Inhibition of Elastase by *N*-Sulfonylaryl β-Lactams: Anatomy of a Stable Acyl−Enzyme Complex[†,‡]

Rupert C. Wilmouth, Nicholas J. Westwood, Kerry Anderson, William Brownlee, Timothy D. W. Claridge, Ian J. Clifton, Gareth J. Pritchard, Robin T. Aplin, and Christopher J. Schofield*

The Oxford Centre for Molecular Sciences and The Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, United Kingdom

Received July 8, 1998; Revised Manuscript Received October 6, 1998

ABSTRACT: β-Lactam inhibitors of transpeptidase enzymes involved in cell wall biosynthesis remain among the most important therapeutic agents in clinical use. β-Lactams have more recently been developed as inhibitors of serine proteases including elastase. All therapeutically useful β-lactam inhibitors operate via mechanisms resulting in the formation of hydrolytically stable acyl−enzyme complexes. Presently, it is difficult to predict which β-lactams will form stable acyl−enzyme complexes with serine enzymes. Further, the factors that result in the seemingly special nature of β-lactams versus other acylating agents are unclear—if indeed they exist. Here we present the 1.6 Å resolution crystal structure of a stable acyl−enzyme complex formed between porcine pancreatic elastase and a representative monocyclic β-lactam, which forms a simple acyl−enzyme. The structure shows that the ester carbonyl is not located within the oxyanion hole and the "hydrolytic" water is displaced. Combined with additional kinetic and mass spectrometric data, the structure allows the rationalization of the low degree of hydrolytic lability observed for the β-lactam-derived acyl−enzyme complex.

The catalytic mechanism of the serine proteases is among the best studied processes in enzymology. The mechanisms of other enzymes, including transpeptidases involved in bacterial cell wall biosynthesis (penicillin binding proteins, PBPs)[1] and resistance to β-lactam antibiotics (β-lactamases), also employ a mechanism involving nucleophilic attack by an active-site serine residue. While there are considerable variations in the active-site architectures and mechanisms of the various subfamilies of serine enzymes, it likely that in all cases catalysis proceeds via acyl−enzyme complexes and (probably) that activation of the cleaved peptide bond and acyl−enzyme involves hydrogen bonding between the oxygen of the ester carbonyl and the oxyanion hole. Similarities between the catalytic machinery of enzymes of the serine family with little or no overall sequence similarity (e.g., chymotrypsin/subtilisin) are powerful arguments for convergent processes during enzyme evolution (*1*−*3*).

Inhibitors that form stable acyl−enzyme complexes with serine enzymes are of medicinal importance. Most prominent among these compounds are the β-lactams, e.g., penicillins and cephalosporins, which were first developed as antibacterials (PBP inhibitors) but whose use has now been extended to serine protease inhibition (*4*, *5*). Bacterial resistance to β-lactams is an increasing clinical problem and is primarily mediated via serine β-lactamases, which hydrolyze β-lactam antibiotics to inactive β-amino acids. β-Lactam inhibitors of β-lactamases have been developed (e.g., clavulanic acid), but even their use is threatened by the development of resistance mechanisms, e.g., β-lactamases with altered selectivities (*6*). Common to the mechanisms of all clinically used β-lactam inhibitors is the formation of a hydrolytically stable acyl−enzyme complex.

Comparison of the structures of acyl−enzyme complexes with varying degrees of hydrolytic liability may lead to a better understanding of the factors required for both efficient catalysis and inhibition (via acyl−enzyme complexes) of serine enzymes. Such an understanding may assist in the design of serine protease inhibitors and antibacterials resistant to serine β-lactamases. We are particularly interested in developing non-β-lactam inhibitors that form stable acyl−enzyme complexes as antibiotics/β-lactamase inhibitors (*7*).

The only structural technique that presently allows detailed high-resolution studies on enzyme−substrate/inhibitor complexes is X-ray crystallography. Commercially available porcine pancreatic elastase (PPE) was chosen as a model system since it crystallizes readily and its structure is similar to that of human leukocyte elastase (HLE), a therapeutic target (*8*, *9*). Recently, we reported the structure (*10*) of an acyl−enzyme complex, formed between a simple heptapeptide (β-casomorphin-7, BCM7), discovered by mass spectrometric screening, and PPE. This led to insights into the stereoelectronics of the acyl−enzyme complex hydrolysis during PPE-catalyzed peptide hydrolysis.

[†] The work was supported by the BBSRC, EPSRC, MRC, and Zeneca Pharmaceuticals (via a CASE award to N.J.W.).

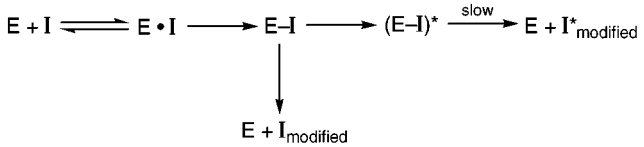
[‡] Coordinates for the structure described in this paper have been deposited with the Brookhaven Protein Data Bank (accession number 1BTU).

* To whom correspondence should be addressed: E-mail christopher.schofield@chem.ox.ac.uk; Tel and Fax 44-1865-275675.

[1] Abbreviations: BCM7, β-casomorphin-7; CNBr, cyanogen bromide; DMSO, dimethyl sulfoxide; ESIMS, electrospray ionization mass spectrometry; HLE, human leukocyte elastase; PBP, penicillin binding protein; PMSF, phenylmethanesulfonyl fluoride; PPE, porcine pancreatic elastase; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid; Wat, water.

