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Crystallization and preliminary analysis of neurolysin

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Neuropeptidases inactivate or modify the activity of peptide neurotransmitters and neurohormones. The neuropeptidase neurolysin acts only on short peptides and accepts a variety of cleavage-site sequences. Structures of the enzyme and enzyme–substrate complexes will help to determine the mechanisms of substrate selectivity used by this enzyme. Crystals of recombinant neurolysin have been grown in the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 157.8, b = 88.0, c = 58.4 Å. Data have been collected to 2.3 Å at 110 K with observed diffraction to 1.8 Å. Circular dichroism measurements suggest that the enzyme is primarily α-helical, with little β-strand secondary structure. Sequence-based secondary-structure prediction supports this conclusion.

1. Introduction

Over 100 biologically active peptides, which range in size from two to 40 residues, have now been identified (Eipper *et al.*, 1986; Konkoy & Davis, 1996). These peptides serve as neurotransmitters and neuromodulators in the central nervous system and as neurohormones in the periphery. The signals from these neuropeptides are terminated or altered through hydrolysis by a collection of enzymes known as neuropeptidases (McKelvy & Blumberg, 1986; Turner, 1987; Littlewood *et al.*, 1988; Checler, 1993; Cusuhai *et al.*, 1998).

Some neuropeptidases are active only on short (<25 residues) substrates and a few of these enzymes cleave at a limited set of sites that are nonetheless diverse in sequence. One such neuropeptidase is neurolysin (Checler *et al.*, 1995; Barrett *et al.*, 1995; Csuhai *et al.*, 1998), which acts as a monomer of 78 kDa (681 residues) and belongs to the M3 family of zinc metallopeptidases (Barrett *et al.*, 1998). The mechanisms by which neurolysin is restricted to short peptides and is able to accommodate a variety of cleavage-site sequences are unknown. Prolyl oligopeptidase (81 kDa, 710 residues), a serine peptidase, is also a neuropeptidase restricted to short peptides. The crystal structure of this enzyme (Fulop *et al.*, 1998) suggests that substrates can only reach the active site by threading through the center of a β-propeller domain, preventing the cleavage of large proteins. This enzyme has no sequence relationship with neurolysin, however, and the relevance of this mechanism of size limitation to neurolysin is not established. Recently, the crystal structure of the extracellular portion of neprilysin (85 kDa, 750 residues), a neuropeptidase of the zinc metallopeptidase class, was reported (Oefner *et al.*, 2000). This enzyme, which is a member of the M13 family of metallopeptidases, also bears no sequence relationship to neurolysin (except for the zinc-binding motif), nor is it apparent from its crystal structure how longer peptides are excluded.

The nine identified members of the M3 family of metallopeptidases (Barrett *et al.*, 1998) all possess a zinc-binding motif consisting of the sequence HE*xx*H, which is found in a number of other families of zinc metalloproteases. The two histidine residues of the motif coordinate the zinc ion; the glutamate residue hydrogen bonds with the catalytic water molecule. Besides this motif, however, there are no other sequence relationships with known proteins. Within the family, all the members are close in size and show a high degree of identity throughout the sequence, with only small gaps or insertions.

Crystallographic studies of neurolysin have been initiated in order to define how the enzyme is restricted to short peptides and how it is able to recognize a variety of cleavage-site sequences. We report here the crystallization of neurolysin and the preliminary characterization of these crystals.

2. Materials and methods

2.1. Expression and purification

Recombinant rat neurolysin was produced in *Escherichia coli* using a pBAD/His vector system (Invitrogen). Bacterial culture was carried out at 310 K in a 10 l fermentor (New Brunswick) using RM media (Invitrogen).

