Novel Mechanism of Inhibition of Elastase by β-Lactams Is Defined by Two Inhibitor Crystal Complexes*

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Two structurally related β-lactams form different covalent complexes upon reaction with porcine elastase. The high resolution x-ray structures of these two complexes provide a clear insight into the mechanism of the reaction and suggest the design of a new class of serine protease inhibitors that resist enzyme reactivation by hydrolysis of the acyl intermediate. The presence of a hydroxyethyl substituent on the β-lactam ring provides a new reaction pathway resulting in the elimination of the hydroxyethyl group and the formation of a stabilizing entity that is resistant to deacylation and hence regeneration of enzyme activity. In this study, we report the discovery of a novel mode of action of a class of monocyclic β-lactams, which results in a particularly stable acyl enzyme intermediate. The proposed mechanism, which is based on the x-ray structures of the elastase-ligand complexes presented below, may be generally applicable to other related serine proteases.

β-Lactam-related compounds are well established irreversible inhibitors of a wide range of serine proteases including elastase (1), β-lactamase (2), phospholipase A2 (3), and bacterial signal peptidases (4). Moreover, their efficacy as orally active inhibitors has led to their widespread use in the clinic. In all cases a first step in the inhibition process is acylation of the active site serine by the β-lactam ring to generate a covalently bound acyl enzyme intermediate. Thereafter, the inhibitor may undergo a further fragmentation resulting in a more stable entity that is resistant to deacylation and hence regeneration of enzyme activity. In this study, we report the discovery of a novel mode of action of a class of monocyclic β-lactams, which results in a particularly stable acyl enzyme intermediate. The proposed mechanism, which is based on the x-ray structures of the elastase-ligand complexes presented below, may be generally applicable to other related serine proteases.

Human leukocyte elastase is an important therapeutic target in view of its implied role in diseases such as emphysema (5), cystic fibrosis (6), and rheumatoid arthritis (7). Two x-ray structures of β-lactams bound to porcine pancreatic elastase (PPE)† have been reported, the first involving a cephalosporin sulfone (8) and more recently an N-sulfonfurylaryl β-lactam (9). In the latter case, the monocyclic β-lactam simply acylates the active site serine residue without further fragmentation. The class of monocyclic β-lactams reported herein contain an aroyloxy substituent at the C-4 position (Fig. 1) that departs subsequent to acylation of the serine residue. We have compared two related members of this class of β-lactam that differ primarily in the nature of the C-3 substituent. This difference results in distinct modes of action upon reaction with elastase.

EXPERIMENTAL PROCEDURES

The crystals were grown by the hanging drop method at room temperature. Porcine pancreatic elastase at 10 mg/ml was incubated with a 3-fold molar excess of inhibitor for 1.5 h at room temperature before drops were laid. The well solution contained 0.1 M sodium acetate buffer at pH 5.1 and 55 mm sodium sulfate. The drops consisted of 2 μl of protein and 2 μl of well solution. Crystals of approximate dimensions 0.5 × 0.3 × 0.3 mm grew within 4 days. The crystals belong to the space group P2₁2₁2₁, with cell constants a = 50.0 Å, b = 57.8 Å, c = 74.3 Å and contain 1 elastase molecule per asymmetric unit. Crystals of the complex were soaked in a freezing solution consisting of 25% glycerol and 75% well solution (ν/ν) for approximately 5 s and then flash frozen by immersing in liquid nitrogen.

X-ray data for the EI116-elastase crystal complex were collected at 100 K using a MAR300 image plate mounted on an Enraf Nonius rotating anode generator operating at 40 kV and 80 mA to a resolution of 1.8 Å. Data collection statistics are given in Table I. X-ray data for the JD261-elastase complex were collected on station 7.2 at the Daresbury synchrotron source using a MAR 300 image plate to a resolution of 1.6 Å (Table I).