10.1021/bi9816249 CCC: $15.00
Published on Web 11/20/1998

Scheme 1



Elastases have been shown to be inhibited by a variety of mono- and bicyclic β-lactams (*11*), some of which have been shown to act via double hit (i.e., covalent cross-linking) mechanisms (*12*). Initially, due to the stability of the acyl−enzyme complex, simple *N*-sulfonylaryl β-lactam inhibitors of HLE (e.g., **1** and **2**; Chart 1) were believed to operate via such a mechanism (*5*, *13*), but a subsequent study demonstrated they were not fragmented (*14*). Kinetic studies indicating a bifurcating pathway (*13*) were consistent with this and other mechanisms involving partitioning of an intermediate between turnover and formation of a relatively stable enzyme−inhibitor complex (Scheme 1). Studies on β-lactams with a leaving group at the C-4 position demonstrated that the mechanism of elastase inhibition by monocyclic β-lactams is dependent upon both the substitution pattern and stereochemistry of the β-lactam template (*15*, *16*). For our studies on the factors governing the stability of acyl−enzyme complexes, we chose to examine the interaction of *N*-sulfonylaryl β-lactams, without a leaving group at the C-4 position, with PPE since it seemed likely that their mechanism of action is likely to be among the simplest of known β-lactam protease inhibitors. Herein, we report studies leading to the crystal structure of a stable acyl−enzyme complex formed by reaction of one such β-lactam with PPE and comparison of this structure with that formed between PPE and BCM7.

MATERIALS AND METHODS

Electrospray Ionization Mass Spectrometry. Electrospray ionization mass spectra were recorded on a VG Bio-Q triple-quadrupole atmospheric pressure mass spectrometer equipped with an electrospray interface. Samples (10 μL) were introduced into the electrospray source via a loop injector as a solution with a final protein concentration of typically 20 pmol μL^{-1} in water/acetonitrile (1:1 v/v) containing 1% (v/v) formic acid. Mass spectra typically consisted of 15 10-s scans over the range 700−1900 M_r. Mass spectra were acquired with a cone voltage of 50 V and a source temperature of 50 °C. The instrument was calibrated with horse heart myoglobin or bovine ubiquitin. All ESI mass spectra of modified PPE samples were calibrated relative to PPE itself.

Inhibition, Reduction, and Alkylation of PPE. PPE (0.5 mg, 0.02 μmol) was dissolved in Milli-Q water (100 μL). The β-lactam inhibitor (3 mM in acetonitrile) was added to this solution to give either a 2:1 or 10:1 ratio of inhibitor:PPE. After incubation at room temperature (20 min), an aliquot was removed for ESIMS analysis and 0.1 M ammonium acetate/acetonitrile (9:1 v/v) at pH 4 (200 μL) was added to the remainder. TCEP (100 mM), in the same solvent system (50 μL, 65-fold excess over disulfide groups), was then added and the solution was heated, with stirring, at 50 °C for 45 min. *N*-Phenylmaleimide (50 mM) in *n*-propanol (150 μL, 50-fold excess over thiol groups) was then added and heating was continued for a further 45 min. Nonprotein byproducts were removed by extraction with dichloromethane (3 × 500 μL) and the resulting white solid was removed by centrifugation and dissolved in Milli-Q water (250 μL).

Cyanogen Bromide Digests of Modified PPE. Modified PPE (0.5 mg) from the above transformation was dissolved in 50% (v/v) formic acid (500 μL). A solution (100 μL) of cyanogen bromide (0.5 mg, 5 μmol) in 50% (v/v) formic acid was added to the PPE solution and incubated in the dark at 20 °C for 6 h. The cyanogen bromide used was colorless and free from nonvolatile polymeric material. The incubated mixture was diluted with 15 volumes (9 mL) of Milli-Q water and freeze-dried. The resulting solid was redissolved in a 1:1 (v/v) water/acetonitrile solution containing 1% (v/v) formic acid and analyzed by M-HPLC-ESIMS using a Hypersil reversed-phase C4 column (5 μm particle size, 300 Å pore, 1.0 × 250 m). A gradient of 25−76% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA was used. Peak fractions were monitored at 218 nm and collected manually for sequence determination by Edman degradation.

NMR Experimental Procedures. NMR experiments were performed at 499.98 MHz on a Bruker AMX500 instrument equipped with a 3 mm microprobe. Sample volumes were 130−150 μL and the sample temperature was regulated at 303 K. All spectra were referenced to internal MeCN at 2.05 ppm. The phosphate buffer (0.1 M) was prepared by dissolution of Na_2DPO_4 (1.107 g) and NaD_2PO_4 (276 mg) in D_2O (100 mL).

Experimental Procedures for Kinetic Studies. Enzyme assays were performed with a Shimadzu 1601 spectrophotometer equipped with a thermostated multicell transport system. The rate of hydrolysis of the MeOSuc-Ala-Ala-Pro-Val-pNA substrate (4 mM concentration) was measured at 410 nm and at a constant temperature of 25 °C. The kinetic analyses were conducted at pH 7.5 in a solution containing 0.1 M HEPES buffer, 0.5 M NaCl, and 10% (v/v) DMSO. Final concentration ranges for analysis of the β-lactams **1** and **2** were 75−400 nM and 10−200 nM, respectively. The rate of hydrolysis of the *p*-nitroanilide substrate was determined by using a curve-fitting algorithm, and from these data an IC_{50} value was calculated.

Synthesis of β-Lactams. Synthesis of the β-lactam inhibitors and their derivatives was carried out by a modified version of the reported protocols (*5*, *17*). Details will be reported elsewhere.

Crystal Preparation and Soaking. The PPE crystals were prepared at pH 5 as previously described (*8*). The β-lactam (**3**) (20 mg mL^{-1}) was dissolved in a solution containing sodium acetate buffer (pH 5.0, 50 mM), sodium sulfate (25 mM), PPE (25 mg mL^{-1}), and DMSO (10% v/v). The mother liquor containing the crystals was exchanged with this solution in a stepwise manner over a 2 h period. After 24 h of soaking in the inhibitor solution, the crystals were placed in a cryoprotectant solution containing 20% (v/v) glycerol and flash-frozen in liquid nitrogen.

Data Collection and Processing. Data were collected at beamline 9.6 of the Synchrotron Radiation Source (Daresbury, U.K.) tuned to a wavelength of 0.87 Å. Data collection was performed at 100 K with a 30 cm imaging plate (MarResearch) to a resolution limit of 1.6 Å. The data were processed with the programs DENZO and SCALEPACK

(*18*). R_{merge} was 5.7% for all data. Data were 98.7% complete [96.9% complete for the highest resolution shell (1.60−1.65 Å)] with 165 584 reflections, of which 29 200 were unique after reduction.

