‘pH-jump’ crystallographic analyses of γ -lactam–porcine pancreatic elastase complexes

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β -Lactams inhibit a range of enzymes via acylation of nucleophilic serine residues. Certain γ -lactam analogues of monocyclic β -lactams have also been shown to be reversible inhibitors of porcine pancreatic elastase (PPE), forming acyl-enzyme complexes that are stable with respect to hydrolysis. Crystallographic analysis at pH 5 of an acyl-enzyme complex formed with PPE and one of these inhibitors revealed the ester carbonyl located in the oxyanion hole in a similar conformation to that observed in the structure of a complex formed between a heptapeptide (β -casomorphin-7) and PPE. Only weak electron density was observed for the His-57 side chain in its ‘native’ conformation. Instead, the His-57 side chain predominantly adopted a conformation rotated approx. 90° from its normal position. PPE– γ -

lactam crystals were subjected to ‘pH-jumps’ by placing the crystals in a buffer of increased pH prior to freezing for data collection. The results indicate that the conformation of the γ -lactam-derived acyl-enzyme species in the PPE active site is dependent on pH, a result having implications for the analysis of other serine protease–inhibitor structures at non-catalytic pH values. The results help to define the stereoelectronic relationship between the ester of the acyl-enzyme complex, the side chain of His-57 and the incoming nucleophile during the reversible (de)acylation steps, implying it is closely analogous to the hydrolytic deacylation step during catalytic peptide hydrolysis.

Key words: enzyme inhibition, β -lactam, serine protease.

INTRODUCTION

The penicillins and related β -lactam antibiotics exert their antibacterial activity by acylation of serine residues at the active site of enzymes (penicillin-binding proteins) involved in the biosynthesis of bacterial cell walls [1]. All members of the extended family of penicillin and cephalosporin antibiotics contain an ‘activated’ β -lactam ring, as well as other structural features required for recognition by their target enzymes. β -Lactam derivatives have also been shown to inhibit a range of other enzymes with nucleophilic serine residues, including mammalian serine proteases such as elastase [2–5] and prostate-specific antigen [6].

Compared with that of analogous γ -lactams, the release of ring strain energy during ring opening of a β -lactam by an alcohol renders reformation of the β -lactam relatively unfavourable [7]. It was speculated that the relative lack of activity of simple γ -lactams as inhibitors of ‘serine’ enzymes might, in part, be due to an unfavourable equilibrium position between the free γ -lactam and the acyl-enzyme complex [8]. This idea was explored by studying the interaction of various analogues of β - and γ -lactams with the serine protease, porcine pancreatic elastase (PPE). The effect on elastase inhibition of ‘inserting’ an extra methylene group into three different bonds of the ring of a monocyclic β -lactam inhibitor [9] of elastase was investigated (Figure 1) [10]. PPE catalysed the hydrolysis of the first series, with the methylene group inserted between C-3 and C-4 of the β -lactam template, but this series proved to be inactive or weakly active as inhibitors. The second series, with the methylene group between C-4 and the nitrogen of the β -lactam template, reacted reversibly with PPE to form acyl-enzyme complexes, which were

stable with respect to hydrolysis. Three second-series compounds (1–3)

(1) R = SO₂C₆H₄CH₃; (2) R = SO₂C₆H₄NO₂; (3) R = SO₂C₆H₄CF₃

with a carboxylate group on C-4 were found to be inhibitors of PPE with K_i values of 1.32 ± 0.13 , 0.20 ± 0.09 and 0.60 ± 0.03 mM respectively. The third series, with the methylene group inserted between C-2 and C-3, were not hydrolysed and were not inhibitors, consistent with a lack of binding to PPE, due to the absence of an alkyl group next to the lactam carbonyl capable of ‘locking’ the γ -lactam into the active site via insertion into the P₁ binding pocket.

