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Structure of the RWJ-51084–bovine pancreatic β-trypsin complex at 1.8 Å

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The three-dimensional structure of bovine pancreatic trypsin complexed with the inhibitor RWJ-51084 has been determined at 1.8 Å resolution. These crystals belong to the trigonal space group $P3_121$, with unit-cell parameters $a = b = 53.43$, $c = 107.76$ Å. The refined R and $R_{\rm free}$ values are 0.175 and 0.237, respectively. The carbonyl group bonded to the benzothiazole group of the inhibitor is covalently linked to the hydroxyl O atom of Ser195, forming a tetrahedral intermediate hemiketal structure. The other carbonyl O atom of the inhibitor forms a hydrogen bond with the Gln192 side-chain amide group. The benzothiazole group is oriented with the aromatic N atom of RWJ-51084 accepting a hydrogen bond from His57 NE2. The arginine side chain of the inhibitor extends into the deep and narrow pocket of the S1 specificity site of trypsin, forming a network of hydrogen bonds.

1. Introduction

Trypsin-like serine proteases play a central role in many physiological processes, ranging from digestion to key regulatory mechanisms such as peptide hormone release and blood coagulation (Hörn & Heidland, 1992; Cunningham & Long, 1987). The imbalance of their activities may lead to serious diseases, including tumors, inflammatory processes, pulmonary emphysema, glomerulonephritis, acute pancreatitis and muscular dystrophy (Nakayama *et al.*, 1997). There is considerable interest in developing selective and potent inhibitors of these enzymes. In the present work, we report the structure of bovine pancreatic β-trypsin complexed with a benzothiazole-containing inhibitor, RWJ-51084 ((*S*)-*N*-{4-[(aminoiminomethyl)amino]-1-(2-benzothiazolylcarbonyl)butyl}-cyclopentanecarboxamide; Fig. 1). RWJ-51084 potently inhibits trypsin with a K_i value of 30 ± 14 nM ($n = 6$) and is selective relative to other serine proteases such as kallikrein, thrombin, factor Xa and chymotrypsin (P. Andrade-Gordon *et al.*, unpublished results).

As with other serine-protease inhibitors, RWJ-51084 lacks the scissile amide bond of a protease substrate, but has a carbonyl group capable of forming a hemiketal bond which resembles the tetrahedral intermediate for amide hydrolysis. The peptide-like inhibitor also has a heterocycle at the C-terminal end, the presence of which activates the carbonyl group toward nucleophilic addition of the active-site serine hydroxyl group (Edwards *et al.*, 1992; Tsutsumi *et al.*, 1994; Costanzo *et al.*, 1996; Matthews *et al.*, 1996). The N atom of the benzothiazole ring participates in stabilizing the enzyme–inhibitor complex by providing a hydrogen-bonding interaction with the His57 residue of the catalytic triad of trypsin. Given the potency of RWJ-51084, it is particularly noteworthy

Table 1
Crystallographic data for bovine pancreatic β-trypsin–RWJ-51086 complex.

Space group	$P3_121$
Unit-cell dimensions (Å)	$a = b = 53.43$, $c = 107.77$
Number of reflections	236936
Number of unique reflections	17832
Overall completeness of data (99–1.8 Å) (%)	99.7
Completeness in the highest resolution shell (1.86–1.80 Å) (%)	98.6
R_{merge} (overall)	0.039
R_{merge} (highest resolution shell)	0.204
I/σ(overall)	21.9
Percentage of reflections with $I/\sigma > 3$ in the highest resolution shell	67
Resolution range for refinement (Å)	8.0–1.8
Number of reflections used for refinement	16515
Number of reflections used for R_{free} calculation	1637
R	0.18
R_{free}	0.24
Number of water molecules	207
Mean B value (all atoms) (Å^2)	21.0
R.m.s. deviations from ideal values	
Bond lengths (Å)	0.01
Bond angles (°)	1.3
Dihedral angles (°)	27.6

that this inhibitor is comprised of just a single amino-acid residue.