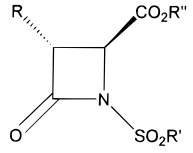
Structure Refinement. The 1.65 Å native PPE structure obtained by Meyer et al. (*8*) was used as the starting model. The structure was refined using a combination of XPLOR 3.1 (*19*) and REFMAC [CCP4 suite (*20*)]. A total of 4% of the reflections in the entire data set were randomly selected in order to provide a test set for the R_{free} calculations (*21*). These reflections were not used during refinement but were included in the electron density map calculations. Models and electron density maps were displayed with the program O (*22*). At the stage in refinement when the $F_o - F_c$ map showed clear and unambiguous density for the ring-opened β-lactam, it was included in the model and appropriate topology files for REFMAC and XPLOR were created. The ester bond was simulated solely with bond length restraints. During the latter stages of refinement, 210 water molecules were added along with the bound calcium and sulfate ions (*8*), giving a final *R*-factor of 19.2% and a free *R*-factor of 22.0%. The average *B*-factor for the atoms in the β-lactam inhibitor molecule was 14.7 Å^2. The rms deviations of the bond lengths and angles in the final structure were 0.021 Å and 2.32°, respectively. There were no unusual outliers in the Ramachandran plot and the final $F_o - F_c$ difference map did not demonstrate any large areas of unexplained electron density.

RESULTS AND DISCUSSION

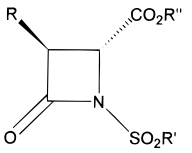
Detailed studies by the Merck group on the interaction of various types of monocyclic β-lactam with HLE (*15*, *16*) have demonstrated that the mechanism of inhibition is dependent upon the structure and stereochemistry of the β-lactam. Since we wished to investigate the factors governing the stability of simple acyl enzyme complexes, i.e., with the only covalent point of attachment being via an ester link to the active-site serine, we initially used ESIMS and NMR to investigate the interaction of *N*-sulfonylaryl β-lactams without a leaving group at C-4 with PPE. The HLE inhibitor L-652117 is a racemic mixture of **1** and **2** (Chart 1). Optically active forms of **1** and **2** were synthesized via modification of precedented methodology from aspartic acid (*5*, *17*). Both enantiomers **1** and **2** were inhibitors of PPE with IC_{50} values of 115 and 65 nM, repectively.

Solution ESIMS and NMR Studies. ESIMS analysis of the reaction between the *N*-sulfonylaryl β-lactams and PPE was carried out in order to provide evidence for the structure of the enzyme−inhibitor complex. In particular, we wished to demonstrate that there was no cleavage of the sulfonamide bond during the inhibition process, as had been originally proposed for inhibition of HLE by **1** and **2** before revision of the mechanism (*14*). Incubation of C-3-ethylated β-lactams (**1**−**4**) followed by ESIMS analysis led to the observation of mass increments of within 0.5 Da of the calculated values for addition of intact inhibitor (Table 1). Similarly, mass shifts corresponding to intact inhibitor were observed for the benzyl esters (**5** and **6**), but the degree of complex formation was less (<10%), as anticipated for the larger ester. No complex was observed upon incubation of **7** with PPE. Experiments on a ca. 1:1 mixture of native PPE and PPE

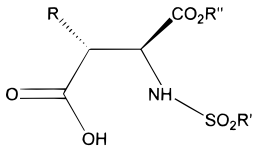
Chart 1: Structures of β-Lactam Inhibitors and Products



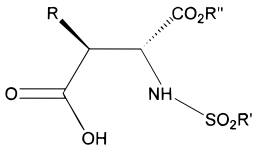
(**1**) R, R" = Et
R' = *p*-$NO_2C_6H_4$
(**3**) R = Et; R" = H
R' = *p*-$CH_3C_6H_4$
(**4**) R, R" = Et
R' = PhCO
(**5**) R = Et; R" = CH_2Ph
R' = *p*-$CH_3C_6H_4$
(**6**) R = Et; R" = CH_2Ph
R' = *p*-$NO_2C_6H_4$
(**7**) R, R" = Et; R' = H
(**13**) R = Et; R" = H
R' = *p*-$NO_2C_6H_4$



(**2**) R, R" = Et
R' = *p*-$NO_2C_6H_4$



(**8**) R, R" = Et; R' = *p*-$NO_2C_6H_4$
(**10**) R, R" = Et; R' = PhCO
(**14**) R = Et; R" = H
R' = *p*-$NO_2C_6H_4$



(**9**) R, R" = Et; R' = *p*-$NO_2C_6H_4$

Table 1

compound	averaged observed mass shifts[a] (Da)	theoretical mass shift for acyl−enzyme complex (Da)
1	355.6 (26)	356.36
2	355.6 (26)	356.36
3	297.9 (4)	297.33
1 + **2**[b]	359.6 (4)	356.36
5	384.3 (2)	387.46
6	418.0 (3)	418.43
4	275.7 (10)	275.31

[a] The number of ESIMS experiments over which the data were averaged is shown in parentheses. [b] Racemic mixture prepared from **1** and **2**.

inhibited by **1** in which the cone voltage of the mass spectrometer was varied between 50 and 90 V did not lead to changes in the ratio of uninhibited:inhibited enzyme, consistent with a covalent link being formed between enzyme and inhibitor. These ESIMS results implied that the inhibitors analyzed were not fragmented by PPE.

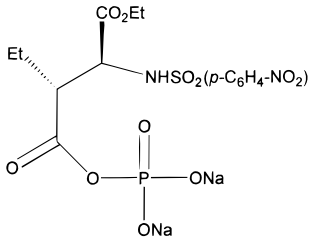
During the studies on the reaction of **1**, **2**, and **5** with PPE, mass shifts corresponding to formation of PPE−inhibitor complexes in a 1:2 ratio were observed at low intensities (<15% compared to the 1:1 PPE−inhibitor complexes). Since the intensities of the 1:2 adducts were reduced at higher cone voltage, it seems likely that the second inhibitor molecule was bound noncovalently to PPE. Attempts to increase the intensity of the 1:2 signals by increasing the inhibitor:PPE ratio to 10:1 were unsuccessful. The observed binding of a second inhibitor molecule may result from the ESI process itself (*23*) and/or from the relatively exposed PPE active site being able to accommodate a second intact inhibitor molecule, at least under ESIMS conditions. However, it would seem unlikely that binding of a second

molecule of inhibitor is important under physiological conditions.

