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Left handed α-helix formation by a bacterial peptide

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The α-helix is a common element of secondary structure in proteins and peptides. In eukaryotic organisms, which exclusively incorporate L-amino acids into such molecules, stereochemical interactions make such α-helices, invariably right-handed. *Pseudomonas tolaasii* Paine is the causal organism of the economically significant brown blotch disease of the cultivated mushroom *Agaricus bisporus* (Lange) Imbach. *P. tolaasii* produces an extracellular lipodepsipeptide toxin, tolaasin, which causes the brown pitted lesions on the mushroom cap. Circular dichroism studies on tolaasin in a membrane-like environment indicate the presence of a left-handed α-helix, probably formed by a sequence of 7 D-amino acids in the peptide. *P. tolaasii* represents the first reported example of an organism which has evolved the ability to biosynthesize a left-handed α-helix.

Left-handed α-helix, Brown blotch disease, Bacterial peptide toxin

1. INTRODUCTION

Approximately 30% of amino acid residues in a wide selection of proteins are involved in α-helical structure [5]. Both right-handed and left-handed forms of an α-helix are conformationally accessible for sequences composed of L-amino acids, but steric interactions are estimated to make the left-handed form energetically less favourable by about 2.0 kcal/residue [6]. The opposite is true for synthetic α-helices constructed from D-amino acids, which adopt a left-handed conformation [7].

Whereas higher organisms use exclusively L-amino acids in constructing peptides and proteins, the ability of bacteria to synthesise peptides non-ribosomally [8] allows them to incorporate both L and D forms of the common amino acids, as well as other components such as fatty acids [9], dehydroamino acids [10] and other unusual amino acids [11]. Tolaasin, one such peptide produced by *P. tolaasii*, is the toxin responsible for lesion formation in brown blotch disease [1] of the edible mushroom *A. bisporus*. We have recently determined the primary structure (Fig. 1) of tolaasin by a combination of chemical and spectroscopic methods [12]. Tolaasin forms voltage-gated ion channels in planar lipid bilayers [4] and disrupts membrane integrity in a variety of prokaryotic and eukaryotic cell types [13]. In this paper, we report the results of circular dichroism (CD) spectroscopic studies on the 3D structure of tolaasin, undertaken as an aid to understanding its mode of action.

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2. MATERIALS AND METHODS

2.1. *Preparation and purification of tolaasin*

Tolaasin was isolated and partially purified using a modification of the method of Peng [3]. A 3 l flask containing 1 l of *Pseudomonas* agar F (PAF, Difco) was inoculated with 8 ml of an overnight culture of *P. tolaasii* NCPPB 1116 and grown in an orbital incubator (200 rpm) for 36 h at 25°C. Cells were removed by centrifugation (10 000 × *g*, 10 min) and the supernatant frozen overnight. The thawed filtrate was filtered through Whatman no. 42 paper, acidified to pH 3 with concentrated HCl, left overnight at room temperature and the precipitate removed by centrifugation (8000 *g*, 20 min). Tolaasin was precipitated by addition of 150 g $CaCl_2$, collected by centrifugation (8000 × *g*, 25 min) and dried at 45°C. The pellet was suspended in 50 ml methanol, centrifuged (10 000 × *g*, 15 min) and the supernatant evaporated to dryness in vacuo at 45°C. The residue was dissolved in 20 ml methanol, evaporated to dryness as described above, redissolved in 10 ml methanol and dried again. 10 ml water was added to the residue and the suspension centrifuged (10 000 × *g*, 15 min). The precipitate was dissolved in 50 ml water and lyophilized. The resulting powder was dissolved in water at a concentration of 50 mg ml^{-1} before passing down a Dowex-1 anion exchange column (15 × 25 mm) at a flow rate of 35 ml h^{-1}. The eluate was monitored at 206 nm and fractions were assayed for activity using the rapid pitting test [14]. All fractions displaying increased absorbance at 206 nm were active on mushroom tissue and were pooled and lyophilized to yield a white powder.

2.2. *Reverse-phase HPLC*

A Varian LCS000 chromatograph with detection at 214 nm and a Spherisorb S5P (phenyl) columns were used. A solvent system of water (0.1% TFA, v/v) and CH_3CN (0.08% TFA, v/v) was employed for elution, with a flow rate of 2.5 ml min^{-1}. Water was obtained from a Millipore Milli-Q water purification system and far UV grade CH_3CN was purchased from Fisons. All solvents were degassed by sonication before use. Crude tolaasin was purified by injection onto the column at 50% organic phase and elution using a gradient to 100% CH_3CN over 25 min.

2.3. *CD spectroscopy*

CD spectra were obtained using a JASCO J600 spectro-polarimeter at a peptide concentration of 0.29 mM, pH 7.5 (Tris buffer) and at 25°C in a 0.5 mm pathlength cell.

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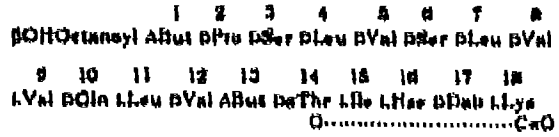

Fig. 1. Primary structure of tolaasin. Abbreviations: a, allo; 4But, 2-amino-*trans*-Δ³-butyryl; Dab, 2,4-diaminobutyryl; Hse, Homoseryl.

