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INHIBITION OF SERINE PROTEINASES BELONGING TO THE CHYMOTRYPSIN SUPERFAMILY BY THE CYCLIC THIOLIC COMPOUND YS3025: A COMPARATIVE CRYSTALLOGRAPHIC STUDY

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SUMMARY. The synthetic cyclic thiolic compound 3-[2-(2-thiophencarboxythio)]-propanoyl-4-thiazolidin carboxylic acid (YS3025) acts as an effective inhibitor of bovine α-chymotrypsin. In the present communication YS3025 binding studies are extended to bovine β-trypsin and porcine pancreatic elastase, by means of crystallographic difference Fourier techniques. For all the enzymes considered, the thiophencarbonyl moiety of YS3025 is located at the entrance of the inhibited proteinase primary specificity pocket (S_1), covalently linked to the catalytic Ser195 OG atom, and forming an acyl-enzyme complex. These observations allow to select between alternative binding (and inhibition) mechanisms for YS3025 and related molecules to serine proteinases belonging to the chymotrypsin superfamily.

Proteases play a central role in several biological processes, spanning from digestion to key regulatory mechanisms such as coagulation and hormone release, being also recognized in many diseases (1, 2). Therefore, the possibility of selectively influencing enzyme activities by specific inhibitors appears of considerable interest in view of their potential terapeutic value as drugs (3). Serine proteinase activity can be modulated by protein enzyme inhibitors, as well as by low molecular weight synthetic molecules, such as the cyclic thiolic compounds 2-[3-thiophencarboxythio]-N-[dihydro-2(3H)-thiophenone-3-il]-propionamide (MR889; see Figure 1*a*) and 3-[2-(2-thiophencarboxythio)]-propanoyl-

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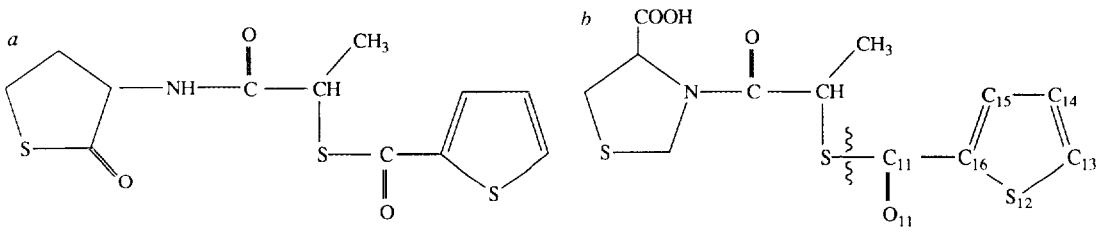

Figure 1. Schematic structures of the cyclic thiolic compounds MR889 *(a)* and YS3025 *(b)*. The atom numbering and the cleavage site of YS3025 are shown.

4-thiazolidin carboxylic acid (YS3025; see Figure 1*b*) (1-9). These acylating agents react preferentially with serine proteinases belonging to the chymotrypsin superfamily acting on noncationic substrates (5-9). MR889 and YS3025 display apparent dissociation equilibrium constants ranging between 10^{-6} M and 10^{-5}M for bovine α-chymotrypsin (α-chymotrypsin), porcine pancreatic elastase (p.p. elastase) and human leukocyte elastase (h.l. elastase) (5-9). The inhibitory effect of MR889 has been tested using insoluble elastin as substrate, showing efficient modulation of the elastolytic activity in the presence of the natural insoluble target of the serine proteinase (6-8). Furthermore, MR889 has been tested in vivo for potential toxicity, without observing any mortality after oral administration of up to 5g/kg body weight, in mice (6). Such findings are prerequisites for the therapeutical application of these low molecular weight serine proteinase inhibitors (3, 6).

Concerning the inhibition mechanism of serine proteinases by MR889, Powers *et al.* (8) have proposed the thiophencarbonyl moiety of the inhibitor as the acylating agent of the enzyme Ser195 catalytic residue on the basis of titration of free thiol groups in solution, after enzyme:inhibitor incubation. Furthermore, Rizzi *et al.* (9) have shown, in a recent crystallographic investigation, that the covalent α-chymotrypsin:YS3025 acyl-enzyme adduct contains the thiophencarbonyl moiety of the inhibitor.

