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HYDROGEN EXCHANGE KINETICS AND DYNAMIC STRUCTURE OF ERABUTOXIN B FROM 1H NMR AND INFRARED SPECTROMETRY

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SUMMARY

As an approach to the conformational dynamics of erabutoxin b, the $^{1}H^{2}H$ exchange kinetics have been measured in $^{2}H_{2}O$ at 30°C, pH 4.75, by means of infrared spectrometry and high resolution ^{21}H NMR. With a solvent exposure coefficient higher than 10^{-3} for 90 % of its backbone amide hydrogens, erabutoxin b shows a relatively large solvent accessibility : this is consistent with an open and/or flexible molecule. 17 backbone hydrogens and 9 side chain hydrogens exchange slowly. These results compare fairly well with the number of potential hydrogen bonds recently reported from X-ray analysis.

INTRODUCTION

Erabutoxin b, a sea snake venom neurotoxin, is a single chain polypeptide of 62 amino acids which acts at the neuromuscular junction by blocking the acetylcholine receptor, specifically and with a high affinity constant.

Solution (1-5) and crystal (6-8) structure of this molecule reveals the presence of β sheets and β turns, implying the formation of hydrogen bonds which, together with the four disulfide bonds, ensure the stability of the peptide chain. However, in acidic solution (1,9) (Menez, A., Montenay-Garestier, T., Fromageot, P. and Hélène, C., to be published) or in the presence of organic solvents (1,2) the molecule can undergo local and/or global reversible conformational transitions which suggests the flexible nature of the chain. In the same way, NMR and circular dichroism data on short homologous neurotoxins indicate an appreciable flexibility in some regions of the molecule (1,2,10-12).

A well suited method to test the overall molecular motility of a polypeptide in solution, is to measure the ^{1}H-^{2}H exchange rates of its labile hydrogens, which reflects their solvent accessibility. In order to obtain further information on the conformational dynamics of erabutoxin b molecule (13,14), hydrogen exchange kinetics studies have been carried out by (i) infrared spectro-

Abbreviations : ^{1}H NMR : proton nuclear magnetic resonance ; IR : infrared spectroscopy ; ppm : parts per million.

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metry (IR) to measure the rate of exchange of the peptide hydrogens (ii) high resolution ^1H NMR to visualize the exchange of all labile hydrogens. Data are interpreted in terms of intramolecular hydrogen bonding and conformational dynamics.

MATERIALS AND METHODS

Purified erabutoxin b (MW 6870) from Laticauda semifasciata was extracted from dried venom glands kindly supplied by Professor N. Tamiya (Sendai,Japan). The same batch was used for IR and NMR exchange experiments. ^2H_2O (99.7 % enrichment) was provided by the "Commissariat à l'Energie Atomique". The ^1H-^2H exchange reaction was initiated by dissolution in ^2H_2O of lyophilized erabutoxin b at a concentration of 3.10^{-3}M(IR) or 7.10^{-3}M ^{2}(NMR). A check experiment has shown that, in this range, the exchange kinetics parameters are not dependent on concentration. The reported pH values for ^2H_2O solutions are direct pH-meter readings.

IR : the ^1H-^2H substitution in the peptide groups of erabutoxin b was monitored by infrared absorption (Perkin-Elmer 180 spectrophotometer with matched thermostated 0.1 mm pathlength CaF2 cells) following the previously described experimental procedure (15-17).

^1H NMR : the exchange was followed by continual scanning of the NH resonance region (10.2 to 5.7 ppm) with a 250 MHz ^1H NMR Cameca spectrometer (18) coupled with a Nicolet 1080 24 k computer. Most spectra were accumulated on a disk unit in the continuous wave mode. Some Fourier Transform spectra were recorded as a control of the overall stability of the protein during the exchange experiment.

Quantitative analyses were performed both on the Nicolet computer with the T2CAM program (19) and on a PDP 12 with the SPECTRAL System (20,21).

RESULTS

IR data.