2. Experimental procedure

2.1. Preparation of the RWJ-51084–trypsin complex

Bovine pancreatic trypsin was purchased from Worthington Biochemical. Trypsin was dissolved in 50 mM ammonium acetate buffer (pH 5.5) containing 0.1%(w/v) $CaCl_2$. The inhibitor, previously dissolved in DMSO, was added in a 1.5-fold molar excess. Crystals of trypsin–RWJ-51084 were grown at room temperature using the hanging-drop vapor-diffusion technique, where drops of 2 µl of protein solution mixed with 2 µl of reservoir solution were equilibrated against a reservoir solution of 0.1 M Tris–HCl buffer in the pH range 8.0–8.7 containing either 1.75 M $(NH_4)_2SO_4$ or 1.6 M $MgSO_4$. Crystals suitable for X-ray diffraction were obtained after 10 d with $MgSO_4$ as a precipitant and after three months using $(NH_4)_2SO_4$ as a precipitant.

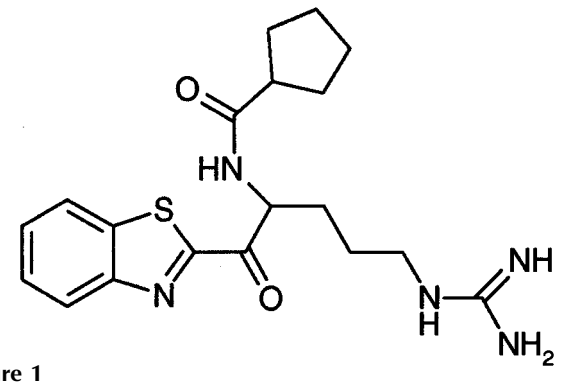

Figure 1
Chemical formula of RWJ-51084.

2.2. Data collection and processing

Crystals of trypsin–RWJ-51084 complex belong to the trigonal space group $P3_121$, with unit-cell parameters $a = b = 53.43$, $c = 107.76$ Å. Diffraction data were collected at 103 K using 25% glycerol as cryoprotectant. The best data, obtained with crystals grown in ammonium sulfate, were collected on an R-AXIS IV imaging plate and processed with *DENZO* (Otwinowski & Minor, 1997) to 1.8 Å. The completeness of data at this resolution was 98.6%, the R_{merge} was 3.9% and the overall I/σ was 21.9.

2.3. Structure determination and refinement.

The structure is isomorphous with a previously reported bovine β-trypsin structure (PDB entry 3ptn; Walter *et al.*, 1982). This model, stripped of solvent molecules and calcium ions and with uniform thermal and occupancy parameters, was used for the initial refinement using *X-PLOR* (Brünger *et al.*, 1987). Rigid-body minimization followed by simulated annealing with slow cooling (Brünger, 1990) and conjugate-gradient positional refinement were carried out. Distinction between $P3_121$ and $P3_221$ was made from R factors calculated after rigid-body refinement using each space group. The molecular model of the inhibitor RWJ-51084 (Fig. 1) was constructed and built into electron density using *CHEM-NOTE* of the *QUANTA* program. The ideal parameters for the inhibitor were derived from small-molecule structure information from the Cambridge Structural Database (Allen *et al.*, 1979). Manual corrections of the model complex were

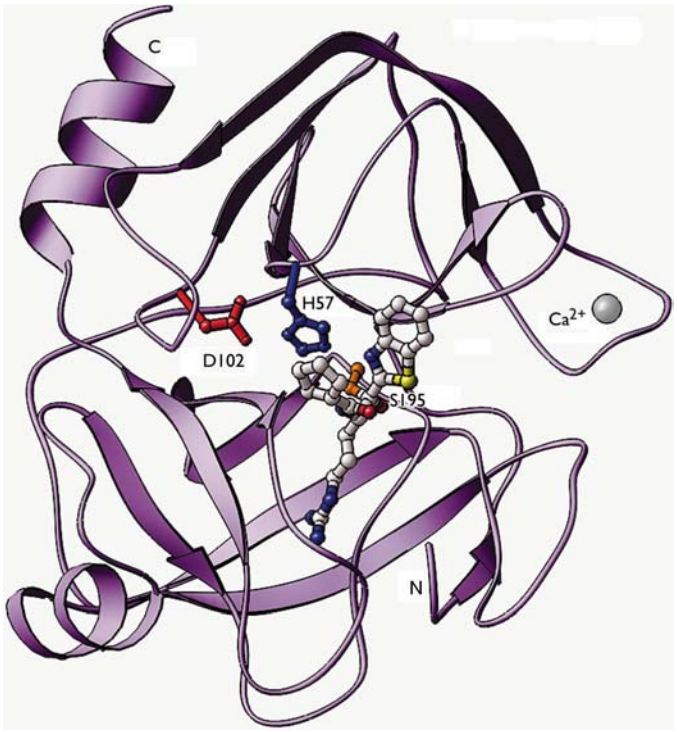

Figure 2
Ribbon plot of the complex formed between trypsin and RWJ-51084. Side chains of the catalytic triad residues His57, Asp102 and Ser195 are explicitly shown, as well as the calcium cation on the right. All figures prepared with *Ribbons*, http://www.cmc.uab.edu/ribbons (Carson, 1997).

