^{19}F NMR DETERMINATION OF INTRAMOLECULAR DISTANCES IN SPIN- AND FLUORINE-LABELLED PROTEINS

Neurotoxin II *Naja naja oxiana*

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1. Introduction

Determination of intramolecular distances between selected groupings gives crucial information on the conformation of proteins in solution and characterizes the topography of the sites responsible for biological activity. It also enables a comparison of the protein's spatial structure in solution and in the crystal to be made on a quantitative basis, which is essential for understanding the dynamic features and conformational potentialities of the molecule. Evaluation of the intramolecular distances in proteins in solution can be done by optical spectroscopy, EPR and NMR techniques. For instance, detailed information can be obtained through the analysis of shift and relaxation effects of paramagnetic metal ions [1] or covalent spin labels (stable nitroxyl radicals) [2] in the NMR spectra of proteins. However, there arises a serious problem of signal assignment in overlapping proton spectra of proteins. In this respect it appears promising to use line-broadening evoked by spin labels in ^{19}F NMR spectra of fluorine-containing protein derivatives. Such derivatives are obtained via chemical modification or biosynthetically, whereas their spectra usually consist of a limited number of signals [3,4]. Feasibility of such an approach is demonstrated here with neurotoxin II (NT-II) isolated from the venom of Central Asian cobra (*Naja naja oxiana*). A spin label was incorporated at the ϵ-amino group of the Lys 27* residue. The amino groups of Leu 1, Lys 15, Lys 26, Lys 45 and Lys 47 residues were trifluoroacetylated. The distances between the spin label and the trifluoroacetyl groups were evaluated by ^{19}F NMR. Comparison of the results on the spatial structure of neurotoxins in solution [2,6] and in the crystal [7] points to adequacy of the utilized approach.

Abbreviations: NT-II, neurotoxin II; SL, spin label

* The numeration of the amino acid residues is according to the homologous series [5]. In neurotoxin II *Naja naja oxiana* there is a residue deletion at position 19

2. Materials and methods

NT-II was isolated from Central Asian cobra venom as in [8]. The Lys 27 spin-labelled derivative of NT-II was obtained as in [6] and the material was then trifluoroacetylated with phenyltrifluoroacetate: 0.6 μM spin-labelled NT-II in 1 ml anhydrous dimethylformamide was stirred for 20 h at 22°C in the presence of 270 μM of phenyltrifluoroacetate and 28 μM triethylamine. The mixture was diluted with water to 70 ml and chromatographed on a carboxymethylcellulose CM-32 column using an ammonium acetate (pH 4.5) 0.01–0.05 M gradient. In doing so, derivatives A and B were isolated, containing 4 and 5 trifluoroacetyl groupings, respectively, according to ^{19}F NMR data (fig.1). The ^{19}F NMR spectra (94.2 MHz) were obtained using an XL-100-15 Varian NMR spectrometer operated in Fourier transform mode. The chemical shifts were measured relative to trifluoroacetic acid as an external standard. The X-band EPR spectra were taken with an E-109 Varian EPR spectrometer. The correlation times of the spin labels (τ_c) in derivative A and B were found from the EPR spectra through equation [9]:

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$$\tau_c = 6.65\ \Delta H_+ (\sqrt{I_+/I_-} - 1) \times 10^{-10}\ \text{s}$$

where ΔH_+ represents the downfield component width; I_+ and I_- are the intensities of the downfield and upfield spectrum components, respectively.

3. Results and discussion

Earlier we showed that the trifluoroacetylation of NT-II with ethylthiotrifluoroacetate leads to modification of 6 amino groups [10]. Since this reaction proceeds with the formation of β-mercaptoethanol, which in the case of spin-labelled NT-II could cause the reduction of the spin label in the course of isolation of the product, here we used phenyltrifluoroacetate.