Expression was induced at the araBAD promoter by the addition of arabinose to a final concentration of 1 m*M* and the cells were maintained in the fermentor for 6 h after induction. The soluble protein was produced with an N-terminal polyhistidine metal-binding sequence which permitted purification by affinity chromatography on nickel-chelating resin (Qiagen). Cells were suspended in 50 m*M* sodium phosphate buffer pH 8.0 and lysed by two passes through a French press. After clearing cell debris by centrifugation at 10 000*g* for 60 min, 0.3 ml of affinity resin were added per liter of original culture and the mixture was incubated for 2 h at 277 K. The resin was washed four times in the phosphate buffer with increasing NaCl concentrations (0.1, 0.5, 0.7, 1.0 *M*). Bound enzyme was then eluted from the resin by removing the polyhistidine tag with enterokinase. 20 units of EnterokinaseMAX (Invitrogen) were added per liter of original culture and the mixture was allowed to shake at 277 K for 72 h. The resin was removed from the released protein by centrifugation and the enterokinase was bound to EK-Away (Invitrogen) resin, which was then separated with a low-speed spin. Some samples were further purified by anion-exchange chromatography on Poros HQ resin (PerSeptive Biosystems), eluting with a NaCl gradient. The purified protein was dialized against 50 m*M* Tris pH 7.4, 100 m*M* NaCl and was concentrated to 0.1 m*M* by ultrafiltration. Concentrations were determined by UV spectroscopy or calorimetric assay, which were calibrated by quantitative amino-acid analysis.

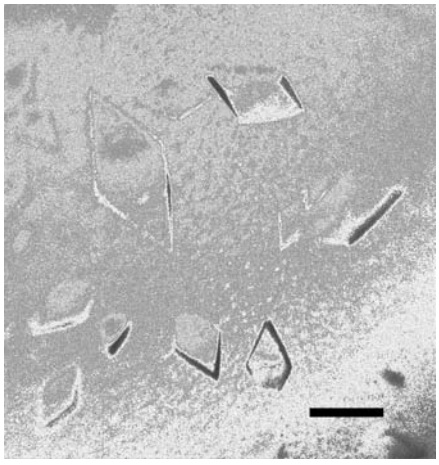

Figure 1
Photomicrograph of neurolysin crystals. The bar indicates 0.05 mm.

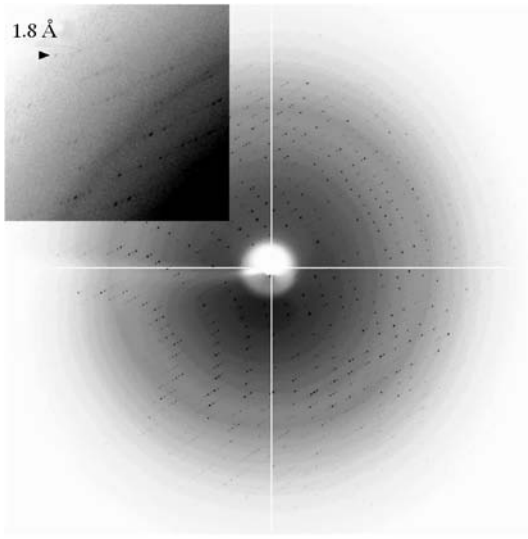

Figure 2
Diffraction pattern from a crystal of neurolysin. Data were collected on station 14-BM-C of the BioCARS beamline at the Advanced Photon Source, Argonne National Laboratory using an ADSC Q4 CCD detector. The crystal was maintained at 110 K and the exposure time was 30 s for the 1° oscillation shown. The inset shows a magnified view of the upper right corner of the pattern. Reflections at 1.8 Å resolution are indicated.

2.2. Crystallization

Crystals of neurolysin were initially obtained by hanging-drop vapor diffusion using a standard matrix search (McPherson, 1982) with commercially available reagent kits (Hampton Research). Conditions were refined to produce high-quality crystals (Fig. 1), which were grown by mixing 2 µl of the protein solution with 2 µl of well solution containing 9–11%(*w*/*v*) polyethylene glycol 8000, 0.1 *M* cacodylate pH 6.5, 0.1 *M* $MgCl_2$, 0.1 m*M* $ZnCl_2$ and 1 m*M* 2-mercaptoethanol.