Table 2
Interactions of the inhibitor in the active site of bovine pancreatic β-trypsin.

O, oxygen atom of the carbonyl group bonded to the benzothiazole ring; OP, oxygen atom of the carbonyl group bonded to the pentanyl ring.

Inhibitor	Trypsin/water	Distance (Å)
NH2	Asp189 OD2	2.70
NH1	Asp189 OD1	2.80
NE	Wat504 OH2	2.98
NH2	Gly219 O	2.85
NH1	Ser190 OG	2.80
O	Ser195 N	2.53
O	Gly193 N	2.91
N5	His57 NE2	2.77
N	Ser214 O	3.45
OP	Wat594 OH2	2.59
OP	Gln192 NE2	2.99

performed during the refinement with the *QUANTA* graphics program. The refinement data were gradually extended to 1.8 Å resolution. Solvent molecules were inserted at reasonable positions where the difference electron density exceeded 2.5σ. Finally, the individual atomic B values were refined. Crystallographic statistics for the structure are reported in Table 1.

To provide a rigorous test of the accuracy of the model, omit maps covering a portion of the structure were computed following a simulated-annealing procedure in which coordinates of the portion of the model to be tested were removed (Brünger, *X-PLOR*, v.3.85). For the complex structure including 208 water molecules and one calcium ion, the R and $R_{\rm free}$ values are 0.175 and 0.237, respectively, for 16515 reflections (resolution limits 8–1.8 Å with a cut-off of 3σ). The root-mean-square deviation from ideality for bond lengths, bond angles and torsion angles are 0.01 Å, 1.22° and 27.6°, respectively. The mean B-factor value for all atoms is 21.0 Å^2.

PROCHECK (Laskowski *et al.*, 1993) shows that 85.6% of the residues, excluding glycines and prolines, are in the most favoured regions of the Ramachandran plot. 13.8% are found in additional allowed areas, whereas one amino acid (Asn115) localized in the generously allowed regions has diffuse density.

3. Results

The overall folding of RWJ-51084-inhibited trypsin (Fig. 2) was almost identical to that of a previously analyzed native trypsin structure (PDB entry 3ptn). The root-mean-square difference between the two structures was 0.29 Å for backbone atoms. This value indicates that the overall structure of trypsin is not influenced by the binding of small-molecule inhibitors (Kurinov & Harrison, 1994). The catalytic triad which is formed by His57, Asp102 and Ser195 is shown in Fig. 2. As in the intact trypsin, the Asp102 carboxyl O atom forms a hydrogen bond with the imidazole N atom of His57 (the distance between Asp102 OD2 and His57 ND1 is 2.55 Å).

The trypsin–RWJ-51084 binary complex is well defined in the final $2F_o - F_c$ electron density, except for the side chains of residues Asn115 and Arg117 of trypsin. These residues have significantly higher thermal parameters than the remainder of the model. The most significant differences between the present structure and the native bovine trypsin occur at Gln192, with a maximum displacement of over 4 Å of Gln192 NE2. The orientation of the Gln192 side chain is favored for the hydrogen bond between the amide group of this residue and the carbonyl OP atom of the inhibitor (Table 2).

The benzothiazole and the cyclopentyl groups of RWJ-51084 are found at the entry of the cleft of the trypsin-binding site, making few interactions with the enzyme (Fig. 3). As a result, although these groups are well defined in the electron-density map, they have high temperature factors (Fig. 4). The cyclopentyl ring presents a flexible conformation. The benzothiazole phenyl ring is within van der Waals distance of the disulfide bond Cys42—Cys58, as part of a π/S—S interaction, and the cyclopentyl ring interacts with Leu99 in the S2 specificity pocket. The benzothiazole phenyl ring is bordered by solvent molecules.