An X-ray crystal structure (at $\approx$ pH 5) was obtained for the acyl-enzyme complex formed between a second-series inhibitor (1) and PPE [10]. The structure revealed a covalent link between the inhibitor and Ser-195, with good occupancy for atoms derived from the γ -lactam ring. The ethyl group was located in the P₁ binding pocket, but, unlike the complex formed with the analogous β -lactam [11], the ester carbonyl was located in the oxyanion hole in a similar conformation to that observed in the structure of an ester complex formed between PPE and the C-terminal isoleucine of the heptapeptide β -casomorphin-7 (YPFVEPI) [12]. In the γ -lactam-inhibited PPE structure there

Abbreviations used: LBHB, low-barrier hydrogen bond; PPE, porcine pancreatic elastase.

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Figure 1 Structures of the three series of monocyclic γ -lactams

Scheme 1 Proposed mechanism for the inhibition of PPE by the monocyclic γ -lactams

was no density observed for the substituent on the lactam nitrogen, a *p*-toluenesulphonyl group. The structure also revealed partial disorder of His-57 in the active site. Only weak electron density was observed for the His-57 side chain in its 'normal' conformation, i.e. that observed in the native structure of PPE [13] and in the PPE- β -casomorphin-7 structure [12]. Instead, the electron-density maps revealed the side chain of His-57 adopted predominantly a conformation rotated approx. 90° from its normal position. Thus, at least under the conditions of this crystallographic analysis, it appeared that His-57 was forced out of its 'normal' conformation by the presence of the inhibitor. The lack of hydrolysis of the acyl-enzyme complex derived from this inhibitor was rationalized by displacement of the hydrolytic water from the active site by the *p*-toluenesulphonyl group (Scheme 1).

In reactions involving general acid-base catalysis, the pH of the medium can profoundly influence the course of an enzyme mechanism. At acidic pH, the catalytic activities of serine proteases are decreased and crystalline acyl-enzyme complexes are stabilized. To investigate the deacylation of the γ -lactams **1** and **2** of the second series and the existence of catalytic intermediates during this mechanism, four PPE- γ -lactam crystals were subjected to 'pH-jumps' by placing the crystals in a buffer of increased pH prior to freezing for data collection. The results indicate that the predominant conformation of the γ -lactam-

derived acyl-enzyme species in the PPE active site is dependent on pH.

EXPERIMENTAL

Crystal preparation and soaking experiments

PPE crystals were prepared as described previously [10]. The γ -lactams (**1** or **2**) ($20 \text{ mg} \cdot \text{ml}^{-1}$) were dissolved in a solution containing sodium acetate buffer (pH 5.0, 50 mM), sodium sulphate (25 mM), PPE ($25 \text{ mg} \cdot \text{ml}^{-1}$) and DMSO (10%, v/v). The mother liquor containing the crystals was exchanged with this solution in a stepwise manner over 2 h. After 24 h of soaking with the inhibitor solution, the crystals were either placed in a cryoprotectant solution at pH 5 containing a mixture of the soaking solution and 20% (v/v) glycerol, or placed in a cryoprotectant buffer {100 mM Ches [2-(*N*-cyclohexylamino)ethanesulphonic acid]/25 mM sodium sulphate} at pH 9 or pH 10 containing 25% (v/v) glycerol for a defined period of time, and then flash-frozen in liquid nitrogen. The assignment of pH values inside the crystal should be regarded as tentative. Although racemic γ -lactams were used [10], only the 3*S*,4*S* forms of the γ -lactams were observed in the crystal structures. (Note: in our previous paper [10], the stereochemistry of the γ -lactam in the active site was incorrectly assigned as 3*R*,4*S*).