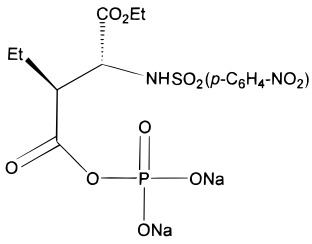
Time course experiments involving ESIMS analysis in which PPE was incubated with 2 equiv of **1** or **2** for between 3 min and 16 h indicated that rapid reaction to form a 1:1 complex occurred with no uninhibited PPE being observed after either 3 min or 1 h incubations. In the case of **1**, with longer incubation times (5 and 16 h) re-formation of a small amount of uninhibited PPE was observed, which increased with time. In the case of **2**, uninhibited PPE was not observed even after a 24 h incubation time. Compound **4** behaved similarly to **1**, with which it shares the same absolute stereochemistry. In parallel with the positive ion ESIMS experiments, negative ion ESIMS analysis was used to demonstrate the PPE-mediated production of ring-opened β-lactams **8**, **9**, and **10** from β-lactams **1**, **2**, and **4**, consistent with the proposal that the final acyl−enzyme inhibitor complex is hydrolyzed slowly (*13*). The negative ion ESIMS results were difficult to quantify due to the lability of the β-lactams under the incubation conditions and the low levels of material. It is noteworthy that in the cases of **1** and **4** no residual intact inhibitor was observed after 20 h but that in the case of **2** only ca. 50% hydrolysis of the β-lactam was observed. This is consistent with the kinetic analyses indicating that **2** is a better inhibitor than **1** and with the prior conclusion that the stability of the final enzyme−inhibitor complex depends on the stereochemistry of the β-lactam (*4*).

HPLC-ESIMS Studies. Evidence that reaction of the β-lactam inhibitors **1** and **2** with Ser-195 was the only covalent modification to PPE came from HPLC-ESIMS studies. In an optimized procedure, incubation of the β-lactam **1** with PPE was followed by lowering the pH to 4, reduction of the disulfides, and *S*-alkylation with *N*-phenylmaleimide. ESIMS on the resultant protein indicated the presence of a major species (27 660 ± 5 Da) compared to native PPE (25 898 Da). The observed mass shift of 1762 Da is close to that predicted for PPE inhibited by **1** in which the four disulfide bonds of PPE have been reduced and alkylated by *N*-phenylmaleimide (calc 1750 Da). Digestion of the inhibited and alkylated PPE with CNBr in formic acid followed by ESIMS analysis indicated that one of the resultant fragments had a mass of 8102.1 ± 2.0 Da. This corresponds to fully *S*-alkylated PPE residues 181−245 (calc 7746.7 Da) plus inhibitor (355.4 Da). Purification of this peptide by HPLC followed by Edman degradation gave the sequence Val(residue181)-Cys(modified)-Ala-Gly-Gly-Asn-Gly-Val-Arg-Ser-Gly-Cys(modified)-Gln-Gly-Asp-(no serine found at position 195)-Gly-Gly-Pro-Leu. This demonstrated that, apart from the *S*-alkylated cysteines, Ser-195 was the only modified residue. Unmodified residues included another serine (Ser-189), demonstrating that the absence of Ser-195 was not artifactual. The CNBr digests also led to isolation of an N-terminal peptide (residues 16−53) but not to the isolation of the third predicted fragment corresponding to residues 54−180. This may reflect the presence of a threonine after Met-53 since this is known to hinder CNBr cleavage (*24*). Similar results were obtained with **2**, except that low levels of unmodified Ser-195 were observed. These may reflect incomplete inhibition in the initial incubation or the relative stabilities of the diastereomeric acyl−enzyme complexes formed from enantiomeric β-lactams **1** and **2** at some stage in the derivatization/purification processes. The results are consistent with Ser-195 being the only covalently modified residue during inhibition of PPE by **1** or **2**.

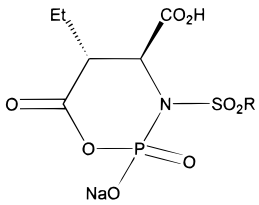
Chart 2: Possible Structures of Unassigned Metabolites



(**11**)



(**12**)



(**15**)

NMR Studies. ^{1}H NMR analyses in phosphate buffer of the incubations of **1** or **2** (30−40 equiv) with PPE led to confirmation of **8** and **9**, respectively, as the major hydrolysis products. Quantitative analysis of the results in buffer was complicated by the fact that hydrolysis of *N*-sulfonylaryl β-lactams apparently proceeds, at least in part, via an acyl-phosphate intermediate (e.g., **11** from **1**) and that the half-life of **1** or **2** in phosphate buffer is ca. 14 h, compared to ca. 6 days in D_2O/CD_3CN (*17*). Indeed, resonances assigned to the acyl-phosphate derivatives **11** and **12** of **1** and **2** (see Chart 2) were observed in incubations with PPE, although at significantly reduced levels compared to incubations in the absence of PPE. In the phosphate buffer incubations of **1** and **2** with PPE, analysis of the aromatic regions of the spectra also revealed the presence of unassigned broad resonances ca. 0.05 ppm upfield of both sites of AA′BB′ resonances of the sulfonylaryl group at ca. 20% of the intensity of ring-opened β-lactam.

