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# The secondary structure of the insect defensin A depends on its environment. A circular dichroism study

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**Summary** — Defensin A is an inducible antibacterial protein isolated from the larvae of *Phormia terranova*. The conformation of defensin A has been previously determined by two-dimensional <sup>1</sup>H-NMR for concentrations in the range of 4–8 mM in water (Bonmatin JM *et al* (1992) *J Biomol NMR* 2, 235–256). CD spectroscopic data of defensin A at lower concentrations (10<sup>-5</sup> to 10<sup>-3</sup> M) are reported herein. The ellipticity in the 200–240 nm wavelength range for various solvents varies as follows: acetonitrile < water < methanol < HFIP. The magnitude of [ $\Theta_{222}$ ] is strongly dependent on defensin concentration in a buffer solution, suggesting an aggregation process. The helical content of defensin A is maximum at a pH value range (7.5–8) for which the optimum antibacterial activity was observed (Cociancich S *et al* (1993) *J Biol Chem* 268, 19239–19245).

**defensin A / insect defensin / antibacterial protein / secondary structure / oligomerisation / circular dichroism**

## Introduction

Insect defensins are inducible antibacterial proteins produced in response to bacterial injuries and secreted in the insect hemolymph [1]. Defensin A has been isolated and characterized from the larvae of the fleshfly *Phormia terranova* [2]. It is a small (40 residues) cationic protein, representative of a large family of insect defense proteins. Its conformation in water has already been determined by using 2D <sup>1</sup>H-NMR associated with modeling techniques [3, 4]. Defensin A is organized in three distinct regions: a C-terminal antiparallel  $\beta$ -sheet linked to an amphipathic  $\alpha$ -helix via two S-S bridges and a N-terminal loop linked to the  $\beta$ -sheet by the third S-S bridge (fig 1). Defensin A behaves as an amphipathic protein able to spread as a monolayer at the air/water interface, and interacts with membrane lipids [5] by forming voltage dependent channels [6].

Here we report circular dichroism (CD) data showing that the secondary structure of defensin A depends on the solvent nature and on the protein concentration, pH and presence of salts in aqueous solutions.

## Materials and methods

Recombinant insect defensin A was prepared by Transgène (Strasbourg, France). Pure water was obtained from a Millipore

(Milli Q) apparatus. Methanol and acetonitrile were purchased from BDH and hexafluoroisopropanol (HFIP) from Merck.

