SOLUTION SPATIAL STRUCTURE OF APAMIN AS DERIVED FROM NMR STUDY

V. F. BYSTROV, V. V. OKHANOV, A. I. MIROSHNIKOV and Yu. A. OVCHINNIKOV
M. M. Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, 117988 Moscow, USSR

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1. Introduction

Apamin, the minor active component of bee venom, is unique in being the smallest known peptide neurotoxin with an excitatory effect on the central nervous system and is also the only known polypeptide that passes the blood–brain barrier [1]. It becomes a very useful instrument in the study of mechanism of nerve pulse transmission [2]. The chemical structure of apamin, an octadecapeptide with two disulphide bridges, has been determined [3–5]:

Cys–Asn–Cys–Lys–Ala–Pro–Glu–Thr–Ala–Leu–Cys–Ala–Arg–Arg–Cys–Gln–Gln–His–NH_2

Apamin properties in solution have been extensively studied by optical [6,7] and NMR [8–11] spectroscopy. Different spatial structures of apamin were theoretically proposed on the basis of energy minimum calculation using atomic [12] and residual [13] representation, and by secondary structure prediction [14].

This paper presents a solution conformation of apamin derived from the results of spectroscopic techniques, mainly proton NMR [8,11].

2. Materials and methods

Apamin was isolated from the venom of honeybee *Apis mellifera* as in [6]. The ^1H and ^{13}C NMR spectra were recorded on a Varian SC-300 spectrometer in Fourier transform mode at 300 and 75 MHz, respectively. The pH measurements were done directly in the 5 mm NMR tubes using a 180 mm Ingold 405 M3 combination microelectrode. For D_2O solution the values used are the direct reading (pH*) of a digital pH meter (Orion Research model 601). The experimental pH dependences of chemical shifts were analyzed by multiparametric non-linear least squares curve-fitting computer software [11].

3. Results

Signals in the proton and ^{13}C NMR spectra of apamin have been identified [11] by two levels of assignment:

(1) The proton signals are interconnected by homonuclear proton–proton selective spin decoupling and assigned to a particular spin multiplet system according to the specific NMR type of amino acid residue [8,10];
(2) The individual spin systems are attributed to a definite position of the corresponding amino acid residue in the primary structure of apamin by heteronuclear ^{13}C $\{^1H\}$ selective decoupling of carbonyl signals in the ^{13}C NMR spectrum [10].

The results of proton signal assignment are shown in fig.1, and the identification of signal parameters with particular amino acid residues is presented in table 1. Due to signal overlap we were not able to differentiate directly by the second level of assignment between Cys^3 and Cys^{15} residues, Arg^{13} and Arg^{14} residues, and Gln^{16} and Gln^{17} residues. The assignment of Cys^3 and Cys^{15} signals shown in table 1 is tentative as based on the influence of N-terminal α-amino group deprotonation on the signal positions of the former residue and of His^{18} imidazole ring deprotonation on the signals of the last residue.

