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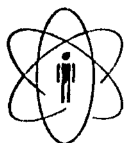
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STRUCTURE AND CONFORMATIONAL CHANGES OF CROTAMINE

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ABSTRACT

The secondary structure of crotamine is predicted by the simultaneous use of the statistical method of Chou and Fasman and the method of hydrofobicity profiles resulting in 19% of helix- and 45% of β-structures, in agreement both with previous results obtained from ORD and laser spectroscopy. It allows for the three disulphide bridges and is consistent with a long ellipsoidal shape for the molecule. From SAXS curves measured at several concentrations the radius of gyration, volume and surface of the molecule are obtained. The knowledge of these parameters enables one to conclude that the overall shape of the crotamine molecule can be described by a prolate (long) ellipsoide of semi-axis a,a and νa with $\nu = 2.3$, in agreement with the above prediction. The radius of gyration of crotamine is also determined as a function of pH. An important change found for pH values from 9.5 to 12.5 is atributed to a dominant effect of molecular aggregation.

Key words: Crotamine; SAXS; Macromolecules.

1 INTRODUCTION

Crotamine is a neurotoxin isolated from the brazilian snake crotalus durissus terrificus. It is a polypeptide toxin, strongly basic ($pH_i = 10.3$), with molecular weight of 4870 daltons. It is composed of 42 residues of 15 common amino acids including six half-cystines. It is very high lysine (9 residues) and low arginine (2 residues). The N-terminus is tyrosine and the C-terminus glycine. It has been sequenced by J.C.Laure (1) who also localized the 3 cysteine bridges (2).

Recent laser Raman spectroscopy (3) studies related to the determination of the secondary structure of crotamine give indication that crotamine may contain β-sheet and α-helix structure with slight predominance of the first. By spectropolarimetric titration (4) it was possible to detect conformational changes in crotamine at several pH and temperature values. Three pH-isomers were identified: a neutral isomer (I) found at pH between 4 and 8.5 corresponding to the native conformation, an acid isomer (II) occurring at pH values lower than 2.0 and a basic isomer (III) above pH 9.5.

To further elucidate these problems the authors decided to investigate the secondary structure by the statistical method of Chou and Fasman (5-9) and by the method of hydrofobicity profiles (10). The structure of crotamine in aqueous solution and some structure changes were also studied by using the small angle X-ray technique under dif-

ferent concentration and pH conditions, at room temperature.

2 PREDICTION OF THE SECONDARY STRUCTURE OF CROTAMINE

2.1 Methods of prediction

Denaturation-renaturation experiments on proteins have provided experimental evidence that nature and sequence of aminoacids are the main factors responsible for the very special folding of the polypeptide chain that give rise to the secondary and tertiary structures of a protein. A whole series of methods that attempt to predict the secondary structure of a protein on the basis of the information contained in the primary structure have been devised. Two of them, that have proved to give a reliability of prediction of the order of 80%, were used to predict the secondary structure of crotamine, these were the Chou and Fasman's method (5-9) and the method of hydrophobicity profiles (10).

Chou and Fasman's technique to predict secondary structure consists of a series of rules based on the assignment of conformational parameters to each one of the 20 natural aminoacids (1-4). These conformational parameters P_α, P_β and P_t represent the probability that each aminoacid in a sequence would be participating in a α-helix, a β-structure or a β-turn respectively. They are empirical probabilities since they represent the normalized occurrence of each aminoacid in a certain type of structure, as obtained from 29 proteins whose

tertiary structure are fully known from X-ray diffraction methods. A probability average greater than 1.05 obtained for a group of aminoacids taken in sequence (6 for a helix, 5 for a β-strand and 4 for a β-turn) is an indication that a certain type of structure is likely to occur in that region. The original method was modified by Dufton and Hider (9) to obtain a probability product instead of a probability average for the group of aminoacids in order to improve the sensibility of the method in the vicinity of 1.0. Also, in the case of β-turns, each aminoacid shows preferences for each of the positions in the tetrapeptide, which can give rise to four different conformational parameters. Proline, for example, has a conformational parameter of 1.36 if located in the first position of the turn, 3.42 for the second, 0.59 for the third and 0.77 for the fourth positions respectively (3).

