Crystal structure of the complex of porcine pancreatic elastase with TEI-8362

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The crystal structure of porcine pancreatic elastase (PPE) complexed with a new benzoxazinone inhibitor, TEI-8362, of human neutrophil elastase (HNE) was determined at 1.8 Å resolution. The hydroxyl oxygen of Ser195 opened the benzoxazinone by nucleophilic attack and formed a covalent bond with the carbonyl carbon. Hydrophobic interaction between the terminal benzene of TEI-8362 and the S4 pocket is reinforced by the side chain of Arg217 and has an impact on the ligand binding conformation. Two additional interactions with the oxyanion hole and His57 are introduced to the benzoxazinone structure of TEI-8362. These combinatorial interactions will also exist in HNE and cause high preference of TEI-8362 for HNE.

Keywords: elastase inhibitor; benzoxazinone; crystal structure.

1. Introduction

Human neutrophil elastase (HNE, EC 3.4.21.37) is a serine protease of the chymotrypsin family and consists of 223 amino acids. HNE is present in the azurophilic granules of neutrophils and is implicated as a causative or contributory agent in several pathological states. Since HNE digests various components of the extracellular matrix, HNE is suggested to play a role in the metabolism of connective tissue and decomposition of foreign substances. Therefore, HNE is an attractive therapeutic target for pulmonary disease, and many compounds have already been developed as inhibitors (Kuraki *et al*., 2002).

We have developed a novel synthetic inhibitor TEI-8362, 4-(N-(3-((3-carboxypropyl)amino)-8-methyl-1-oxo-4-azaisochromen-6-yl)carbamoyl)-4-((phenylmethoxy) carbonylamino) butanoic acid, $C_{26}H_{28}N_4O_9$, with K_i value of 1.38 nM with respect to HNE (Mitstuhashi *et al.*, 1999). This compound was derived from the benzoxazinone structure, which is known to be an inhibitor for serine proteases (Spencer *et al*., 1986). Compared to other benzoxazinone compounds, TEI-8362 is water-soluble, so it offers advantages for intravenous administration and oral administration.

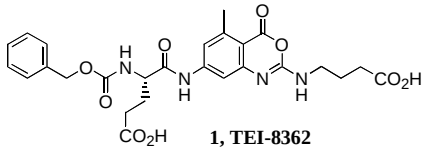
The best way to understand the inhibition mechanism is to determine the complex structure of the target protein with its inhibitors. Porcine Pancreatic Elastase (PPE, EC 3.4.21.36) is a popular target protein when examining the binding mode of HNE inhibitors (Peisach *et al*., 1995). Radhakrishnan *et al.* (1987) reported PPE complex structures with two Valine-derived benzoxazinone compounds and confirmed their common binding mode. These benzoxazinone compounds formed a covalent bond to the Oγ of Ser195 and the Valine moiety moiety oriented toward the S' direction. However, the binding mode does not explain why TEI-8362 has potent inhibitory activity.

This study will report on the crystal structure of PPE complexed with TEI-8362 at 1.8 Å resolution and discuss how this compound inhibits PPE and HNE.

2. Materials and methods

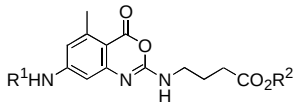
2.1. Chemistry

Compound **3** (Fig. 1) was prepared from phenylmethyl 6-amino-2-methyl-4-nitrobenzoate (**4**) in 5 steps. After amino groups in compound **4** were converted to isocyanate groups, the compound reacted with tert-butyl 4-aminobutanoate to give a urea derivative. After elimination of benzyl groups with palladium, the urea derivative was converted to compound **3** by dehydration/condensation. After compound **2** was synthesized from compound **3** and N-phenylmethoxycarbonyl-glutaric anhydride (Z-Glu anhydride), tert-butyl groups were eliminated with hydrochloric acid. Product **1** (TEI-8362, Fig. 1) that was obtained as a product of the reaction is a hydrochloride and was desalinated by the following procedures. After the product **1** was neutralized with aqueous sodium hydrogen carbonate, the neutral solution was extracted with ethyl acetate. After excess sodium hydrogen carbonate in the extract was neutralized with citric acid and the organic phase was washed with water, crystals were obtained by concentration to a given quantity. The crystals obtained were filtered, washed with cold ethyl acetate, and dried under reduced pressure to produce free-form **1**.