For the structural work it was important to demonstrate that these broad signals did not derive from byproducts of a double-hit type mechanism. Thus, incubations were carried out in D_2O/CD_3CN. Under these conditions the rates of hydrolysis of **1** and **2** were slowed and only ca. 70% hydrolysis of **1** and ca. 50% hydrolysis of **2** were observed after overnight incubation, cf. complete hydrolysis in buffer. The lower level of hydrolysis of **2** is consistent with the ESIMS results. The only signals present in the aromatic part of the ^{1}H NMR spectrum came from **1** or **2** and **8** or **9**. Thus the broad signals observed in phosphate buffer are associated with the use of this buffer, and in D_2O/CD_3CN only simple hydrolysis occurs. Some preliminary experiments were carried out in the hope of assigning the origin of the broad signals observed in phosphate buffer. They were not present in the absence of PPE nor in the presence of PMSF-inhibited PPE but were still present after removal of the PPE, demonstrating that their broad nature did not result from a slow equilibrium with the enzyme. Doping experiments ruled out the possibility that the broad resonances were due

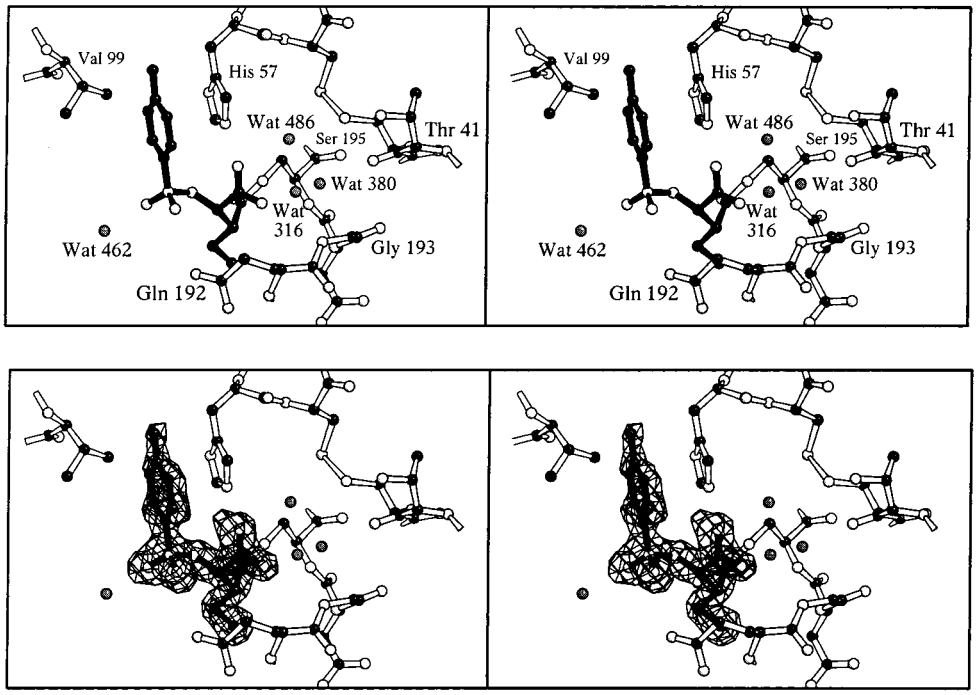


FIGURE 1: Active site of PPE (in white) showing compound **3** (in black) covalently linked via an ester bond to Ser-195. The bottom stereo picture shows an $F_o - F_c$ electron density omit map contoured at 3σ.

to acids **13** or **14** resulting from ethyl ester hydrolysis. On incubation of **13** with PPE in buffer, different unassigned broad resonances in the aromatic region were also observed, demonstrating that an ester is not required for production of the unknown compound(s). Incubation of the *p*-toluenesulfonyl β-lactam **3** also led to the observation of broad resonances in the aromatic region, but shifted upfield relative to those for the *p*-nitrobenzenesulfonyl inhibitors **1**, **2**, and **13**. A further broad signal corresponding to the methyl of the *p*-toluenesulfonyl group was also observed. It is possible that the unassigned compounds giving rise to the broad resonances result from decomposition of an acyl enzyme intermediate via a process other than hydrolysis, e.g., cleavage of the sulfonamide bond as in the originally proposed mechanism of action for these compounds (*5*, *13*). However, doping experiments, using an incubation of (**3**) with PPE, demonstrated that the broad resonances were not due to the formation of *p*-toluenesulfonate, *p*-toluenesulfinate, or *p*-toluenesulfonamide. Since they were only observed in the presence of phosphate buffer and PPE, it seems probable that phosphate ions play a role in their generation. Since the β-amino acids **8**, **9**, **10**, and **14** did not react with phosphate, then such a process would probably have to involve reaction of phosphate with an enzyme-bound complex. To the extent of our knowledge, such a process is unprecedented in serine protease chemistry. However, phosphonate esters are known inhibitors of serine proteases, leading to the phosphorylation of the active-site serine residue and phosphonamides are β-lactamase inhibitors (*25*, *26*). A speculative structure for the unknown compound giving rise to the broad resonances in the NMR spectra is **15**, which might arise via phosphate attack onto the acyl−enzyme complex followed (or preceded) by intramolecular attack onto the resultant intermediate. The broad nature of the NMR resonances might be explained by hindered rotation about the N−S bond.

Studies from the Merck group have shown that breakdown of acyl−enzyme complexes formed from monocyclic β-lactams with leaving groups at the C-4 position can lead to different products depending upon the conditions and inhibitor structure (*27*). Although inconclusive in terms of the identification of the unknown product, the present NMR studies provide evidence that, in phosphate buffer, even in the case of β-lactams without a C-4 leaving group, the results of hydrolysis may be more complex than anticipated. However, the lack of evidence for fragmentation of the β-lactams by ESIMS and HPLC-ESIMS and the observation of a single product in D_2O was sufficient evidence for simple acylation of the active-site serine for us to pursue crystallographic studies.

Crystallographic Studies. The acid **3** rather than the esters **1** or **2** was chosen for crystallographic studies in order to reduce the possibility of complications arising from ester hydrolysis of PPE via the C-4 group. After experimentation, it was found that 10% DMSO had to be included in the soaking solutions (pH 5) to permit dissolution of a sufficiently high concentration of the inhibitor. The soaked crystals were isomorphous with native PPE (*8*) and there was no significant change in unit cell parameters. The resultant 1.6 Å resolution structure shows well-defined electron density for all the atoms in the β-lactam inhibitor (average *B*-factor = 14.7 Å^2). Comparison of this structure with that of the stable acyl−enzyme complex formed between β-casomorphin-7 and PPE (also at pH 5) (*10*) is interesting.

