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Crystallization and X-ray analysis of the Y75N mutant of *Mucor pusillus* pepsin complexed with inhibitor

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Y75N mutant *Mucor pusillus* pepsin has been overexpressed in yeast, purified and cocrystallized with the iodine-containing human renin inhibitor CP-113972 {(2*R*,3*S*)-isopropyl 3-[(L-prolyl-*p*-iodo-L-phenylalanyl-*S*-methyl-cysteinyl)amino-4]-cyclohexyl-2-hydroxybutanoate} for X-ray crystallography. Tetragonal complex crystals with space group $P4_32_12$ were produced by the hanging-drop vapour-diffusion method and diffracted to 3.0 Å. The crystals exhibited unit-cell parameters $a = b = 182.5$, $c = 99.1$ Å and contained four molecules in the asymmetric unit. A 96% complete data set was collected at 298 K using Cu $K\alpha$ X-rays from a rotating-anode generator. Solution of the crystal structure of Y75N mutant *M. pusillus* pepsin is under way by molecular replacement using the molecular coordinates of wild-type *M. pusillus* pepsin as a model.

1. Introduction

Aspartic proteinases are widely distributed and are found not only in fungi, plants and vertebrates, but also in the HIV retrovirus, in which the proteinase is involved in particle maturation. These enzymes are very important both commercially and in medical research and have been implicated in illnesses such as hypertension, malaria, amyloid disease and tumorigenesis (Dhanaraj *et al.*, 1992; Dealwis *et al.*, 1994; Metcalf & Fusek, 1993; Silva *et al.*, 1996). Their inhibitors have not only proven to be important for the treatment of AIDS, but are also considered to be potential therapeutic agents for other major diseases. X-ray crystallographic analysis of these enzymes has revealed that their three-dimensional X-ray crystal structures are similar to each other (Andreeva *et al.*, 1984; James & Sielecki, 1983; Miller *et al.*, 1989; Blundell *et al.*, 1990; Dhanaraj *et al.*, 1992; Aguilar *et al.*, 1997; Yang *et al.*, 1997; Cordeiro *et al.*, 1998). They mainly consist of β-sheet structures comprised of two similar domains, each contributing an aspartic acid residue to form a catalytic dyad that acts to cleave the substrate peptide bond (James *et al.*, 1982; Blundell *et al.*, 1990). A flexible flap region is also located at the entrance of the active site and a tyrosine residue on the flap is highly conserved amongst the aspartic proteinases. This residue, Tyr75 (pepsin numbering), has been shown to be involved in inhibitor binding to the active site.

The aspartic proteinases show marked differences both in catalytic activity as well as substrate specificity when changes are made to the residues around the active site. These have given indications of the basis of the specificity and the pH optimum of this class of enzymes (Fruton, 1976; Hofmann & Hodges, 1982; Mantafounis & Pitts, 1990). To this end, recombinant DNA methods have been used to make defined sequence changes in order to test these hypotheses and a number of engineered mutants of chymosin and *Mucor pusillus* pepsin have been characterized (Suzuki *et al.*, 1989; Mantafounis & Pitts, 1990; Cummings & McCumman, 1986; Strop *et al.*, 1990; Jun-ichi *et al.*, 1991). In this paper, we describe the successful cocrystallization of a mutated form (Y75N) of *M. pusillus* pepsin complexed with an iodine-containing human renin inhibitor and the preliminary X-ray analysis of the complex crystals obtained.

M. pusillus pepsin (EC 3.4.23.6) is a milk-clotting fungal aspartic proteinase. In milk clotting, *M. pusillus* pepsin cleaves milk-protein casein with a similar specificity to mammalian calf chymosin, not only having a high milk-clotting activity but also a low proteolytic activity and yielding high clotting material. Mutation of Tyr75 to Asn reduces the non-specific proteolytic activity, leading to a large relative enhancement of the specific clotting activity. X-ray crystal structure analysis of mutant Y75N complexed with the inhibitor CP-113972 will provide information that will explain the observed modulation of the properties of the mutant *M. pusillus* pepsin.