1, TEI-8362

(4S)-4-(N-{2-[(3-carboxypropyl)amino]-5-methyl-4-oxobenzo[3,4-d]1,3-oxazin-7-yl}carbamoyl)-4-[(phenylmethoxy)carbonylamino]butanoic acid



2, R^1= Z-Gul, R^2 = ^{t}Bu
3, R^1= H, R^2 = ^{t}Bu

Figure 1

Chemical structure of TEI-8362.

2.2. Crystallization

PPE and TEI-8362 were crystallized using the sitting drop vapour diffusion method. PPE was purchased from Sigma and was used without further purifications. 20 mM DMSO solution of TEI-8362 was added to the PPE solution, 30 mg/ml in 30mM sodium acetate buffer (pH5.5), with molar ratio 10:1 for co-crystallization. The reservoir solution consisted of 0.9M Na_2SO_4, 100 mM sodium acetate buffer (pH5.5) and 5%(v/v) DMSO. All crystallization trials were carried out at a constant temperature of 20°C. The droplets were allowed to equilibrate for a day before they were seeded from native PPE crystals (Sawyer *et al*., 1978).

2.3. Data collection and processing

A crystal with approximate dimensions of 50 μm x 70 μm x 70 μm was used for data collection with an R-AXIS IV imaging plate detector (Rigaku) at the BL24XU-A, Hyogo beamline (λ=0.834 Å), in SPring-8. The crystal was mounted on a cryoloop (Hampton Research) at 100 K during the data collection. The data set was processed using the program PROCESS (Rigaku). The crystallographic data are shown in Table 1.

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2.4. Refinements

Refinements were carried out with Refmac5 in the CCP4 suite (1994). The isomorphous PPE crystal coordinate (PDB code: 1EAS) was initially used for rigid body refinement (Bernstein *et al*., 1994). QUANTA2000/X-ray (Accelrys) was used for map fitting and for determination of the inhibitor binding. Restrained refinements were gradually expanded to 1.8 Å resolution. The connecting electron density over the Oγ of Ser195 indicated a covalent bond between TEI-8362 and hydroxyl oxygen of Ser195. The modified serine residue was created by Monomer library sketcher in the CCP4 suite (Murshudov *et al*., 1997). A sulphate ion and a calcium ion were added to the model by manual assignment and 98 water molecules by ARP/wARP (Asselt van *et al*., 1998) in the CCP4 suite.

Table 1

Crystallographic data and refinement statistics of the TEI-8362 complexed PPE.

Data collection and processing	
Space group	$P2_12_12_1$
Unit cell parameters (Å)	a=50.04, b=57.75, c=74.26
Resolution (Å)	40-1.8
R_{merge} (%)	6.7
Completeness (%)	99.0 (96.9)
Observations	76,972
Unique reflections	18,655
Redundancy	4.13
$I/\sigma(I)$	13.6 (3.48)
Refinement	
Wilson B factor (Å^2)	18.3
R_{cryst} (%)	16.3 (23.9)
R_{free} (%)	21.2 (30.7)
RMS deviations from ideal values	
Bond length (Å)	0.028
Angle (°)	2.083
Estimated coordinate error (Å)	0.136

Values in parentheses are for the outer shell (1.85-1.80Å).

3. Results and discussions

3.1. Binding conformation of TEI-8362

Based on the electron density of the PPE complex structure, TEI-8362 is found to cover the active site with a stretched form (Fig. 2). Two carboxyl groups, which are hydrophilic parts of TEI-8362, oriented for the solvent region and have no specific interactions with the protein structure. This means that all carboxyl groups contribute only to solubility of the inhibitor. As a characteristic interaction in the benzoxazinone structure, TEI-8362 opened the ring in the benzoxazinone moiety by nucleophilic attack *via* Oγ of Ser195 and formed a covalent bond (Fig. 3). This chemical reaction was also observed in the PPE complex structure with the Valine-derived inhibitor 5CL, (1-(5-chloro-4-oxo-4H-3, 1-benzoxazine-2-yl)-2-methyl-propyl) carbamic acid 1,1-dimethylethylethyl ester (Radhakrishnan *et al.,* 1987). Therefore it should be the principal factor of inhibitory activity of the benzoxazinone structure.