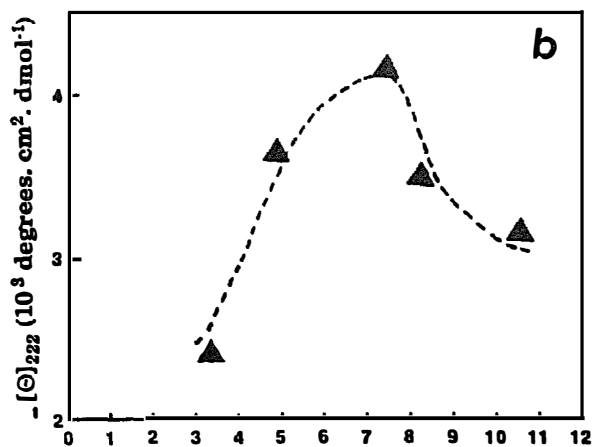
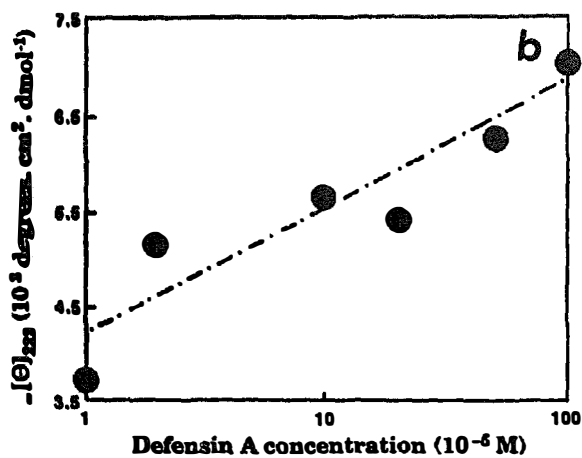
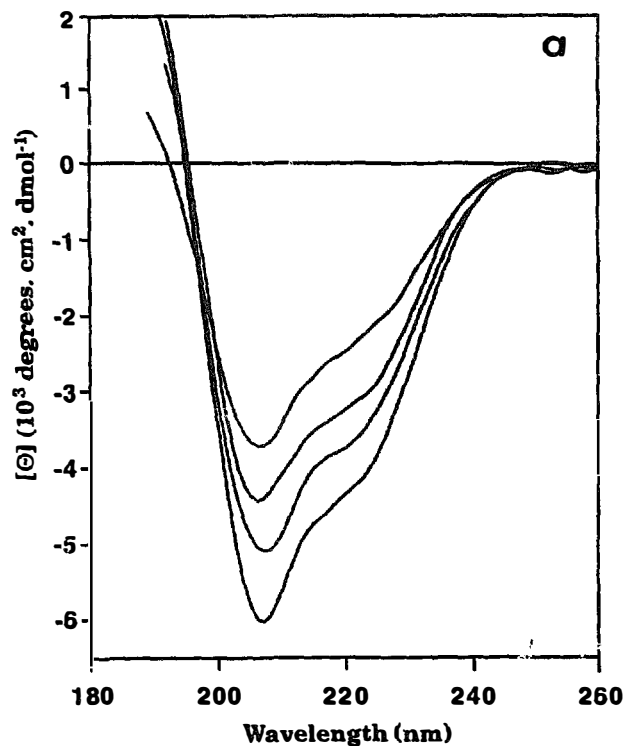
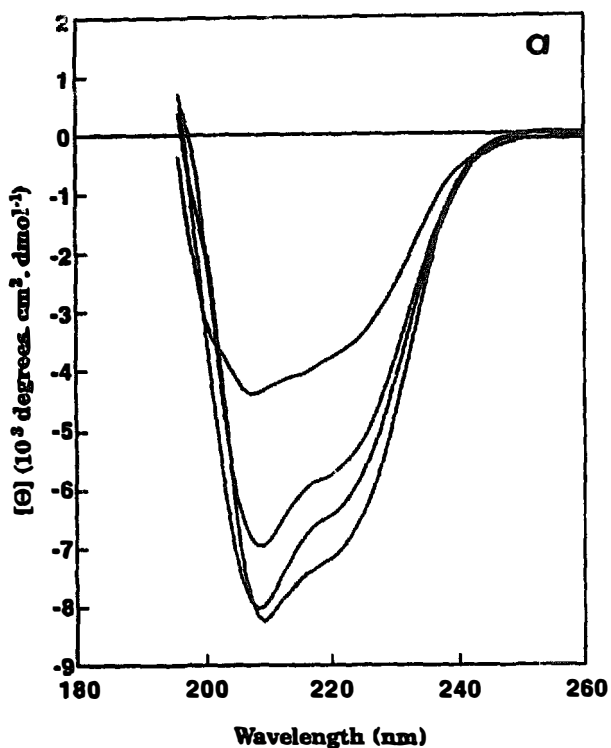
Concentrations of defensin A were calculated from the absorbance at 275 nm using  $\epsilon = 1400 \text{ M}^{-1} \text{ cm}^{-1}$  for the single Tyr29 chromophore. CD spectra were recorded at room temperature on a Jobin-Yvon IV autodichrograph using 0.1 or 0.01 cm path length in order to measure absorbance values less than 1 on the whole range of wavelengths. Optical activities were reported as ellipticity *per* amino acid residue:  $\Theta_{\text{res}}$  (unit:  $\text{deg cm}^2 \text{ dmol}^{-1}$ ). The  $\alpha$ -helical content was roughly estimated from CD data according to the formula: %  $\alpha$ -helix =  $\Theta_{222}/3298 \times (-10)$  given by Zhong and Johnson on the basis of a 26-protein data set [7].