Table 1 Data collection and refinement statistics

	γ -Lactam–PPE complex				
	PPE–(1), pH 9, 1 min	PPE–(1), pH 9, 2 min	PPE–(2), pH 5	PPE–(2), pH 9, 1 min	PPE–(2), pH 9, 2 min
Resolution (Å)	1.80	1.90	1.70	1.75	1.55
Unit cell (Å)					
<i>a</i>	50.43	50.50	50.17	50.93	51.07
<i>b</i>	57.54	57.75	57.56	57.96	57.91
<i>c</i>	74.39	74.64	74.41	74.74	75.59
<i>R</i> _{merge} (%)	4.7	7.1	3.7	7.5	5.5
Completeness (%)	95.6	96.7	98.5	98.2	95.6
Completeness in last shell (%)	84.6	75.6	96.3	99.2	92.0
Total reflections	140 172	110 101	188 087	422 170	349 414
Unique reflections	20 798	17 858	24 440	22 794	33 072
<i>R</i> factor (%)	19.0	18.9	18.3	19.8	19.5
Free <i>R</i> factor (%)	21.8	22.5	22.0	23.0	20.8
Protein Data Bank ID code	1e34	1e35	1e36	1e37	1e38

Data collection and processing

Data for the PPE–(1), pH 9, 2 min and PPE–(2), pH 5 structures were collected at 100 K using a Rigaku rotating anode generator (CuK α radiation) and a 30 cm imaging plate (MAR Research, Norderstedt, Germany). Data for the PPE–(1), pH 9, 1 min structure, the PPE–(2), pH 9, 1 min structure and the PPE–(2), pH 9, 2 min structure were collected at 100 K using beamline BM14 at the ESRF (Grenoble, France) equipped with a Princeton charge-coupled-device (CCD) detector. The CCD images were corrected for spatial distortion using Fit2D [14]. The crystals all belonged to the space group $P2_12_12_1$. Raw data were processed with the programs DENZO and SCALEPACK [15].

Structure refinement

The 1.65 Å (0.165 nm) native PPE structure obtained by Meyer et al. [13] was used as the starting model. The structures were refined using a combination of REFMAC (CCP4 suite [16]), XPLOR 3.851 and SHELXL-97 [17]. A total of 4% of the reflections in the entire data set were randomly selected in order to provide a test set for the R_{free} calculations [18]. These reflections were not used during refinement, but were included in the density-map calculations. Models and electron-density maps were displayed with the program O [19]. At the stage in refinement, when the $F_o - F_c$ maps showed clear and unambiguous density corresponding to the γ -lactam-derived species, it was included in the model, and appropriate topology files for REFMAC, XPLOR or SHELXL were created. The ester bond was modelled only with bond-length restraints. During the latter stages of refinement, water molecules were added, along with the bound calcium and sulphate ions [13] (see Table 1 for refinement statistics). There were no Ramachandran outliers, and the final $F_o - F_c$ difference maps did not demonstrate any large areas of unexplained electron density.

RESULTS

PPE–(1) complex, pH 9, 1 min and 2 min structures

Both structures showed well-defined electron density for all the atoms in the γ -lactam inhibitor, except for the toluene ring of the *p*-toluenesulphonyl group (Figure 2). The relevant atoms of the toluene group were modelled in with zero occupancy using the conformation of the sulphonamide group as a guide to its

likely orientation. In contrast, the structures of PPE–(2) complexes had well-defined electron density corresponding to the nitrobenzene substituent (see below), the conformation of which supported the proposed location of the toluene group in the PPE–(1) complexes. In the PPE–(1), pH 5 structure [10], only weak electron density was observed for the side chain of His-57 in its normal conformation [13]. Instead, the His-57 side chain appeared to be rotated approx. 90° from its normal position. Following soaking for 1 min of the PPE–(1) complex at pH 9, the side chain of His-57 appeared to have rotated back to its 'normal' position, with much less electron density observed in the 'rotated' conformation. After soaking another crystal for 2 min at pH 9, electron density for the His-57 side chain was apparent in both the 'normal' and 'rotated' conformations, with the electron density in the 'normal' position being slightly stronger. In all cases, the His-57 side chain was modelled in both conformations, each set to an occupancy of 0.5. As the resolution of the data was not sufficiently high to permit refinement of the occupancy, the average *B* factors for the two positions of the imidazole ring were used as a rough estimate of likely occupancy. These were 8.8 and 17.6 Å² for the normal and rotated conformations respectively in the case of the 1 min structure and 16.2 and 18.7 Å², respectively, for the 2 min structure. This *B* factor analysis agrees with relative strength of electron density observed in the two conformations.