Considering that the substantial degree of structural conservation in the active site of serine proteinases belonging to the chymotrypsin superfamily should allow inhibitor cross-reactivity, we have undertaken a crystallographic study on the bovine β–trypsin (β-trypsin): and p.p.elastase:YS3025 adducts. The present communication brings crystallographic evidence that thiophencarbonyl moiety of the inhibitor binds to the Ser195 OG atom of the homologous enzymes with comparable but not identical binding modes, partly reflecting the different substrate specificities displayed by α-chymotrypsin, β-trypsin and p.p. elastase.

MATERIALS AND METHODS

β-trypsin was purified from commercial enzyme preparations (from SIGMA Chemical Co., St.Louis, MO, U.S.A.) as detailed elsewhere (10). P.p. elastase was obtained from

FLUKA Chemie, AG (Buchs, CH). YS3025 was synthesised as described elsewhere (11). All the other chemicals (from Merck AG, Darmstadt, FRG) were of analytical grade, and used without further purification. Orthorhombic (space group $P2_12_12_1$) crystals of β-trypsin were grown by vapour diffusion techniques against 1.8 M ammonium sulfate solutions, at pH 5.8-6.2 (12). Similarly p.p.elastase crystals (belonging to the orthorhombic space group $P2_12_12_1$) were grown by vapour diffusion against 0.1 M sodium sulfate reservoirs, at pH 5.0 (13). Reaction of YS3025 with the crystalline enzymes was achieved by soaking of the crystals in their mother liquor solutions, saturated with YS3025, at pH 8.0 and 4° C in the case of β-trypsin, and at pH 5.0 and room temperature in the case of p.p.elastase, for 2-3 days (9).

Diffracted intensities were collected for both β-trypsin: and p.p. elastase:YS3025 adducts with very similar experimental protocols, using an ENRAF-NONIUS CAD4 four circle X-ray diffractometer, equipped with a helium flushed 2θ extension arm (368 mm). For the β-trypsin acyl-enzyme complex, 10,849 intensities (with $I > 1.0\ \sigma(I)$) were collected from three crystals. These were merged to a data set of 6,655 unique reflections in the 15.0-2.7 Å resolution range (82.1% completeness of this shell). For the p.p. elastase acyl-enzyme complex, a total of 7,219 intensities (with $I > 1.0\ \sigma(I)$) were measured from two crystals in the 15.0 - 2.7 Å resolution shell (75.4% completeness). In this case, a deviation of the unit cell edge *a* of 1.4 Å from published cell dimensions (14) was observed. The p.p.elastase crystals used for data collection had unit cell edges : $a = 50.7$, $b = 58.1$, $c = 75.4$ Å. For both enzyme:YS3025 adducts measured intensities were processed as described by Rizzi et al.(9). For the calculation of crystallographic phases atomic co-ordinates of β-trypsin and p.p.elastase were recovered from the Brookhaven Protein Data Bank (data sets 1TLD and 3EST, respectively) (15). Solvent molecules located in the strict neighbourhood of the enzyme:YS3025 binding site (i.e. the proteinase central region) on both enzymes were omitted from structure factor calculations. Difference Fourier maps with 2Fd - Fn and Fd - Fn coefficients (Fd is the acyl-enzyme observed structure factor, and Fn is the calculated native protein structure factor) were computed using calculated phases. In the case of β–trypsin the starting crystallographic R-factor was 0.257; for p.p. elastase the observed data yielded a R-factor value of 0.422. Upon rigid body refinement of the p.p. elastase model, in the 10.0 - 4.0 Å resolution range the R-factor dropped to 0.251.

The crystallographic refinement was run in both cases using the routines from the TNT program package (16); inspection of the electron density maps and correction of the molecular models were performed on an Evans & Sutherland PS390 graphics system with the graphics program FRODO (17).

RESULTS

a) β–trypsin:YS3025 acyl-enzyme complex

The electron density for the thiophencarbonyl moiety of YS3025, covalently linked to the β-trypsin Ser195 OG atom, was evident from the first inspection of difference Fourier maps, with unrefined phases, and therefore easily modeled. The crystallographic refinement of the complex was considered at convergence after 57 cycles, with a R-factor value of 0.180 for the 6,655 observed reflections in the 15.0 - 2.7 Å resolution range. In the final stage of the conventional refinement of the β-trypsin:YS3025 complex, the occupancy of Ser195-acyl group was refined, yielding an average value of 60% for the bound inhibitor moiety, while the atoms of Ser195 maintained the expected 100% occupancy of the site. The overall stereochemistry of the refined model is close to ideality; r.m.s. deviation from ideal bond lengths is 0.018 Å, while the corresponding figure for ideal bond angles is 2.59°.