The second method employed is that of the hydrophobicity profiles (6). This method postulates that the folding of the polypeptide chain will occur in such a way to allow the location of the hydrophilic aminoacids in the protein surface and to bury the hydrophobic aminoacids in the interior of the molecule. The "bulk hydrophobic character" for each one of the 20 natural aminoacids, as defined by Ponnuswamy et al. (12), is employed to draw the hydrophobicity profile of the protein. Four typical hydrophobicity profiles illustrated in Fig. 1, are defined for an exposed α-helix, an exposed and a buried β-strand, and a β-turn. The structure prediction by this method consists simply in the identification of these basical patterns in the hydrophobicity profile of the protein.

2.2 Results

The Chou and Fasman's method gives low probabilities for helical, and β-structures, as compared to the high probabilities found for the β-turns. Helical regions are likely to occur in aminoacid sequences 2-7 (1.05), 13-20 (1.06), 24-29(1.06), 27-32(1.06) β-strands are possible in the sequence 1-5 (1.07), 16-20(1.08) and 30-38(1.1).

Probabilities greater than 1.08 for β-turn structure are found practically for the whole sequence, with the exception of the zones 13-16, 14-20 and 25-28, when only one average P_t coefficient for each aminoacid was used. When the program was run with four conformational parameters for each aminoacid residue, the following zones were selected for β-turns: 5-9(1.40), 8-11(1.20), 12-16(1.35), 19-27(1.42). The hydrophobicity profile for crotamine is given in the Fig. 2. Table 1 lists the two independent and the combined prediction for the secondary structure of crotamine.

An "arrow-and-cyllinder" representation of the predicted secondary structure of crotamine is given on Fig. 3. It can be seen that the structure proposed is rather compact and it allows the near contacts for the disulphide bridges reported (12) between Cys 4-Cys 37, Cys 11-Cys 36, and Cys 18-Cys 30. The predicted structure has 19% of helix, and 45% of β-structure, which agrees both with the results obtained from ORD and from laser Raman spectroscopy (9). The proposed secondary structure allows Tyr 1 to be shielded and Trp 32 and Trp 34 to be exposed to the solvent. The structure is consistent with

a rather elongated ellipsoidal molecule.

3 SMALL-ANGLE X-RAY SCATTERING

3.1 Materials and methods

The pure protein used in the experiments was obtained from the snake venon purified by the same method as described previously by one of the authors (1).

The SAXS intensity was recorded by means of two electronics systems. The several curves at constant pH and varying concentrations were obtained by using a Tennelec position sensitive detector and the scattering curves for constant concentration and different pH by means of a small angle goniometer, scintillation detector and automatic step scanning device (13). CuKα filtered radiation and slit collimation were used in both kinds of measurements.

The protein solutions were placed in Lyndemann glass capillary tubes (1.0 mm diameter) and kept at room temperature. The concentration of the solutions were of 10% by weight of protein and the varying pH adjusted by the addition of HCl or NaHO to the desired values.

The scattering due to the solvent and capillar was subtracted from the total experimental scattering intensity. The resulting SAXS curves were normalised to equivalent sample absorption and thickness.

The radius of gyration R of the proteine was

obtained by using the Guinier law, which holds for very dilute solutions of macromolecules (11). To correct for the influence of finite dilution, several SAXS curves were obtained from solutions having different concentrations and constant pH. The radius of gyration was obtained by extrapolating the R values to zero concentration (14).

The equations that allow one to determine the radius of gyration R, volume V and external surface S of the macromolecules are (11):

$$I(s) = I(0)\, e^{-\frac{4}{3}\pi R^2 s^2} \quad (1)$$

$$V = K_1 \frac{I(0)}{Q} R \quad (2)$$

and

$$S = K_2 \frac{P}{Q} \quad (3)$$

In equation 1,2 and 3, $I(s)$ is the SAXS intensity as a function of the modulus of the scattering vector $\vec{s}$, $I(0)$ is the extrapolated intensity to $s = 0$, Q is the integral of the scattering intensity in reciprocal space, $P = \lim_{s \to \infty} |s^3\, I(s)|$ at high s (Porod invariant) and K_1 and K_2 are numerical constants equal to $1/\sqrt{3}\pi$ and 4π respectively. Equation 1 holds for pinhole collimation and can also be used as an approximation for beams with linear cross-section. Equation 2 applies for beams with "infinite" linear cross-section (11).