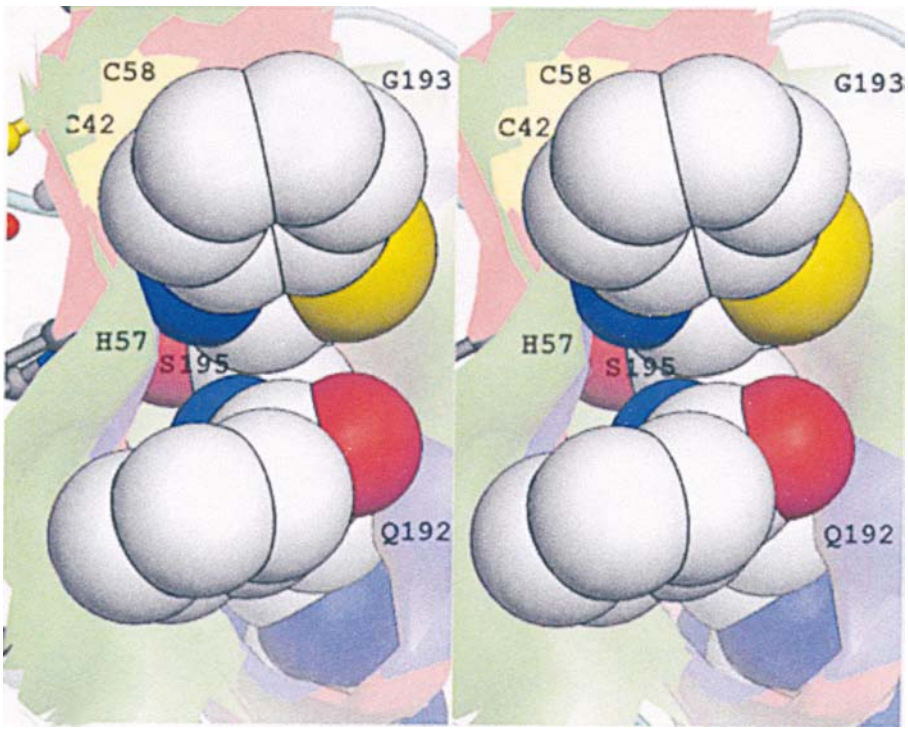

Figure 3
Stereoview of the entrance of the active-site pocket. The location of the residues of trypsin forming the entrance are indicated on the surface.

