Crystal structure of the insect neuropeptide proctolin.†‡

Judith A. K. Howard,[a] Dmitry S. Yufit,[a] Olga V. Chetina,*[a] Simon J. Teat,§[b] Silvia C. Capelli¶[c] and Philip Pattison[d]

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The crystal structure of the neuropeptide proctolin (Arg-Tyr-Leu-Pro-Thr) is reported revealing the solid-state conformation of its molecules and their association in the crystal.

An enormous increase in the number of malaria victims over the past 20 years awakens a renewed attention of scientists all over the world.[1] One of the main reasons for the recent rise in the incidence of malaria is insecticide resistance of mosquitoes.[2] A new type of insecticides highly selective, safe and environmentally friendly is urgently required to solve the problem. Neuropeptides, biologically active peptides produced in neurosecretory cells of insects, are able to control many aspects of an insect life. Therefore neuropeptide antagonists are considered as a basis for the design of new insect control agents.[3]

Proctolin (Arg-Tyr-Leu-Pro-Thr), the first structurally identified myotropic insect neuromodulator,[4] due to extensive studies performed by insect physiologists, neuroscientists, biochemists, synthetic and physical chemists became the best known and most thoroughly investigated insect neuropeptide.[5–7] Proctolin and some of its analogues appeared to have significant biological effects not only in insects and crustaceans, but also in mammals and even on human blood cells.[6] Eventually proctolin emerged as a model peptide in neuroscience research.[8] Hundreds of proctolin analogues were synthesised in an attempt to elucidate the role of each of its five amino acid residues in the myotropic activity in insects. Their structure/activity relationship studies have confirmed that each amino acid residue of proctolin is important for its bioactivity.[6] Theoretical conformational analysis of proctolin and its active analogues revealed five families of low energy conformations of the molecule but failed to propose a biologically active conformation of this small flexible pentapeptide.[9,10] The solid state conformation of proctolin obtained by X-ray crystal structure analysis has not been reported hitherto and we herein report the full three dimensional molecular structure.

The asymmetric unit of the orthorhombic crystal structure contains four independent molecules of proctolin (Fig. 1). The conformation of molecules **A** and **C** is similar except for a partial disorder in the pyrrolidine ring of the Pro residue in **A**. The other two molecules, **B** and **D**, are much more disordered but their conformations are similar to one another in Tyr-Leu-Pro-Thr. Thus, the conformation of the peptide chain in all four molecules remains principally the same.

The most flexible section appeared to be the guanidine side chain of Arg residue. In molecule **D** it shows two completely different positions equally occupied in the crystal. The side chain of the

[a]*Department of Chemistry, University of Durham, Durham, UK DH1 3LE*
[b]*Daresbury Laboratory, Warrington, UK WA4 4AD*
[c]*European Synchrotron Radiation Facility, BP220, F-38043, Grenoble, France*
[d]*Laboratory of Crystallography, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015, Lausanne, Switzerland*

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§ Present address: Advanced Light Source, Lawrence Berkeley National Lab, Berkeley, CA 94720, USA.

¶ Present address: Institut Laue-Langevin, BP 156, 6, rue Jules Horowitz, 38042 Grenoble Cedex 9, France.

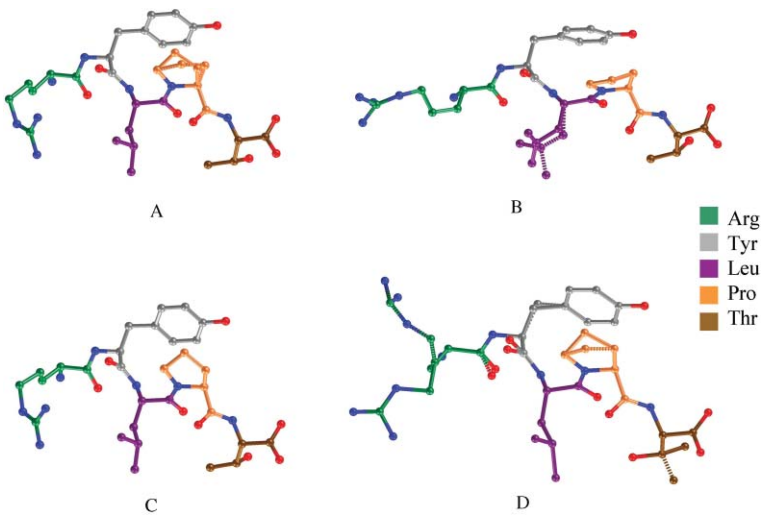

Fig. 1 Conformation of molecules in crystal (hydrogen atoms are omitted for clarity).

