JOURNAL OF MOLECULAR RECOGNITION, VOL. **10**, 26–35 (1997)

Crystal Structure of the Bovine α-Chymotrypsin:Kunitz Inhibitor Complex. An Example of Multiple Protein:Protein Recognition Sites†

Clemente Capasso,[1,2] Menico Rizzi,[3] Enea Menegatti,[4] Paolo Ascenzi[5] and Martino Bolognesi[1,3]*

[1] Centro Biotecnologie Avanzate IST, and Dipartimento di Fisica, Università di Genova, Largo Rosanna Benzi 10, 16132 Genova, Italy
[2] C.N.R. Istituto di Biochimica delle Proteine ed Enzimologia, Via Guglielmo Marconi 10, 80125 Fuorigrotta Napoli, Italy
[3] Dipartimento di Genetica e Microbiologia, Università di Pavia, Via Abbiategrasso 207, 27100 Pavia, Italy
[4] Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Via Fossato di Mortara 17/19, 44100 Ferrara, Italy
[5] Dipartimento di Biologia, Terza Università di Roma, Via Ostiense 173, 00154 Roma, Italy

The crystal structure of bovine α-chymotrypsin (α-CHT) in complex with the bovine basic pancreatic trypsin inhibitor (BPTI) has been solved and refined at 2.8 Å resolution (*R*-factor = 0.18). The proteinase:inhibitor complex forms a compact dimer (two α-CHT and two BPTI molecules), which may be stabilized by surface-bound sulphate ions, in the crystalline state. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor's *C*-terminal region. Specific recognition between α-CHT and BPTI occurs at the (re)active site interface according to structural rules inferred from the analysis of homologous serine proteinase:inhibitor complexes. Lys15, the P_1 residue of BPTI, however, does not occupy the α-CHT S_1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217. © 1997 John Wiley & Sons, Ltd.

J. Mol. Recogn. **10**, 26–35 (1997) No. of Figures: 5 No. of Tables: 4 No. of References: 51.

Keywords: **bovine *α*-chymotrypsin; bovine basic pancreatic trypsin inhibitor (Kunitz-type inhibitor); serine proteinase:Kunitz inhibitor complex; crystal structure**

Received 13 May 1996; revised August 1996; accepted 14 August 1996

Introduction

Bovine basic pancreatic trypsin inhibitor (BPTI) is the prototype model molecule for several biophysical and biochemical studies. Its crystal structure was solved in 1970, at 2.5 Å resolution (Huber *et al.*, 1970), and subsequently analysed in molecular complexes with bovine *β*-trypsin (*β*-TRP), bovine anhydrotrypsin, porcine pancreatic kallikrein-A, bovine trypsinogen, and bovine trypsinogen:Ile-Val and :Val-Val adducts (Rühlmann *et al.*, 1973; Huber *et al.*, 1974 and 1975; Bode and Huber, 1976 and 1992; Huber and Bode, 1978; Bode, 1979; Chen and Bode, 1983; Bode *et al.*, 1984). Different crystal forms of BPTI have been isolated and studied both by X-ray crystallography and neutron diffraction (Huber *et al*., 1970; Wlodawer *et al.*, 1984 and 1987; Parkin *et al.*, 1996). The inhibitor structure has also been thoroughly characterized through nuclear magnetic resonance (Wüthrich *et al.*, 1982). Low molecular weight proteins or peptide fragments related to BPTI have been identified in bovine plasma (Fioretti *et al.*, 1983), in the Alzheimer's amyloid *β*-precursor protein (Hynes *et al*, 1990) and as snake toxins (Lancelin *et al.*, 1994). Moreover, BPTI has been used as a molecular model for protein folding studies (Creighton *et al.*, 1993).

Bovine *α*-chymotrypsin (*α*-CHT) three-dimensional structure has been known for several years (Tsukada and Blow, 1985) and, together with those of trypsin and elastase, extensively used for modelling and inhibitor design studies in this serine proteinase homology superfamily (Creighton and Darby, 1989). Crystallographic studies on *α*-CHT:protein proteinase inhibitor complexes are more limited in number than in the case of bovine *β*-TRP. In fact, only the

Contract grant sponsor: Ministry of University, Scientific Research and Technology of Italy.
Contract grant sponsor: National Research Council of Italy.
Contract grant sponsor: European Union Human Capital and Mobility Program: *contract grant number:* CT 940 69.
† This paper is dedicated to Professor A. Ballio on the occasion of his 75th birthday.
* Correspondence to: M. Bolognesi, Centro di Biotecnologie Avanzate IST, Largo Rosanna Benzi 10, 16132 Genova, Italy.