## Results

### Nature of the solvent

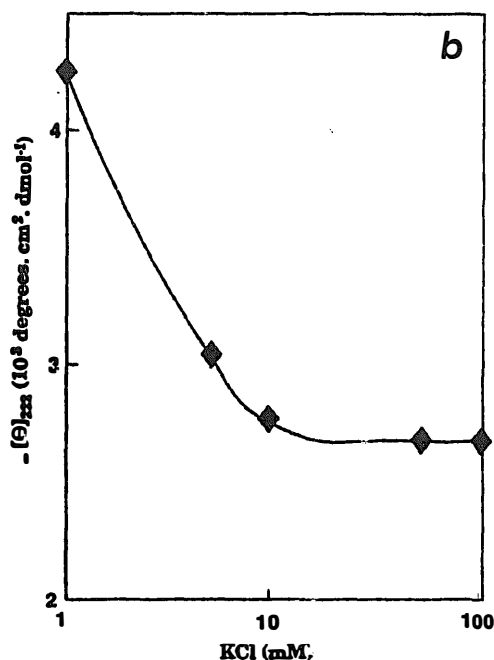
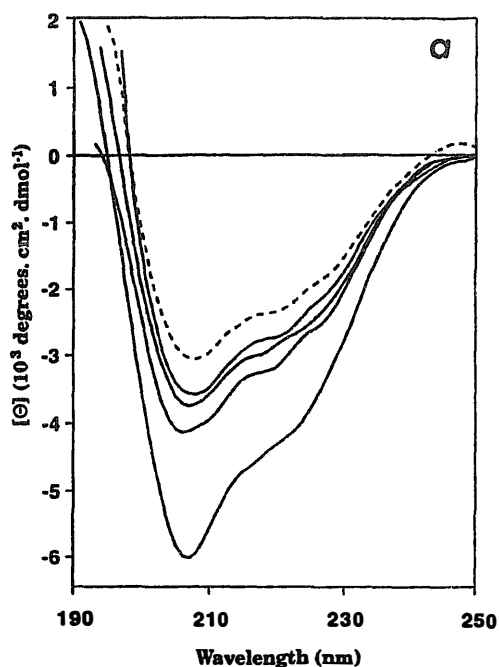
CD spectra of defensin A in various solvents are presented in figure 2. In acetonitrile the spectrum was characterized by a weak positive band near 200 nm and a weak negative band centered at 228 nm. The spectrum obtained in water presented a negative band at 207 nm and a shoulder near 221 nm. Spectra obtained in HFIP and methanol had comparable shapes: a negative band centered near 208 nm (210 nm for methanol) and a negative shoulder near 218 nm (221 nm for methanol).





**Fig 3.** Effect of concentration on the secondary structure of defensin A. **a.** CD spectra of defensin A as a function of concentration. From top to bottom:  $10^{-5}$  M,  $10^{-4}$  M,  $5 \times 10^{-4}$  M, and  $10^{-3}$  M in 30 mM phosphate buffer (pH 7.5). **b.**  $|\Theta_{222}|$  as a function of defensin A concentration.

**Fig 4.** Effect of pH on the secondary structure of defensin A. **a.** CD spectra of defensin A as a function of pH in 5 mM Tris buffers. From top to bottom: pH 3.3, 10.6, 4.9, and 7.5. Defensin A concentration:  $2 \times 10^{-5}$  M. **b.**  $|\Theta_{222}|$  as a function of pH.