3.2. Effects of hydrophobic interaction

Compared to the 5CL binding conformation, the benzene moiety produced from the benzoxazinone scaffold is inclined +36.8° towards the S4 pocket (Fig. 4). The S4 pocket of PPE consists of three hydrophobic residues (Phe215, Val99a and Trp172). Terminal benzene of TEI-8362 is accommodated there and covered by the side chain of Arg217. There are no significant differences between the native PPE (Wurtele *et al*., 2000) and TEI-8362 complexed PPE structures except the Arg217 position (Fig. 5). The CZ atom of Arg217 has moved 2.76 Å from the position of native structure. The side chain movement of Arg217 was also observed in the PPE complex with FR136706 (Kinoshita *et al*., 2003). In addition, carbonyl oxygen of TEI-8362 forms a hydrogen bond with Val216 NH (3.21 Å, Fig. 6). These two interactions determine the binding conformation of TEI-8362 and produce two additional interactions with the benzoxazinone moiety of TEI-8362. One is an interaction with the oxyanion hole (Henderson, 1970), and the other one is a hydrogen bond with His57 catalytic triad.

3.3. Oxyanion hole interaction

As seen in the PPE/5CL complex, simple benzoxazinone derivatives have difficultly orienting the carbonyl group towards the oxyanion hole. One reason is that the benzene ring of the benzoxazinone moiety tends to come parallel to the S1 pocket and insert a small hydrophobic portion into the S1 pocket. However, under the influence of hydrophobic interaction, TEI-8362 can locate carbonyl oxygen in the oxyanion hole (Fig. 6). The carbonyl oxygen forms two hydrogen bonds with Ser195 NH (2.88 Å) and Gly193 NH (2.79 Å). On the other hand, 5CL oriented the carbonyl group in the direction opposite the oxyanion hole. The interaction with oxyanion hole was often observed in the peptidyl inhibitor complexes with PPE (Mattos *et al*., 1994) and HNE (Wei *et al*., 1988).

3.4. Hydrogen bond with His57

To avoid the steric repulsion with the carbonyl group located at the oxyanion hole, the adjacent carbonyl group, produced from the benzoxazinone moiety, is oriented towards His57. Thus, TEI-8362 forms a hydrogen bond between carbonyl oxygen and His57 NE2 (2.82 Å). Since the position of His57 is almost the same in the native PPE structure, the carbonyl oxygen seems to play the role of Ser195 hydroxyl oxygen and stabilizes the catalytic triad in the TEI-8362 complex. In the PPE/5CL complex, on the contrary, the Valine moiety of 5CL pushed away the side chain of His57 to bind the active site of PPE.

4. Conclusion

A covalent bond formation with Oγ of Ser215 is an important interaction for TEI-8362 to inhibit the elastase function. In addition, four hydrogen bonds were newly observed in the bezoxazinone structure and caused by the hydrophobic interaction in the S4 pocket. The hydrophobic interaction in the S4 pocket would also effect upon the specificity of the inhibitor against PPE. Thus, the hydrogen bonding network and the hydrophobic interaction seems to enhance the inhibitory activity of TEI-8362 and stabilize the binding conformation. These combinatorial interactions observed in the PPE/TEI-8362 complex may occur in the HNE complex as well.

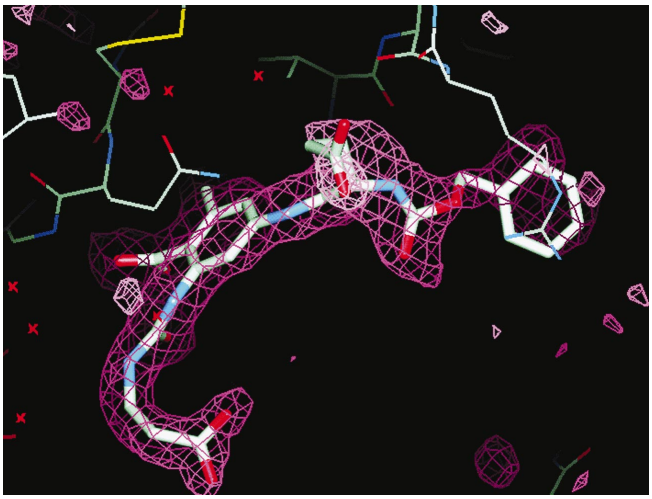

Figure 2
Omit map (*Fo-Fc*) for bound inhibitor (TEI-8362) contoured at 2.5σ.