The ring-opened β-lactam is clearly located in the active site of PPE with well-defined electron density consistent with the presence of an ester linkage between the O_γ atom of Ser-195 and the C-2 atom of the β-lactam (Figures 1 and 2). Significantly, the carbonyl oxygen of the ester is not located within the oxyanion hole, but instead is rotated by ca. 120° away from the arrangement observed in the PPE−BCM7

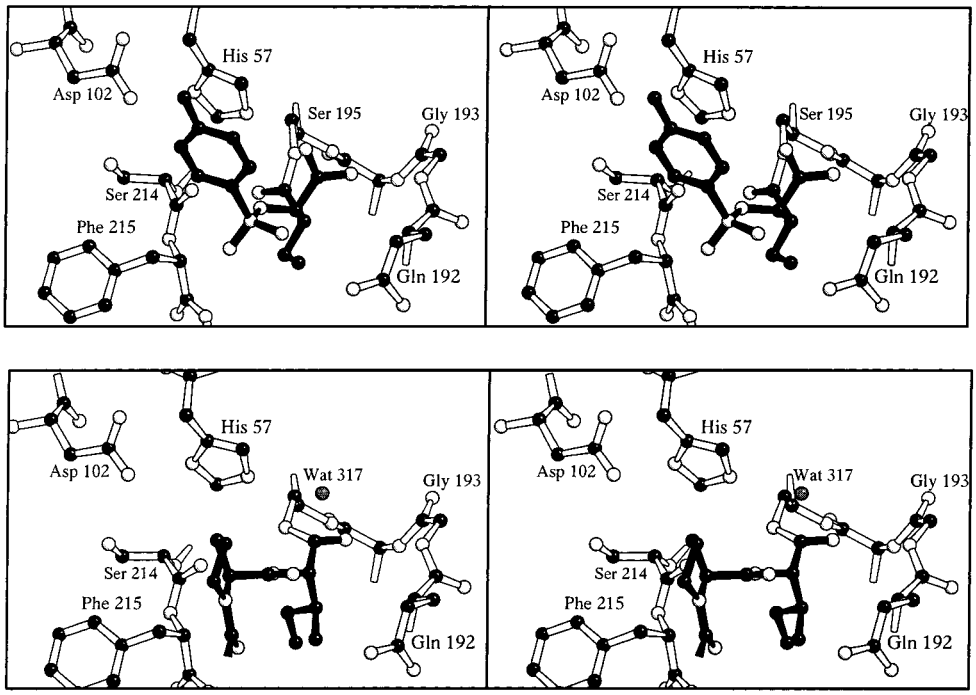


FIGURE 2: Comparison of the location of the ester carbonyl in the case of the acyl−enzyme complex formed with the monocyclic β-lactam **3** in the top stereo picture and β-casomorphin-7 in the bottom stereo picture. In both cases the inhibitor molecule is shown in black and the PPE active site in white. The figures were produced with the program BOBSCRIPT (*31*).

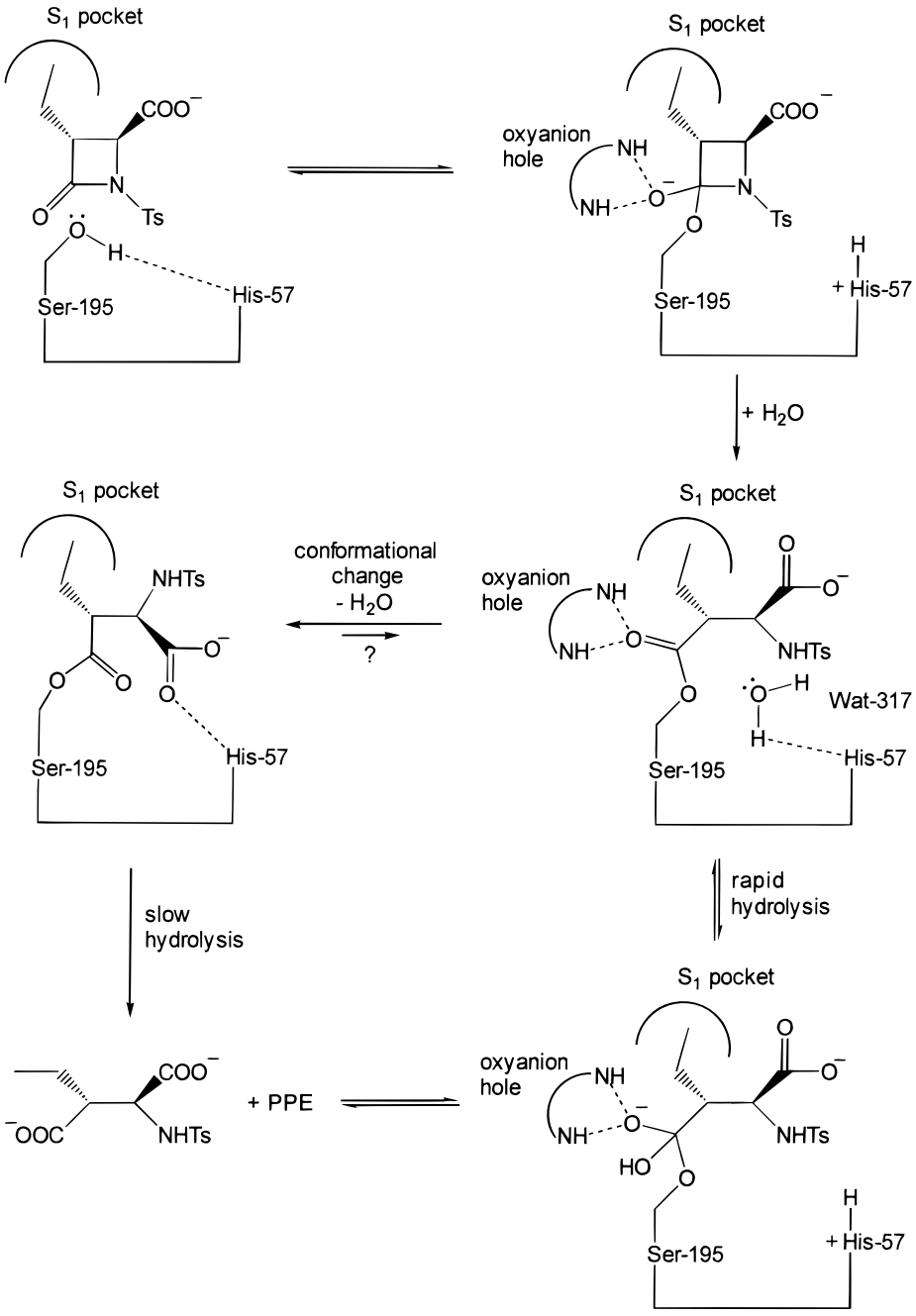
structure toward the active-site cleft. In this rotated conformation, the carbonyl oxygen of the ester does not appear to be in position to form hydrogen bonds with the protein. The ethyl side chain on C-3 is located in the S_1 subsite as anticipated, since in this aspect the β-lactam inhibitors are designed to mimic a peptidic substrate (*4*). One oxygen atom of the C-4 carboxylate group is in position to form hydrogen bonds with $N_{\epsilon 2}$ of His-57 (2.68 Å) and Wat-486 (2.64 Å), whereas the other carboxylate oxygen atom is in position to make hydrogen bonds with Wat-316 (2.70 Å) and Wat-380 (3.23 Å). Wat-380 lies close to the oxyanion hole and is in position to make hydrogen bonds with the amido nitrogens of Gly-193 (2.91 Å) and Ser-195 (3.02 Å) and the carbonyl group of Thr-41 (2.96 Å).

