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# AGGREGATION AND ION TRANSFER INDUCED BY TENTOXIN

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It is shown that tentoxin, a cyclic tetrapeptide with two *N*-methylated residues, is able, when added to lipid bilayers, to increase the transmembrane current through discrete events. Conformational investigations involving $^1$H-NMR, infrared and circular dichroism studies show that, at concentrations above $7\times10^{-5}$ M, the cyclic tetrapeptide aggregates in chloroform. We suggest that the aggregates could form a pore through a stacking of cycles.

## 1. Introduction

Tentoxin is a phytotoxic cyclic tetrapeptide, cyclo(*N*-Me-Ala-Leu-*N*-Me-ΔPhe-Gly), which is thought to act on the synthesis of ATP at the chloroplast level [1]. It is characterized by the presence of unusual amino acids, namely, *N*-methylalanine and *N*-methyldehydrophenylalanine [2] and a conformational model has been proposed mainly on the basis of NMR data [3]. Owing to the presence of two *N*-methylated residues leading to at least two peptide carbonyl groups which cannot be hydrogen bonded, it was tempting to assume that tentoxin would form complexes with ions and that these could act as a transmembrane ion carrier like valinomycin.

We report here preliminary investigations showing that tentoxin facilitates the diffusion of ions through lipid bilayers and propose a model which accounts for the physico-chemical properties observed in a hydrophobic medium.

## 2. Experimental

Tentoxin used in our experiments was synthesized as described by Jacquier and Verducci [4]. Some bilayer experiments were duplicated using commercial natural tentoxin (Sigma) in order to check that the unexpected behavior observed on incorporation into lipid bilayers was not due to impurities.

Lipid bilayers were formed from 2% solutions of lipid (monooleoyl- or monopalmitoleoylglycerol, soybean lecithin or dioleoylphosphatidylcholine (Sigma) in decane using Teflon cells filled with aqueous electrolyte solutions. The membrane area was 0.1–0.2 mm$^2$.

Infrared spectra were recorded on a Perkin-Elmer model 521 spectrometer or a Bruker IFS 85 using 0.1–1 mm thick $CaF_2$ cells according to the concentration used. Circular dichroism (CD) experiments were performed on a Roussel-Jouan mark V dichrograph. $^1$H-NMR spectra were recorded at 18°C on a Bruker WM 360 spectrometer working in the Fourier transform mode.


Fig. 1. Single channel trace of tentoxin in a 1-monopalmitoleoylglycerol-decane bilayer; 1 M KCl; applied voltage, 150 mV.

## 3. Results and discussion

Contrary to the hypothesis which was at the origin of this work, addition of tentoxin to a lipid bilayer membrane does not generate an immediate increase in the transmembrane current. However, after a while (10 min–1 h depending on the experiments) the transmembrane current begins to fluctuate through discrete events (fig. 1) the number of which increase with time. It should also be mentioned that the intensity of the fluctuations depends on the amount of material added to the medium.

Indeed, while the first events are of low intensity ($5 \times 10^{-13}$ A in 1 M KCl, applied voltage 160 mV) an increase in conductance of the fluctuations ($5 \times 10^{-12}$ A under the same conditions) is observed as incubation continues for a further 3 or 4 h or with a further addition of tentoxin; in fact, these two distinct intensities can be observed simultaneously.

Concerning the conductances of these fluctuations, both show a linear voltage dependence: for example, for the low-conductance fluctuations, $\lambda = 0.5 \times 10^{-11}$ and $1 \times 10^{-11}$ $\Omega^{-1}$ at 50 and 150 mV applied voltage, respectively. This behavior could be detected in KCl as well as RbCl media but not in $CaCl_2$, suggesting that the pores are selective for monovalent cations. Such behavior differs strongly from that of valinomycin for which, under identical conditions, no discrete fluctuations could be detected within the experimental sensitivity used ($10^{-14}$ A).

A detailed analysis of the tentoxin-induced transmembrane current fluctuations will be given elsewhere. Nevertheless, the finding of discrete fluctuations is not only reminiscent of the observations made on single-channel forming peptides or

