Stabilization of porcine pancreatic elastase crystals by glutaraldehyde cross-linking

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Elastase is a serine protease from the chymotrypsin family of enzymes with the ability to degrade elastin, an important component of connective tissues. Excessive elastin proteolysis leads to a number of pathological diseases. Porcine pancreatic elastase (PPE) is often used for drug development as a model for human leukocyte elastase (HLE), with which it shares high sequence identity. Crystals of PPE were grown overnight using sodium sulfate and sodium acetate at acidic pH. Cross-linking the crystals with glutaraldehyde was needed to resist the soaking procedure with a diethyl *N*-(methyl)pyridinyl-substituted oxo- β -lactam inhibitor. Crystals of PPE bound to the inhibitor belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 51.0$, $b = 58.3$, $c = 74.9$ Å, and diffracted to 1.8 Å resolution using an in-house X-ray source.

1. Introduction

Human neutrophil elastase (HNE; EC 3.4.21.37), also known as human leukocyte elastase (HLE), is a serine protease that plays a major role during inflammation, when massive migration of neutrophils to the lungs and the subsequent release of proteolytic enzymes can cause several pulmonary dysfunctions (Korkmaz *et al.*, 2010). Elastases are proteolytic enzymes that break down elastin and collagen, which determine the mechanical properties of connective tissues (Bieth & Wermuth, 1973). Inflammatory diseases such as adult respiratory distress syndrome (ARDS), chronic bronchitis, chronic obstructive pulmonary disease (COPD; Donnelly & Rogers, 2003), pulmonary hypertension (Cowan *et al.*, 2000) and rheumatoid arthritis (Lucas *et al.*, 2013) are the consequence of an imbalance between HNE and its endogenous inhibitors, such as α_1 -antitrypsin and secretory leukocyte proteinase inhibitor (Barnes, 2000).

Porcine pancreatic elastase (PPE; EC 3.4.21.36) has been used for several years as a valid structural model for HNE (Wilmouth *et al.*, 1999; Bode *et al.*, 1989) and shares the same catalytic triad of His–Ser–Asp residues. With regard to the pressing need for low-molecular-weight inhibitors of elastases (Bernstein *et al.*, 1994; Malhotra *et al.*, 2006), β -lactam-based molecules appear to be promising candidates (Veinberg *et al.*, 2013) that have been shown to act as potent inhibitors of serine proteases (Leung *et al.*, 2000; Konaklieva & Plotkin, 2004). Previous kinetic and structural characterizations of HNE/PPE with β -lactam inhibitors (Oliveira *et al.*, 2007; Mulchande *et al.*, 2007, 2010) have highlighted the interest of

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the 4-oxo- β -lactam scaffold, which is also under investigation in this study. However, attempts to obtain a PPE-inhibitor complex failed using both co-crystallization (no crystal growth) and soaking (crystal damage). Cross-linking the crystals with glutaraldehyde was successful in allowing the crystals to endure the high concentrations of dimethyl sulfoxide required for inhibitor solubilization. Glutaraldehyde cross-linking was first described by Quiocho & Richards (1964) and has since been used in various applications, namely to enhance crystal stability (Glumoff *et al.*, 1996; Wang *et al.*, 2003; Cohen-Hadar *et al.*, 2006; Wine *et al.*, 2007), to prevent lattice damage upon crycooling (Haas & Rossmann, 1970), to increase crystal diffraction quality (Vetting *et al.*, 2009; Duan & Ye, 2009), to permit solvent exchange (Lusty, 1999) and to allow ligand soaking (Andersen *et al.*, 2009). Furthermore, this technique has also been employed to produce catalytically active and insoluble chloroperoxidase crystals (Ayala *et al.*, 2002), to obtain highly robust and active lipase crystals (Rajan & Abraham, 2008) and to study urea-driven denaturation using ribonuclease S crystals (Ratnaparkhi & Varadarajan, 1999). More recently, Shaw Stewart *et al.* (2011) described the use of glutaraldehyde cross-linking for the stabilization of seed crystals for random microseed matrix screening. To the best of our knowledge, this technique has not yet been described for PPE crystals. Using this methodology, crystals of PPE in complex with a novel activity-based substituted oxo- β -lactam inhibitor of HNE (JM102) were obtained. The inhibitor is expected to show ring opening upon the nucleophilic attack of Ser195. This result paves the way for the use of JM102 as a starting point for the design of activity-based probes (ABPs) to track protease activity; ABPs targeting HNE may lead to the development of a potent methodology for the quantification of HNE activity *in vivo* (Kasperkiewicz *et al.*, 2014; Sanman & Bogyo, 2014).