Abbreviations used: *α*-CHT, bovine *α*-chymotrypsin; *β*-TRP, bovine *β*-trypsin; BPTI, bovine basic pancreatic trypsin inhibitor (Kunitz-type inhibitor); eglin *c*, serine proteinase inhibitor from the leech *Hirudo medicinalis*. Amino acid residues of BPTI bound to *α*-CHT are identified by the 300 suffix. For the *β*-TRP:BPTI complex, the E and I labels (within parentheses) have been adopted for the enzyme and the inhibitor, respectively (Rühlmann *et al.*, 1973). Sulphate groups have been indicated as 500, 600, 700 and 800. The specificity subsites surrounding the catalytic centre of the serine proteinases considered have been identified as $S_n \ldots S_1$, $S_{1'} \ldots S_{n'}$. The amino acid residues forming the reactive site of BPTI have been labeled as $P_n \ldots P_1$, $P_{1'} \ldots P_{n'}$, P_1–$P_{1'}$ being the scissile peptide bond (Schechter and Berger, 1967).

 CCC 0952–3499/97/010026–10 $17.50

structures of the α-CHT complexes with eglin *c* (Frigerio *et al.*, 1992) and with the turkey ovomucoid third domain (Fujinaga *et al.*, 1987) have been solved. Moreover, the three-dimensional models of bovine chymotrypsinogen-A complexed with synthetic mutants of the human pancreatic secretory trypsin inhibitor (Hecht *et al.*, 1991) have been determined.

Structural, thermodynamic and kinetic studies have shown that the well known specificity difference between α-CHT (selectively cleaving the substrate peptide bond on the *C*-side of apolar-aromatic residues) and β-TRP (cleaving on the *C*-side of positively charged residues) can be primarily ascribed to the chemical nature of residue 189, sitting at the closed end of the enzyme primary S_1 specificity subsite (Schechter and Berger, 1967). This residue is a neutral Ser in α-CHT, and a negatively charged Asp in β-TRP (Greer, 1990; Bode and Huber, 1992). Thus, small cationic substrates and inhibitors, such as *N*-α-carbobenzoxy-L-lysine *p*-nitrophenyl ester and benzamidine, display higher affinity for β-TRP than for α-CHT (Ascenzi *et al.*, 1982). The strong conservation of the active site geometry in the homologous superfamily, however, allows sufficient enzyme:inhibitor affinity even in the case of macromolecular inhibitors endowed with suboptimal specificity. Thus, eglin *c*, which displays a dissociation equilibrium constant (K_d) of 2.0×10^{-10} M for α-CHT, can still bind to β-TRP with $K_d=8.3\times10^{-6}$ M (Ascenzi *et al.*, 1991). Moreover, protein engineering studies, in which the nature of the inhibitor reactive site residue P_1 was changed, have allowed the alteration of the enzyme specificity of the human pancreatic secretory trypsin inhibitor, and the conversion of this protein into a potent inhibitor of α-CHT and human leucocyte elastase (Hecht *et al.*, 1991).

In the case of BPTI, similar studies have shown that the very high inhibitor affinity for β-TRP ($K_d=6.0\times10^{-14}$ M) drops by five orders of magnitude when the inhibitor is tested against α-CHT (Vincent and Lazdunski, 1972; Antonini *et al.*, 1983). On the other hand, semisynthetic variants of BPTI bearing *nor*-Leu or Phe at the reactive site P_1 residue (Jering and Tschesche, 1976; Tschesche *et al.*, 1987) display a decreased affinity for β-TRP, inhibiting α-CHT. These studies indicate that serine proteinase inhibition by BPTI involves the proteinase primary specificity recognition subsite, locating the inhibitor P_1 residue, and the surrounding secondary subsites. The proteinase:inhibitor contact area is approximately 700 Å^2 (Bode and Huber, 1992).