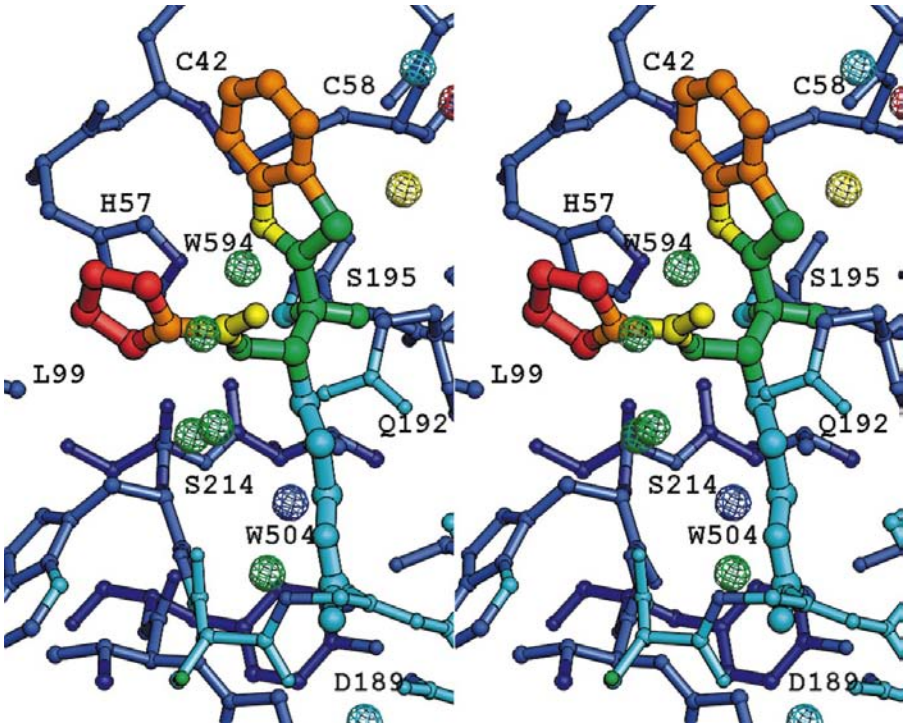

Figure 4
Stereoview of the *B*-factor plot. The inhibitor atoms are colored according to their respective *B* factors. The lowest *B* factors, <24 Å^2, are colored in blue and correspond to the arginine side chain inserted in the S1 pocket. The atoms with *B* factors <30 Å^2, corresponding to the arginine main-chain and some atoms of the thiazole ring, are colored in green. The S atom and the cyclopentyl carbonyl group, whose *B* factors are <36 Å^2, are colored yellow. The benzyl and cyclopentyl groups, which are the most exposed parts of the inhibitor, present the highest *B* factors, <42 (orange) and <48 Å^2 (red), respectively.

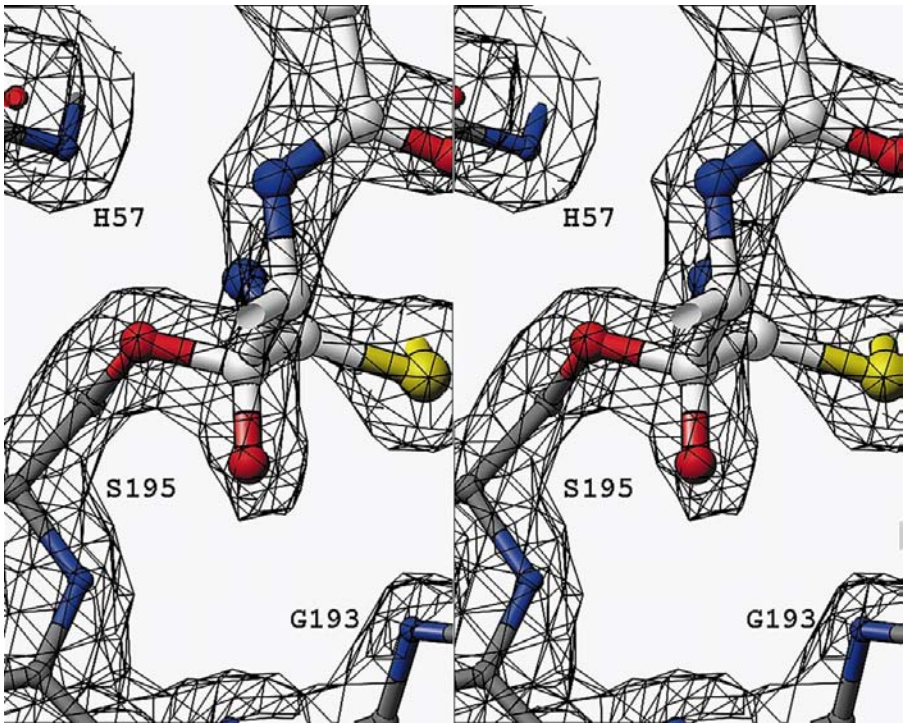

Figure 5
Stereoview of the electron-density map ($2F_o - F_c$, at 1.5σ) for the tetrahedral model of bovine pancreatic trypsin and RWJ-51084. The carbonyl group joined to the benzothiazole group of the inhibitor is covalently linked to the hydroxyl O atom of Ser195 forming a tetrahedral intermediate hemiacetal structure.

The location of the calcium ion is similar to that in other trypsin structures (Huang *et al.*, 1993). However, in the present structure, a water molecule occupies the position of Glu77 OE1 (Bartunik *et al.*, 1989). The coordinating O atoms show a distorted octahedral geometry, with a longer than average distance to Glu77 OE2. The coordination distances are as follows: Asn72 O, 2.35 Å; Val75 O, 2.14 Å; Glu80 OE2, 2.40 Å; Glu70 OE2, 2.36 Å; Wat553 OW, 2.44 Å; Wat601, 2.34 Å; Glu77 OE2, 3.14 Å. The calcium ion has full occupancy and a *B* factor of 24.6 Å^2.