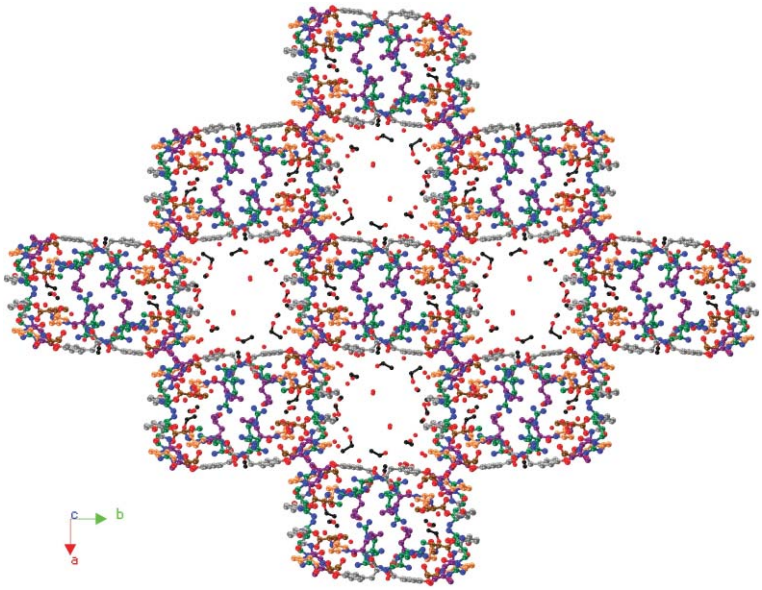

Fig. 2 The packing of proctolin molecules viewed in the *ab* plane.

Leu residue is also quite flexible and disordered but its overall conformation does not change significantly.

The side chain of tyrosyl residue in all independent molecules is bent towards the proline in such a way that the plane of its aromatic ring is almost parallel to the pyrrolidine ring of the Pro residue. It was reported earlier[11] that elimination of the methylene group between the aromatic ring of the Tyr residue and the peptide chain as well as addition of an extra methylene group results in lack of myotropic activity in insects. Obviously in both cases, the conformation of the molecule observed in crystal structure wouldn't be possible. Therefore the conformation revealed from the crystal structure should be similar to the biologically active one.

The proline residue has *trans* geometry in every molecule. The same proline conformation was indicated by NMR studies in some proctolin analogues.[10] However, the γ-turn conformation with a hydrogen bond between Leu-C*O* and Thr-N*H* are not observed in crystal structure. Moreover both C$^{\gamma}$-*endo* and C$^{\gamma}$-*exo* conformations of the pyrrolidine ring of Pro are present in solid state proctolin.

The packing of proctolin molecules in the crystal (Fig. 2) is quite remarkable and very unusual for a small molecule crystal structure. There are three-dimensional polymeric nanotubes stretching along the *c* axis of the crystal, which are packed in a chequerboard order. The channels inside the nanotubes, as well as the cavities formed between them, are filled with a number of disordered solvent and water molecules stabilizing the whole structure. This feature is quite common for a protein crystal structure.

A closer look at the structure of a nanotube in the direction of *c* and *a* axis is shown in Fig. 3. Beside the solvent and water molecules (omitted in this picture) the main channel of a nanotube contains the side chains of Arg and Leu residues belonging to molecules **B** and **D** (Fig. 1). It is well visible that the nanotube is porous in *a* axis direction. Guanidine residues of the same two molecules are the only ones present inside these cavities. Another notable feature is that all Tyr residues of the structure are situated on the outer side of nanotubes.

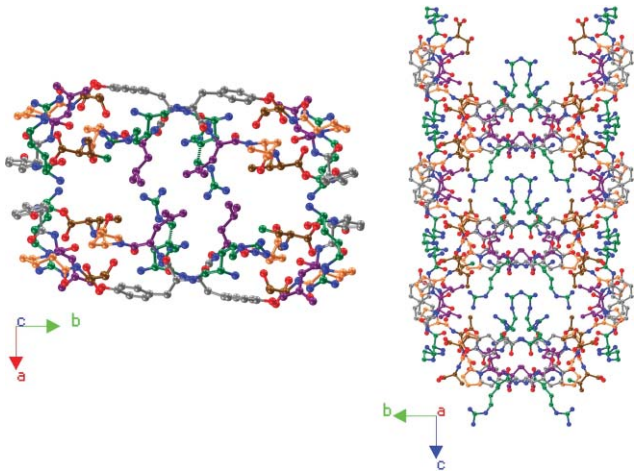

Fig. 3 A view of a polymeric nanotube in *c* (left) and *a* (right) direction.