Two IR spectra of erabutoxin b in ^2H_2O are shown on Fig 1. The 1644-1643 cm^{-1} maximum of the asymmetric amide I peak (due to the C=O stretching vibration of the peptide bond) and the shoulder at 1684-1675 cm^{-1} indicate the coexistence of aperiodic and β antiparallel structures (22,23). The slight shift with time of the Amide I shoulder may correspond to the progressive deuteration of the cross β structure (24). The poor resolution of the tyrosine band detected at 1510 cm^{-1} and its shift down from its normal position at 1515 cm^{-1} suggest that the single Tyr^{25} is buried inside the molecule.

The exchange rate of peptide protons was followed at 1541 cm^{-1} on the maximum of the Amide II band (due to the coupled $-N^1H$ bending and -CN stretching vibrations). The percentage X of unexchanged peptide hydrogens is estimated as $X = 100\ (A_{\text{amide II}})/(w\ A_{\text{amide I}})$, where $A_{\text{amide II}}$ and $A_{\text{amide I}}$ are respectively the absorbances at 1541 cm^{-1} and 1644 cm^{-1} : w = 0.45 for undeute-

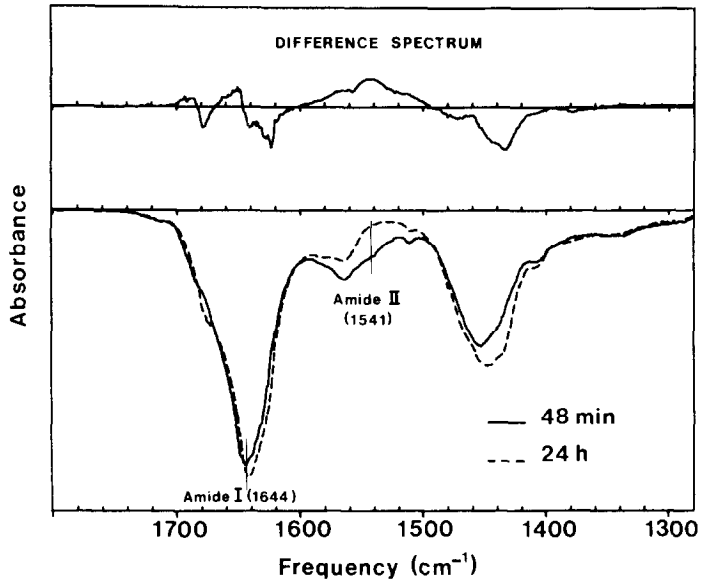

Fig 1 IR spectra of erabutoxin b (3.10^{-3} M) in ^2H_2O at 30°C, pH 4.75. Upper graph is the computed difference spectrum.

rated proteins (15,16,25). Complete deuteration of the exchangeable hydrogens was achieved by heating the solution at 55°C for 90 minutes.

NMR Data

The 250 MHz NMR spectrum of erabutoxin b is shown on Fig 2 A,B. The spectrum is similar to that published by Inagaki et al. (9). In addition, it contains numerous resonances corresponding to labile hydrogens : their wide distribution (10.2-5.9 ppm) is comparable to that observed on homologous toxins (12) or on highly structured proteins such as BPTI (27), and reflects the complex three-dimensional structure of erabutoxin b.

The quantitative analysis of the 10.2-5.9 ppm region includes several previously described steps (18) : spectra correlations, phase and base line corrections, computation of difference spectra (Fig. 2 C). Both corrected spectra and difference spectra are integrated in order to get the number of unexchanged protons. The results obtained for the total 10.2-5.9 ppm and for the limited 10.2-7.9 ppm areas will be considered in the following sections.

Exchange kinetics

1 - Comparison of IR and NMR data. The IR exchange kinetics of erabutoxin b in ^2H_2O at 30°C is shown on Fig. 3. Two kinds of plots represent the NMR data, computed on the total and on the limited exchange areas. This graph