Fig.1 and 2 show the ^{19}F NMR spectra of the modified derivatives A and B, their spectra after quenching the spin label with ascorbic acid, as well as the spectrum of the earlier obtained NT-II derivative trifluoroacetylated at all 6 amino groups [10] (derivative C). Quenching of the nitroxyl spin label allowed us to study in detail the pH-dependence of the ^{19}F chemical shifts and to assign the signals for derivatives A and B. Earlier [10], for the derivative C, a full

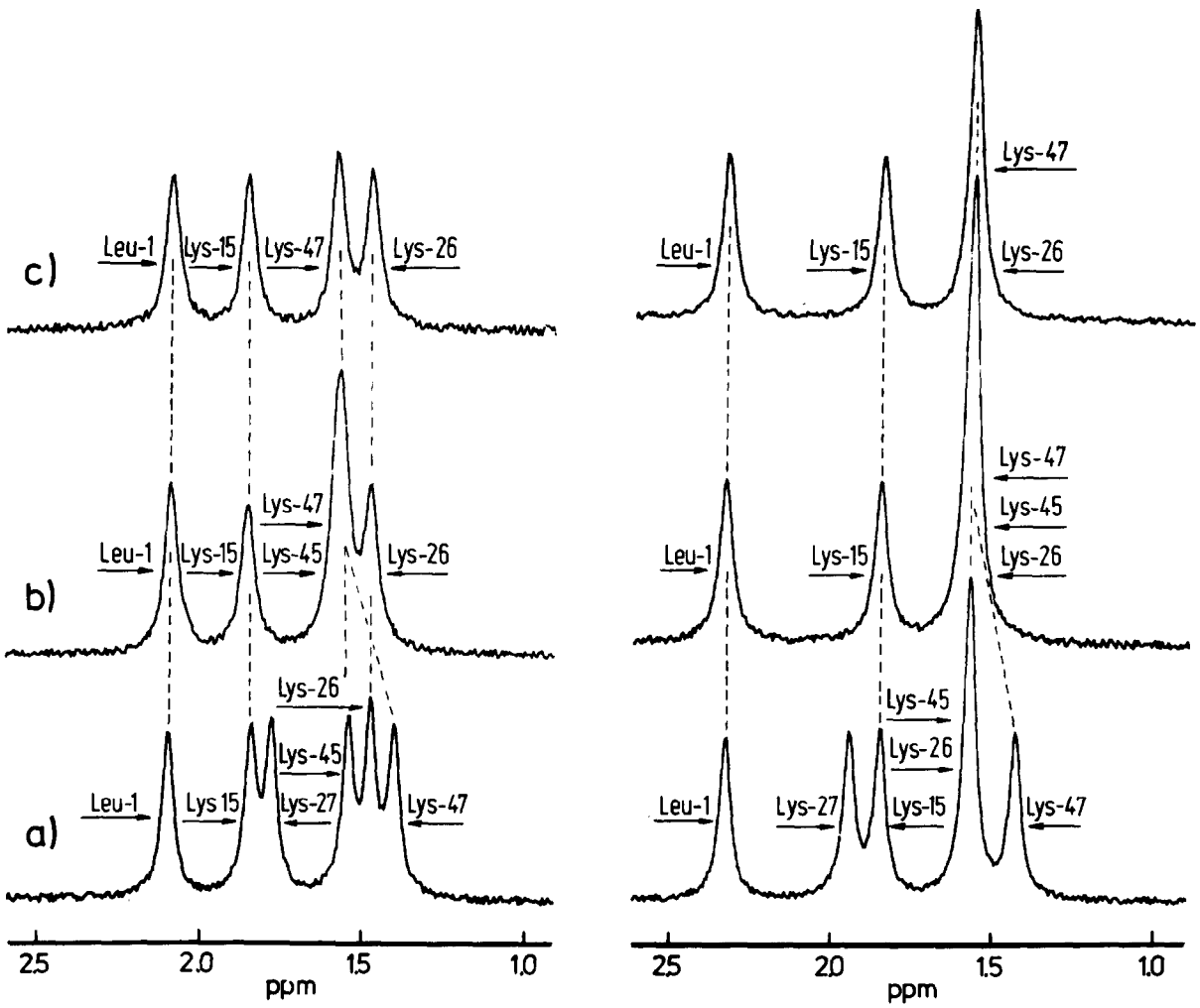


Fig.1. ^{19}F NMR spectra (94.2 MHz) of NT-II derivatives A, B and C (5×10^{-4} M, ^2H_2O, 0.3 M NaCl, 32°C): (left) at pH 2.5; (right) at pH 6.5; (a) derivative C; (b) derivative B with quenched (when 10 equivalents of ascorbic acid is added) spin label; (c) derivative A with quenched spin label.