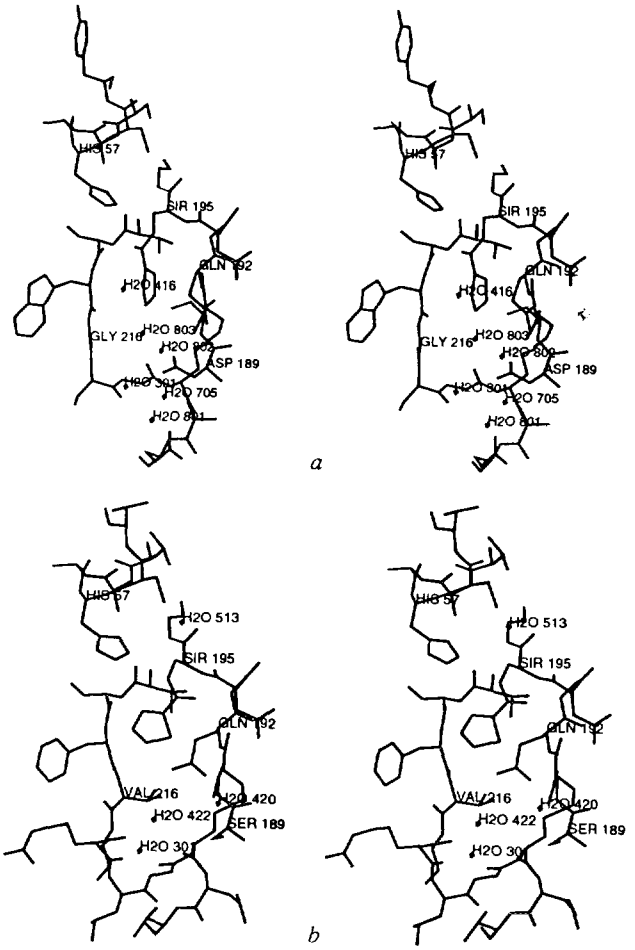

Figure 2. *a)* Stereo view of the active site region of the β–trypsin:YS3025 acyl-enzyme complex. The thiophencarbonyl moiety of the inhibitor is evident in the central part of the picture, accommodated at the entrance of the S_1 subsite, which is closed at the lower end by residue Asp189.

b) Stereo view of the p.p.elastase:YS3025 acyl-enzyme complex. The orientation of the complex is comparable with that of Figure 2*a*.

The thiophencarbonyl moiety of YS3025 is accommodated at the entrance of the β–trypsin primary specificity subsite (S_1), sitting among the polypeptide backbone of residues 190-193 and 213-215, forming a series van der Waals contacts (see Figure 2*a*). Moreover, a number of polar enzyme:inhibitor contacts involve O and O11 atoms of the Ser195-acyl group, and side chain or main chain atoms of residues Gly43, His57, Gly197 and Ser214. In particular, the carbonyl group of the acyl bond is forming a weak hydrogen bond (3.24 Å) with His57 NE2 atom. Owing to the contained dimensions of the thiophene ring of YS3025 the β-trypsin S_1 subsite is not entirely occupied. Indeed, atom C13 of the inhibitor, which is the most deeply buried in the enzyme S_1 subsite is 5.39 Å from Asp189 OD1 atom, thus leaving a cavity which is filled by water molecules 414,

416, and 803; these solvent molecules are hydrogen bonded to the backbone atoms of residues Ser214, Trp215, Ser217, Gly219, Lys224 and Val227, in the proteinase S_1 subsite. Moreover, the negative charge of Asp189 carboxylate group is partially delocalised by hydrogen bonds to solvent molecules 801 and 802.