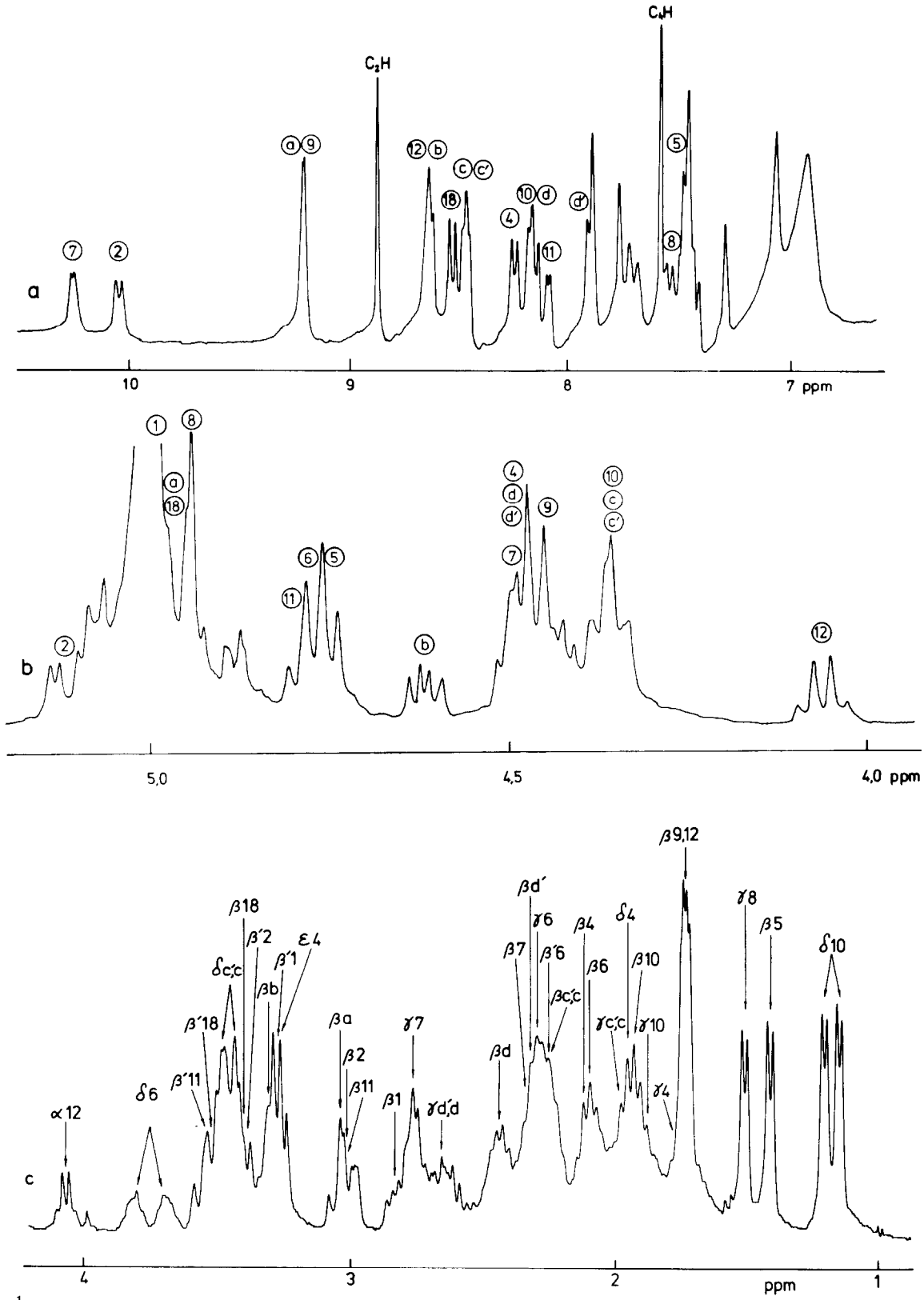

Fig. 1

Table 1
Proton NMR signal parameters and approximate torsional angles ϕ and ψ for apamin solution conformation

Apamin residue		$^3J_{HNC^\alpha H}$[a] (Hz)	$^3J_{HC^\alpha C^\beta H}$[b] (Hz)	$t_{1/2}$[c] (h)	ϕ[d] (grad)	ψ[e] (grad)
Cys^1		–	7.8, 7.8	–	–	−150
Asn^2		9.5	11.2, 4.2	<0.1	−120	110
Cys^3(a)		5.2	11.9, 2.8	0.2	−75	−30
Lys^4		7.6	[f]	4	−90	−10
Ala^5		6.8	6.1	11	−85	160
Pro^6		–	6.2, 6.2	–	−60[e]	−60
Glu^7		6.9	9.6, 4.4	0.1	−85	−60
Thr^8		8.5	1.9	4	−100	−40
Ala^9		2.7	6.1	<0.1	−60	−60
Leu^{10}		5.5	[f]	20	−75	−45
Cys^{11}		5.9	7.6, 7.6	14	−80	−60
Ala^{12}		3.0	6.1	26	−60	−50
$Arg^{13,14}$	(c)	4.2	[f]	>100	−70	−30
	(c′)	3.5	[f]	2.7	−65	−30
Cys^{15}(b)		5.4	9.6, 4.2	26	−75	−30
$Gln^{16,17}$	(d)	7.1	[f]	0.5	−85	−10
	(d′)	6.8	[f]	11	−85	−60
His^{18}		7.9	8.6, 6.1	<0.1	not fixed	

[a] In H_2O solution; [b] In ^2H_2O solution
[c] Backbone NH deuterium exchange $t_{1/2}$ in ^2H_2O at pH* 2.9 and 14°C
[d] Evaluated from ϕ-angle dependence of the $^3J(H-NC^\alpha-H)$ and $^3J(^{13}C'-NC^\alpha-H)$ couplings [15] in accordance with the Dreiding model of fig.3
[e] Estimated from the Dreiding model of fig.3
[f] The parameters were not measured because of considerable signal overlap