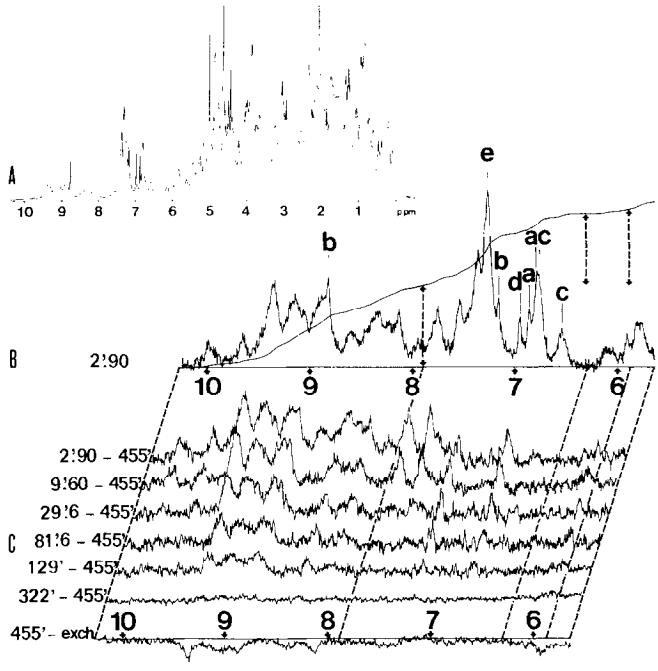

Fig 2A 10-1 ppm 250 MHz ^1H NMR spectrum of erabutoxin b ($7.10^{-3}M$) at 30°C, pH 4.82, 3 hours after dissolution in ^2H_2O. Chemical shifts are given downfield from DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt). Fig 2B 10.2-5.7 ppm NMR region, recorded 2.9 min after the exchange initiation. Assignments are as follows (ref. 9) : His^7 C-2 and C-4 (a) ; His^{26} C-2 and C-4 (b) ; Tyr^{25} C-2,6 and C-3,5 (c) ; Trp^{29} C-2 (d) ; Phe^4 and Phe^{32} (e). The 10.2-7.9 ppm integral corresponds to His^{26} C-2H + 31.4 labile protons ; the 7.9-6.3 ppm integral to 22 aromatic + 5.9 labile protons ; the 6.3-5.9 ppm integral to 1.1. labile + several Cα hydrogens abnormally shifted downfield (ref. 26). Fig 2C 10.2-5.7 ppm NMR difference spectra.

shows a good agreement of IR and 10.2-7.9 ppm NMR data : it corresponds to the amide hydrogens exchange. The first part of the graph, enlarged on the inset, points out additional exchanging hydrogens detected only with NMR below 7.9 ppm : therefore, they are assigned to labile protons of amino acid side chains.

2 - Kinetics analysis. Table I presents a computer analysis of the exchange kinetics. For the amide hydrogen kinetics, a valuable convergence is obtained with a three-class decomposition : 27.7 peptide hydrogens are readily solvent accessible : the slow exchange of 16.8 other protons indicates their shielding from the solvent. The number of detected side chain hydrogens may be tentatively approximated to 9 ± 2 from the total kinetics, but we must keep in mind the quite low precision of this result obtained from the first ten NMR experimental points. The erabutoxin b 7.9-6.3 ppm exchange region is well comparable with the NMR chemical shifts (7.6-6.6 ppm) observed for side chain