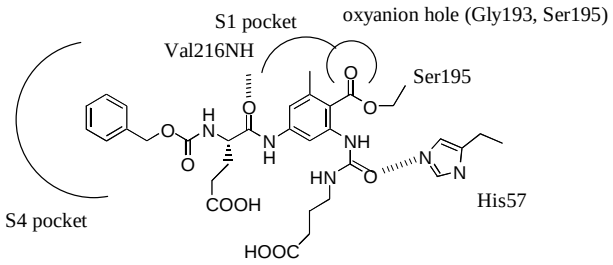

Figure 3
Interactions of TEI-8362 in the active site. A covalent bond is formed between TEI-8362 and the Oγ of Ser195. The hydrophobic interaction in the S4 pocket determines the binding conformation to form the hydrogen bonding network.

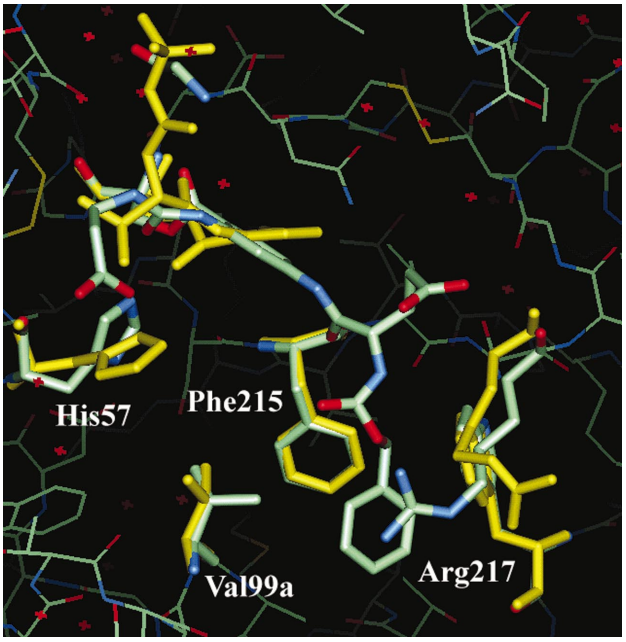

Figure 4
Binding conformations of TEI-8362 and 5CL. The yellow and the atom coloured stick models represent the 5CL and the TEI-8362 complex structures, respectively.

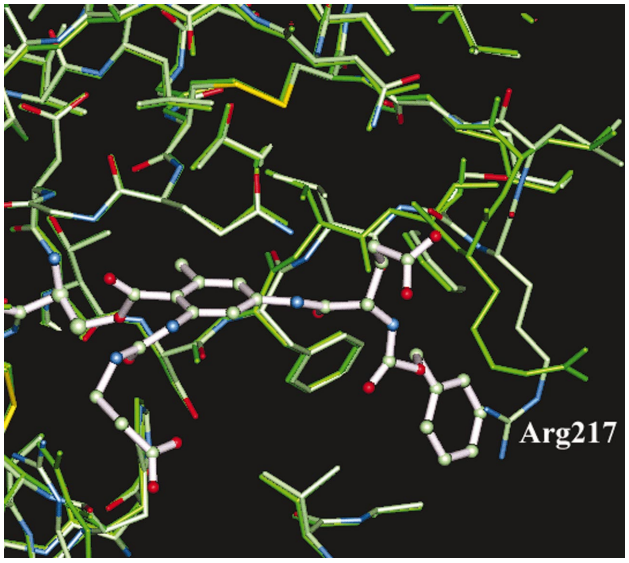

Figure 5
Superposition of the native PPE (1QNJ; green) and the TEI-8362 complexed PPE. TEI-8362 is drawn in ball and stick model.