RWJ-51084 is a reversible tight-binding inhibitor (Knight, 1986). It inhibits the enzyme by forming a transition-state analog complex. It forms a covalent complex with trypsin. There is continuous well defined electron density between Ser195 OG and the Arg carbonyl group of the inhibitor (Fig. 5). The keto carbonyl C atom of the inhibitor and the Ser195 OG are joined, with a bond length of 1.42 Å. This gives rise to a nearly tetrahedral arrangement around the carbonyl (hemiketal) C atom.

To describe the interactions between trypsin and the inhibitor, the nomenclature introduced by Schechter & Berger (1967) is used. The position of the reactive site of the inhibitor is known as the primary binding site or P1 position. The inhibitor binds tightly to the enzyme by means of the covalent union and the interactions of the guanidinium group, which will be discussed later. This tight binding stabilizes the structure of this portion of the inhibitor, as can be seen from the low *B* values of the corresponding atoms (Fig. 4). This is one of the structural features of the transition-state analog-type inhibitors, which are more potent than other small molecules which simply fill an active-site pocket of the enzyme (Kurinov & Harrison, 1994, 1996).

There are 11 hydrogen bonds/ion pairs between trypsin and the inhibitor. A summary of all polar contacts between the enzyme and inhibitor is given in Table 2. The P1 arginine residue is involved in eight hydrogen bonds. The arginine occupies trypsin's

deep narrow specificity pocket, making full use of the hydrogen-bonding capabilities of the guanidinium ion. The guanidinium ion forms two salt bridges with the Asp189 side-chain carboxylate ion at the bottom of the pocket. It also forms hydrogen bonds with Gly219 O and Ser190 OG and a weak hydrogen bond with Ser190 O (Taylor *et al.*, 1984). One water molecule (Wat504) bridges Gly216 O, Gly219 O and the inhibitor Arg NE through hydrogen bonds (distances: Arg NE···Wat504, 2.98 Å; Wat504···Gly216 O, 2.82 Å; Wat504···Gly219 O, 2.87 Å). The presence of this arginine is not favorable for binding of RWJ-51084 to chymotrypsin, which prefers an aromatic residue in the substrate-binding pocket.

One of the interactions involved in the antiparallel β-strand binding which characterizes serine-proteinase substrate recognition motifs is the hydrogen bond between the amido N atom of the P1 arginine and the carbonyl O atom of Ser214. However, in the present structure these atoms are not at a favorable distance (3.5 Å) and orientation to form a hydrogen bond. This is commonly seen in other complexes of this group of enzymes (Takahashy *et al.*, 1989). Neither does the cyclopentyl carbonyl O atom at P2 contribute to the β-pleated sheet arrangement observed in other serine-proteinase–inhibitor complexes (Edwards *et al.*, 1992; Takahashy *et al.*, 1989). Nevertheless, the carbonyl O atom forms hydrogen bonds with the amido N atom of the Gln192 side chain (2.99 Å) and with a water molecule (Wat594, distance 2.59 Å). The carbonyl O atom of the hemiketal accepts a hydrogen bond from the main-chain amido groups of Gly193 and Ser195 in the oxyanion hole (Fig. 6).

The benzothiazole ring of the RWJ-51084 inhibitor binds at the S1′ subsite. It is surrounded by His57, with which it forms a hydrogen bond involving NE2, as well as Cys42 and Cys58. A similar hydrogen bond involving His57 NE2 and a N atom of a heterocyclic ring was inferred in the crystal structures of the complexes between thrombin and RWJ-50353 (*N*-methyl-D-phenylalanine-*N*-{5-[(aminoiminomethyl)amino]-1-[(2-benzothiazolyl)carbonyl]-butyl}-L-prolinamide trifluoroacetate (Costanzo *et al.*, 1996; Matthews *et al.*, 1996) and between elastase and (*R*)-*N*-acetyl-L-alanyl-*N*-1-(2-benzoxazolylcarbonyl)-2-methylpropyl-L-prolinamide (Edwards *et al.*, 1992).

The active-site region of the human α-thrombin structure overlaid with the transformed model of RWJ-51084 (after energy minimization) is shown in Fig. 7. In this model, the inhibitor molecule fits in the active site of thrombin almost perfectly in its trypsin-binding conformation (Fig. 2). The benzothiazole group of RWJ-51084 is similarly placed in the thrombin pocket to that of RWJ-50353 in its thrombin complex (Matthews *et al.*, 1996); the arrangement of the main-chain atoms is also similar. However, the cyclopentyl group of RWJ-51084 takes a perpendicular configuration to the proline of RWJ-50353 in the S2-cavity and does not fill it up tightly.