2. Materials and methods

2.1. Crystallization, cross-linking and soaking experiments

A concentrated stock solution (14 mM) of JM102, a diethyl *N*-(methyl)pyridinyl-substituted oxo- β -lactam, was prepared in 100%(v/v) dimethyl sulfoxide (DMSO; Fig. 1; Mulchande *et al.*, 2010). Elastase from porcine pancreas was purchased from SERVA Electrophoresis GmbH, Heidelberg, Germany

Figure 1

Structure of the diethyl *N*-(methyl)pyridinyl-substituted oxo- β -lactam inhibitor JM102.

Table 1

Crystallization conditions and experimental setup.

Method	Vapour diffusion (hanging drop)
Plate type	24-well Linbro plate (Crystalgene)
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	40
Buffer composition of protein solution	Double-distilled water
Composition of reservoir solution	200 mM sodium sulfate, 100 mM sodium acetate pH 5.1
Volume (μ l) and drop ratio	4 (1:1 protein:precipitant)
Volume of reservoir (μ l)	500

(catalogue No. 20929) and was used without further purification. The protein (25.9 kDa) was dissolved in double-distilled water to a concentration of 40 mg ml⁻¹. The initial crystallization conditions were based on previous reports (Shotton *et al.*, 1968; Weiss *et al.*, 2002; Würtele *et al.*, 2000). 2 μ l protein solution was mixed with 2 μ l reservoir solution consisting of 200 mM sodium sulfate, 100 mM sodium acetate pH 5.1 and was equilibrated against 500 μ l reservoir solution. Crystallization droplets were set up at 293 K by the hanging-drop vapour-diffusion method in 24-well Linbro plates (Crystalgene).

For co-crystallization experiments, PPE was first incubated with JM102 inhibitor dissolved in 100%(v/v) DMSO (up to a fivefold molar excess) for 15–30 min at room temperature. The crystallization conditions were similar to those used for the apoprotein, with various protein:precipitant drop ratios tested at two temperatures: 277 and 293 K. Streak-seeding experiments (Bergfors, 2003) were carried out in hanging drops as described above, which were pre-equilibrated for 1–24 h. A cat whisker was used to touch a single large crystal ($\sim 0.3 \times 0.2 \times 0.2$ mm) and was then consecutively passed through several drops in a smooth motion. The cat whisker was then cleaned with double-distilled water and ethanol.

For soaking trials, 0.4 μ l JM102 solution [dissolved in 100%(v/v) DMSO] was added to a 4 μ l drop containing single PPE crystals. Several tests were performed to dissolve JM102 (a hydrophobic compound) in alcohols, *e.g.* ethanol at various ratios and combined with DMSO. Moreover, DMSO and ethanol at different concentrations and in different combinations were directly added to apo PPE crystals to assess their sensitivity towards these solvents.

Cross-linking experiments with glutaraldehyde were performed based on the protocol of Quiocho & Richards (1964) and the subsequent reports by Morozov & Morozova (1981) and Lusty (1999). Crystals of PPE were exposed to glutaraldehyde vapour by adding 5 μ l of a 50%(v/v) concentrated solution to a bridge in the well plate containing a reservoir volume of 500 μ l of the abovementioned crystallization solution for 4–5 h. Glutaraldehyde cross-linked crystals were then transferred three times between 4 μ l drops of sulfate-free washing solution comprising 5%(v/v) DMSO, 5%(v/v) ethanol, 100 mM sodium acetate pH 5.1 and ultimately into a 4 μ l drop of soaking solution consisting of 10%(v/v) DMSO, 10%(v/v) ethanol, 100 mM sodium acetate pH 5.1. Soakings were further carried out at 273 K for 30–60 min using similar procedures as described above for PPE crystals. A volume of 0.8 μ l JM102 solution at 14 mM in

100%(v/v) DMSO was added to a 4 μ l drop containing single glutaraldehyde cross-linked PPE crystals so that the final DMSO and ethanol concentrations were around 25 and 8%(v/v), respectively. Crystals were cryoprotected using the soaking solution supplemented with 30%(v/v) glycerol. This solution comprised the inhibitor at the same ratio as mentioned above. The crystals were flash-cooled either in a

nitrogen-gas stream or by plunging directly into liquid nitrogen. Crystallization information is summarized in Table 1.