2.3. Data collection

Crystals were characterized on an R-AXIS IV detector mounted on a Rigaku rotating-anode generator (λ = 1.5418 Å, 46 kV, 92 mA). Initial characterization was performed at 278 K, but all subsequent data collection was carried out at 108 K. The crystals proved sensitive to all cryoprotectants normally used for flash-cooling (Rodgers, 1994). However, a brief exposure (<10 s) to 20% polyethylene glycol 400 in the normal crystallization solution permitted successful cooling by plunging the loop-mounted crystals into liquid nitrogen (Rodgers, 1997). Native data sets were collected from flash-cooled crystals on the R-AXIS IV detector and on a CCD detector (ADSC Q4) at station 14-BM-C of the BioCARS sector at the Advanced Photon Source, Argonne National Laboratory. All data reduction was performed with the *HKL* package (Otwinowski & Minor, 1997)

Table 1
Statistics for native data.

Resolution (Å)	Completeness (%)	Average redundancy	$I > 3\sigma$ (%)	R_{shell}
4.93	95.6	3.49	98.0	0.031
3.93	99.9	3.90	98.5	0.028
3.43	99.9	3.94	98.0	0.032
3.12	99.8	3.96	97.0	0.039
2.90	99.9	3.96	94.9	0.053
2.73	100.0	3.96	92.4	0.066
2.59	100.0	3.96	90.5	0.088
2.48	99.9	3.95	87.0	0.108
2.38	100.0	3.94	84.8	0.126
2.30	95.5	3.66	82.3	0.156
Overall	99.0	3.87	92.4	0.036

† R_{shell} for symmetry-related reflections is defined as $R_{shell} = \sum_{ij}|I_{ij} - \langle I_i\rangle|/\sum I|I_{ij}|$, where I_{ij} is the intensity of an individual measurement and $\langle I_i\rangle$ is the mean value of all measurements for each independent reflection.

Circular dichroism measurements were made on a Jasco 710 spectropolarimeter at room temperature. Neurolysin was at a concentration of 68 n*M* in a solution containing 50 m*M* sodium phosphate pH 7.0. The protein was placed in a 1 cm path length quartz cell and 50 spectra in the wavelength range 195–240 nm were collected and averaged. Data were analyzed according to Andrade *et al.* (1993).

3. Results and analysis

Crystals of neurolysin obtained from protein produced in *E. coli* using the pBAD overexpression system are shown in Fig. 1. The crystals have either a diamond or truncated diamond shape. They are typically 0.15 mm in the longest dimension and 0.02 mm thick. Crystals appear and grow to full size in 2–4 d.

An N-terminal polyhistidine sequence used for purification was removed from the expressed neurolysin by enterokinase treatment. Crystals were first obtained with a construct that did not allow removal of the polyhistidine tag. These crystals suffered from varying degrees of packing disorder, with most (but not all) crystals unsuitable for data collection. The packing disorder was not encountered after removal of the polyhistidine tag.

Cryo-cooled neurolysin crystals typically diffract to 2.8 Å on a laboratory X-ray source. Native crystals diffract to beyond 2.0 Å (Fig. 2) on station 14-BM-C of the BioCARS facility at the Advanced Photon

Source. Statistics for data sets collected on the laboratory source and at the synchrotron facility are given in Table 1. Data were collected to only 2.3 Å at the station 14-BM-C because time restrictions prevented repositioning of the detector. The space group of the crystals is $P2_12_12$, with unit-cell parameters a = 157.8, b = 88.0, c = 58.4 Å. One molecule of neurolysin in the asymmetric unit gives a solvent content of approximately 50% and is therefore the only possibility. Rotation functions peformed using the *CNS* package (Brunger *et al.*, 1998) with thermolysin (34 kDa), a well studied metallopeptidase from the M1 family, or astacin, another well studied metallopeptidase, as search models gave no solutions above background. Neprilysin coodinates are not yet available.

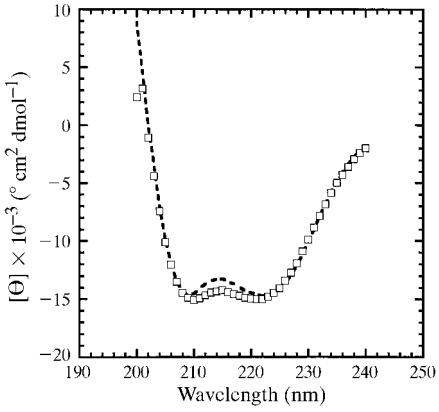

Figure 3
Plot of molar ellipticity against wavelength for a solution of recombinant neurolysin. The spectrum is an average of 50 spectra taken on a Jasco 710 spectrapolarimeter at room temperature. Open boxes indicate data points taken at nanometer intervals and the dashed line is the best fit to the observations by the algorithm used to estimate secondary-structure content.