2. Materials and methods

2.1. Purification

As described elsewhere (Jun-ichi *et al.*, 1991), both native and mutated *M. pusillus* pepsin were correctly processed and secreted in large quantities from *Saccharomyces cerevisiae* MC16 carrying the *Escherichia coli*

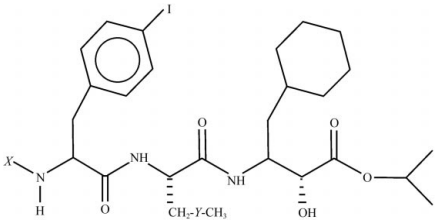

Figure 1
Chemical structure of CP-113972 (X = L-Pro and Y = S).

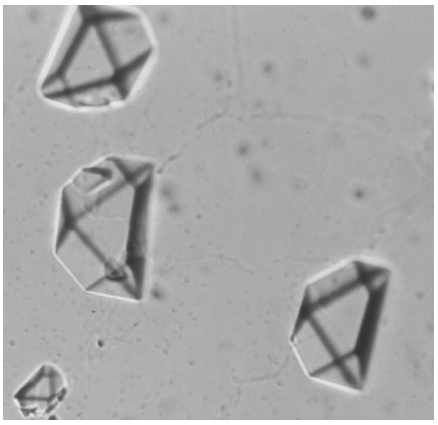

Figure 2
Crystals of mutant Y75N complexed with CP-113972 grown using ammonium sulfate in phosphate buffer pH 5.5.

shuttle plasmid JP1. This includes the yeast GAL7 promoter upstream of the *M. pusillus* pepsin gene. Briefly, *S. cerevisiae* MC16 strain containing JP1 was grown at 293 K in LB medium containing 2%(*w*/*v*) Difco Bacto tryptone, 1%(*w*/*v*) Bacto yeast extract and 2%(*w*/*v*) glucose and the supernatant of the culture containing the enzyme was directly applied onto a DEAE-Sephadex A-50 ion-exchange column. The absorbed protein was eluted with 50 m*M* sodium phosphate buffer pH 5.5 containing 150 m*M* sodium chloride. The fraction containing the mutant enzyme was then concentrated in an Amicon stirred cell with a PM-30 membrane and applied onto a Superdex-200 gel-filtration column on an FPLC system (Pharmacia Biotech, Sweden). The protein eluted as a single peak and SDS–PAGE showed a single major band of protein. Pure and homogenous mutant *M. pusillus* pepsin was concentrated for crystallization using Centricon-30 concentrators.

2.2. Inhibitor

CP-113972 is a norstatine-type inhibitor (Cooper *et al.*, 1989) provided by the Pfizer pharmaceutical company for study with human renin. It was previously cocrystallized with both human and mouse renin and inhibited human plasma renin at pH 7.4 with an IC_{50} value of 310 nm (Badasso, 1994). It is synthesized in a stepwise fashion as described by Hoover *et al.* (1989) from Boc-SMe-Cys, Boc-*p*-I-Phe and Boc-Pro. The chemical structure of CP-113972 is given in Fig. 1.

2.3. Crystallization

Crystallization of the Y75N mutant enzyme complexed with the inhibitor CP-113972 was carried out using the hanging-drop vapour-diffusion method (McPherson, 1999). The conditions that gave crystals were similar to those that proved effective with the wild-type *M. pusillus* pepsin (Newman *et al.*, 1991). The mutant *M. pusillus* pepsin–CP-113972 cocrystals were obtained by mixing 5 µl of enzyme–inhibitor complex solution with an equal volume of reservoir solution. A tenfold excess of inhibitor was dissolved in enzyme solution buffered with 50 m*M* sodium phosphate pH 5.5 and ammonium sulfate was used as the precipitant. The mutant enzyme concentration was 10 mg ml^{-1} and the ammonium sulfate concentration varied between 30 and 35%(*w*/*v*). Crystals appeared in 2–3 d at 293 K and grew to maximum dimensions of 0.5 × 0.3 × 0.1 mm after a week. The complex crystals were tetragonal and have an estimated solvent content of 58.67% (Matthews, 1968), corresponding to four molecules per asymmetric unit (Fig. 1).