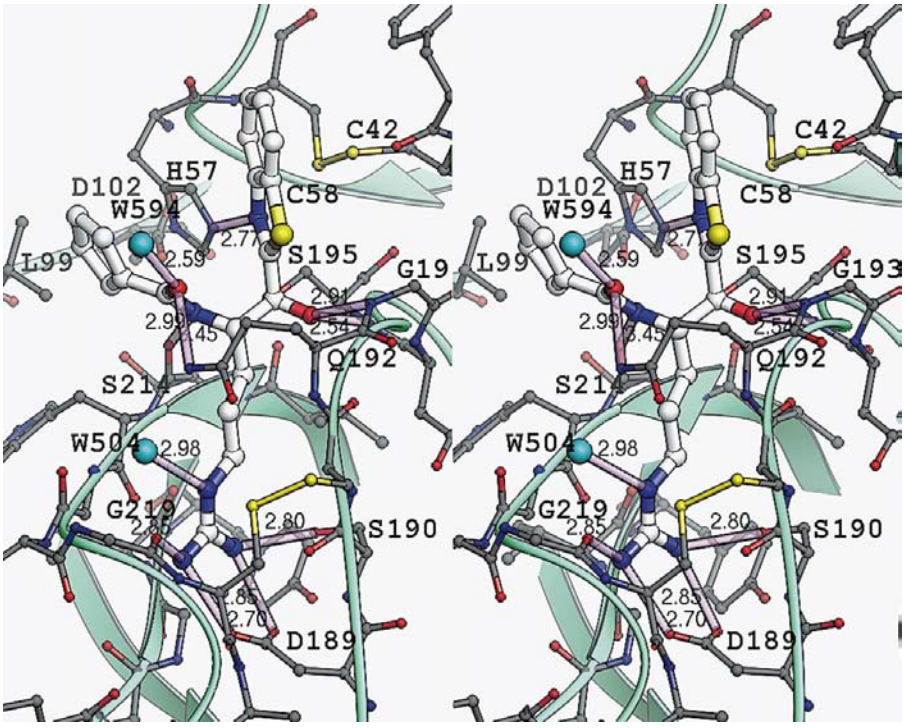

Figure 6
Interactions of the RWJ-51084 inhibitor with the bovine pancreatic β-trypsin. Hydrogen bonds are shown as transparent purple cylinders. Distances are labeled (see Table 2). (i) The arginine side chain of the inhibitor extends into a deep and narrow pocket of the S1 specificity site of trypsin, forming a network of hydrogen bonds with the carboxylate group of Asp189, the main-chain carbonyl groups of Ser190 and Gly219 and two water molecules. (ii) The O atom of the carbonyl group bonded to the benzothiazole group is oriented towards the oxyanion hole, forming hydrogen bonds with the main-chain NH groups of Gly193 and Ser195. (iii) The other inhibitor carbonyl group forms hydrogen bonds with the Gln192 amide group and a water molecule. (iv) The configuration of the benzothiazole group is disposed such that the aromatic N atom of the inhibitor accepts a hydrogen bond from His57. (v) The rings of the inhibitor make hydrophobic interactions with Leu99 and the disulfide bond between Cys42 and Cys58.

4. Discussion

The crystal structure of bovine β-trypsin complexed with the RWJ-51084 inhibitor presented in this paper reveals some general features of the interaction of the guanidino/thiazole-containing inhibitors with trypsin as well as with thrombin.

(i) The guanidino group is placed adjacent to the carboxylate group of Asp189, allowing a favorable salt-bridge interaction.