The parameter V was determined as a function of concentration by using equation 2. The extrapolated value of V to zero concentration gave us a measure of the molecular volume.

The Porod invariant (equation 3) was calculated by averaging (and not by extrapolating) the P values corresponding to the several concentrations because of the negligible concentration effect on the high angle domain of the scattering curve.

3.2 Experimental results

The slopes of the SAXS plots in log I versus s^2 scale (Guinier plots) lead to different apparent molecular radii of gyration for each protein concentration. The experimental values of R, which are plotted in Fig. 4, show a linear dependence on molecular concentration.

By extrapolating the different structural parameters to c = 0, the radius of gyration of the crotamine molecule was found to be R = 13.5 Å, its volume V = 13,200 $Å^3$ and its surface S = 1,040 $Å^2$. The next step was to establish a molecule shape compatible with these three structural parameters.

From the radius of gyration R, which was obtained from equation 1 and assuming a spherical shaped molecule, the molecular volume $V = \frac{4}{3}\pi(\frac{5}{3})^{3/2} R^3$ was found to be equal to 22,000 $Å^3$. This volume is much higher than the obtained by means of equation 2 (V = 13,200 $Å^3$). Therefore the experimental R and V values are not compatible with a spherical shaped molecule.

The next assumed molecular shape was that of an ellipsoide of revolution having semi-axis a, a and c. Fig. 5 shows the volume V of ellipsoides of R = 13.5 Å as a function of the parameter $\nu = \frac{c}{a}$. The horizontal line represents the

volume V obtained from equation 2. Two ellipsoides have R and ν which are consistent with the volume determined by equation 2: an oblate (flat) with $\nu = 0.32$ and a prolate (long) with $\nu = 2.3$. Their surface area are 3310 $Å^2$ and 1210 $Å^2$ respectivelly. The surface area of the prolate ellipsoide agree better with the value $S = 1040$ $Å^2$ deduced from equation 3. Therefore, these results suggest that crotamine is a prolate (long) ellipsoide shaped molecule, with a semi axes quotient ν approximately equal to 2.3.

The SAXS curves I(s) for various pH are plotted in Fig. 6. The various scattering curves in Fig. 6 can be approximated by two straight lines. This feature is expected from scattering objects constituted by two kinds of molecules with different radii of gyration R_1 and R_2. The different R values obtained for the solutions with different pH are given in Table 2. These radii of gyration (apparent radius of gyration) are not corrected from effects of finite concentration. Nevertheless, they can help us to detect conformational changes and/or aggregation processes related to the crotamine molecules in different pH conditions.

The R_1 values are represented as a function of the pH in Fig. 7. Assuming that R_1 is related to monomers with constant volume its variation means that the ellipsoidal shape of the monomers approaches a more spherical one, when the pH increases from 2 to approximately 9. The minimum of R_1 corresponds to a shape which is the closest to the spherical one.

An important change of R_1 is observed for pH varying from 9.5 to 12.5. The sudden increase of the molecular radius of gyration may be due to a dominant effect of aggregation which

leads to a system without monomers. The SAXS results in pH = 12.5 are consistent with a mixture of dimers and more complex molecular aggregates.

The second radius of gyration, deduced from the SAXS results (Table 2) must be considered as a rather crude approximation because of the narrow domain of validity of the Guinier law in the very small angle portion of the scattering curves (Fig. 6). Nevertheless the SAXS results bring clear evidence of the heterogeneity of the macromolecular solution. The R_2 values corresponding to pH ranging from 2 to 9.5, are consistent with the presence of dimers and/or trimers composed by molecular units with apparent radius of gyration of 10 Å.

4 DISCUSSION

The secondary structure of crotamine predicted by the combination of the statistical Chou and Fasman's method and the method of hidrofobicity profiles and the SAXS results are in agreement: both are consistent with an elongated ellipsoidal shape of the molecule at pH = 4.5.

The SAXS study showed the coexistence of isolated molecules and aggregates in the different solutions at various pH. At low pH(pH < 9) slight conformational variation in the ellipsoidal molecule occurs, leading to a more spheroidal shape for increasing pH. At higher pH(pH = 12.5), the high R suggests a dominant aggregation process.