As Wat-380 is also the closest water molecule to the carbon of the ester carbonyl (4.22 Å), it may represent the hydrolytic water responsible for the slow hydrolysis of the acyl−enzyme complex in solution. However, Wat-380 is not in the plane of the ester carbonyl and there is no general base proximate to it. Further, the angle subtended between Wat-380 and the ester carbonyl is 128°, which is considerably more than the optimum (ca. 100−110°) for nucleophilic attack onto a carbonyl group (*28*). It is therefore likely that a significant movement of Wat-380 and/or the ester carbonyl would be required for it to be located in a suitable position to effect hydrolysis. Hydrolysis of the stable acyl−enzyme complex may occur via re-formation of the productive conformer, or another conformer in which the ester carbonyl is in the oxyanion hole.

The excellent definition of the electron density for every atom derived from the inhibitor suggests that the degree of conformational flexibility is low. However, apart from the carboxylate group on C-4, the only other part of the β-lactam inhibitor readily able to form electrostatic interactions with the protein are the oxygen atoms of the sulfonyl group, which are in position to form hydrogen bonds with Wat-462 (2.92 Å) and the side chain of Gln-192 (3.38 Å). This suggests that hydrophobic interactions contribute significantly to the stability of the final inhibitor complex. It is noteworthy that the phenyl ring of the tosyl group is situated slightly deeper within the S_2 subsite than the proline ring in the PPE−BCM7 complex. Two hydrophobic residues, Trp-94 and Val-99, border the *p*-toluene ring and the isopropyl side chain of Val-99 appears to have rotated slightly (relative to the PPE−BCM7 structure) to accommodate the bulkier tosyl group. The *p*-toluene ring also lies close to and parallel with the imidazole ring of His-57, suggesting the possibility of a $\pi-\pi$ interaction which could further add to the conformational stability of the inhibitor.

Hydrolysis of the β-lactam-derived acyl−enzyme complex is disfavored as the ester carbonyl is rotated out of the oxyanion hole, thus precluding any stabilization of the tetrahedral intermediate. Second, as the location of the closest water (Wat-380) is incompatible with a hydrogen bond to His-57, this water molecule is unlikely to be polarized to such a degree that its nucleophilicity would be significantly enhanced. The kinetic studies of Knight et al. (*13*) (Scheme 1) led to the proposal that two acyl−enzyme complexes are formed with the first being the more easily hydrolyzed. This leads to our proposal of the following mechanism (Scheme 2). Initially the β-lactam binds to the PPE active site noncovalently in a productive conformation with the PPE. For this to occur, the C-3 hydrophobic group must be located in the S_1 subsite, and the ester carbonyl must be positioned in the oxyanion hole. Formation of an initial acyl−enzyme complex, which acts as the bifurcation point in the mechanism, would be expected to occur via a tetrahedral intermediate. Water can then bind to the complex and effect hydrolysis to give a β-amino acid, which diffuses away. Alternatively, a conformational change involving

Scheme 2



rotation of the ester carbonyl out of the oxyanion hole occurs. Although this would result in the breaking of two hydrogen bonds between the carbonyl oxygen and the amido nitrogens of Gly-193 and Ser-195, it must be assumed that a combination of other effects are sufficient to render the conformational change favorable. Possible important contributing interactions include the formation of a hydrogen bond between the carboxylate group on C-4 and His-57 and the $\pi-\pi$ interaction between the tosyl ring and His-57. Other effects that could influence the relative stability of the two conformational states are the level of strain present in the opened β-lactam or the relative levels of hydrophobicity in the environment of the C-4 carboxylate group.

Comment should also be made on the observation that **2**, with the opposite stereochemistry to **1** and **3**, is a PPE inhibitor. The relative positions of the ester carbonyl and the C-3 ethyl group are of interest in the PPE−**2** complex. The detailed mechanism of the inhibition by **2** may well be different from that of **3**, and modification of the β-lactam substituents for optimal inhibition is probably best carried out on separate enantiomers.

The structure of the acyl−enzyme complex formed from (**3**) and PPE suggests that the design of a β-lactam inhibitor, even one operating via an apparently simple acylation process, must take into account a number of factors. These should include the fit of the intact β-lactam into the active site and the structure of the initially formed acyl−enzyme complex. It might be possible to design inhibitors that react to form a hydroytically stable acyl−enzyme complex in which the ester carbonyl is located in the oxyanion hole, e.g., by displacing or perturbing the hydrolytic water. Work directed to this objective has been reported in studies on the β-lactamases (*29*). Naturally occurring 5,5-*trans*-lactone inhibitors of human α-thrombin, which are believed to form

stable acyl−enzyme complexes in which the hydrolytic water has been displaced, have also been recently reported (*30*). Alternatively, if a conformational change rotating the ester carbonyl out of the oxyanion hole occurs, then the hydrolytic stability of the complex should increase. To design the propensity for such a change into an inhibitor of a protease is an interesting challenge since it requires predictions of the relative stabilities and rates of interconversion of the initial noncovalent complex, productive acyl−enzyme complex, and the conformationally changed stable acyl−enzyme complex.