2.2. Data collection and processing

A single crystal was used for data collection at cryogenic temperatures after flash-cooling it in a nitrogen-gas stream at

Figure 2

Porcine pancreatic elastase crystals grown in 200 mM sodium sulfate, 100 mM sodium acetate pH 5.1 (*a–f*), after streak-seeding (*b, e*), incubated with 50% DMSO/ethanol (*c*) or 100% DMSO (*d*), cross-linked with glutaraldehyde (*e, f*) and soaked with JM102 (*f*).

Table 2

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	X8 Proteum
Wavelength (Å)	1.54
Temperature (K)	100
Detector	PLATINUM ¹³⁵ CCD
Crystal-to-detector distance (mm)	60
Rotation range per image (°)	0.5
Exposure time per image (s)	30
Space group	$P2_12_12_1$
<i>a</i> , <i>b</i> , <i>c</i> (Å)	51.0, 58.3, 74.9
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.45
Resolution range (Å)	46.03–1.80 (1.90–1.80)
Total No. of reflections	222913
No. of unique reflections	19425
Completeness (%)	94.0 (67.9)
Multiplicity	10.4 (1.3)
$\langle I/\sigma(I) \rangle$	30.4 (5.2)
R_{merge} (%)	4.76 (14.75)

100 K (Oxford Cryosystems 700). X-ray diffraction images were collected at a wavelength of 1.54 Å using an X8 Proteum X-ray diffractometer with a PLATINUM¹³⁵ CCD detector. The data were indexed, integrated and scaled using the *SC-XRD* software (Bruker). Phasing was performed by molecular replacement with *Phaser* (McCoy *et al.*, 2007) using the coordinates of the PPE–JM54 complex as a search model (PDB entry 2v35; Mulchande *et al.*, 2007) without any solvent or ligand molecules.

2.3. Results and discussion

We have crystallized PPE in 200 mM sodium sulfate, 100 mM sodium acetate pH 5.1 at room temperature. Crystals grew to approximate dimensions of $0.3 \times 0.2 \times 0.2$ mm (Fig. 2a). This is the most commonly used crystallization condition, although others have been reported (Supplementary Table S1).

No crystals were obtained in co-crystallization trials of PPE with the inhibitor JM102, in contrast to previous work on PPE with JM54, another lipophilic compound (Oliveira *et al.*, 2007). The droplets were further subjected to streak-seeding with microseeds of apo PPE crystals using a cat whisker, but this still failed to promote nucleation. These negative results may be owing to interference of the β -lactam inhibitor and/or the DMSO/ethanol mixture, which had to be present at higher than 10% to ensure a molar excess of JM102 inhibitor, in the crystallization process.

We therefore proceeded to soaking experiments with pre-formed PPE crystals. As only one to a few crystals were grown per drop, we used streak-seeding to control the nucleation rate and to obtain a reasonable number of good-quality crystals per drop for soaking trials. A line of smaller single crystals were observed after a few hours, and the best results were achieved when the cat whisker touched the crystal once and was passed through several drops (Fig. 2b). Otherwise, the newly formed crystals were smaller and prone to agglomeration. The addition of JM102 (dissolved in DMSO) to the drops

containing PPE crystals led to its prompt precipitation and induced phase separation in the drop and cracks in the crystals, which slowly started to dissolve. These soaked PPE crystals diffracted poorly (to ≥ 3 Å resolution), so no data were collected. Other combinations of solvents for JM102 were also tried, for example a lower DMSO concentration with the addition of alcohols, but similar damage to the PPE crystals was observed upon JM102 soaking. Furthermore, the effect of the solvents DMSO and ethanol alone (at various concentrations and in various combinations) on apo PPE crystals was also evaluated. The addition of these solvents *per se* is clearly harmful to the protein crystals (Figs. 2c and 2d).