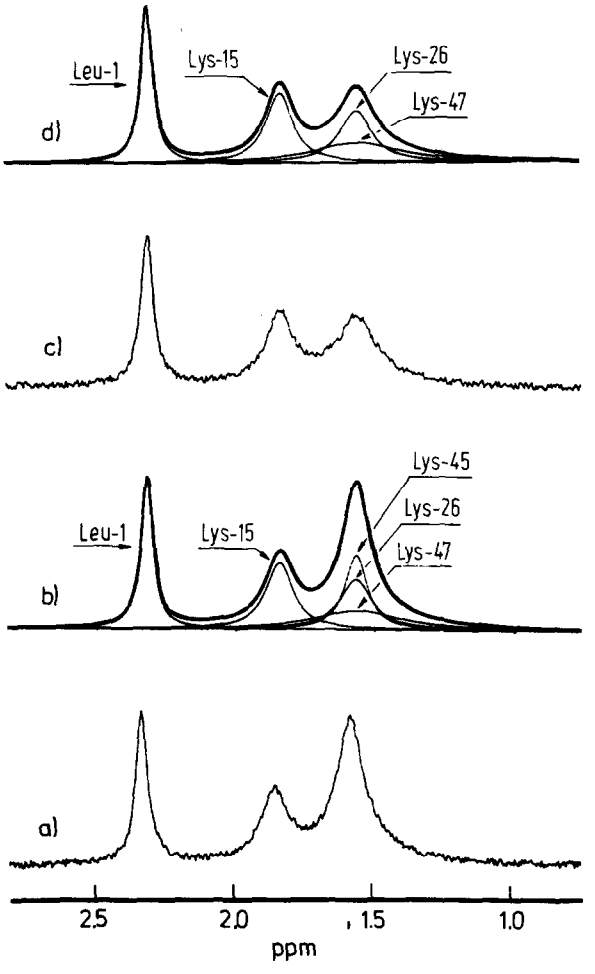


Fig.2. ^{19}F NMR spectra (94.2 MHz): (a) derivative B; (c) derivative A (5×10^{-4} M ^2H_2O, 0.3 M NaCl, 32°C); (b,d) computer-stimulated spectra of derivatives B and A, respectively.

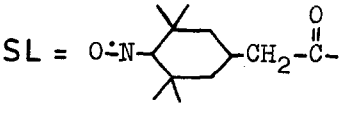
assignment of ^{19}F signals was performed (as indicated in fig.1) and the pH-dependence of the chemical shifts was also thoroughly studied.

As could be expected, one signal (1.94 ppm at pH 6.5 and 1.78 ppm at pH 2.5) corresponding to the CF_3CO group at the Lys 27 residue in derivative C is absent in the spectra of derivatives A and B. The comparison of pH-dependence of the chemical shifts for derivatives A, B and C allows us to unambiguously assign the signals of the CF_3CO groups at Leu 1, Lys 26 and Lys 15 residues in spectra of A and B (fig.1). The spectrum of derivative B at pH 2.5 differs from derivative A spectrum in that the signal at 1.56 ppm has twice the intensity of the rest of the signals. Hence it follows that the Lys 45 and Lys 47 residue signals are present in the derivative B spectra at 1.56 ppm, whereas for derivative A one of them is absent. Amino acid analysis of tryptic peptides of the reduced and carboxymethylated derivative A (isolated by the column chromatography on Sephadex G-25) has shown that the modification did not effect the amino group of the Lys 45 residue. This makes it possible to identify the structure of A and B derivatives (table 1) and to fully assign the signals for corresponding compounds with quenched spin label. From these data it follows that the substitution of the CF_3CO groups for a quenched spin label causes a 0.14 ppm downfield shift of the Lys 47 signal. A similar shift by 0.07 ppm was observed when the CF_3CO group at Lys 27 was substituted for the CH_3CO group [10], the result being explained by the spatial proximity of the Lys 47 and Lys 27 side chains. However, this finding does not afford sufficient grounds for a qualitative determination of the distance between the specified groups.