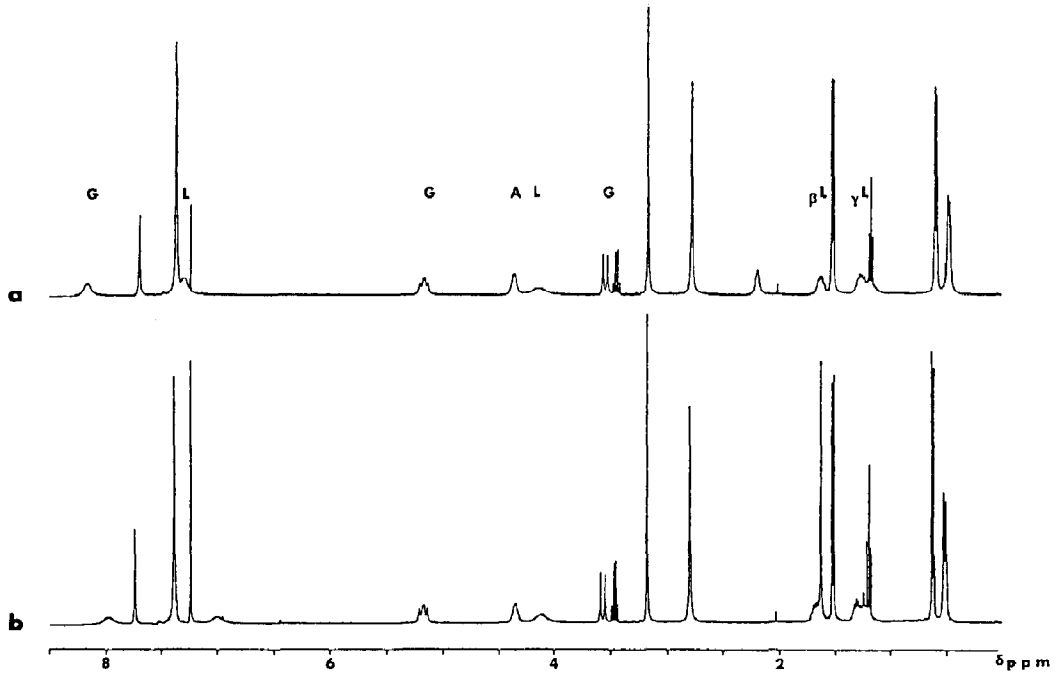


Fig. 2. $^1$H-NMR spectra of tentoxin in chloroform: (a) $1.2 \times 10^{-1}$ M, (b) $8 \times 10^{-3}$ M.

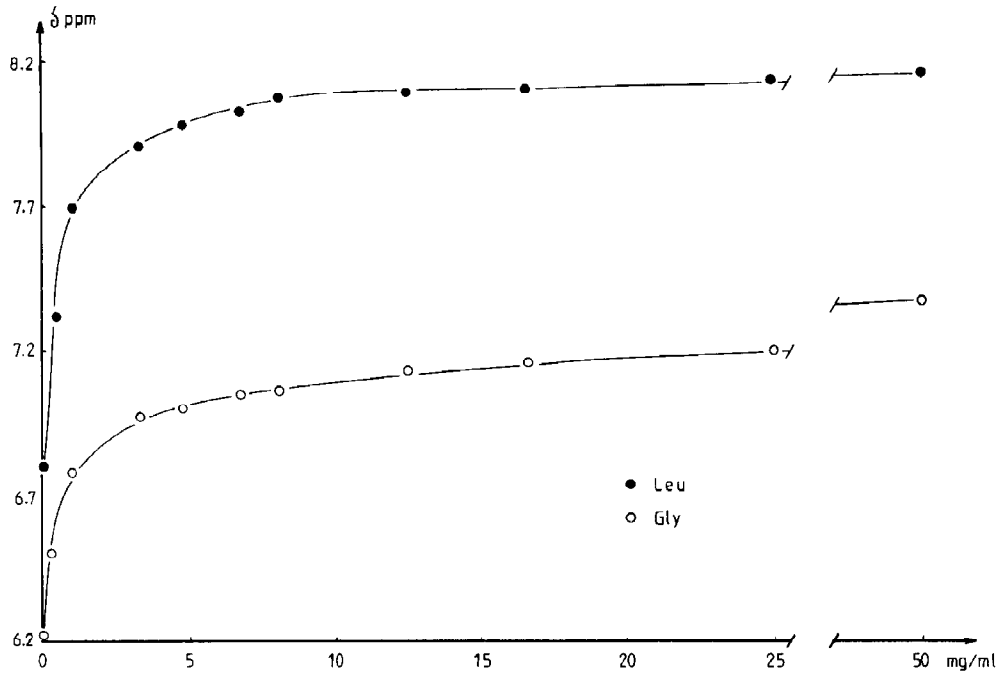


Fig. 3. Variation of the NH proton chemical shifts as a function of tentoxin concentration in chloroform.

polypeptides such as linear gramicidins [5], alamethicin [6] or other transmembrane proteins, but also of amphotericin or nystatin [7], suggesting that tentoxin is able to induce the formation of transmembrane ionic channels. Now, how could tentoxin participate in the formation of channels?