PPE–(2) complex at pH 5

This structure shows well-defined electron density for all of the atoms in the γ -lactam inhibitor, including the aromatic ring of the *p*-nitrobenzenesulphonyl group (Figure 3). The overall conformation of the ring-opened γ -lactam is very similar to that of the pH 5, PPE–(1) complex. The ester carbonyl is located in the oxyanion hole and is in position to form hydrogen bonds with the amido nitrogens of Gly-193 (3.09 Å) and Ser-195 (2.86 Å). The ethyl group on C-3 is located in the S₁ subsite. One oxygen atom of the C-4 carboxylate is in position to form a hydrogen bond with the side chain of Gln-192 (2.83 Å), and the other oxygen atom via a bridging water molecule (3.04 Å) is in position to form a hydrogen bond to the main chain carbonyl of Ser-214 (2.72 Å). Both atoms of the C-4 carboxylate are also in position to hydrogen bond via another bridging water molecule (3.11 and 2.96 Å) to the main chain carbonyl of Val-216 (2.69 Å). The electron density assigned to the side chain of His-57, like that of

Figure 2 Stereo-view pictures showing compound (1) (in yellow) bound in the active site of PPE (in green) at pH 5 (A), and after a pH 9 jump for 1 min (B) and 2 min (C)

The rotated position of His-57 is shown in thin lines. The $2F_o - F_c$ electron density maps were contoured at 1σ .

the pH 5 PPE-(1) complex, was best defined in a conformation rotated 90° away from its normal position. The average B factors for the imidazole ring in the normal and rotated conformations were 17.0 and $15.2 \text{ \AA}^2$ respectively. However, unlike the pH 5 structure of the PPE-(1) complex, the electron density for the side chain of Arg-61 was not disordered, and its guanidino group was in position to interact with the nitro group of the inhibitor, perhaps explaining the much better visualization of the *p*-nitrobenzene group of 2 compared with that of the *p*-toluenesulphonyl group of 1.

PPE-(2) complex, pH 9, 1 min and 2 min structures

Both structures showed well-defined electron density corresponding to all of the atoms in the γ -lactam inhibitor, including the aromatic ring of the *p*-nitrobenzenesulphonyl group (Figure 3). Soaking of the PPE-(2) complex at pH 9 for 1 min resulted in

visualization of electron density for the His-57 side chain in both 'normal' and 'rotated' conformations, with slightly stronger density apparent in the former. After soaking for 2 min at pH 9, the electron density for the histidine side chain in the 'normal' position was much stronger than after 1 min, consistent with further reaction after the longer soak time. The average B factors for the imidazole ring in the normal and rotated conformations were 21.6 and $22.2 \text{ \AA}^2$ respectively in the case of the 1 min structure, and 12.4 and $20.8 \text{ \AA}^2$ respectively for the 2 min structure. These results indicate that the electron density in the normal location of His-57 increases from the pH 5 structure to the pH 9, 1 min structure to the pH 9, 2 min structure. In the pH 9, 1 min structure, the orientation of the Arg-61 side chain is unchanged compared with that of the pH 5 structure, consistent with the relative lack of movement of the His-57 side chain at this time point. After 2 min at pH 9, the Arg-61 side chain appears to have rotated away from its original position and can no longer

Figure 3 Stereo-view pictures showing compound (2) (in yellow) bound in the active site of PPE (in green) at pH 5 (A), and after a pH 9 jump for 1 min (B) and 2 min (C)

The rotated position of His-57 is shown in thin lines. The $2F_o - F_c$ electron density maps were contoured at 1σ . Figures 2 and 3 were produced using the programs Bobscript [28], Raster3D [29], ImageMagick, Illustrator 8.0 (Adobe Inc.) and Photoshop 5.0 (Adobe Inc.).

form an interaction with the nitro group of the inhibitor, consistent with an increased extent of reaction in this structure.

