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Crystallization and preliminary crystallographic characterization of three peptidic inhibitors in complex with α-thrombin

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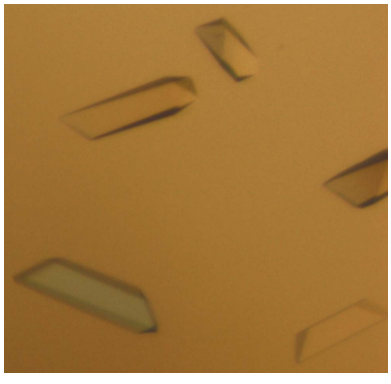

The serine protease thrombin plays a major role in thrombosis and haemostasis. This has driven interest in thrombin inhibitors as potential antithrombotic drugs. Here, the crystallization and preliminary crystallographic analysis of human α-thrombin in complex with three noncovalent peptide inhibitors of the general sequence D-Phe-Pro-D-Arg-P1′-$CONH_2$ are reported. The crystals belonged to the orthorhombic space group $P2_12_12_1$ and diffracted to beyond 1.3 Å resolution.

1. Introduction

Physical damage to the vascular system triggers the coagulation cascade, a series of activation reactions of precursor proteins and regulating factors circulating in the blood that ultimately prevent blood loss without compromising blood flow through either the uninjured or the damaged vessels.

α-Thrombin (EC 3.4.21.5), the last proteolytic enzyme of the coagulation cascade, arises from the cleavage of prothrombin by serine protease factor Xa in the context of the prothrombinase complex. Given its multiple roles in blood clotting, thrombin plays a major part in haemostasis (Stubbs & Bode, 1993), where it catalyses the conversion of soluble plasma fibrinogen to insoluble fibrin (Doolittle, 1984), which then forms the scaffold for the growth of the thrombus, but also cleaves and activates coagulation factors V, VIII, XI and XIII (Gailani & Broze, 1991; Muszbek *et al.*, 1999; Kane & Davie, 1988) and stimulates platelet aggregation through activation of platelet receptors (Vu *et al.*, 1991). The entire system must be strictly regulated to restrict clotting to the site of injury. As it diffuses away from the site of damage, thrombin associates with the endothelial surface receptor thrombomodulin, becoming an anticoagulant factor by activating protein C (Esmon, 1995).

Thrombosis-related disorders such as myocardial infarction, stroke and pulmonary embolism remain major causes of mortality and morbidity worldwide (Rauch *et al.*, 2001). This has driven interest in thrombin inhibitors as potential antithrombotic drugs (Paoli *et al.*, 2005). However, to date the discovery of safe, selective and orally available inhibitors has proven difficult to accomplish, therefore limiting their therapeutic use (Hauptmann, 2002; Kikelj, 2003).

In order to acquire and digest their blood meal, haematophagous animals must counteract the coagulation system of their hosts. This is often achieved by resorting to specific and highly selective thrombin inhibitors, of which only six have been structurally characterized to date (Macedo-Ribeiro *et al.*, 2008; Richardson *et al.*, 2000; Fuentes-Prior *et al.*, 1997; van de Locht *et al.*, 1995, 1996; Grütter *et al.*, 1990), revealing six distinct modes of inhibition. Recently, a novel and sequence-unrelated thrombin inhibitor has been identified in the hard tick *Haemophysalis longicornis* (Iwanaga *et al.*, 2003; Nakajima *et al.*, 2006). The mode of inhibition of this cysteine-less molecule, termed chimadanin, is expected to provide essential clues for the design of improved synthetic anticoagulants.

Several small-molecule synthetic thrombin inhibitors have been described (Hauptmann, 2002; Kikelj, 2003) that mimic the binding

Table 1
Data-collection statistics.

Values in parentheses are for the outermost shell.