3. RESULTS AND DISCUSSION

In aqueous solution, the CD spectrum of tolaasin contains no readily identifiable elements of secondary structure (Fig. 2a). The addition of sodium dodecyl sulphate (SDS) to a point above its critical micellar concentration gave rise to the spectrum shown (Fig. 2b). Further experiments using diphosphatidylcholine liposomes gave similar spectra (data not shown). The general shape of this spectrum, with maxima at 192 nm, 207 nm and 225 nm is characteristic of an α-helix [15], but is essentially a mirror image of that expected for the respective right-handed α-helix (Fig. 2d) [16]. A synthetic β-endorphin analogue made from D-amino acids exhibits a similar spectrum at high concentrations of 2,2,2-trifluoroethanol [7]. We conclude that in a membrane-like environment, the conformation of tolaasin includes a region of left-handed α-helix. Inspection of the structure of tolaasin reveals a sequence of 7 consecutive D-amino acid residues extending from Pro^2 to Val^8. Val^9 has L-stereochemistry, and unfavourable steric interactions between $Val^{9\beta}$ and $Leu^{12\delta}$ protons might be expected to prevent inclusion of Leu^{12} in an α-helix formed by this region of the peptide.

Spectra 2a, 2b and 2c were analysed for secondary structure contributions using CONTIN [17]. The spectrum of tolaasin in aqueous solution contains little contribution from α-helical forms. For tolaasin in 150 mM aqueous SDS, the percentage of α-helical content was calculated from the CD spectrum to be 20%. A modified form of tolaasin (from genetically engineered strain PT144), lacks one Ser-Leu-Val unit [11] and exhibits the CD spectrum in aqueous SDS shown in Fig. 2c. The α-helical content of this molecule was calculated to be 10%, supporting the hypothesis that the α-helix extends over the sequence of D-amino acids. Values of 20% and 10% are lower than might be expected from the primary sequences of tolaasin and the PT144 variant; it is not clear whether this reflects ensemble averaging or partial ordering, although the latter seems unlikely since 10% of the PT 144 toxin represents only 14 residues.

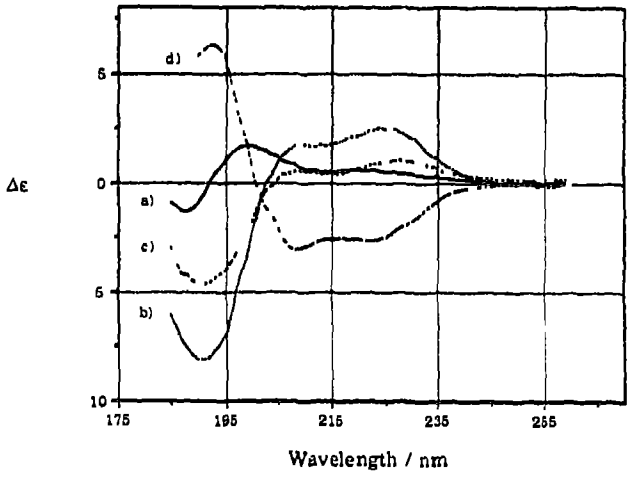

Fig. 2. The differential extinction coefficient ($\Delta\epsilon$ in units of 10^3 cm^2 mol^{-1}) is plotted: (a) tolaasin in aqueous solution, (b) tolaasin in presence of ca 150 mM sodium dodecyl sulphate (SDS), (c) an altered form of tolaasin in which one SLV unit is deleted, also in ca 150 mM SDS, (d) CD spectrum of polylysine in α-helical form (CD intensity divided by 5 for comparison) [11].

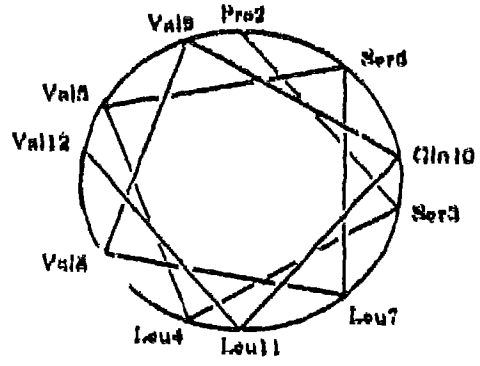

Fig. 3. Schiffer-Edmundson α-helical wheel plot [18] of tolaasin residues 2 to 12.

We note further that the sequence Ser^3 to Gln^{10} contains hydrophilic amino acids in i, $i+3$, $i+7$ (Ser, Ser, Gln, respectively) positions interspersed by hydrophobic residues, giving an α-helix formed by this sequence amphiphilic character (Fig. 3) [18]. Such amphiphilic α-helices are often involved in intracellular targetting of proteins [19], ion channel formation and disruption of membrane function [20]. *P. tolaasii* is the first reported example of an organism which has been shown to produce the functionally equivalent, yet stereochemically inverted, form of the amphiphilic α-helix.

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