A more detailed study of the conformational

changes with pH may be done by measuring the SAXS curves for a range of concentrations to obtain the extrapolated radius of gyration and correcting the experimental intensity from collimation effects. This should give more accurate informations on the nature of the aggregates and, consequently, more quantitative details of the molecular conformational variations.

ACKNOWLEDGEMENTS

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FIGURE E CAPTIONS

Fig. 1: The four basic hydrophobicity profiles.

Fig. 2: The hydrophobicity profile of crotamine.

Fig. 3: Predicted secondary structure of crotamine.

Fig. 4: Extrapolation to obtain the molecular radius of gyration R and the volume V.

Fig. 5: Volume as a function of the ν parameter for ellipsoides of revolution.

Fig. 6: SAXS for different pH and constant concentration ($c = 10\%$). The curves were vertically displaced for clarity.

Fig. 7: Radius of gyration of monomers as a function of pH.

TABLE CAPTIONS

Table 1: Prediction of the secondary structure of crotamine.

Table 2: Apparent radii of gyration of the molecules and aggregates at different pH (molecular concentration $c = 0.10$).

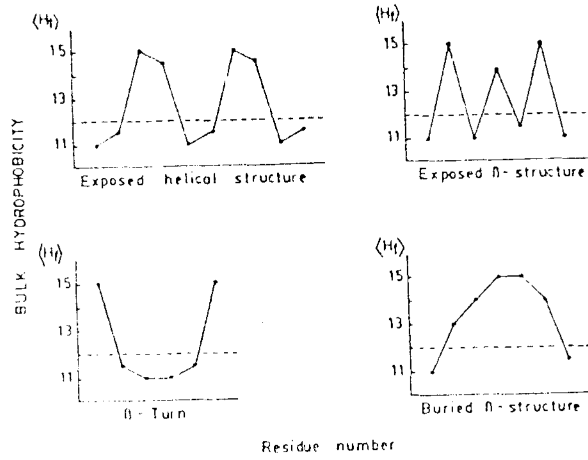

Fig. 1

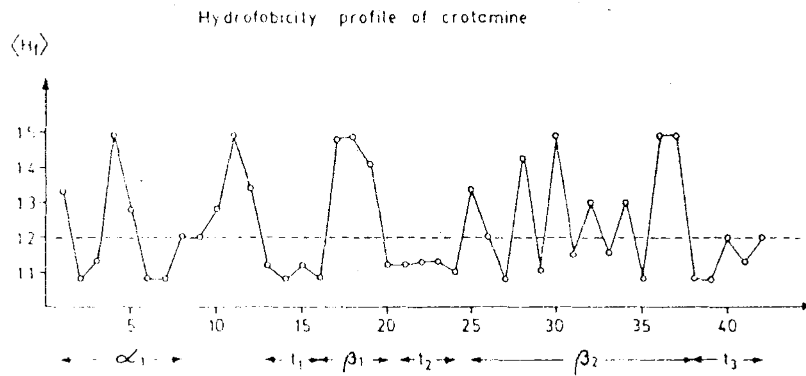

Fig. 2

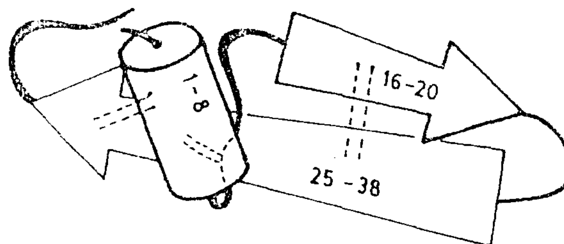

Fig. 3

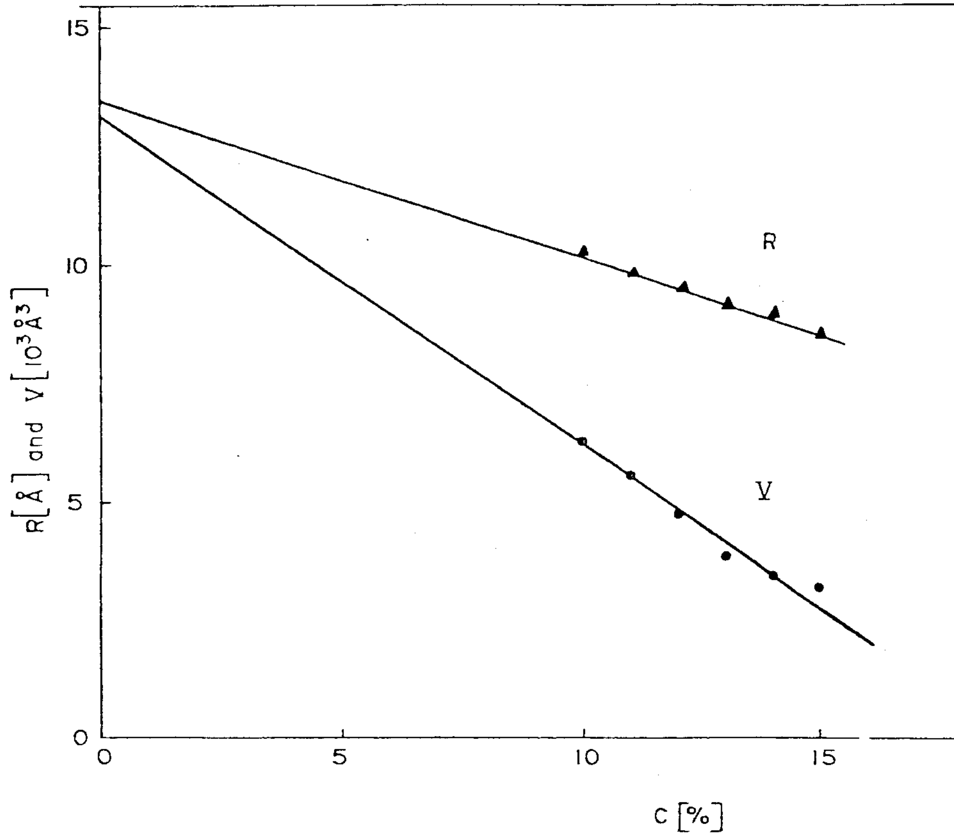

Fig. 4