The structures were solved with the molecular replacement program AMoRe (10) using the model of porcine pancreatic elastase from a published crystal structure (entry 8EST in the Brookhaven Protein Data Base). All water and ligand molecules were removed from the model structure. Initial difference Fourier maps using phases calculated from the molecular replacement solutions showed clear electron density for both ligands. Subsequent refinement of all atom positions and individual isotropic thermal parameters was carried out using SHELXL-97 (11) (Table I). A total of 282 water molecules were added to JD261 and 275 to EI116 using the program SHELXLWAT (11). Both structures contained a calcium and a sulfate ion as described in other published structures. Despite the short (5 s) soaking time in the cryoprotectant solution, a well ordered glycerol molecule was located in the JD261-elastase complex. Ranges of conguadient wedge squares refinement were alternated with examination of sigma weighted (2Foobs – F(calc)) Fourier maps. This, along with the excellent quality high resolution data, assured that any model bias was minimized. Once the R-factor had refined to a value of less than 0.18 using all data, hydrogen atoms were added in calculated positions. This reduced both the R-factor and the free R-factor for both structures by more than 1% leading to final R-factors of 0.16 with excellent geometries (Table I). Coordinates of both structures have been deposited in the Brookhaven Protein Data bank.

RESULTS

Geometry of JD261 and Interactions with the PPE Active Site—The ligand was clearly visible in an initial electron density difference map. The excellent quality of the electron density calculated with data to a resolution of 1.60 Å shows that the ligand derived from JD261 is planar between the ester

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‡ The abbreviations used are: PPE, porcine pancreatic elastase.
carbon attached to the serine and the nitrogen preceding the naphthalene ring system (Fig. 2). Furthermore, there is no trace of substituents on positions 3 and 4 of the β-lactam. These observations are consistent with the structures and mechanism shown in Fig. 1a.

The amide nitrogen atoms from Gly-201(193) and Ser-203(195) act as hydrogen bond donors to the ester carbonyl oxygen atom, which lies in the oxyanion pocket. The planar all trans configuration of JD261 places constraints upon the orientation of the urea moiety and only one other direct hydrogen bond can be formed between the urea oxygen atom and Gln-200(192) (Fig. 3). The urea nitrogen (N-1) is hydrogen bonded to a water molecule that forms a bridging hydrogen bond to the amide nitrogen of Val-224(216). The active site His-60(57) does not make any direct hydrogen bonds with JD261 but does form a hydrogen bond to W751 (Ne...W = 2.7Å). This water molecule is only 3.3 Å from the carbon of the ester carbonyl and is well positioned to carry out a nucleophilic attack along the Burgi-Dunitz trajectory (Fig. 3).

**DISCUSSION**

**Mechanism of β-Lactam Inhibition**—The x-ray structures of the complexes are consistent with the mechanisms outlined in Fig. 1. The mechanism shown for the reaction of EI116 with...
elastase is similar to that proposed for a related series of compounds binding human leukocyte elastase (12). In that work, mass spectrometry and NMR were used to show acylation of the Ser-203(195), loss of the substituent on C-4 of the lactam ring, and subsequent formation of the carbinolamine. We can now show that the addition of water to the intermediate imine results in a carbon with (S) configuration as predicted by modeling studies (12). This hydroxyl group forms a hydrogen bond to His-60(57) and may play a role in the reactivation mechanism. Indeed this hydroxyl group may be derived from the hydrolytic water molecule that is required for deacylation and hence reactivation of elastase.

The new mechanism revealed by the binding of JD261 to elastase involves initial acylation of Ser-203(195) followed by loss of the OAr group from C-4. Thereafter, the hydroxyethyl substituent on C-3 provides an alternative pathway for reaction by a retro-aldol reaction to generate the acyl-enzyme intermediate with the concomitant release of acetaldehyde. This results in the planar conjugated intermediate shown in Fig. 1 and as a consequence there is no possibility of addition of water to C-4. The identity of the acylated enzyme complexes have both been verified using electrospray ionization mass spectrometry, and the masses of these covalent intermediates correspond to the major molecular species.

Comparison of Binding Modes of EI116 and JD261 with Peptide Analogue Compounds—Atomic coordinates are available for 26 PPE x-ray structures. Protein inhibitor-PPE complexes include those with elafin (13), chymotrypsin elastase inhibitor (14), and a heptamer from β-casomorphin-7 (15). Small molecule inhibitor complexes include: peptidyl fluoromethyl ketones (16–19), peptidyl chloromethyl ketones (20), peptidyl boronic acids (21), isocoumarin derivatives (22–24), peptidylketobenzoxazoles (25), and a peptidomimetic aminimide (26). In all cases the elastase active site maintains the same shape, and typical root mean square differences between backbone atoms are less than 0.3 Å. The side chains Gln-200(192) and Arg-226(217A) can adopt different conformations and these changes are particularly pronounced in the complex with JD261 where a very different conformation of the Gln-200(192) is required to make way for the naphthyl group (Figs. 3 and 4). The unique placement of this ligand compared with all other published classes of inhibitor gives an indication of the relative lack of flexibility caused by its all-trans configuration and charge delocalized structure. The x-ray structure of trifluoroacetyl-Lys-Phe-p-isopropylanilide/PPE defined a new subsite for the anilide group, which was located under the side chain of Gln-200(192) (19). One ring of the naphthyl group