We have determined the crystal structure of the 1:1 complex formed by α-CHT and wild-type BPTI, bearing Lys15 at site P_1, in order to investigate the details of an intermolecular complex, which, at the primary specificity subsite, is expected to violate the recognized structural rules for serine proteinase:inhibitor recognition. This study shows that the polar interaction of the P_1 lysyl residue of BPTI with Asp189 of β-TRP (Rühlmann *et al.*, 1973) is replaced by hydrogen bonds occurring between the inhibitor and α-CHT residues outside the specificity S_1 pocket. Moreover, the α-CHT:BPTI complex adopts an unprecedented compact dimeric structure (two complex moieties related by a non-crystallographic two-fold axis), the two inhibitor molecules being roughly antiparallel to each other, each of them simultaneously contacting both α-CHT molecules.

Materials and Methods

α-CHT was purchased from Sigma Chemical Co. (St Louis, MO, USA) and used without further purification. BPTI was obtained from Lepetit S.p.A. (Milano, Italy) and purified as reported elsewhere (Kassel, 1970).

Crystals of the α-CHT:BPTI complex were obtained by vapour diffusion techniques, mixing 3.0 μl of a stock protein solution, containing 20.0 mg/ml α-CHT and 8.0 mg/ml BPTI (in water), with 3.0 μl of the precipitating medium, containing 2.0 M $MgSO_4$ in 0.05 M sodium acetate buffer at pH 6.0. The crystallization drops were equilibrated, at 22°C, against 0.5-ml reservoir wells in Linbro plates. Large bipyramidal hexagonal crystals (0.6 mm × 0.3 mm × 0.3 mm) appeared in about 3 months, and were harvested in solutions containing 2.2 M $MgSO_4$, 0.05 M sodium acetate at pH 6.0, for further characterization.

X-ray diffraction data collection was conducted on an R-axis IIC imaging plate system, mounted on a Rigaku RU-200HB rotating anode generator. The crystals of the α-CHT:BPTI complex belong to the hexagonal space group $P6_1$ (or enanthiomorph), with unit cell constants $a=b=102.4$ Å, $c=207.6$ Å, and contain two binary complexes *per* asymmetric unit ($V_M=4.7$ Å^3/Da) (Matthews, 1968). The whole data set collected consisted of 106 612 intensities, which were subsequently reduced to 27 254 independent reflections (90.3% complete in the 20.0–2.8 Å resolution range; $R_{merge}=0.096$). The diffracted intensities decayed abruptly beyond the 2.8 Å resolution threshold, the average $I/\sigma(I)$ ratio being 0.85 at 2.75 Å resolution. Under the same experimental conditions, crystals of the bovine γ-chymotrypsin:BPTI complex can be grown. However, their diffraction pattern is limited to a resolution lower than 10 Å (Capasso, unpublished results).

As a first step, in the structural analysis, the self-rotation function was calculated in the 15.0–3.5 Å resolution range, locating a prominent peak at $\varphi=26°$, $\psi=90°$, $\kappa=180°$, with a correlation coeficient of 67.4 (Navaza, 1994). Next, the cross rotational search was run employing as search molecule a locally built model of the α-CHT:BPTI complex (Cutruzzolà *et al.*, 1993; Oddone *et al.*, 1994), based on the superposition of α-CHT [monomer A from the dimeric α-CHT structure; PDB code 2CHA (Tsukada and Blow, 1985)] onto the β-TRP:BPTI complex [PDB code 2PTC (Rühlmann *et al.*, 1973)]. The rotational search was run in the 15.0–3.5 Å resolution range, providing two solutions (2.5 r.m.s. above the next rotation peak), one for each of the two complex moieties. Similarly, the translational search allowed discrimination between the two enantiomorphic space groups ($P6_1$ and $P6_5$), and to locate both complex moieties with respect to the symmetry elements. In the space group $P6_1$ only one solution for the translational search of both complex units was found, with a final correlation coefficient of 51.9 (Navaza, 1994). The crystallographic *R*-factor, calculated in the 15.0–3.5 Å resolution range, had, at this stage, a value of 0.404. Rigid body refinement of the four molecular components present in the asymmetric unit (two α-CHT and two BPTI units) dropped the crystallographic *R*-factor to 0.374; inspection of the corresponding electron density map allowed to confirm an overall good agreement between the proposed molecular model and the observed density.