ACKNOWLEDGMENT

We thank Mr. A. C. Willis for N-terminal sequencing and Professor J. Hajdu and Dr. P. L. Roach for helpful discussions.

REFERENCES

1. Bode, W., Meyer, E., and Powers, J. C. (1989) *Biochemistry 28*, 1951−1963.
2. Blow, D. M. (1976) *Acc. Chem. Res. 9*, 145−152.
3. Wright, C. S., Alden, R. A., and Kraut, J. (1969) *Nature 221*, 235−242.
4. Knight, W. B., Green, B. G., Chabin, R. M., Gale, P., Maycock, A. L., Weston, H., Kuo, D. W., Westler, W. M., Dorn, C. P., Finke, P. E., Hagmann, W. K., Hale, J. J., Liesch, J., MacCoss, M., Navia, M. A., Shah, S. K., Underwood, D., and Doherty, J. B. (1992) *Biochemistry 31*, 8160−8170.
5. Firestone, R. A., Barker, P. L., Pisano, J. M., Ashe, B. M., and Dahlgren, M. E. (1990) *Tetrahedron 46*, 2255−2262.
6. Frere, J.-M. (1995) *Mol. Microbiol. 16*, 385−395.
7. Westwood, N. J., Claridge, T. D. W., Edwards, P., and Schofield, C. J. (1997) *Bioorg. Med. Chem. Lett. 4*, 2973−2978.
8. Meyer, E., Cole, G., Radhakrishnan, R., and Epp, O. (1988) *Acta Crystallogr. B44*, 26−38.
9. Bode, W., Wei, A. Z., Huber, R., Meyer, E., Travis, J., and Neumann, S. (1986) *EMBO J. 5*, 2453−2458.
10. Wilmouth, R. C., Clifton, I. J., Robinson, C. V., Roach, P. L., Aplin, R. T., Westwood, N. J., Hajdu, J., and Schofield, C. J. (1997) *Nat. Struct. Biol. 4*, 456−462.
11. Mascaretti, O. A., Boschetti, C. E., Danelon, G. O., Mata, E. G., and Roveri, O. A. (1995) *Curr. Med. Chem. 1*, 441−470.
12. Knight, W. B., Maycock, A. L., Green, B. G., Ashe, B. M., Gale, P., Weston, H., Finke, P. E., Hagmann, W. K., Shah, S. K., and Doherty, J. B. (1992) *Biochemistry 31*, 4980−4986.
13. Knight, W. B., Chabin, R., and Green, B. (1992) *Arch. Biochem. Biophys. 296*, 704−708.
14. Knight, W. B., Swiderek, K. M., Sakuma, T., Calacay, J., Shively, J. E., Lee, T. D., Covey, T. R., Shushan, B., Green, B. G., Chabin, R., Shah, S., Mumford, R., Dickinson, T. A., and Griffin, P. R. (1993) *Biochemistry 32*, 2031−2035.
15. Shah, S. K., Finke, P. E., Brause, K. A., Chandler, G. O., Ashe, B. M., Weston, H., Maycock, A. L., Mumford, R. A., and Doherty, J. B. (1993) *Bioorg. Med. Chem. Lett. 3*, 2295−2298.
16. Hagmann, W. K., Shah, S. K., Dorn, C. P., O'Grady, L. A., Hale, J. J., Finke, P. E., Thompson, K. R., Brause, K. A., Ashe, B. M., Weston, H., Dahlgren, M. E., Maycock, A. L., Dellea, P. S., Hand, K. M., Osinga, D. G., Bonney, R. J., Davies, P., Fletcher, D. S., and Doherty, J. B. (1991) *Bioorg. Med. Chem. Lett. 1*, 545−550.
17. Westwood, N. J., Schofield, C. J., and Claridge, T. (1997) *J. Chem. Soc., Perkin Trans.1*, 2725−2729.
18. Otwinowski, Z. (1993) in *Data Collection and Processing* (Sawyer, L., Isaacs, N. W., and Bailey, S., Eds.) pp 55−62, Daresbury Laboratory, Warrington, U.K.
19. Brunger, A. T., Kuriyan, J., & Karplus, M. (1987) *Science 235*, 458−460.
20. Bailey, S. (1994) *Acta Crystallogr. D50*, 760−763.
21. Brunger, A. T. (1992) *Nature 355*, 472−475.
22. Jones, A. T., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. A47*, 110−119.
23. Aplin, R. T., Robinson, C. V., Schofield, C. J., and Westwood, N. J. (1994) *J. Chem. Soc., Chem. Commun.* 2415−2417.
24. Schroeder, W. A., Shelton, J. B., and Shelton, J. R. (1969) *Arch. Biochem. Biophys. 130*, 551−556.
25. Kovach, I. M., and Enyedy, E. J. (1998) *J. Am. Chem. Soc. 120*, 258−263.
26. Page, M. I., Laws, A. P., Slater, M. J., & Stone, J. R. (1995) *Pure Appl. Chem. 67*, 711−717.
27. Underwood, D. J., Green, B. G., Chabin, R., Mills, S., Doherty, J. B., Finke, P. E., MacCoss, M., Shah, S. K., Burgey, C. S., Dickinson, T. A., Griffin, P. R., Lee, T. E., Swiderek, K. M., Covey, T., Westler, W. M., and Knight, W. B. (1995) *Biochemistry 34*, 14344−14355.
28. Burgi, H. B., Dunitz, J. D., & Shefter, E. (1973) *J. Am. Chem. Soc. 95*, 5065−5067.
29. Maveyraud, L., Massova, I., Birck, C., Miyashita, K., Samama, J. P., and Mobashery, S. (1996) *J. Am. Chem. Soc. 118*, 7435−7440.
30. Weir, M. P., Bethell, S. S., Cleasby, A., Campbell, C. J., Dennis, R. J., Dix, C. J., Finch, H., Jhoti, H., Mooney, C. J., Patel, S., Tang, C.-M., Ward, M., Wonacott, A. J., and Wharton, C. W. (1998) *Biochemistry 37*, 6645−6657.
31. Esnouf, R. M. (1997) *J. Mol. Graphics 15*, 133−138.

BI9816249