The distance from Lys 27–Lys 47 as well as to the

Table 1
Structure of derivatives of *Naja naja oxiana* neurotoxin II

Derivative	Substituent grafted and amino group of the residue					
	Leu 1	Lys 15	Lys 26	Lys 27	Lys 45	Lys 47
A	CF_3CO	CF_3CO	CF_3CO	SL	–	CF_3CO
B	CF_3CO	CF_3CO	CF_3CO	SL	CF_3CO	CF_3CO
C	CF_3CO	CF_3CO	CF_3CO	CF_3CO	CF_3CO	CF_3CO



Scheme 1

rest of the CF_3CO groups can be determined from the broadening of the ^{19}F signals due to the dipole–dipole interaction with the Lys 27 spin label. The spectra of derivatives A and B containing the spin label are shown in fig.2. The chemical instability of the nitroxyl radical at the Lys 27 residue in NT-II at pH < 4 does not allow one to obtain the spectra of derivatives A and B at pH 2.5, when the signals from the CF_3CO groups are not overlapped. To determine the linewidths of the overlapping signals from Lys 27 and Lys 47 residues in derivative A, as well as from Lys 26, Lys 45 and Lys 47 in derivative B, the spectra (fig.2) were simulated on the computer assuming the Lorentz line-shape up to the best fit with the experimental spectra. The calculated half-width of the signals are given in table 2. For derivatives A and B the half-widths of the Leu 1 and Lys 15 residue signals are 5 and 11 Hz, respectively. Fig.2 shows that the broad signal at 1.57 ppm in the derivative B spectrum is fairly well approximated by the sum of 3 signals with half-widths of 9.0, 13.6 and 30–35 Hz, while in the derivative A spectrum by the sum of the latter 2 signals. Since in derivative A the Lys 45 residue is not modified, the signal with a half-width of 9.0 Hz in the derivative B spectrum should be assigned to the Lys 45 residue. This assignment is supported by the fact that the subtraction of the derivative A spectrum from the derivative B spectrum produces the difference spectrum representing one signal at 1.57 ppm with a half-width of 9.2 Hz. It was shown earlier for derivative C that the nearest of all the lysine residues are Lys 27 and Lys 47 [10]. Due to such a proximity the substitution of CF_3CO group at the Lys 27 position for a spin label leads to a substantial change in the chemical shift of the CF_3CO group at the Lys 47 position, whereas the chemical shifts of the rest of the signals in derivatives A and B do not change. In the ^1H NMR spectra of the NT-II derivatives spin-labelled at the lysine residues, one can observe an extensive broadening of those very signals whose chemical shifts undergo the largest change with the introduction of the spin label [2]. Thus, on the basis of the above-indicated facts, we assigned the signal with a half-width of 30–35 Hz to the CF_3CO group at the Lys 47 residue, and the signal with a half-width of 13 Hz to the Lys 26 group.

The line broadening of ^{19}F signals in derivative A and B spectra (fig.2) is due to the magnetic dipole–dipole interaction of the spin label with fluorine nuclei. On the assumption of an isotropic movement of the radius vector linking the unpaired electron and ^{19}F nucleus, the broadening of the signal ($\Delta H_{1/2}$) is described by Blombergen's equation [11]:

$$\Delta H_{1/2} = \frac{C}{r^6}\left[4\tau_c + 3\tau_c/(1+\omega_I^2\tau_c^2) + 13\tau_c/(1+\omega_s^2\tau_c^2)\right]$$

$C = 3.7 \times 10^{15}$ Å^6/s^2 for the case of electron ^{19}F nucleus interaction; ω_I and ω_s, the Larmor frequencies for the nucleus and electron; r, the nucleus–

Table 2
Data on ^{19}F NMR for spin-labelled trifluoroacetylated NT-II, EPR for dispin-labelled NT-II derivatives, and X-ray diffraction analysis for erabutoxin b [7]

Signal assignment	^{19}F (ppm)[a]	Signal width at half height (Hz)				Distance to Lys 27 (Å)		
		A[b]	B[b]	A	B	^{19}F NMR[c]	EPR [6]	X-Ray [7]
Leu 1	2.31	5.0	5.0	5.0	5.0	≳18	–	19
Lys 15	1.87	4.5	4.5	11.0	11.0	13.5 ± 0.6	14.2	21
Lys 26	1.58	4.5	4.5	13.0	13.0	12.5 ± 0.5	15.3	14[d]
Lys 45	1.57	–	4.5	–	9.0	14.5 ± 0.7	14.5	11[d]
Lys 47	1.56	4.5	4.5	30–35	30–35	10.2 ± 0.5	13	8