b) P.p. elastase:YS3025 acyl-enzyme complex

The unusually high crystallographic R-factor calculated at the beginning of refinement for the p.p. elastase:YS3025 complex is to be ascribed to the large changes in unit cell edges, observed for the crystals employed. The quick drop of the R-factor value upon rigid body refinement of the protein location in the unit cell allowed to start the subsequent restrained refinement of this adduct with an electron density map (R = 0.251) comparable to that of the β-trypsin:YS3025 complex described above. A prominent electron density peak, compatible with the thiophencarbonyl moiety of YS3025, was observed at the entrance of the proteinase S_1 subsite, covalently bound to the Ser195 OG atom, and thus model built in this position. After completion of the crystallographic refinement of the acyl-p.p.elastase complex (28 cycles) the R-factor value is 0.149 for the 5,034 observed reflections in the 15.0 - 2.7 Å resolution shell. The stereochemistry of the refined complex is close to ideal, showing r.m.s. deviations of bond lengths and bond angles of 0.018 Å and 1.93°, respectively, from their target values. Refinement of atomic occupancies indicates that the Ser195 acylation by YS3025 is 100% complete in the crystal.

The contacts of the Ser195-thiophencarbonyl moiety with the p.p. elastase S_1 specificity subsite include a number of interactions between the inhibitor's O11 and S12 atoms with the main chain and side chain atoms of the residues Cys191, Gln192, Gly193, Asp194, Ser214, and Val216. The inhibitor O11 atom is at hydrogen bonding distance from the peptide N atoms of residues 192 and 193; moreover a hydrogen bond is observed between S12 and Gln192 NE2 atom (3.17 Å). The His57 NE2 - O11 hydrogen bond observed in the β-trypsin:YS3025 complex is replaced by a His57 NE2 - Ser195 OG interaction (3.32 Å), due to the different orientation of the inhibitor in the active site pocket. Additionally, water molecules 420 and 422, along with the solvent molecules 301 and 513, that were located by difference electron density maps in the enzyme central region, fill the cavity and stabilise the active site protein structure with a number of hydrogen bonded interactions to main chain and side chain atoms.

DISCUSSION

The binding mode of YS3025 to β-trypsin and p.p. elastase, reported in the present study, is coherent with our previous results on the α-chymotrypsin:YS3025 acyl-enzyme complex structure (9). As shown in Figures 2*a* and 2*b*, the thiophencarbonyl moiety is equivalently bound to β−trypsin and p.p. elastase active sites, but with differing orientations. A least squares overlay of the active sites of β-trypsin and p.p.elastase (run imposing equivalence of the protein backbone segments 56 - 60, 189 - 196 and 213 - 220)