DISCUSSION

At pH 5, the complexes formed between the γ -lactams (1, 2) and PPE are relatively stable (at least in the crystalline form), whereas at higher pH the acyl-enzyme complexes are formed reversibly [10]. Crystallographic analysis of 'pH-jumped' crystals of enzyme–inhibitor complexes provided a means of visualizing the early stages of the deacylation process and, assuming microscopic reversibility, the late stages of the acylation process. Using the current techniques it was not possible to visualize intermediates after longer pH soaks, since upon exposure of PPE–(1) or PPE–(2) complexes to pH 10 for more than 10 s, or to pH 9 for several minutes, the crystals were observed to crack and subse-

quently dissolve, suggesting that conformational changes stress the crystalline lattice as the pH is raised.

In all the refined crystal structures, the atoms derived from the γ -lactam rings were in good occupancy, the ethyl substituent of the inhibitors was located in the P_1 binding pocket, and continuous density between Ser-195 and the lactam carbonyl demonstrated that the ester bond remained intact. In the cases of both the PPE–(1) and PPE–(2) complexes, the carboxylate of the inhibitor is in position to hydrogen-bond with the side chain of Gln-192, an interaction that might help to stabilize the complexes.

At pH 5, the electron-density maps for the PPE–(1) and PPE–(2) complexes revealed that the predominant conformation of the His-57 side chain is at an orientation rotated approx. 90° away from its 'normal' conformation in native PPE [13]. In the rotated conformation, the His-57 side chain is not in a suitable position to catalyse deacylation via deprotonation of the sul-

phonamide group of the enzyme-inhibitor complex. The possibility of an alternate His-57 conformation may also affect its pK_a value, which in turn might influence the rates of recyclization and/or hydrolysis. In each of the four 'pH-jumped' structures, changes in the electron-density maps indicated movement of the His-57 side chain from the rotated position, predominant in the pH 5 structures, back to its 'normal' position. This movement places N_{ε2} of His-57 being within hydrogen-bonding distance of the sulphonamide group (Figures 2 and 3). This distance is 2.92, 2.87 and 2.75 Å in the pH 5, pH 9, 1 min and pH 9, 2 min PPE-(2) structures respectively, and 2.68 and 2.55 Å in the pH 9, 1 and 2 min PPE-(1) structures. These distances are all significantly less than the N...N van der Waals contact distance of 3.1 Å [20], especially in the case of the PPE-(1) complex. To our knowledge, this type of hydrogen bond is unusual in protein structures, and because the pK_a values of the histidine side chain (≈ 6.6) and sulphonamide group (≈ 7) in the active site are likely to be similar, there is potential for formation of a low-barrier hydrogen bond (LBHB) [21] during deacylation. Such a proposal should be regarded as speculative, due to lack of a precedent. An LBHB has been proposed to exist between Asp-102 and His-57 of the serine protease catalytic triad [22], but its importance in catalysis remains controversial [23,24].

The 'pH-jump' crystallographic analyses demonstrate that the side chain of His-57 can 'flip' between two conformations, the occupancy of which depends upon the pH, illustrating the need for care in drawing conclusions from structures obtained under non-native conditions. It will be a challenge to prepare inhibitors that cause the His-57 side chain to occupy a rotated position, thereby hindering deacylation via recyclization and by hydrolysis. It is notable that highly potent bicyclic γ -lactam and γ -lactone inhibitors of serine proteases have been reported recently [25–27], and crystallographic analyses on these structures will be of interest with respect to the conformation of the active site residues.

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