[a] The chemical shifts were measured at pH 6.5 relative to trifluoroacetic acid as external standard
[b] The spin label is quenched with ascorbic acid
[c] The distance errors are given with allowance for determination error for half-width of the signals (±0.5 Hz) and correlation time of the spin label
[d] The distances are measured for the NT-II model constructed in accordance to the X-ray analysis of the erabutoxin b

electron distance; τ_c, the dipole–dipole interaction correlation time for which one can use the spin-label correlation time determined from EPR spectra [2]. The correlation time of the spin label at the Lys 27 residue in derivatives A and B is $(2.0 \pm 0.5) \times 10^{-9}$ s. The distances between the spin label at Lys 27 and the CF_3CO groups of NT-II, determined by Blombergen's equation are listed in table 2.

These values are compared with data obtained by other techniques. The distances measured for derivatives A and B in solution are in good agreement (allowance being given for measurement errors and possible deviation due to the size of the introduced labels) with the data derived by the EPR technique for solutions of dispin-labelled NT-II derivatives frozen at liquid nitrogen temperature (see table 2). The data obtained also show that due to the highly stable spatial structure of NT-II in solution, its derivatives, modified at 1 or 2 [2,6] or even the whole of the amino groups, are adequate models for studying the native NT-II conformation.

The homologous neurotoxins of postsynaptic action have on the whole a similar conformation in solution [2,6,8,10,12–14] which in turn possesses a considerable likeness to erabutoxin b structure in the crystal [7]. This is confirmed by the distances measured here. A spatial structural model was constructed according to the coordinates given for erabutoxin b [7], the orientation of the Lys 26 and Lys 45 side chains in NT-II being chosen the same as of His 26 and Thr 45 residues in erabutoxin b. Table 2 illustrates a fairly good agreement for most of the measured distances with the X-ray diffraction model. However, the distance between the labels at the Lys 27 and Lys 15 residues (13.5 ± 0.6 Å) turned out to be substantially smaller than the distance between the corresponding ϵ-amino groups in the erabutoxin b crystal (21 Å) [7]. It may be felt that this difference is due to the dynamic properties of the neurotoxin structures which manifest themselves, for instance, in the mobility of the side chains in solutions and in the local conformational differences between the homologous proteins [10]. Analysis of the spatial structural model of erabutoxin b constructed according to the coordinates from the X-ray diffraction analysis indicates that one can easily change the position of the Lys 15 side chain by directing it towards the Lys 27 residue in accordance with the distance measured in solution.

The data obtained gave also new information on the spatial structure of NT-II in solution. Thus, it has been established that the N-terminal amino group is located at a distance of >18 Å from the spin label at position 27. The distance between the spin label at the Lys 27 residue and the CF_3CO group at Lys 26 is consistent with the conclusion that these residues are located in the antiparallel β-structure [2,12]. The relatively short distance from the Lys 27 spin label to Lys 47 can be realized only in case of the spatial closeness of the two corresponding loops confined by the disulfide bridges, as was assumed earlier [10].

The determination of the mutual disposition of NT-II amino groups is important for understanding the molecular basis of interaction of the neurotoxins with their biological target, the nicotinic acetylcholine receptor of the postsynaptic membrane. Earlier, it was shown that the N-terminal amino group and ϵ-amino groups of Lys 15, Lys 26, Lys 27 and Lys 47 residues contact the receptor surface when it binds the respective NT-II derivatives [2,15]. This work demonstrates that the lysine amino groups are located relative to one another at a comparatively close distance. The side chains of Lys 27, Lys 47 and Lys 15 residues lie at one side of the neurotoxin molecule [7]; if we position the side chain of Lys 15 in accordance with distance measured in solution, we will observe that these 3 residues form a cluster of positive charges which can play an important part in binding the neurotoxin to the receptor. On the other hand, the remoteness of the Leu 1 and Lys 27 side chains, both of which interact with the receptor, testify to the sufficiently extended size of the binding site on the acetylcholine receptor.

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