(ii) The presence of a highly electron-withdrawing benzothiazole group in the trypsin inhibitor RWJ-51084 and in the thrombin inhibitor RWJ-50353 (Matthews *et al.*, 1996) increases the

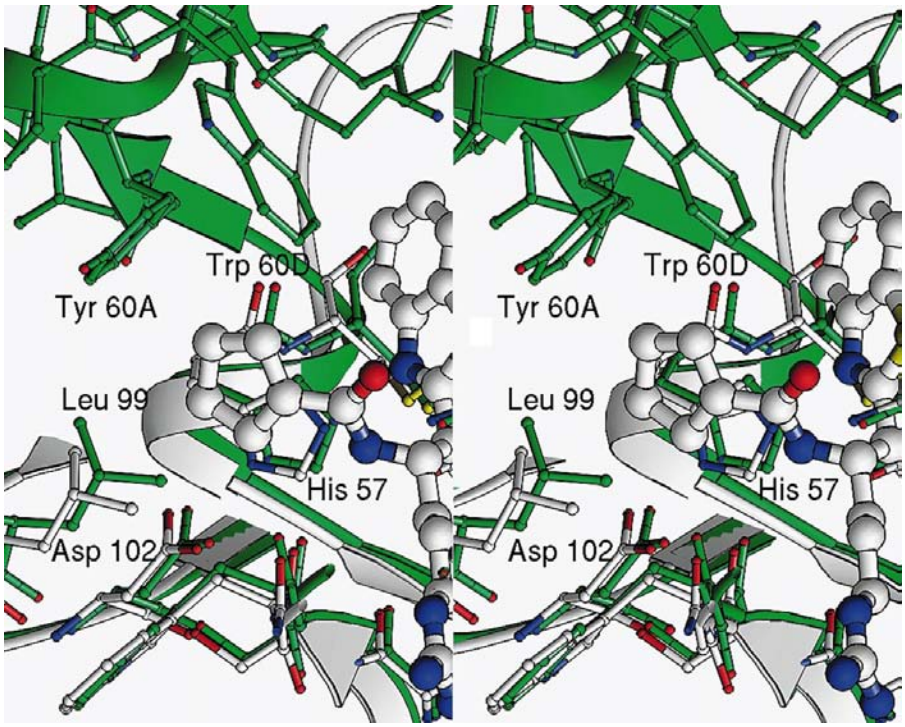

Figure 7
Active-site region of thrombin (green) superposed on trypsin (white) with proposed model of RWJ-51084.

electrophilicity of the arginine carbonyl C atom, leading to a stronger interaction with Ser195 OG (Edwards *et al.*, 1992; Costanzo *et al.*, 1996; Matthews *et al.*, 1996). The covalent union of the inhibitor with the Ser195 OG is also stabilized by the presence of a heteroatom (N3) capable of functioning as a hydrogen-bond acceptor with the protonated active-site residue His57.

(iii) The sp^3 hybridization of the carbonyl C atom with a tetrahedral geometry is also stabilized by specific interactions in the oxyanion hole (Presnell *et al.*, 1998). The carbonyl O atom in this position has its acidity increased and can therefore take maximum advantage of the stabilization afforded through hydrogen-bonding interactions with Ser195 NH and Gly193 NH.

As described above, interactions of the guanidinium and benzothiazole group are equally possible in trypsin and in thrombin. Thrombin, however, offers additional docking sites for interactions with the peripheral inhibitor groups, namely the 'aryl-binding site' (Bode *et al.*, 1990) and a cavity-like S2 subsite. In most thrombin–inhibitor/substrate complexes, the S3 aryl-binding site is generally occupied by an aromatic or hydrophobic residue. However, in trypsin there is an increased polarity at this site and it is filled by water molecules in the present structure. This explains the fact that RWJ-51084 inhibits trypsin more efficiently than it inhibits thrombin. On the other hand, RWJ-50353, which has the benzothiazole group joined to the D-Phe-Pro-Arg moiety, binds to thrombin with a K_i of 0.13 n*M* (Matthews *et al.*, 1996; Costanzo *et al.*, 1996).

In thrombin, as is shown in Fig. 7, the cyclopentyl ring is shielded from bulk solvent and is situated in a hydrophobic cavity formed by the residues His57, Trp215, Tyr60A, Trp60D and Leu99. In the trypsin structure, the lack of the insertion loop (Tyr60A–Thr60I) exposes the cyclopentyl ring to solvent. This would explain the high *B* factors of the cyclopentyl ring atoms (Fig. 4). High *B* factors are observed for atoms in the phenyl ring of the benzothiazole group in the trypsin structure, where it is surrounded by water molecules. In the thrombin structure, the phenyl ring presents more interactions with the Tyr60A–Thr60I loop (see Matthews *et al.*, 1996).

This structural study of bovine α-trypsin complexed with RWJ-51084 provides important information for the design of tight-binding inhibitors of serine proteases.

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