CRYSTALLISATION AND PRELIMINARY CRYSTALLOGRAPHIC DATA OF A PORCINE NEUROPHYSIN I–TYR-PHE-NH_2 COMPLEX

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1. Introduction

The hormones oxytocin and vasopressin are stored in the neurosecretory granules of the neurohypophysis bound to acidic 10 000 mol. wt proteins called neurophysins. Three major neurophysins can be extracted from the porcine pituitary. The amino acid sequences of forms I and III are very similar, form I being a truncated form of III with three residues removed from the C-terminus [1]. The physiological role of neurophysins is poorly understood. They are secreted with the hormones into the blood-stream where they circulate in an uncomplexed form. Intracellularly, they may stabilise the hormones during intra-axonal transport and storage by preventing leakage, enzymatic degradation and reduction of the susceptible 1–6 disulphide bond. The protein also concentrates the hormones in an insoluble form until required and reduces osmotic problems in the storage compartment.

Neurophysins represent a family of proteins that show a highly conserved central core sequence with marked variability at the amino- and carboxyl-termini both within and between species. The chemical specificity for ligand binding has been thoroughly studied and there is a large amount of kinetic information derived from spectroscopic and thermodynamic studies [2].

The structure analysis of neurophysin bound to a hormone peptide analogue should contribute to the understanding of many biochemical questions. This structure might provide a rare opportunity to define a peptide hormone/acceptor interaction of the kind one might expect to observe with cell surface receptors. The amino acid sequence contains an internal homology indicative of a gene duplication but the equivalence of two apparent binding sites of the neurophysin protomer for hormone and peptide analogues is still unclear. Neurophysin can form dimers and there are indications that the protomer/protomer interface may involve the burial of non-polar residues. The binding of either hormone or analogues seems to favour the association of neurophysin molecules, and aggregates higher than dimers can form. There is some indication of positive cooperativity in ligand binding.

We have purified large quantities of porcine neurophysin I (pNP I) and prepared cyrstals with a bound dipeptide fragment of vasopressin (residues 2 and 3), Tyr-Phe-amide. This report describes the preliminary crystallographic characterisation of these new crystals.

2. Materials and methods

Crude neurophysin was obtained from a side fraction of a commercial porcine pituitary extraction process. A waste fraction (100 g) was chromatographed on an industrial Sephadex G-50 column (bed vol. 75 l) in 0.1 M formic acid, which separated neurophysins I and III from neurophysin II and other material. The fractions were lyophilised to yield 10.92 g neurophysin I and III. This material was subjected to ion-exchange chromatography to separate the neurophysins. Usually 1 g was loaded onto a 3 cm × 30 cm column of CM-cellulose (Whatman) at pH 4 in acetate buffer and the pNP I was eluted with a pH gradient to pH 6 in a similar manner to that reported in [3]. The purified NP I fractions were

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freeze-dried and shown to be homogeneous on disc gel electrophoresis at pH 8.9. The dipeptide Tyr-Phe-amide was made by a conventional condensation of an 'activated ester' of Boc-Tyr (butyl) with Phe-amide. The deprotected peptide was freeze-dried and used without further purification.

To crystallise the complex 5 mg pNP I were dissolved in 0.3 ml 0.1 M Tris buffer with the pH adjusted to 5.4 with acetic acid. Dipeptide (1 mg) was also dissolved in 0.3 ml buffer and mixed with the protein solution. The pH was adjusted to 5.4 and the solution became slightly turbid. A saturated solution of $MnCl_2$ in buffer had been prepared and 0.01 ml quantities were added until a heavy precipitate formed. The protein solution was heated to 55°C where the precipitate dissolved and then the solution was filtered while hot. It was allowed to cool slowly over 14 days to room temperature in an insulated dewar. Crystals grew singly and in clusters on the test tube wall.

The density of the crystals was measured in a calibrated toluene/bromobenzene density gradient. Single crystals were removed, dried and dropped into a measuring cylinder containing the density gradient. The density of the mother liquor was also obtained in this gradient system.

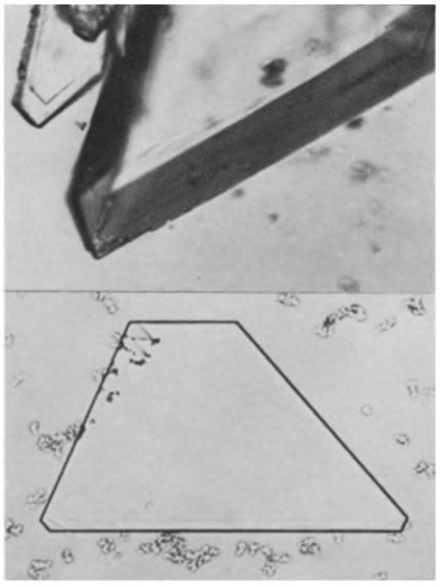

Fig.1. Crystals of the porcine neurophysin I–Tyr-Phe-NH_2 complex grown from 0.1 M Tris/acetate buffer at pH 5.4 in the presence of $MnCl_2$.

3. Results

The slow approach to room temperature in the 'hot box' allowed good quality single crystals of the protein–dipeptide complex to be grown. The crystals are plates of variable thickness, the optimal dimensions being in the range 1.5 × 1.5 × 0.3–0.6 mm (fig.1). The greatest crystal length was found to be ~5 mm in the largest plates grown.