Thrombin complex	Unliganded	p3	p4	p6
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)				
a	57.5	51.6	51.8	57.5
b	73.0	76.5	77.1	72.7
c	83.0	83.3	83.4	83.1
Resolution range (Å)	72.93–1.55 (1.63–1.55)	51.6–1.47 (1.55–1.47)	35.0–1.86 (1.96–1.86)	47.3–1.28 (1.35–1.28)
Reflections measured	500945	495888	166128	1002793
Unique reflections	50373	56417	28722	87023
Completeness (%)	98.1 (96.6)	100.0 (100.0)	99.9 (100)	96.7 (93.4)
Multiplicity	9.9 (7.6)	8.8 (5.8)	5.8 (5.6)	11.5 (5.8)
Average mosaicity (°)	0.20	0.39	0.88	0.15
R_{merge}†	0.067 (0.349)	0.061 (0.286)	0.072 (0.181)	0.048 (0.183)
$R_{p.i.m.}$‡	0.020 (0.135)	0.018 (0.130)	0.018 (0.130)	0.011 (0.083)
$\langle I/\sigma(I)\rangle$	5.5 (2.2)	8.0 (2.6)	6.8 (4.1)	8.7 (4.1)
Monomers per asymmetric unit	1	1	1	1
Matthews coefficient (Å^3 Da^{-1})	2.36	2.25	2.28	2.38
Solvent content (%)	47.9	45.3	46.1	48.2

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity of multiple observations of symmetry-related reflections. ‡ $R_{p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity of multiple observations of symmetry-related reflections.

modes of natural peptide ligands (for reviews on the inhibition modes of some natural anticoagulants, see Corral-Rodriguez *et al.*, 2009, 2010). They can be broadly divided into two major groups: covalent electrophilic inhibitors (*e.g.* PPACK), which form a covalent tetrahedral intermediate upon nucleophilic attack of the activated carbonyl group of the P1 residue by Ser195 OG from the catalytic triad of the enzyme (Bode *et al.*, 1989), and noncovalent inhibitors that interact with thrombin solely through hydrophobic and polar interactions.

Previous isothermal titration calorimetry (ITC) and structure–activity relationship (SAR) studies towards thrombin for peptides with the general sequence D-Phe-Pro-D-Arg-P1′-$CONH_2$ (Clement *et al.*, 2009; Clement & Philipp, 2006) identified these compounds as reversible thrombin inhibitors and potent anticoagulants. Furthermore, at concentrations of 5–15 times their K_i (in an *in vitro* inhibition assay) these peptides were also able to completely inhibit thrombin-activated platelet aggregation (Clement *et al.*, 2007). Here, we report the crystallization and preliminary crystallographic analysis of human α-thrombin in complex with three of these noncovalent peptide inhibitors with L-isoleucine (p3), L-cysteine (p4) or D-threonine (p6) at the P1′ position (Clement & Philipp, 2006).

2. Materials and methods

2.1. Purification of recombinant chimadanin

Chimadanin (GenBank accession No. BAE00177) was expressed in *Escherichia coli* BL21 CodonPlus (Stratagene) transformed with pET44-chimadanin (Iwanaga *et al.*, 2003; Nakajima *et al.*, 2006). Cells were grown in LB medium supplemented with 50 µl ml^{-1} ampicillin and 30 µl ml^{-1} chloramphenicol and were induced with IPTG (final concentration 1 m*M*) at mid-exponential growth (OD_{600} = 0.5). After 3 h at 310 K, the cells were harvested by centrifugation at 5000*g* at 277 K, resuspended in 20 m*M* bis-tris pH 6.3 (buffer *A*) supplemented with 50 µl ml^{-1} lysozyme, 10 µl ml^{-1} DNase and 5 m*M* $MgCl_2$ and lysed by freezing and thawing. Cell debris was removed by centrifugation at 15 000*g* for 30 min at 277 K. The crude protein extract was filtered through a 0.22 µm pore low-protein-binding membrane and loaded onto a 5 ml Bio-Scale Mini UNOsphere Q Cartridge (Bio-Rad) pre-equilibrated with buffer *A*. Elution was performed with a linear NaCl gradient (0–0.4 *M*) in buffer *A*. Fractions were analysed by SDS–PAGE and probed for their ability to inhibit thrombin in an *in vitro* activity assay. Chimadanin-containing fractions were pooled, diluted tenfold in buffer *A* and loaded onto a UNO Q-1 column (Bio-Rad) pre-equilibrated with buffer *A*. The sample was eluted with a linear NaCl gradient (0–0.18 *M*) in buffer *A*. Fractions containing purified chimadanin were pooled and concentrated to 17 mg ml^{-1} on a centrifugal concentration device with a 3 kDa molecular-weight cutoff membrane.