The first indication was provided by Rich and Bhatnagar [3] who showed that an analogue, namely, L-Pro$^1$-tentoxin, aggregates in chloroform. Therefore, we undertook structural investigations in hydrophobic media similar to a lipid bilayer.

The $^1$H-NMR spectra of tentoxin in chloroform show that the chemical shifts of the NH protons strongly depend on the concentration while the other resonance lines are less affected (figs. 2 and 3).

Indeed, increasing concentration of tentoxin from 0.05 mg/ml ($\sim 10^{-4}$ M) to 5 mg/ml ($\sim 10^{-2}$ M) is accompanied by a strong downfield shift of the NH protons from 6.21 and 6.80 to 7.00 and 7.99 ppm for Leu and Gly, respectively (fig. 3). Further additions of tentoxin to the solution do not generate major modifications of the spectrum. This behavior, which is similar to that reported for L-Pro$^1$-tentoxin [3], has been attributed to the formation of aggregates probably through hydrogen bonds involving the NH group even at concentrations as low as $10^{-4}$ M.

That the two NH groups participate in the formation of the aggregates through CO---NH hydrogen bonds is corroborated by infrared observations. The infrared spectrum of tentoxin in chloroform shows, at a concentration of 12 mg/ml ($\sim 3 \times 10^{-2}$ M), an amide A band at 3360 cm$^{-1}$ and two amide I bands at 1660 and 1623 cm$^{-1}$ (fig. 4) indicating the presence of both nonbonded (1660 cm$^{-1}$) and bonded (1623 cm$^{-1}$) carbonyl groups and that all the NH protons are hydrogen bonded.

While no major modification of the spectrum occurs upon dilution down to 1 mg/ml, a further lowering of the concentration to 0.4 mg/ml ($10^{-3}$ M) is characterized by the appearance of an amide A band at 3420 cm$^{-1}$. Simultaneously, the intensity of the low wave number amide I band decreases whereas that at 1660 cm$^{-1}$ increases. This indicates that dilution favors the existence of nonbonded carbonyl and NH groups and therefore

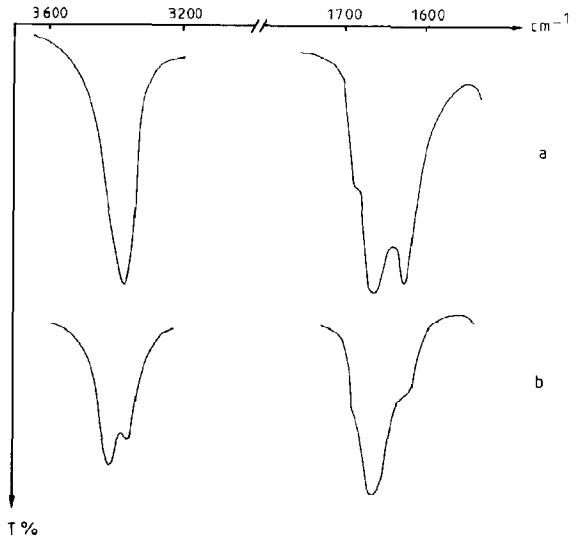


Fig. 4. Infrared spectra of tentoxin in chloroform: (a) $3\times10^{-2}$ M, (b) $10^{-3}$ M.

the monomeric form of tentoxin.

When the same experiments are carried out by measuring the optical activity in the 260–340 nm region, which corresponds to the absorption bands

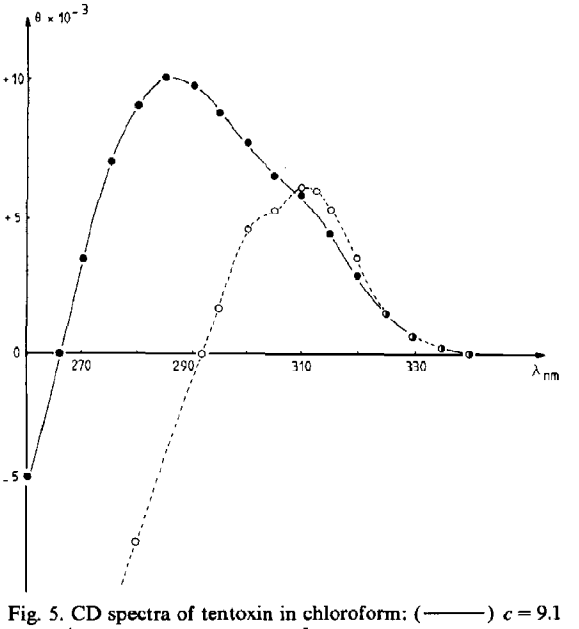


Fig. 5. CD spectra of tentoxin in chloroform: (———) $c = 9.1\times10^{-4}$ M, (- - - - -) $c = 6.8\times10^{5}$ M.

