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Crystals of Ligand-Free Bovine Neurophysin II

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Abstract

A modified neurophysin, des 1-6 bovine neurophysin II, has been crystallized in the absence of bound hormone or hormone analogue. These crystals represent the first crystals of ligand-free neurophysin, and are essential for understanding neurophysin–hormone recognition as well as hormone-induced neurophysin dimerization. The crystals diffract to beyond 1.8 Å resolution, belong to space group $P3_121$ (or $P3_221$) with $a = 48.86$, $c = 78.61$ Å, and contain one molecule per asymmetric unit.

1. Introduction

Neurophysins (NP's) are small (93–95 residues) disulfide-rich proteins synthesized together with the hormones oxytocin (OT) and vasopressin (VP) as part of a common precursor (Ivell & Richter, 1984; Land *et al.*, 1983; Land, Schutz, Schmale & Richter, 1982). After synthesis, the hormone–NP precursor is transported along the cell's axon to the neurosecretory granules (NSG's) of the posterior pituitary for storage until release into the bloodstream. During transport, a three-residue (Gly-Lys-Arg) segment linking the hormone and NP is removed, yielding a non-covalent complex between the hormone and NP. Within the NSG's, the concentration of the hormone–NP complex is quite high [greater than 0.1 *M* (Dreifuss, 1975)] and it has been suggested that the intragranular hormone–NP complex is in the precipitated or crystalline state (Gainer, Peng Luo & Sarne, 1977; Livingstone & Lederis, 1971). While the physiological properties of the hormones have been identified, the neurophysins appear to be essential to the correct targeting of the precursor and the packaging, storage and stabilization of the hormone complex in the NSG prior to release into the bloodstream (Breslow, 1993; Breslow & Burman, 1990; Rholam, Nicolas & Cohen, 1982). Mutations of the neurophysin segment of the precursor are associated with deficiencies in vasopressin production (Bahnsen *et al.*, 1992; Ito, Mori, Oiso & Saito, 1991; Schmale & Richter, 1984).

Two closely related classes of neurophysins are known reflecting the synthesis of the two hormones and their associated NP *via* separate precursors. They are denoted NP-I and NP-II based on their electrophoretic mobility at pH 8.1 (Uttenthal & Hope, 1977). The two NP's show a very high sequence homology (Chauvet, Colne, Hurpet, Chauvet & Acher, 1983) with the majority of the sequence variation occurring at the N and C termini. *In vitro*, both NP's are capable of binding either hormone as well as small peptide analogs (Breslow, 1979; Breslow & Walter, 1972) and the interactions between NP and small peptide analogs have been extensively studied in solution (Breslow & Buman, 1990) as a model for protein–peptide interactions. The crystal structures of bovine NP-II complexed with the hormone surrogate *para* iodo-Phe-Tyr-amide (Chen *et al.*, 1991) and oxytocin (Rose, Wu, Hsiao, Breslow & Wang, 1996) have been determined and have confirmed most of the deductions about NP–hormone bonding made from spectroscopic and thermodynamic studies (Breslow & Burman, 1990). Hydrodynamic studies (Rholam *et al.*, 1982) suggest that the NP dimer undergoes considerable conformational change upon hormone binding. Additionally, the dimerization constant is markedly increased (Benatan, Rose, Breslow & Wang, 1991; Pitts *et al.*, 1980; Rose *et al.*, 1988; Yoo, Wang, Sax & Breslow, 1979). Therefore, the structure of ligand-free NP is essential to the understanding of NP–peptide recognition and the basis of the ligand-facilitated dimerization. Crystals of NP–peptide (Benatan *et al.*, 1991; Pitts *et al.*, 1980; Rose *et al.*, 1988; You *et al.*, 1979) and NP–hormone (Rose, Breslow, Huang & Wang, 1991) complexes have been reported but crystals of the 'unliganded' NP have proved difficult to obtain until now.

Analysis of the NP–hormone (Rose *et al.*, 1996) and NP–peptide (Chen *et al.*, 1991) crystal structures shows that the complexes consist of a very stable peptide core (residues 8–86) with large conformational variability observed for the N and C termini (see Fig. 1). It was felt that removal of residues 1–6 and 87–95 of the protein, which are not involved in hormone binding, could lead to more favorable crystal packing interactions thus producing crystals of higher diffraction quality. As a first step towards this goal, des 1–6 bovine NP-II was prepared and crystal screens were conducted with both NP alone and with NP complexed with the hormones

vasopressin and hydrin-I. This work describes the crystallization of ligand-free des 1–6 bovine NP-II. A knowledge of the 'ligand-free' structures is essential to the understanding of NP–peptide recognition and the conformational changes accompanying hormone binding.