We then decided to cross-link PPE crystals using glutaraldehyde. After 4–5 h of exposure to glutaraldehyde vapour, the crystals became yellowish (Fig. 2e). Cross-linked crystals were soaked with different DMSO/ethanol concentrations and were found to be stable in up to 30% DMSO and 10% ethanol. As sulfate was observed to bind at the active site in some of our previous studies, we removed sodium sulfate from the soaking solution. Cross-linked crystals remaining in the soaking solution consisting of 10%(v/v) DMSO, 10%(v/v) ethanol, 100 mM sodium acetate pH 5.1 for several days showed no visible damage. This is not surprising since it has been described that some glutaraldehyde cross-linked crystals displayed increased internal strength and could even withstand soaking in pure water for several hours (Andersen *et al.*, 2009). Glutaraldehyde cross-linked PPE crystals were then soaked for 30–60 min with a solution containing JM102. Some

Figure 3

X-ray diffraction image. A pattern collected from a single glutaraldehyde cross-linked PPE–JM102 crystal using an in-house X8 Proteum X-ray diffractometer. The image corresponds to a 30 s exposure and 0.5° crystal rotation collected in two passes. The circle highlights the 2.0 Å resolution edge and higher resolution reflections are clearly visible in the top left corner of the detector.

of these drops showed light precipitation around the crystals and phase separation in the outer border of the droplet (Fig. 2f).

X-ray diffraction data were collected in-house using a PPE–JM102 cross-linked crystal that diffracted well (Fig. 3). Initially, a strategy was devised to collect diffraction data to ~ 2 Å resolution. However, during the integration and scaling steps it was decided to increase this limit since the $\langle I/\sigma(I) \rangle$ value in the last resolution shell was high (~ 13 at 2.00 Å). This increment in the high-resolution limit was obtained at the expense of completeness and multiplicity in the last resolution shell. Data-collection and processing statistics are displayed in Table 2. The data set has an overall R_{merge} of 4.76%, is 94.0% complete and displays a mean $\langle I/\sigma(I) \rangle$ of 5.2 in the last resolution shell. Overall, the data-collection strategy could have been slightly optimized, with the crystal showing an ability to diffract to close to 1.6 Å resolution. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 51.0$, $b = 58.3$, $c = 74.9$ Å. Crystals of apo PPE (Weiss *et al.*, 2002; Würtele *et al.*, 2000) and PPE-inhibitor complexes (Oliveira *et al.*, 2007; Wilmouth *et al.*, 1997; Kinoshita *et al.*, 2005; Åy *et al.*, 2003) grown under the same experimental conditions also showed similar unit-cell parameters. There is one protein macromolecule per asymmetric unit, corresponding to a Matthews coefficient (Matthews, 1968) of $2.1 \text{ Å}^3 \text{ Da}^{-1}$ and an approximate solvent content of 41%. The calculated initial map clearly shows electron density for JM102, which is covalently bound to the catalytic Ser195. Further model fitting and refinement are in progress.

3. Conclusions

In the present work, we describe the procedures used to surpass the problems caused by the addition of a hydrophobic compound dissolved in organic solvents, such as DMSO or alcohols, to PPE crystals. Cross-linking the crystals with glutaraldehyde stabilized the crystals, allowing them to be transferred to solutions that differed from the initial crystallization conditions without damaging the crystals. It also permitted a sufficient amount of the lipophilic ligand to remain soluble in the soaking solution without losing the crystal diffraction quality. This work illustrates the successful use of glutaraldehyde to cross-link PPE crystals and further corroborates the usefulness of this technique, which has been known for a long time (Quiocho & Richards, 1964). Our initial electron-density maps revealed successful binding of JM102 to PPE.

4. Related literature

The following references are cited in the Supporting Information for this article: Dementiev *et al.* (2006), Gilliland *et al.* (1994), Hilpert *et al.* (2004), Kinoshita *et al.* (2006) and Tsunemi *et al.* (1996).

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