2.2. Preparation of thrombin–chimadanin complexes

For complex preparation, 5 mg human α-thrombin (Haematologic Technologies, USA) was mixed with a 10% molar excess of purified chimadanin and incubated on ice for 1 h. The complex was separated from the isolated components by size-exclusion chromatography on a Superdex 75 HR10/30 column (GE Healthcare) equilibrated in 20 m*M* Tris pH 8.5, 150 m*M* NaCl. The complex-containing fractions were pooled and concentrated to 7.2 mg ml^{-1} on a centrifugal concentration device with a 10 kDa molecular-weight cutoff membrane.

2.3. Crystallization of human α-thrombin

Initial crystallization conditions were screened at 293 K using the sitting-drop method with commercial sparse-matrix crystallization screens. The drops contained identical volumes (2 µl) of complex solution (at 7.2 mg ml^{-1}) and precipitant solution and were equilibrated against a 300 µl reservoir. Crystals were obtained after 2 d using 50 m*M* Tris pH 8.5, 50 m*M* Bicine, 30 m*M* sodium fluoride, 30 m*M* sodium bromide, 30 m*M* sodium iodide supplemented with 11.5%(*v*/*v*) MPD, 11.5%(*w*/*v*) PEG 1000 and 11.5%(*w*/*v*) PEG 3350 as precipitant.

The calculated Matthews coefficients considering the presence of one molecule of the protease (2.36 Å^3 Da^{-1} for 36.7 kDa) or a single equimolar complex (1.97 Å^3 Da^{-1} for 44.2 kDa) in the asymmetric unit raised doubts about the actual composition of the crystals. It was

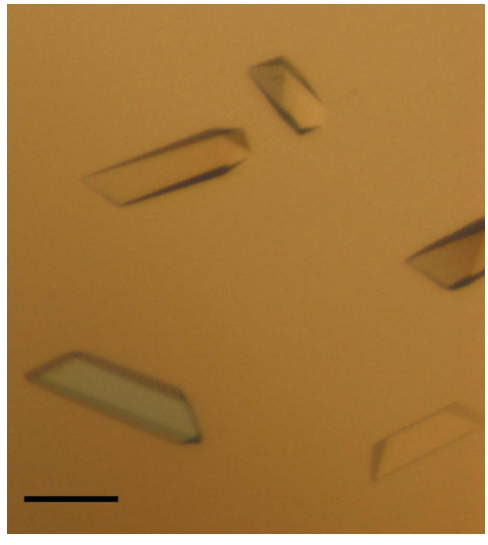

Figure 1
Single crystals of human α-thrombin belonging to the orthorhombic space group $P2_12_12_1$. The scale bar is 100 µm in length.

later verified that only thrombin had crystallized, with no evidence for the presence of chimadanin in the electron density.

2.4. Preparation of complexes of α-thrombin with synthetic inhibitors

The α-thrombin crystals obtained were subsequently used to prepare complexes with three synthetic inhibitors of general sequence D-Phe-Pro-D-Arg-P1′-$CONH_2$ with L-isoleucine (p3), L-cysteine (p4) or D-threonine (p6) at the P1′ position.

Thrombin complexes were prepared by soaking thrombin crystals at 293 K in a pre-equilibrated (24 h) drop consisting of 2 µl crystallization solution and 1 µl of a 10 m*M* solution of the inhibitor (p3, p4 or p6) in water. For all inhibitors, soaking times of 3 and 48 h were tested. Although the longer soaking time with peptides p3 and p4 did not seem to negatively affect the crystals, they were severely damaged upon 48 h soaking with peptide p6. Therefore, the crystals used for data collection were soaked with peptides p3 or p4 for 48 h and with peptide p6 for 3 h. The crystallization solution contained 38.5% MPD/PEG 1000/PEG 3350 for p3, 36.5% MPD/PEG 1000/PEG 3350

