the following two inner loops



## ACKNOWLEDGEMENTS

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## REFERENCES

- (1) Yang, C. C. *J. Biol. Chem.* **240**, 1616. (1965)
- (2) Yang, C. C. *J. Formosan Med. Assoc.* **63**, 325. (1964)
- (3) Ryden, L., Gabel, D. and Eaker, D. *Int. J. Peptide Protein Res.* **5**, 261. (1973)
- (4) Billeter, M., Braum, W. and Wüthrich, K. *J. Mol. Biol.* **155**, 321. (1982)
- (5) Redfield, A. G. and Kunz, S. D. *J. Magn. Reson.* **19**, 250. (1975)
- (6) Marion, D. and Wüthrich, K. *Biochem. Biophys. Res. Commun.* **113**, 967. (1983)
- (7) Infinity Systems, 14810 216th Avenue, NE, Woodinville, Washington 98072, U. S. A.
- (8) Chazin, W. J., Rance, M. and Wright, P. E. *J. Mol. Biol.* **202**, 603. (1988)
- (9) Eich, G., Bodenhausen, G. and Ernst R. R. *J. Amer. Chem. Soc.* **104**, 3731. (1982)
- (10) Bax, A. and Drobny, G. P. *J. Magn. Reson.* **61**, 306. (1985)
- (11) Kumar, A., Ernst, R. R. and Wüthrich, K. *Biochem. Biophys. Res. Commun.* **95**, 1. (1980)
- (12) Englander, S. W. and Wand, A. J. *Biochemistry* **26**, 5958. (1987)