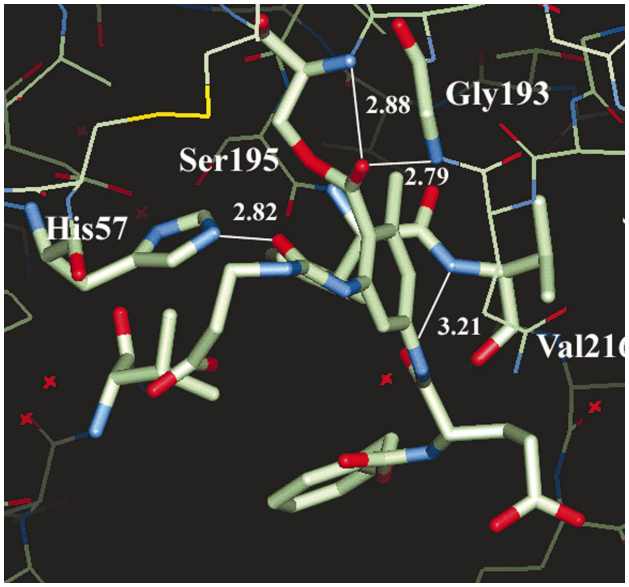

Figure 6
The hydrogen bond network in the active site. White lines represent hydrogen bonds between PEE and TEI-8362.

References

Asselt van, E.J., Perrakis, A., Kalk, K.H., & Lamzin, V.S. (1998) *Acta Cryst.* **D54**, 58-73.

Bernstein, P.R., Andisik, D., Bradley, P.K., Bryant C.B., Ceccarelli C., Damewood, J.R. Jr, Earley, R., Edwards, P.D., Feeney, S., Gomes, B.C., Kosmider, B.J., Steelman, G.B., Thomas, R.M., Vacek, E.P., Veale, C.A., Williams, J.C., Wolanin, D.J. & Woolson, S.A. (1994) *J. Med. Chem.* 37(20), 3313-3326.

Collaborative Computational Project, Number 4.1994. *Acta Cryst.* **D50**, 760-763.

Henderson, R. (1970) *J. Mol. Biol.* **54**(2), 341-354.

Kinoshita, T., Nakanishi, I., Sato, A. & Tada, T. (2003) *Bioorg. Med. Chem. Lett.* **13**(1), 21-24.

Kuraki, T., Ishibashi, M., Takayama, M., Shiraishi, M. & Yoshida, M. (2002) *Am. J. Respir. Crit. Care. Med.* **166**(4), 496-500.
Mattos, C., Rasmussen, B., Ding, X., Petsko, G.A. & Ringe, D. (1994) *Nat. Struct. Biol.* **1**(1), 55-58.
Meyer, E., Cole, G., Radhakrishnan, R. & Epp, O. (1988) *Acta Cryst.* **B44,** 26-38.
Mitsuhashi, H., Nonaka, T., Hamamura, I., Kishimoto, T., Muratani, E. & Fujii, K. (1999) *Br. J. Pharmacol.***126**(5), 1147-1152
Murshudov, G.N., Vagin, A.A. & Dodson, E.J. (1997) *Acta Cryst*. **D53**, 240-255.
Peisach, E., Casebier, D., Gallion, S.L., Furth, P., Petsko, G.A., Hogan, J.C. Jr. & Ringe, D. (1995) *Science* **269**(5220), 66-69.
Radhakrishnan, R., Presta, L.G., Meyer, E.F. Jr. & Wildonger, R. (1987) *J. Mol. Biol.* **198**(3), 417-24.
Sawyer, L., Shotton, D.M., Campbell, J.W., Wendell, P.L., Muirhead, H., Watson, & H.C. (1978) *J. Mol. Biol.* **118**(2), 137-208.
Spencer, R.W., Copp, L.J., Bonaventura, B., Tam, T.F., Liak, T.J., Billedeau, R.J.& Krantz, A. (1986). *Biochem. Biophys. Res. Commun.* **140**(3), 928-933.
Wei, A.Z., Mayr, I. & Bode, W. (1988) *FEBS Lett.* **234**(2), 367-373.
Wurtele, M., Hahn, M., Hilpert, K. & Hohne, W. (2000) *Acta Cryst.* **D56**, 520-523.

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