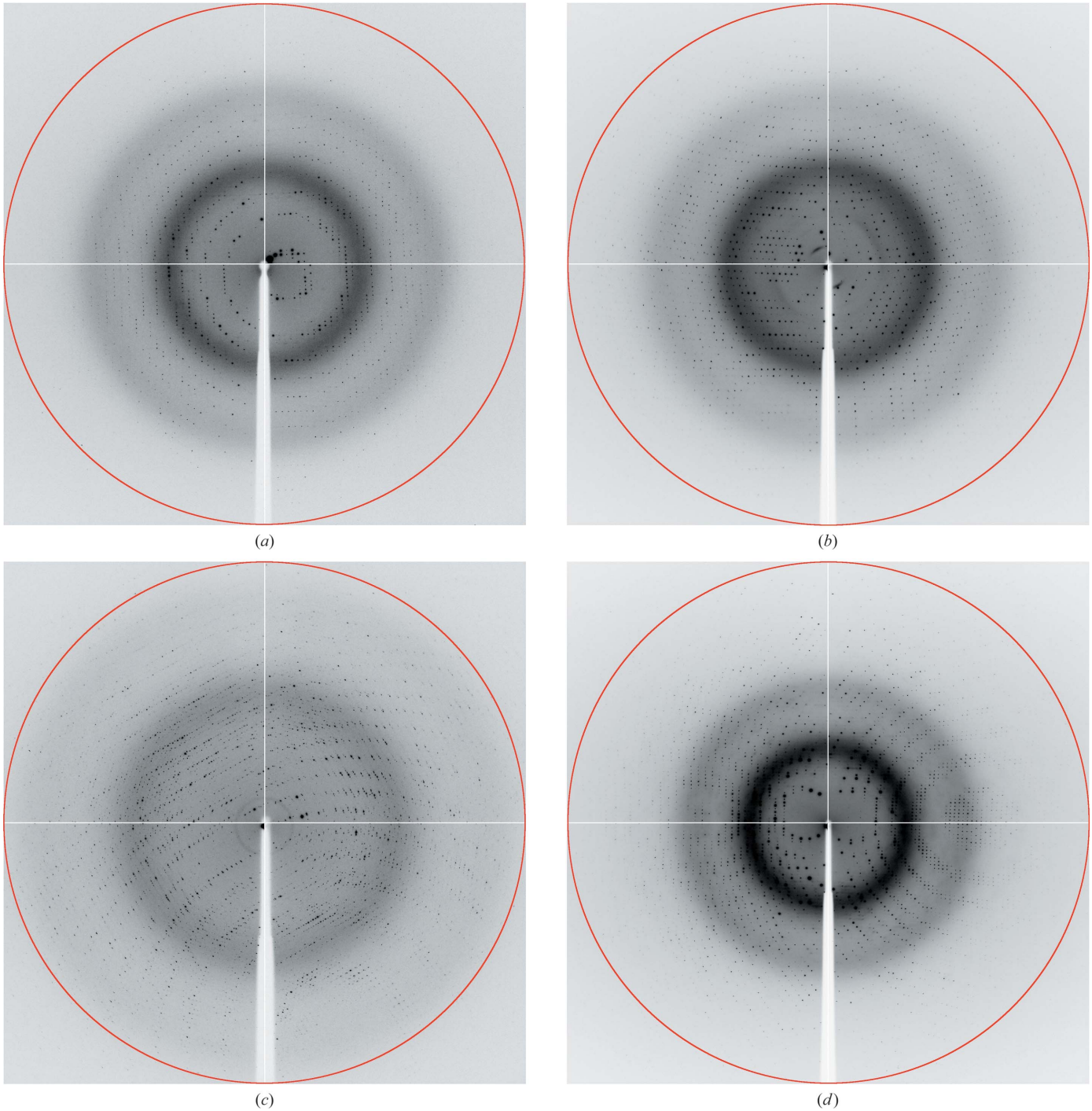

Figure 2
X-ray diffraction images of crystals of unliganded thrombin (*a*) and of thrombin in complex with p3 (*b*), p4 (*c*) and p6 (*d*). The red circles correspond to resolution limits of 1.55 Å (*a*), 1.49 Å (*b*), 1.88 Å (*c*) and 1.29 Å (*d*).

for p4 or 34.5% MPD/PEG 1000/PEG 3350 for p6. The crystals were flash-cooled by plunging them into liquid nitrogen.