**Fig 5.** Effect of salts on the secondary structure of defensin A. **a.** CD spectra of defensin A as a function of KCl concentration.  $2 \times 10^{-5}$  M defensin A in 5 mM Tris (pH 7.5). KCl concentration from bottom to top: 0, 5, 10, and 100 mM. (---) additional effect of 5 mM  $\text{CaCl}_2$  (100 mM KCl + 5 mM  $\text{CaCl}_2$ ). **b.**  $|\Theta_{222}|$  as a function of KCl concentration.

ellipticity ( $-13\%$  for 5 mM  $\text{CaCl}_2$ ) (fig 5a). From all these features, the ellipticity appears related to the presence of salts with a possible specific effect of  $\text{Ca}^{2+}$ , but this independent from the ionic strength change.

## Discussion

This study illustrates the great sensitivity of the secondary structure of defensin A to environmental conditions. CD spectra show clearly the solvent dependence of the helical content as monitored by  $|\Theta_{222}|$ . It is not surprising that the higher helical content was observed in HFIP since this solvent is known as a structure-promoting solvent [8] in contrast to acetonitrile in which defensin A is weakly structured. Intermediate situations which clearly demonstrated the presence of an  $\alpha$ -helix were observed for water and methanol.

The propensity of defensin A to self-associate was previously demonstrated both by surface tension measurements (Maget-Dana, Ptak, in preparation) and by fluorescence polarization experiments of the single Tyr residue as a function of viscosity (Talbot JC, personal communication). In this study, the dependence of the helicity percentage on defensin concentration clearly indicates that oligomerization plays a major role on the secondary structure formation. A helical content of about 20% at  $10^{-3}$  M as extracted from the present study is in good agreement with the 25% value determined unambiguously from NMR data in the same range of concentrations [3]. It is likely that this would be the maximum amount of helical content induced by the defensin oligomerization. In dilute solutions, we can assume that the helix fragment (16–20) between the two S-S bridges is invariant and corresponds to the  $\sim 10\%$  of  $\alpha$ -helix content found at  $10^{-5}$  M. It can be noticed that the well resolved signals detected in NMR experiments carried out at mM concentrations [3] ruled out the presence of large defensin A oligomers. Therefore, the evolution of the dichroic intensity as a function of defensin A concentration would more correspond to an increased amount of small oligomers than to oligomers growing in size.

Secondary structure is also very sensitive to other environmental parameters such as pH or the presence of mono- and divalent cations. As far as the pH is concerned, the maximum helical content was detected in the pH range 7–8 corresponding to the deprotonation of the two His residues ( $pK \approx 7$ ). From NMR data [3], His13 was located just before the beginning of the helix whereas His19 belonged to the first turn of the helix. Thus, the ionisation state of the His residues may have a great influence on the secondary structure of defensin A. The other ionisable group involved in the helix (Arg23) is always protonated ( $pK \approx 12$ ) in

the pH range of this study. We can also consider that, in addition to a direct influence on defensin A folding, the pH of the solution might affect, as a first event, the associated state of defensin and, as a consequence, the helix content. This is consistent with the observation that the pH for the maximum helicity corresponds also nearly to the isoelectric point of defensin A (calculated  $pI \approx 8.3$ ). At this pH the electrostatic repulsions between defensin A molecules are weak and self-association should be favoured. Besides, the bell-shape of the curve (fig 4b) is in favour of a relation between the global charge of the protein and the helicity. Finally, the maximum helicity corresponds to a pH range where His residues are deprotonated and where the global charge of defensin A is weak. The KCl effect might be analyzed with respect to the self-association process as well. As a matter of fact, it has been shown that defensin A has less tendency to self-associate in the presence of monovalent salts [9]. Here, the loss of ellipticity observed in the presence of KCl might be a consequence of the dissociation of defensin A oligomers.

The optimum antibacterial activity has been observed at a pH ranging between 7.5 and 8 [6]. This is the pH range for which we found a maximum helical content. Therefore, the biological activity of defensin A seems to be closely dependent on its secondary structure and may be on its autoassociation state since both appeared correlated.

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