2.4. Data collection and processing.

Several attempts to cryoprotect these crystals for cryocooling with a range of cryoprotectants were initially unsuccessful. The crystals became unstable and cracked while being stabilized in mother liqour containing various concentrations of either polyethylene glycols or glycerol. Single crystals dipped briefly in solutions containing 30% MPD, 50 m*M* sodium phosphate pH 5.5 and 30% ammonium sulfate appeared not to be affected drastically and the crystals were frozen. However, the freezing process did not alter the resolution limits of the data compared with room-temperature images. Data were collected on an in-house system using Cu $K\alpha$ radiation with a FAST area detector, an oscillation angle of 1°, a crystal-to-detector distance of 120 mm and an exposure time of 2 min. The crystals were rather radiation-sensitive even when frozen and only rapid data collection to 3.0 Å was possible, with a total of 87 375 independent measurements which reduced to 19 433 unique reflections. The data set was 96% complete with $R_{\rm merge}$ = 8.4%. All data were processed using *MOSFLM* (Leslie, 1992) and scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994). Data-collection and processing statistics are summarized in Table 1.

Table 1
Summary of data-collection and processing statistics.

Values in parentheses refer to the highest resolution data shell.

Wavelength (Å)	1.5418
Oscillation angle (°)	1.0
Resolution (Å)	30–3.0 (3.15–3.0)
Space group	$P4_32_12$
Unit-cell parameters (Å)	$a = b = 182.5$, $c = 99.1$
Mosaicity (°)	1.22
No. reflections measured	87,375
No. unique reflections measured	19,433
$R_{\rm merge}$ (%)	4.8 (12.4)
Completeness (%)	96.0 (92.0)
Average $I/\sigma(I)$	14.3 (8.1)

3. Results and discussion

Inhibitor-complexed crystals of mutant *M. pusillus* pepsin have been prepared and analyzed using X-ray diffraction. Analysis of the complex crystal data using the autoindexing algorithm of the *CCP*4 suite (Leslie, 1992) showed that the crystals have a primitive tetragonal lattice with unit-cell parameters $a = b = 182.5$, $c = 99.1$ Å and a unit-cell volume of 3.30×10^6 Å^3. Processing and scaling of the data in the *HKL* suite of programs resulted in the statistics given in Table 1. All attempts so far to grow crystals of native Y75N mutated enzyme from yeast without addition of inhibitor have failed. However, we have previously reproduced the crystallization of wild-type *M. pusillus* pepsin (Bunn *et al.*, 1971) and described the structure of *M. pusillus* pepsin at 2.0 Å resolution (Newman *et al.*, 1991). The successful growth of crystals of the Y75N mutant–inhibitor complex gave new cell-packing contacts that differed from those of the fungal derived wild-type *M. pusillus* pepsin. There is no sign of glycosylation in the wild-type *M. pusillus* pepsin.

Inhibitor cocrystallization experiments on the related aspartic proteinases such as human renin, mouse renin and saccharopepsin (previously known as yeast proteinase-A) have all provided clear examples of unit-cell changes and often dramatic enhancements of crystal quality upon inhibitor binding (Foundling *et al.*, 1987; Badasso *et al.*, 1992; Dealwis *et al.*, 1994; Aguilar *et al.*, 1997). In endothiapepsin

enzymes these changes are often associated with a conformational change which has been defined as a rigid-body motion involving residues 190–302 (Sali *et al.*, 1989). It is not clear whether this relates to inhibitor-induced conformational change, crystal packing or a complex interplay of both.

In some enzyme–inhibitor complexes no change in unit cell is observed and conformational changes are limited to the active-site 'flap' region. In the native wild-type *M. pusillus* pepsin structure the active site is not occluded by crystal contacts, thus explaining how lactyl-pepstatin readily diffuses into preformed crystals (Jones, 1984). The unusual perturbation of the crystal lattice observed in these soaking experiments must result in some way from transmission of the conformational disturbances associated with inhibitor binding to affect intermolecular interactions. These changes could be of the rigid-body type or less extensive 'flap' adjustments. The more extensive rigid-body changes within the aspartic proteinase structures have more often been associated with extreme changes in the crystal-packing arrangements, which also goes some way towards explaining the overall variability in structure across the family. The nature of the rigid-body interface and its variation in the family members probably defines the predominant conformer type, one of which may be stabilized by inhibitor binding and thus populated sufficiently to support crystal growth.

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