Table 1 presents the NMR parameters which are most important for the apamin conformational study. Backbone $H-NC^\alpha-H$ and side chain $H-C^\alpha C^\beta-H$ vicinal proton–proton coupling constants provide information on the torsional angles ϕ and χ^1 of the $N-C^\alpha$ and $C^\alpha-C^\beta$ bonds, respectively [15]. Deuterium exchange $t_{1/2}$ discriminates intramolecular hydrogen bonded backbone NH groups [16]. In addition, chemical shift pH-dependence studied in [8] revealed the microenvironment of ionogenic groups: N-terminal α-amino; ε-amino of Lys^4; γ-carboxyl of Glu^7 and imidazole ring of His^{18}. The pK_a values of α-amino group (pK_a 6.7) and His^{18} (pK_a 6.6) are too similar to be differentiated for some weakly pH-dependent chemical shifts. Therefore the chemically modified apamin with blocked α-amino group ([Ac-Cys^1]-apamin) was studied and shifts affected by His^{18} deprotonation were revealed [11].

4. Discussion

The conformational stability of apamin follows from CD spectra [6], which demonstrate persistence against a wide pH range, presence of organic solvents and 6 M guanidinium–HCl, and reasonable chemical modifications [17].

When analyzing the NMR parameters (table 1) attention is captured by the 3 or even 4 residues in succession (Leu^{10}, Cys^{11}, Ala^{12} and possibly Arg^{13}) with both very slow deuterium exchange ($t_{1/2} > 14$ h) and low values of $H-NC^\alpha-H$ proton coupling (3.0–

Fig.1. Signal assignment in 300 MHz proton NMR spectrum of apamin (10 mM, 32°C) as obtained by homo- and heteronuclear multiresonance [11]. Signal numbering corresponds to the position of amino acid residue in the primary structure of apamin. The signals labeled by ⓐ, ⓑ, ⓒ, ⓒ′, ⓓ, ⓓ′ correspond to 6 spin multiplets which are not yet unambiguously assigned to the particular position (see table 1). The greek letters stand for corresponding carbon atom position in the side chain of residue. (a) NH proton signal region (in H_2O, pH 3.4); (b) $C^\alpha H$ proton signal region (in ^2H_2O, pH* 5.1); (c) high field spectrum region (in ^2H_2O, pH* 6.7).

5.9 Hz). Both of these features are inherent to polypeptide right-handed helical structure (3_{10}, α- or π-helix) [16]. Thus it was assumed that the 10–13 fragment is incorporated in a helix and its backbone NH groups are hydrogen bonded to the carbonyls of $n-i$ residues (i = 3,4 and 5 for 3_{10}-, α- and π-helix, respectively). The preceding to this fragment Ala[9] residue has also a low ^3J(H–NC$^\alpha$–H) value.

By model building it was found that only the α-helix could be constructed due to steric hindrances introduced by 1–11 and 3–15 disulphide links. The presence of the α-helical region in apamin is in accord with CD [6] and laser Raman [7] spectra, as well as with secondary structure prediction [14] based on Chou and Fasman method [18].

The model building around the α-helical fragment 6–13 permits the formation of β-turns for other very slow exchanging NH groups of Ala[5], Cys[15] and Gln[16] (or Gln[17]) residues. The most plausible system of intramolecular hydrogen bonds is schematically depicted in fig.2 as consisted of the α-helix and three β-turns. Laser Raman study [7] indicates the presence of at least two β-turns.

In constructing the apamin spatial structure shown in fig.3 the following main features of the NMR study

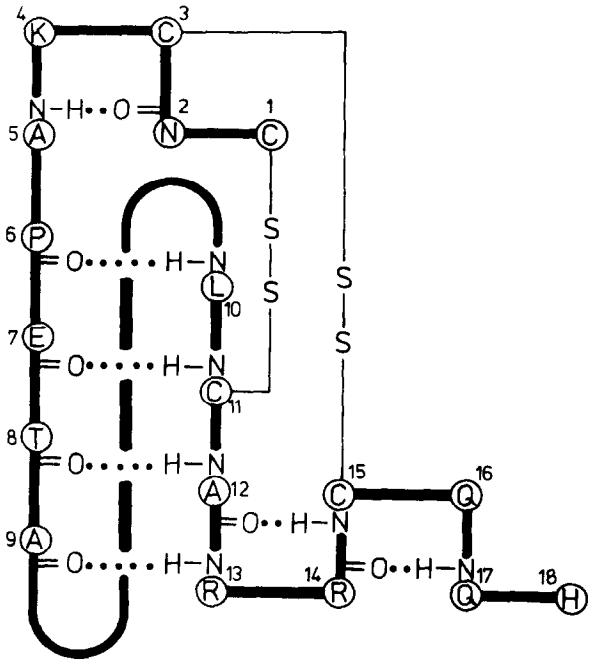

Fig.2. Diagram of intramolecular hydrogen bonds in apamin. The standard one-letter code is used to designate the amino acid residues.