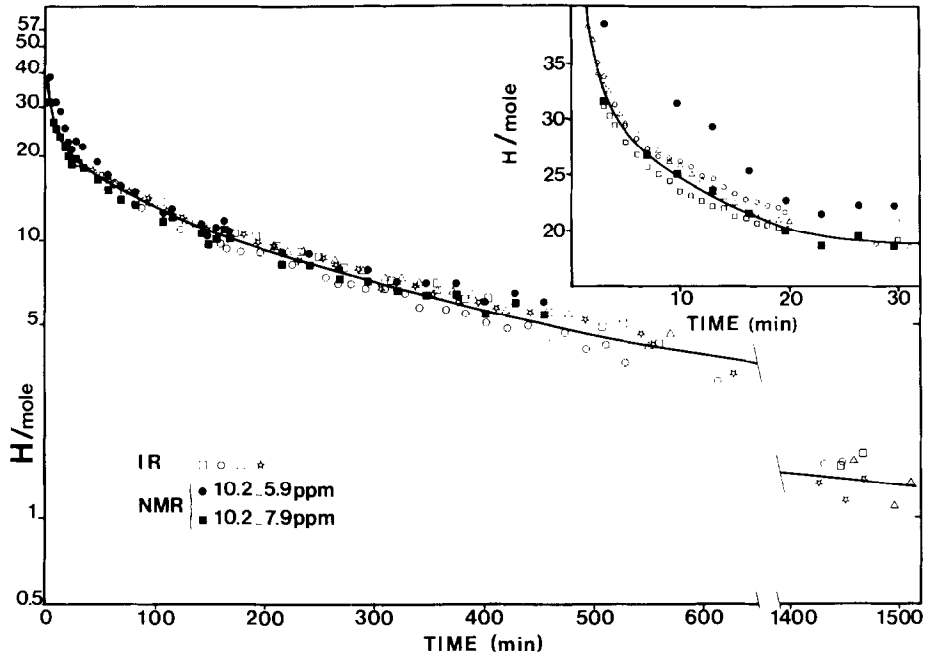

Fig 3 Semi-logarithmic plots of the $^1H^2H$ exchange kinetics of erabutoxin b from IR and NMR data. Inset : linear scale showing the discrepancy between the IR and the 10.2-5.9 ppm NMR data, over the 30 first minutes of exchange.

free amino groups of amino acids like Gln, Asn, Lys and Arg (28) : the NH protons of free Trp or His rings (usually 10-12 ppm) cannot be concerned here.

3 - Erabutoxin b solvent accessibility. The experimental data are interpreted with the "breathing model" (29,30) according to which the i^{th} peptide hydrogen exchanges with a rate constant k_i equal to $\rho_i k_o$: ρ_i is the solvent exposure

TABLE I

EXCHANGEABLE H OF ERABUTOXIN B	PEPTIDE H IR AND NMR (10.2-7.9 ppm)		SIDE CHAIN H NMR (7.9-5.9 ppm)	
	H	$t_{1/2}$	H	$t_{1/2}$
Class I	27.7	1.08	3	9
Class II	12.5	16.4	4.6	19
Class III	16.8	254	1.4	142
Σ H	57	-	9	-

Computation of hydrogen classes from IR and NMR data : H is the hydrogen number, $t_{1/2}$ is the half life (min). For comparison, the half life of a freely solvent exposed peptide hydrogen under identical experimental conditions is calculated equal to 0.115 min.

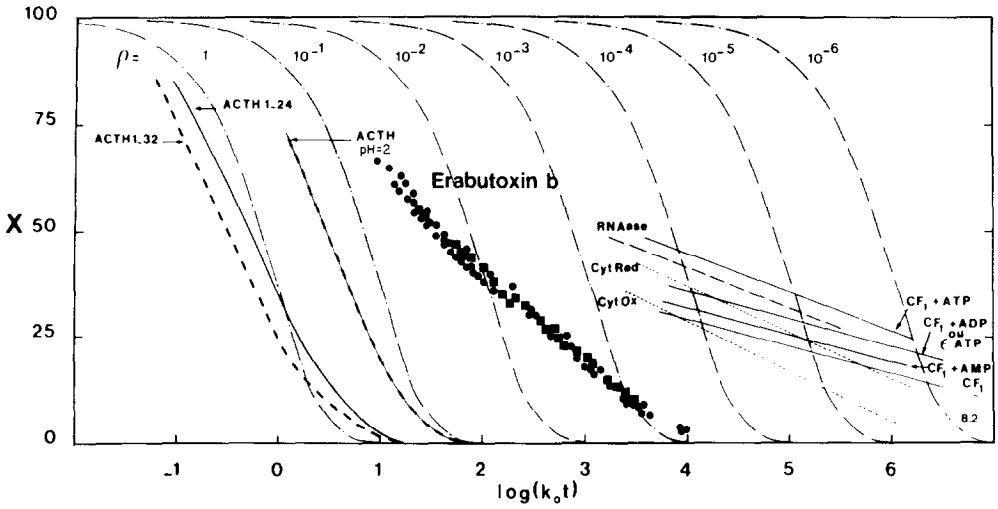

Fig 4 Relative accessibility of erabutoxin b from IR (●) and 10.2-7.9 ppm NMR data (■) compared to other polypeptides investigated in the Laboratory. X is the percentage of unexchanged peptide hydrogens at time t, k_0 is the first order rate constant of a solvent exposed peptide proton ; the S shape curves are calculated for hypothetical polypeptides with the ρ values indicated. Cyt : horse heart cytochrome c (see ref. 16) ; CF1 : coupling factor 1 (see ref. 32).

coefficient and k_0 is the first order rate constant of a freely solvent exposed peptide hydrogen (29,31)

$$k_0 = (10^{-pH} + 10^{pH-6})\ 10^{0.05\ (\theta-25)}\ s^{-1},$$

where θ is the temperature in degree centigrade. The distribution of the ρ values for a protein is representative of its conformational dynamics. Plots of X (percentage of unexchanged peptide hydrogens) versus $\log(k_0 t)$ allow the direct comparison of polypeptides studied under different experimental conditions.