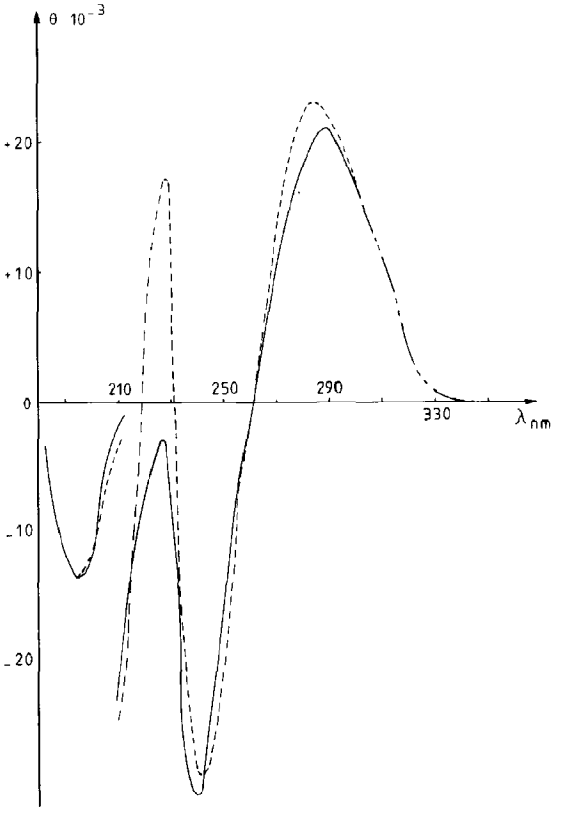


Fig. 6. CD spectra of tentoxin. (———) In water, $c = 3.8\times10^{-4}$ M; (- - - - -) in methanol, $c = 3.8\times10^{-4}$ M; below 210 nm, $\times10^{-4}$.

of the side-chain chromophore of the (ZΔ) Phe residue, drastic modifications of the CD spectrum are observed, the transition occurring at a concentration of approx. $7\times10^{-5}$ M. It is characterized by a reversal of the sign of the ellipticity in the 280 nm region (fig. 5). This indicates that the interactions involving the side chains are modified and, on the basis mainly of the infrared observations, modification of the CD spectrum is attributed to an aggregate-monomer transition. It should also be mentioned that no concentration-induced transition could be detected in the CD spectra when tentoxin was dissolved in water or methanol [3] and that the spectra in these two solvents are similar to those observed in chloroform at high concentrations (fig. 6). Therefore, it can be concluded that in these two latter solvents

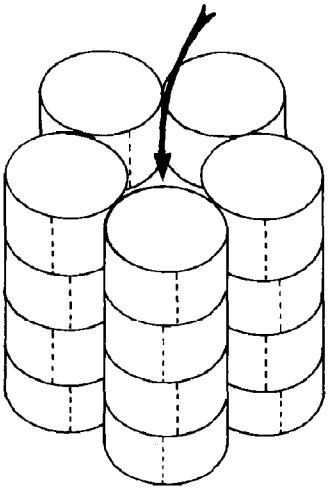

Fig. 7. Proposed schematic model of the pore formed by tentoxin. The number of residues shown was chosen to be arbitrary. Dotted lines correspond to hydrogen bonds. The arrow indicates the channel.

tentoxin is also in an aggregated form.

In conclusion to the present physico-chemical investigations, in order to explain the pore formation induced by tentoxin we suggest a model which takes into account all the spectroscopic results together with the fact that the single channel events are observed only when large amounts of material are added to the medium ($\sim 10^{-5}$ M as compared to $10^{-12}$ M for gramicidin A). It is based on the structure already proposed by Rich and Bhatnagar [3] which allows stacking of the peptide rings through intermolecular hydrogen bonds, thus forming an $\omega$-like helical structure [8] (fig. 7) which could act as a transmembrane channel similarly to alamethicin [9]. The possibility of ring stacking was checked by examination of molecular models (CPK). Thus we propose that the pores could arise from a lateral association of these stacked rings, similarly to alamethicin [9]. Nevertheless, an alternative model of pores formed by micellization of the membrane constituents cannot be ruled out but is less probable owing to the high homogeneity of the single channel amplitude under well-defined experimental conditions.

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