The molecules **A** and **C** (Fig. 1) are bound to each other by hydrogen bonds between guanidine nitrogen atoms of Arg residues and terminal carboxyl group oxygen atoms of Thr residues, forming polymeric chains along the *c* axis of the crystal (Fig. 4a). The chains of **A** molecules are bound to the chains of **C** molecules by hydrogen bonds involving the terminal peptide nitrogen atoms of the Arg residues, thus building the side walls of a nanotube. The molecules **B** and **D** are linked in pairs by four hydrogen bonds between nitrogen and carbonyl atoms of the peptide chain (Fig. 4b). Guanidine nitrogen atoms of molecules **D** and the peptide carbonyl oxygen atoms of Tyr residues of molecules **B** bind these pairs to each other building the porous parts of nanotubes. Additional hydrogen bonds involving OH-groups of the side chains of Tyr and Thr residues, the peptide chain nitrogen atoms of the Arg and Thr residues and also the terminal carboxyl oxygen atoms of Thr residues, join the walls of a nanotube with its top and bottom parts.

Adjacent nanotubes of the structure are held together by hydrogen bonds between the guanidine nitrogen atoms of Arg and

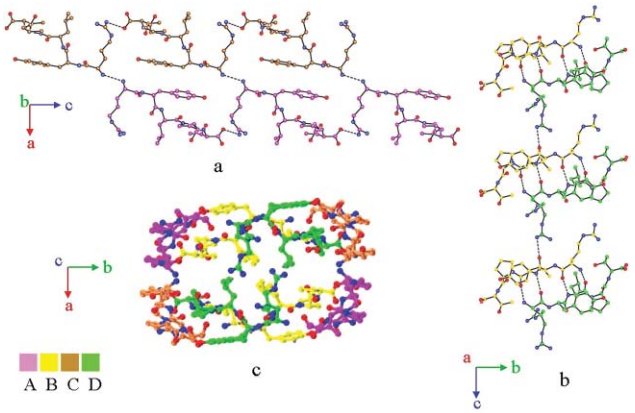

Fig. 4 Hydrogen bonded chains of proctolin molecules **A–C** (a) and **B–D** (b) and their position in a nanotube (c).

peptide chain carbonyl oxygen atoms of Pro residue belonging to **A** and **C** molecules. Solvent and water molecules also participate in some hydrogen bonding providing additional stability of the nanotubes position in crystal.

Summarising the main features of the crystal structure of proctolin we may suppose that the solid-state conformation of this pentapeptide should be similar to the biologically active one. Indeed, all nitrogen and oxygen atoms of the peptide chain of the molecule as well as of the side chains of amino acid residues are engaged in the intermolecular hydrogen bonding within the crystal. The only exception is the tertiary nitrogen atom of the Pro residue that is screened by the overhanging aromatic ring of Tyr. This means that in such conformation every amino acid residue of a proctolin molecule can participate in either binding to, or interaction with, a biological receptor and thus could be responsible for its bioactivity. The flexibility of the side chain of Arg residue in the crystal, its tendency to occupy the cavities in the structure as well as the essential activity in hydrogen bonding, confirm its possible role in promoting high affinity binding with a receptor on the target tissue, which was suggested from structure-function relationship studies of proctolin analogues. The conformations of the Tyr and Pro residues observed in the crystal are in a good agreement with the data on myotropic activity of the peptide analogues with a modified tyrosine residue. Finally, as a whole the crystal structure of proctolin is built of porous polymeric nanotubes filled with stabilising water and solvent molecules and as such it resembles closely the structures of biological macromolecules.

Amorphous proctolin supplied by Sigma has been crystallised from ethanol solution. The crystals were of weak diffraction nature and unstable in absence of ethanol vapour or mother liquor. Despite several attempts to collect sufficient single crystal data on the Swiss-Norwegian Beam line at ESRF, we were not able to elucidate the full 3D structure from these data. The best diffraction data were collected from a single crystal sealed in a quartz capillary on station 9.8[12] at the Synchrotron Radiation Source (SRS), Daresbury Laboratories, Cheshire, UK. The structure was solved by direct method using SHELXD program,[13] visualized and analysed with OLEX program.[14] Crystal system orthorhombic, space group P 2_12_12, $Z = 16$, cell parameters $a = 34.710(7)$ Å, $b = 42.495(9)$ Å, $c = 15.363(3)$ Å, conventional $R_1(F) = 0.1072$ (see ESI for details‡).

We hope that the structure reported here will provide a higher level of understanding of the mode of action of proctolin and its physiological role in arthropods and encourage chemists and applied biologists to make the last decisive step on the way to a new type of insecticide and hence to the relief of malaria.

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