Both IR and 10.2-7.9 ppm NMR erabutoxin b data are displayed on Fig 4, together with the exchange curves acquired in the Laboratory for several other polypeptides (16,25,32). It is seen that :

- the IR and NMR experimental points are superimposed, with an even better agreement than on the direct exchange kinetics, since slight differences in pH and/or temperature are taken into account.

- the solvent exposure coefficient of 90 % of the erabutoxin b amide hydrogens is higher than 10^{-3}, while in globular proteins, ρ values vary from 1, for a freely solvent accessible hydrogen, to 10^{-6}_10^{-8} for buried ones.

DISCUSSION

The solvent accessibility of erabutoxin b can be considered as relatively large, and consistent with an "open" and/or "flexible" conformation of the molecule.

Circular dichroism and NMR titrations have evidenced a reversible acid denaturation process of erabutoxin b at low pH, with a mid-transition point at pH 2.85-2.95 (1,9). At pH 4.75 the Henderson-Hasselbalch titration model predicts at most 1.4 % of the "reversibly denatured" form. This may explain part of the relatively high accessibility of erabutoxin b.

X-ray analysis of erabutoxin b crystals has shown that the molecule is flat, only "one peptide chain thick", and "unusually open" (7,8,33). Among the 57 peptide hydrogens of erabutoxin b, the exchange kinetics fits indicate 16.8 slowly exchanging ones, likely implicated in hydrogen bonds. This is in agreement with the presence of β sheets and β turns in the molecule, and compares fairly well with the 18 potential hydrogen bonds reported by Kimball et al. (8).

Confrontation of IR and NMR results reveals the slow exchange rate of roughly 9 side chain hydrogens. Probably involved in hydrogen bonds, they may act as an additional stability factor of the whole erabutoxin b molecular conformation. Erabutoxin b has one terminal function and 17 amino acid side chains with exchangeable amino groups detectable by NMR :

$$(NH_3)^+ - Arg^1...Asn^5Gln^6His^7...Gln^{10}...Gln^{12}...Lys^{15}...His^{26}Lys^{27}$$

$$Gln^{28}Trp^{29}...Arg^{33}...Arg^{39}...Lys^{47}...Lys^{51}...Asn^{61}Asn^{62}-(COO)^-$$

Four of them, His^{26}, Lys^{27}, $Trp^{29}$and Lys^{47}, are chemically accessible (34-36). It is striking that 8 of the remaining amino functions belong to the first 1-17 β sheet loop. Such a high density may result from a structural requirement. Indeed, Low et al. (33) reported the presence of five hydrogen bonds involving the amino terminal group of Arg^1 and the side chain nitrogens of Gln^6, Gln^{10}, Lys^{51} and Asn^{62}. Further assignments on the NMR spectrum of the slowly exchanging peptide and side chain hydrogens to specific amino acids of erabutoxin b would give an inestimable answer.

In conclusion, the exchange kinetics of erabutoxin b in aqueous solution, at 30°C pH=4.8, indicates a number of slow protons comparable with that of potential hydrogen bonds reported from X-ray crystallography. Such an agreement has already been found in some globular proteins (17 and ref. therein) but should not be necessarily expected for small peptides, possibly involved in a dynamic equilibrium between several conformations (18): a molecular polymorphism would be undetectable by X-ray crystallography.

Work is in progress (Nabedryk-Viala,E. et al., in preparation) to investigate the effect of environmental conditions on the accessibilities of erabutoxin b

and homologous neurotoxins, which have similar predicted structures (erabutoxin b is considered as the prototype (33) postsynaptic snake neurotoxin) but various conformational stabilities. Distinct solvent accessibilities could be related to different degrees of flexibility of the polypeptide chain, or to a polymorphism of the molecule in solution.

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