Results from circular dichroism measurements indicate that neurolysin contains a high proportion of α-helix. A circular dichroism spectrum in the wavelength range 200–240 nm is shown in Fig. 3. Standard analysis of this spectrum based on calibration against a database of proteins with known three-dimensional structure (Andrade *et al.*, 1993) indicates a helical content of 60%, while the β-strand content is estimated at only 7%. The strong peak at 5.4 Å in a plot of average intensity *versus* resolution for the X-ray diffraction data (not shown) also indicates a high content of α-helical secondary structure. In addition, analysis of the sequence by the PredictProtein server (Rost, 1996) predicts a 59% helical content and only 6% β-strand.

4. Conclusions

Neurolysin has been crystallized in a form suitable for high-resolution analysis. These crystals have been used to collect a native data set to 2.3 Å and efforts towards structure determination are under way. We have produced selenomethionine-substituted protein and have possible derivatization in the presence of methylmercury chloride. Both the X-ray data and CD measurements indicate that neurolysin is principally α-helical. Prolyl oligopeptidase, another neuropeptidase, contains a large β-propeller motif that serves to exclude large protein substrates. It seems unlikely that neurolysin contains such a motif, given the low β-strand content indicated by circular dichroism analysis and sequence-based prediction.

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References

Andrade, M. A., Chacon, P., Merelo, J. J. & Moran, F. (1993). *Protein Eng.* **6**, 383–390.
Barrett, A. J., Brown, M. A., Dando, P. M., Knight, C. G., McKie, N., Rawlings, N. D. & Serizawa, A. (1995). *Methods Enzymol.* **248**, 529–556.
Barrett, A. J., Rawlings, N. D. & Woessner, J. F. (1998). *Handbook of Proteolytic Enzymes.* London: Academic Press.
Brunger, A. T., Adams, P. D., Clore, G. M., Delano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J. N., Pannu, N. S., Reed, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.
Checler, F. (1993). In *Methods in Neurotransmitter and Neuropeptide Research*, Part 2, edited by T. Nagatsu, H. Parvez, M. Naoi & S. Parvez. Amsterdam: Elsevier.
Checler, F., Barelli, H., Dauch, P., Dive, V., Vincent, B. & Vincent, J. P. (1995). *Methods Enzymol.* **248**, 593–614.
Csuhai, E., Safavi, A., Thompson, M. W. & Hersh, L. B. (1998). In *Proteolytic and Cellular Mechanisms in Prohormone and Neuropeptide Precursor Processing*, edited by V. Hook. Heidelberg: Springer–Verlag.
Eipper, B. A., Mains, R. E. & Herbert, E. (1986). *Trends Neurosci.* **9**, 463–468.
Fulop, V., Bocskei, Z. & Polgar, L. (1998). *Cell*, **94**, 161–170.
Konkoy, C. S & Davis, T. P. (1996). *Trends Pharmacol. Sci.* **17**, 288–294.
Littlewood, G. M., Iversen, L. L. & Turner, A. J. (1988). *Neurochem. Int.* **12**, 383–389.
McKelvy, J. F. & Blumberg, S. (1986). *Annu. Rev. Neurosci.* **9**, 415–434.
McPherson, A. (1982). *Preparation and Analysis of Protein Crystals.* New York: John Wiley.
Oefner, C., D'Arcy, A., Hennig, M., Winkler, F. K. & Dale, G. E. (2000). *J. Mol. Biol.* **296**, 341–349.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rodgers, D. W. (1994). *Structure*, **2**, 1135–1140.
Rodgers, D. W. (1997). *Methods Enzymol.* **276**, 183–203.
Rost, B. (1996). *Methods Enzymol.* **266**, 525–539.
Turner, A. J. (1987). Editor. *Neuropeptides and their Peptidases.* New York: Ellis Horwood.