2. Preparation and purification

Bovine neurophysin II (NP-II) was purified as previously described (Breslow, Aanning, Abrash & Schmir, 1971). The des 1–6 protein, representing cleavage after Glu6, was prepared by limited digestion by *Staphylococcus* protease V8. NP-II (2 mg ml^{-1}) in pH 5.9 pyridine acetate buffer (0.41 *M* pyridine, 0.08 *M* acetic acid) was treated with 0.5% enzyme by weight for 24 h at room temperature. Digestion was stopped almost completely (~10% residual activity) by the addition of an equal volume of glacial acetic acid and a final concentration of 10 mg ml^{-1} phenylmethyl sulfonyl fluoride (PMSF), with stirring for 5 h. The digestion mixture was diluted with 3 vol of water and insoluble PMSF was removed by centrifugation. The lyophilized supernatant was chromatographed on Sephadex G-50 in 0.2 *M* acetic acid, and the lyophilized protein peak from this step was further chromatographed on DEAE Sephaxex A-50 at pH 5.9 in pyridine acetate buffer (see above). The first protein peak contained principally the des 1–6 protein and was lyophilized and further purified by affinity chromatography (Rabbani, Pagnozzi, Chang & Breslow, 1982). The binding fractions from this step were homogeneous as judged by N-terminal sequencing and native gel electrophoresis (Breslow *et al.*,1971; Rabbani *et al.*, 1982). The final yield of pure des 1–6 protein represented 20% by weight of the initial native protein used.

3. Crystallization

For the crystallization trials, the protein was concentrated to 14 mg ml^{-1} in water and sodium azide (0.25 mg ml^{-1}) was added. The initial crystallization conditions were determined at 291 K using the sparse-matrix screening approach of Jancarik & Kim (Jancarik & Kim, 1991) using 2 µl hanging drops (McPherson, 1982). Crystals of des 1–6 NP were obtained by mixing equal volumes of the protein concentrate and a reservoir solution containing 2.0 *M* sodium chloride, 0.1 *M* MES buffer pH 6.5 and 0.05 *M* sodium/potassium phosphate. Using these conditions, crystals appeared in 4 d. After the crystals appeared, the sodium chloride concentration was then gradually increased to 2.4 *M* over a one week period in order to promote further crystal growth. Crystals suitable for X-ray analysis (Fig. 2) were obtained in two weeks.

4. Space-group determination

For X-ray diffraction analysis, crystals measuring approximately 0.4 × 0.3 × 0.3 mm were sealed in a thin-walled glass capillary containing a small amount of mother liquor to prevent dehydration. Data sets were collected on both a Siemens X100 (Blum, Metcalf, Harrison & Wiley, 1987) and a Rigaku R-AXIS II (Sato, Yamamoto, Imada & Katsube, 1992) area detectors. The X100 data were collected using 2.4 kW (40 kV, 60 mA) mirror (Supper design) focused Cu $K\alpha$ X-rays generated on a Rigaku RU200 rotating-anode generator. Each 0.25° oscillation frame was exposed for 300 s using a crystal-to-detector distance of 120 mm and

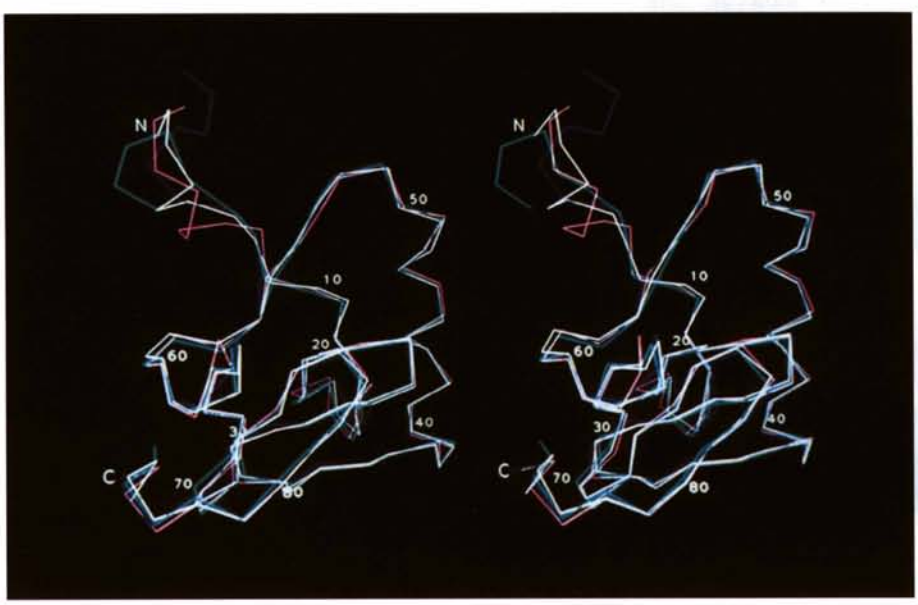

Fig. 1. A stereoview of the superposition of the four neurophysin molecules (Cα's) observed in the neurophysin peptide complex (Chen *et al.*, 1991; PDB entry 1bn2) showing the multiple conformations of the N-terminal residues (1–6). Color scheme, white chain 1, cyan chain 2, blue chain 3 and magenta chain 4.