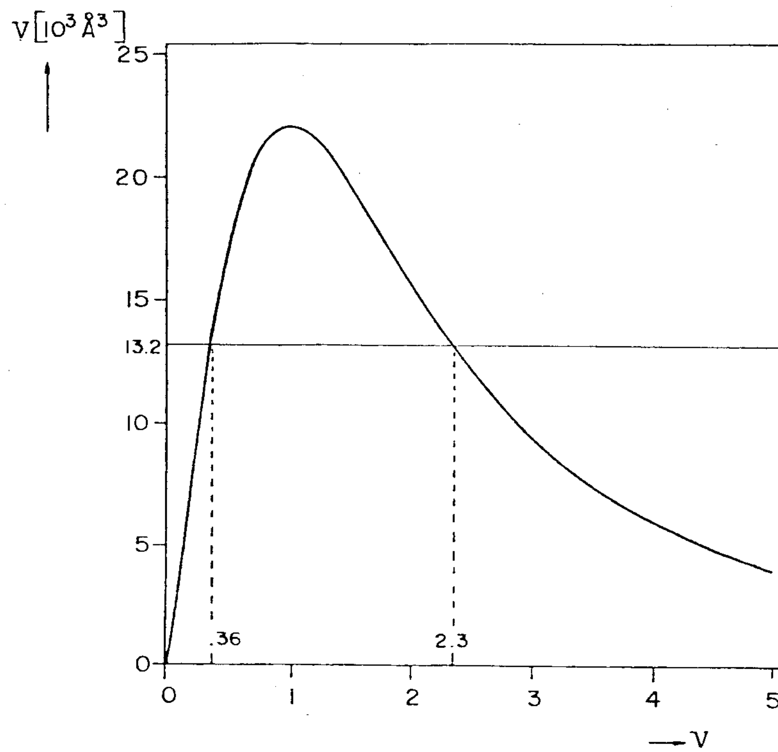

Fig. 5

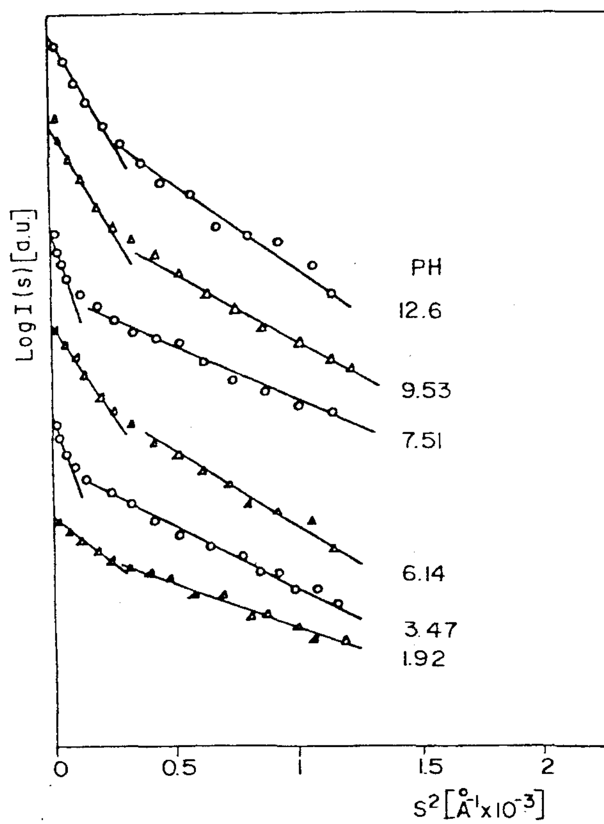

Fig. 6

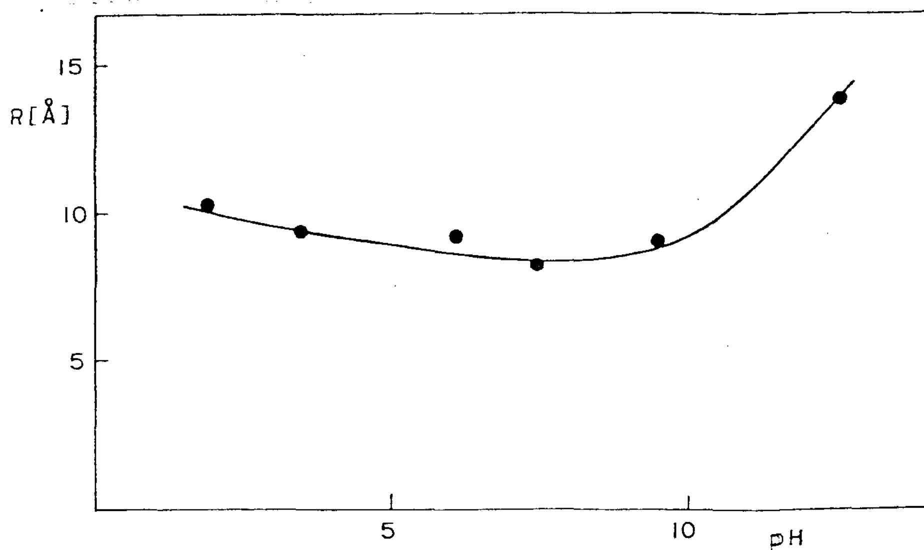

Fig. 7

TABLE 1

Chou and Fasman method		Hydrophobicity profile method		Combined prediction	
AA sequence	Structure	AA sequence	Structure	AA sequence	Structure
2-7	helix	1-8	helix	1-8	helix
8-11	β-turn	8-13	β-strand	9-12	R.C.
12-16	β-turn	13-16	β-turn	13-16	β-turn
16-20	β-strand	16-20	β-strand	16-20	β-strand
20-24	β-turn	21-24	β-turn	21-24	β-turn
25-29	R.C.	24-38	β-strand	25-	
30-38	β-strand			-38	β-strand
37-40	β- turn	38-41	β-turn	37-40	β-turn
41-42	R.C	42	R.C.	41-42	R.C.

TABLE 2

pH	R_1 |Å|	R_2 |Å|
1.92	10.3	15
3.47	9.3	19
6.14	9.2	14
7.51	8.1	19
9.53	9.0	15
12.6	13.9	23