![Figure 2](image_url)

**Figure 2.** A difference electron density map calculated with sigma weighted \((2F_o - F_c)^2\) coefficients and contoured at 1.5σ showing the product of JD261 complexed with PPE. Carbon atoms for the inhibitor and protein are magenta and white, respectively. Oxygen atoms are red, and nitrogen atoms are blue.

| Protein | EI116 Contacts | JD261 Contacts |
|---------|----------------|----------------|
| EI116(O)–W611 | 3.13 | JD261(O)–W759 | 3.16 |
| EI116(O)–Ser-203/N | 2.93 | JD261(O)–Ser-203/N | 2.75 |
| EI116(O)–Gly-201/N | 3.16 | JD261(O)–Gly-201/N | 3.07 |
| EI116(O)–His-60/N | 2.85 | JD261(N1)–W749 | 2.95 |
| EI116(O)(—W702 | 2.71 | JD261(O1)–Gln-200/N | 2.90 |
| EI116(N1)–W570 | 2.96 | W751–W756 | 2.80 |
| EI116(N2)–W570 | 2.96 | W751–W759 | 2.40 |
| W702–W718 | 2.67 | W751–W764 | 2.56 |
| W751–W751 | 2.95 |
| W751–W778 | 3.12 |
of JD261 partly overlaps this site; however, the uniquely different conformation of Gln-200(192) in this structure provides a rather differently shaped pocket.

The position of EI116 in the active site resembles the binding adopted by other peptide-type inhibitors (16) in which the peptide side chains fill the specificity pockets S1 to S4. The closest similarity occurs with the trifluoroacetyl dipeptidyl isopropylanilide family of inhibitors (18). The S1 pocket is filled in this family by the trifluoroacetyl group, whereas EI116 fills the S1 pocket quite efficiently with one of the ethyl groups on what was C-3 of the \( \beta \)-lactam. The S4 subsite accommodates the aromatic anilide group, which lies in the same place as the bromphenyl group of EI116. The presence of the aromatic group causes a significant shift in the position of the side chain of Arg-226(217A), which forms van der Waals contacts with the group.

The main-chain structures of elastase bound to EI116 and JD261 show an unexpected conformational difference from residues Asn-99(95)-Thr-100(96)-Asp-101(97)-Asp-102(98). All 26 available published x-ray structures of PPE complexes show these residues in a type I \( \beta \)-turn. The electron density in the EI116 complex clearly shows a \( \beta \)II turn despite the fact that there are no direct interactions with the ligand that may have induced the change.

**Implications of Structure for Reactivation**—It has been shown that reactivation of human leukocyte elastase-inhibitor complexes, of a series of substituted mono-\( \beta \)-lactams, results in a half-life of between 3 and 15 h. Stability of the complex is insensitive to increasing ionic strength and also to the nature of the leaving group on C-4 but does however depend on the nature of the substituted urea (1).

An unmodified heptapeptide complexed with elastase (15) has been found to form an acyl-complex with Ser-203(195) through the C-terminal carboxy group. The x-ray crystal structure of this complex shows the presence of a water molecule hydrogen bonded to Ne-2 of His-60(57) and in an ideal position to attack the carbon of the ester carbonyl. A mechanism has been proposed that explains the cleavage of the acyl-enzyme intermediate by a hydroxide ion that is derived by deprotonation of a water molecule by His-60(57) (15). The structure of the JD216 complex has an identically positioned water molecule. However, the presence of the conjugated double bond is likely to confer hydrolytic stability on the acyl-enzyme intermediate as has been previously noted by the inhibition of \( \beta \)-lactamase by clavulanic acid (27–29). JD261 is therefore an example of a new class of serine protease inhibitor that provides a novel chemical mechanism for resisting reactivation. We are currently exploring the generality of this mechanism with proteases other than elastase.

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Novel Mechanism of Inhibition of Elastase by \(\beta\)-Lactams

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