a 2θ angle of 18°. The data were indexed, integrated and scaled using *XENGEN*2.1 (Howard *et al.*, 1987). The R-AXIS data were collected using mirror-focused (Yale design) 5 kW Cu $K\alpha$ X-rays generated on a Rigaku RU200 rotating-anode generator. Each 2° oscillation image was exposed for 10 min using a crystal-to-detector distance of 100 mm and a 2θ angle of 0°. The data were indexed, integrated and scaled using both *bioteX* (Chen *et al.*, 1993) and *HKL* (Minor, 1993; Otwinowski, 1993).

A data set to 2.5 Å resolution (95.2% complete, average redundancy 2.6) was collected on the X100 detector from 720 data frames. Initial indexing indicated that the crystals belong to either a trigonal or hexagonal space group with $a = 48.86$ and $c = 78.61$ Å. In order to determine the space group, the data set was scaled in space groups $P3$ and $P6$, representing the lowest symmetry trigonal and hexagonal space groups. The data did not scale well in space group $P6$, giving an initial R_{sym}* before outlier rejection of approximately 46%. The data scaled well, in space groups $P3$ giving an initial R_{sym} before outlier rejection of approximately 14%. Following outlier rejection, the R_{sym} for the data set converged to 6.51%. Analysis of the diffraction pattern [*XPREP* (Sheldrick, 1991)] suggested that the space group was either $P3_121$ or $P3_221$.

A second data set to 2.15 Å resolution (94.2% complete, average redundancy 5:1) was obtained from 47 oscillation images collected on an R-AXIS II. Following scaling and outlier rejection (*HKL*), the R_{sym} for the data set was 5.4%. Analysis of the three-dimensional diffraction pattern (*bioteX* Laue check) confirmed that the crystals belong to the trigonal space group $P3_121$ (or $P3_221$) with unit-cell constants $a = 48.99$ and $c = 78.49$ Å. Based on the above described unit cell containing one NP molecule (one chain) per asymmetric unit, the Matthews coefficient (Matthews, 1968) is calculated to be 2.7 Å^3 Da^{-1} which corresponds to a solvent content of 54%.

One of the goals of making the des 1–6 protein was to determine if removing the six N-terminal residues, which showed large conformational variability in the previous NP crystal structures, could produce crystals of higher diffraction quality. The marked increase in resolution observed for the des 1–6 NP crystals suggests that the deletion of residues 1–6 has indeed resulted in a more favorable crystal packing environment. Improved lattice contacts may also explain why we were able to crystallize the 'ligand-free' des 1–6 protein while numerous attempts to crystallize the full length 'ligand-free' protein were unsuccessful. The observation of only one NP molecule per crystallographic asymmetric unit (ASU) is also interesting since it represents the first time that NP has been crystallized with a monomer in the ASU. However, based on solution studies (Breslow *et al.*, 1971; Nicolas, Wolff, Camier, Di & Cohen, 1978) and the previous crystal structures of NP–peptide (Chen *et al.*, 1991) and NP–hormone (Rose *et al.*, 1996) complexes which show that NP prefers to associate as dimers, we believe that the crystal structure once solved will show an NP–NP dimer sitting across one of the crystallographic twofold symmetry axes. The structure determination using molecular replacement is in progress.

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* $R_{sym} = \sum |I - \langle I \rangle| / \sum I$.

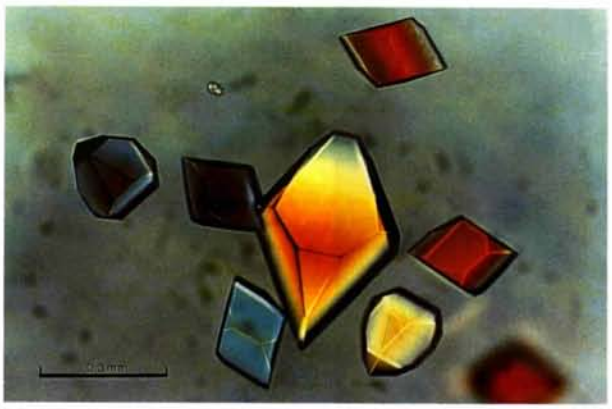

Fig. 2. Crystals of the des 1–6 bovine neurophysin II grown by vapor diffusion, the color results from cross polarization of the crystallization plate.

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