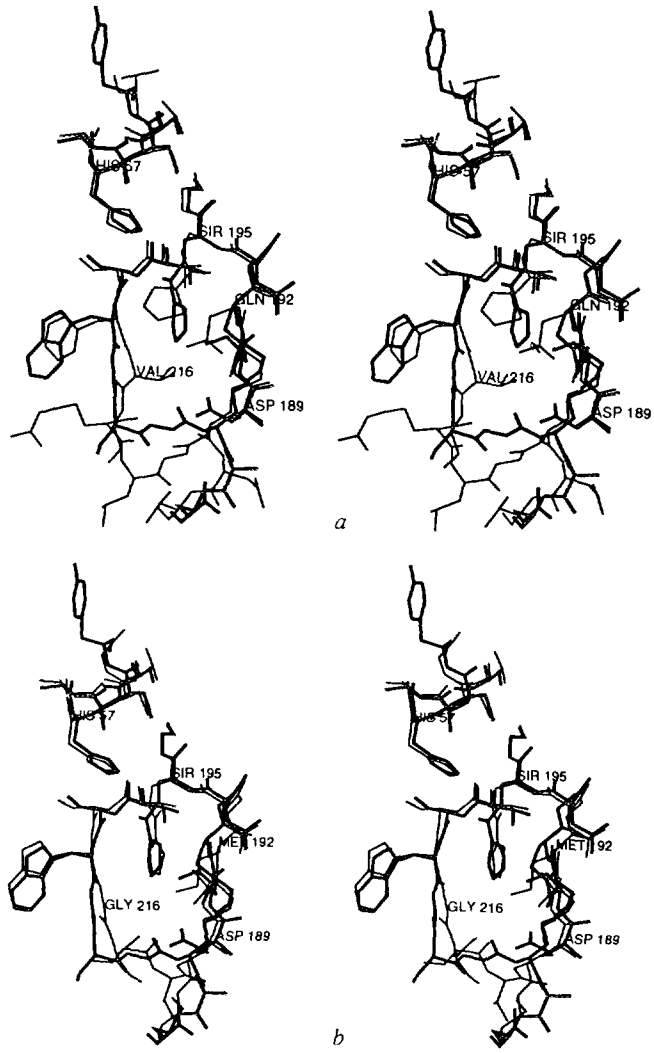

Figure 3. Stereo view of the overlay of: *a)* β–trypsin:YS3025 (thick lines) and p.p.elastase:YS3025 (thin lines) and *b)* β-trypsin:YS3025 (thick lines) and α-chymotrypsin:YS3025 (thin lines) acyl-enzyme complexes, showing the different orientations of the inhibitor moiety in the respective active sites. For ease of comparison water molecules in the neighborhood of the inhibitor have been omitted from the drawing. β-trypsin:YS3025 and α-chymotrypsin:YS3025 complex structures have been overlayed based on equivalence of the 51-59, 189-196 and 213-221 polypeptide segments. The α-chymotrypsin:YS3025 complex structure has been previously reported (9).

brings the thiophencarbonyl ring of YS3025 in quite comparable locations but with nearly perpendicular orientations (see Figure 3*a*). The different conformations of the Ser195-thiophencarbonyl moiety of YS3025 in the two enzymes can be attributed to the Gly216 → Val amino acid substitution present in p.p. elastase. The bulky hydrophobic side chain of residue Val216 is limiting accessibility to the S_1 pocket, and prevents the insertion of

the thiophene ring of YS3025 between residues 191 - 194 and 214 - 216, as observed in the β-trypsin:YS3025 complex (see Figures 2*a*, *b* and 3*a*).

Concerning the orientation of the acyl group C=O bond, in β–trypsin:YS3025 complex (as observed for α-chymotrypsin:YS3025 (9)) it is pointing away from the direction required for hydrogen bonding to the peptide N atoms of the "oxyanion binding hole" (formed by residues 193 and 195) (see Figures 2*a* and 3*b*). Thus it is in a non-productive orientation for nucleophilic attack by an incoming water molecule, during deacylation. Such an orientation, is primarily determined by the constrained orientation of the thiophene ring in the specificity subsite.

The inhibitor electron density in the active site of p.p.elastase is not as flattened as in the case of β-trypsin and α-chymotrypsin, suggesting an increased rotational freedom for the thiophencarbonyl moiety in the former case. This observation is in keeping with the more exposed location of the inhibitor in p.p. elastase, with respect to the other two enzymes. The scissile enzyme:inhibitor acyl bond, although solvent accessible, is not properly positioned with respect to the oxyanion binding site (see Figures 2*b* and 3*a*), possibly decreasing the electrophilic character of the inhibitor C11 atom.

The crystallographic analysis of three pancreatic serine proteinase:inhibitor acyl-enzyme complexes provides a general model for the reaction of YS3025 and related cyclic thiolic compounds (i.e., MR889) with homologous enzymes belonging to the chymotrypsin superfamily. Upon enzyme intact-inhibitor recognition, the thioester bond adjacent to the thiophencarbonyl moiety is cleaved, with the concomitant release of the first product (see Figure 1) (8,9). The acyl-enzyme is stable for a time which is longer ($\geq$ 200 s; (6)) than that of a conventional substrate, due to an unfavourable sterical disposition of the acyl group for the incoming nucleophile, at the start of the deacylation process. In the case of the α-chymotrypsin:YS3025 acyl-enzyme, we have shown that active site bound water molecules may contribute to this unfavourable orientation and in screening the electrophile from the surrounding environment (9). Similar observations and considerations have been raised in the cases of the indoleacryloyl α-chymotrypsin and of the guanidinobenzoyl β-trypsin acyl-enzyme complexes, which show very slow deacylation rates, and for which crystal structures have been reported (18, 19).

The present study shows that YS3025 and MR889 originally designed for h.l. elastase (5-8) can actually inhibit homologous serine proteinases endowed with quite different substrate specificities. The molecular bases for such cross-reactivity have been ascribed to the detailed stereochemistry of the S_1 subsite in the different proteinases, to the contained dimensions and to the stereochemistry of the inhibitor's acylating group.

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