2.5. Data collection and processing

Diffraction data were collected using an ADSC Q210 detector on beamline ID14-EH1 at the European Synchrotron Radiation Facility (ESRF; Grenoble, France). For each complex a single cryocooled crystal was used and all data sets were measured in 1° oscillation steps.

For the thrombin–p3 complex three data sets were collected over a range of 150° with crystal-to-detector distances of 297.5, 227.3 and 141.3 mm and 1, 3 and 6 s exposures per frame, respectively.

For the thrombin–p4 complex a single data set was collected over a range of 150° with a 191.1 mm crystal-to-detector distance and 3 s exposure per frame.

For the thrombin–p6 complex two data sets were collected over a range of 270° with crystal-to-detector distances of 262.6 and 178.9 mm and 0.2 and 0.6 s exposures per frame, respectively. A third, high-resolution data set was collected over a range of 300° with a 114.8 mm crystal-to-detector distance and 6 s exposure per frame.

Diffraction data sets were processed with *MOSFLM* (Leslie, 1992) and scaled using *SCALA* (Evans, 2006) from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Data-collection statistics are summarized in Table 1.

2.6. Structure solution

The structure of unliganded human α-thrombin was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2007) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) using the coordinates of the serine protease (PDB entry 1vzq; Schärer *et al.*, 2004) as the search model. A partially refined model of unliganded human α-thrombin was subsequently used as the search model in the structural determination of the thrombin–p3, thrombin–p4 and thrombin–p6 complexes.

3. Results and discussion

3.1. Crystallization of thrombin–inhibitor complexes

Crystallization experiments were performed with an *in vitro* complex of human α-thrombin and recombinant chimadanin, a specific macromolecular thrombin inhibitor that was first isolated from the salivary gland of fed *H. longicornis* (hard tick; Iwanaga *et al.*, 2003; Nakajima *et al.*, 2006). Surprisingly, the crystals obtained only contained the unliganded protease, indicating that complex formation was not favoured under the crystallization conditions used, although thrombin itself was stabilized. The crystals grew to maximum dimensions of 50 × 185 × 50 µm (Fig. 1), belonged to the orthorhombic space group $P2_12_12_1$ and diffracted to beyond 1.6 Å resolution on a synchrotron source (Fig. 2; Table 1), which prompted us to use them in the preparation of complexes of thrombin with small synthetic inhibitors. Although several orthorhombic thrombin crystals with similar unit-cell parameters have been reported previously, this is the first report of the crystallization of non-mutant unliganded human thrombin in this setting.

For complex preparation, human α-thrombin crystals were incubated in solutions of synthetic peptides with the general formula D-Phe-Pro-D-Arg-Xaa-$CONH_2$, where Xaa is either Ile (p3), Cys (p4) or D-Thr (p6) (Clement & Philipp, 2006; Clement *et al.*, 2007). The crystals of the resulting complexes diffracted X-rays to 1.47 Å (p3), 1.86 Å (p4) and 1.28 Å (p6) resolution (Fig. 2). The data-collection and processing statistics are summarized in Table 1.

3.2. Structure solution

The molecular coordinates of the light chain and heavy chain of human α-thrombin from PDB entry 1vzq (Schärer *et al.*, 2004) were used as a search model to solve the structure of the unliganded protease by molecular replacement. The model was subjected to alternating cycles of manual building with *Coot* (Emsley *et al.*, 2010) and refinement with *PHENIX* (Adams *et al.*, 2010) until an *R* factor of approximately 0.20 was reached. The coordinates of this partially refined model were then used as a search model to solve the structures of the three thrombin–inhibitor complexes by molecular replacement. The initial electron-density difference maps showed interpretable density for all inhibitors. The three-dimensional models are currently under refinement.

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