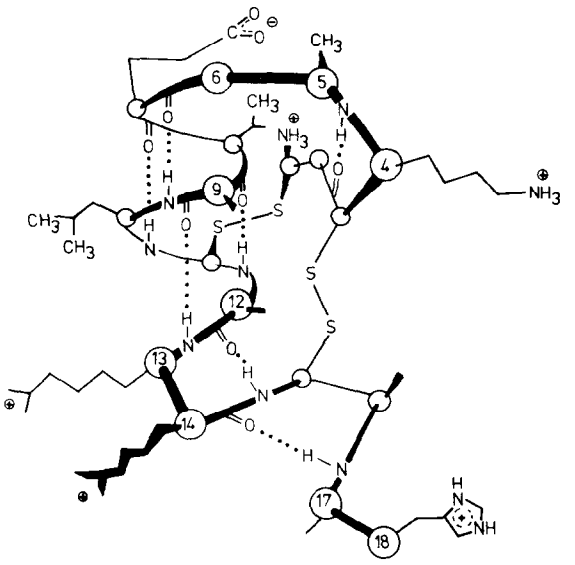

Fig.3. Schematic representation of proposed spatial structure of apamin in solution.

[8–11] were also taken into account:

(i) Carboxyl group of Glu[7] residue is at ~0.5 nm from the N-terminal α-amino group.
(ii) Carboxyl group of Glu[7] is also proximate to the methyl groups of Ala[5] and Thr[8] residues.
(iii) The side chain of at least 1 arginine residue is in the vicinity of Leu[10] methyl group.
(iv) The ϵ-amino group of Lys[4] is distant to the other residues.
(v) The side chains of His[18] and of both arginine residues are far from the other ionogenic residues.
(vi) The backbone NH groups of Lys[4], Thr[8] and presumably Arg[14] are capable of forming weaker intramolecular hydrogen bonds, possibly with side chain functions of the molecule.
(vii) The torsional angles ϕ must be in conformity with the measured vicinal coupling constants ^3J(H–NC$^\alpha$–H) and ^3J(^{13}C′–NC$^\alpha$–H).

The laser Raman spectroscopy data on the similarity in conformation of the both disulphide bridges [7] were also taken into account.

The backbone torsional angles ϕ presented in table 1 for the solution spatial structure (fig.3) were evaluated from the ^3J(H–NC$^\alpha$–H) coupling constants and the ambiguity in the ϕ values was partially put aside by the ^3J(^{13}C′–NC$^\alpha$–H) couplings. The ψ angles in table 1 were estimated from the Dreiding model. The Ramachandran ϕ–ψ energy map indicates that

the apamin residue points fall within the allowed regions.

The rigidity of the apamin conformation is achieved by relative orientation of the α-helical unit 6–13 and two β-turns 2–5 and 12–15 about the disulphide bridges Cys^1–Cys^{11} and Cys^3–Cys^{15}. Additional, but weaker intramolecular hydrogen bonds can be formed by the backbone NH of Lys^4 with hydroxyl group of Thr^8, by the backbone NH of Arg^{14} with the sidechain carbonyl of Gln^{17}, and by the backbone NH of Thr^8 with the sidechain carbonyl of Asn^2, or backbone carbonyl of Ala^5 residue. The C-terminal His^{18} is the only residue not incorporated into the apamin tertiary structure.

The solution of the spatial structure of apamin presented in fig.2 and 3 explains the spectroscopic results [6–9,11] much better than the structures theoretically predicted in [12–14].

5. Conclusions

The spatial structure derived for apamin in solution could be considered as similar to the conformation adopted on a receptor because of its persistence against media conditions and chemical modification as well as the fact that synthetic apamin possesses full biological activity [19–21] and the same CD and NMR spectra [21] which is considered [14] as evidence that the information for the correct folding of the peptide chain is contained in the chain and not in an apamin precursor.

However the overall rigid spatial structure is not the only feature that is important for biological action. The chemical structure of arginine side chain is also crucial as follows from a dramatic loss of neurotoxic activity when both Arg^{13} and Arg^{14} are substituted either by lysine [22] or by ornithine [23] residues.

Further progress in structure–activity relationship study of apamin depends on localization and isolation of receptor system, and studying of its interaction with apamin and its derivatives.

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