Still X-ray photographs taken at room temperature show diffraction to a nominal resolution of 2.5 Å and the crystals are very stable in the X-ray beam. The diffraction pattern has *mmm* Laue symmetry so that the crystals belong to an orthorhombic space group. There are systematic absences of the type $h \neq 2n$ for ($h00$) and $k \neq 2n$ for ($0k0$), (fig.2). The ($h0l$) reflections do not exhibit any absences along l (fig.3) and there are no conditions limiting general reflections. The space group is therefore $P2_12_12$ with four general positions. The unit cell dimensions were measured on a Hilger-Watts 4 circle diffractometer and were $a = 152.78$ Å, $b = 69.08$ Å and $c = 36.30$ Å and $V = 3.83 \times 10^5$ Å^3.

The density of the crystals was found to be 1.27

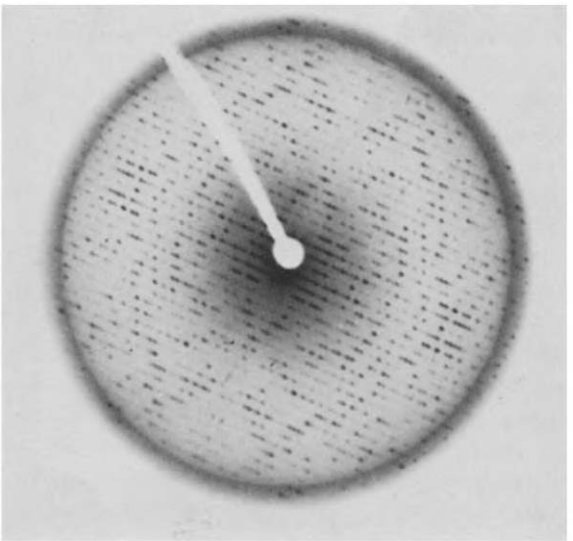

Fig.2. 15° precession photograph of the $hk0$ zone of pNP I–Tyr-Phe-NH_2 complex crystals using CuK_α radiation from a rotating anode tube operated at 37 mA and 40 kV with a crystal-to-film distance of 60 mm.

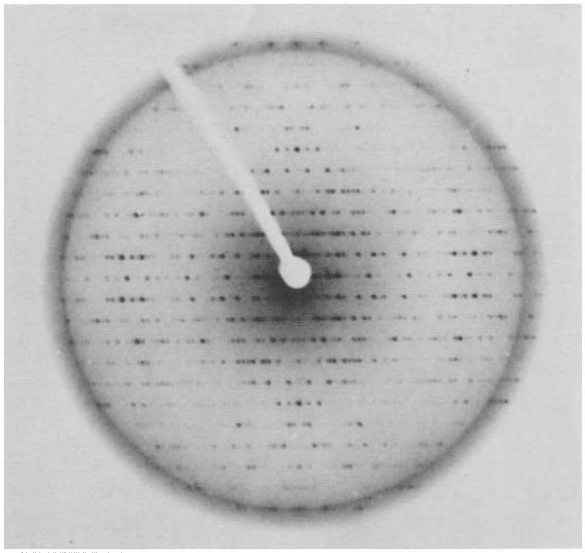

Fig.3. 15° precession photograph of the *h0l* zone of pNP I–Tyr-Phe-NH_2 complex crystals using CuK_α radiation from a rotating anode tube operated at 37 mA and 40 kV with a crystal-to-film distance of 60 mm

g/ml and the mother liquor was 1.087 g/ml. An average partial specific volume, $\bar{v}$ = 0.74 ml/g [4] was used to calculate the percentage by volume of protein (52%) and solvent (48%). There are 4 molecules in the asymmetric unit.

In identical crystallisation conditions a few crystals with rhombic dodecahedral morphology were encountered. Cubic symmetry was confirmed by precession photography with a = 143 Å but the crystals do not diffract strongly.

4. Discussion

The crystals of porcine neurophysin I described here are suitable for a high resolution structure analysis. The protein contains 92 amino acids with 7 disulphide bridges [5] and circular dichroism indicates that the predominant secondary structure is β-sheet [6]. Several crystal forms of neurophysin have been reported but only in [7] has a form suitable for X-ray analysis appeared. Crystallisation of bovine neurophysin II (bNP II) with bound 8-Arg-vasopressin at pH 3.9 was described in [8]. In our hands these crystals can be grown up to 3 mm in length but in spite of extensive efforts the other dimensions remain very small. Breslow [9] described small crystals of bNP II with bound *S*-methyl-Cys-Phe-Ile-amide at pH 8 and more recently bNP II with bound Phe-Tyr-NH_2 [7]. The latter crystals are suitable for X-ray analysis. BNP II, however, has 95 amino acids compared to 92 for pNP I. The deletion of the C-terminal sequence Arg-Arg-Ala for pNP I may explain the lower pH required for its crystallisation since we have obtained rather small crystals of pNP III at pH 8. It will be interesting to see if the crystal forms grown at different pH values contain aggregates of the same type since both the crystal form reported here and that in [7] contain 4 molecules in the asymmetric unit although the density and unit cell dimensions for each form are quite different. Furthermore, although vasopressin is thought to be the natural ligand associating with pNP I and bNP II the order of the amino acids in the peptide analogues used in the two crystallisation methods is reversed. The pH for maximal hormone binding is normally in the region of pH 5.5 and since excess dipeptide has been used it may be possible to observe the weak secondary hormone binding site and to define the mechanism whereby hormone binding is influenced by interactions between protomers. Attempts are continuing to find a crystal form with bound hormone suitable for X-ray analysis.

Acknowledgements

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