Subsequent restrained crystallographic refinement was performed using programs from the TNT package (Tronrud *et al.*, 1987), employing all the observed structure factors in the 20.0–2.8 Å resolution range, and taking advantage of the non-crystallographic twofold axis present in the asymmetric unit. After 30 cycles of atomic coordinates refinement, the *R*-factor was 0.287 [R_{free} = 0.339, calculated on 1030 randomly selected reflections (Brünger, 1992)]. Next, 20 cycles of coordinates and isotropic *B*-factor refinement lowered the *R*-factor to 0.217 (R_{free} = 0.291). Electron density maps calculated with 2Fo-Fc and Fo-Fc coefficients were regularly inspected; map analysis and manual refitting were performed using FRODO (Jones, 1978). Water molecules were added to the model only if they occupied stereochemically reasonable positions. Further refinement cycles, incorporating water molecules, brought the *R*-factor to 0.185 (R_{free} = 0.253); at this stage strong residual density near residues Tyr310 and Tyr335 of both BPTI molecules was interpreted and refined as four sulphate ions.

Atomic coordinates and structure factors for the α-CHT:BPTI complex have been deposited with the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977), from which copies are available (data sets: 1MTN, R1MTNSF).

Results and Discussion

Overall structure of the complex

The α-CHT:BPTI refined molecular model includes 4522 protein atoms, 80 water molecules and four sulphate ions. The corresponding *R*-factor is 0.180, for the 27 254 reflections in the 20.0–2.8 Å resolution range; the R_{free} is 0.248. The overall stereochemistry of the molecular model is satisfactory: as calculated by the program TNT (Tronrud *et al.*, 1987), the r.m.s. bond length deviation from ideal values is 0.013 Å, and the r.m.s. bond angle deviation is 2.5° (see Table 1). Moreover, the quality of the model, as assessed by the PROCHECK program package (Engh and Huber, 1991; Laskowski *et al.*, 1993), is better than average. Only one residue, Leu10 (whose electron density is defined in the cleaved *N*-terminal segment of α-CHT) falls in a disallowed region of the Ramachandran plot.

Table 1. Refinement statistics

Number of protein atoms	4522
Number of solvent atoms	80
Resolution range (Å)	20 to 2.8
Number of reflections	27 254
R-factor	0.180
R_{free}	0.248
Root-mean-square deviation from standard geometry	
Bonds (Å)	0.013
Angles (°)	2.5
Trigonal plane (Å)	0.018
General plane (Å)	0.017
Non bonded contacts (Å)	0.082
B value correlation (Å^2)	4.5
Average temperature factor (Å^2)	
Main-chain α-CHT atoms	42.8
Side-chain α-CHT atoms	50.0
Main-chain BPTI atoms	31.4
Side-chain BPTI atoms	39.9
Solvent atoms	42.1

The two enzyme:inhibitor complexes present in the asymmetric unit of the α-CHT:BPTI $P6_1$ crystalline form display a tight and symmetrical mode of aggregation. As shown in Fig. 1, if the conventional picture of a mushroom (whose head is the proteinase, α-CHT units *A* or *C* in the following) is adopted, the two complex moieties are mostly contacting each other through the elongated BPTI molecules (i.e. their stalks, named BPTI units *B* and *D* in the following), which run roughly antiparallel to each other. Moreover, additional proteinase:inhibitor contacts are achieved at the base of each stalk, such that each BPTI contacts both α-CHT molecules, respectively through the reactive site loop (697 Å^2 contact area) and through its *N*- and *C*-terminal regions, which are neighbours in the three-dimensional structure (340 Å^2 contact area). Such a symmetrical arrangement can be described by a non-crystallographic two-fold axis relating the two complex moieties by an almost exact 180° rotation, located among the two BPTI molecules, aproximately between residues Ala340-Lys341 of BPTI-*B* and Lys341-Ala340 of BPTI-*D* (see Fig. 1). In accordance with the results from the self-rotation function calculation, the local two-fold axis is parallel to the unit cell *ab* plane and forms an angle of 26° with respect to the *a* axis direction. The resulting side-by-side contact of the two BPTI molecules, never observed before, buries 271 Å^2 of each inhibitor surface.

In the crystalline complex, the BPTI region around the non-crystallographic twofold axis is characterized by strong polar interactions, which involve basic residues and two sulphate anions. The carbonyl O atom of BPTI Ala340 is hydrogen bonded to the guanidine NH2 atom of Arg339 (3.3 Å for the BPTI *D*-*B*, and 3.1 Å for the *B*-*D* pairs, respectively). Additionally, a weak hydrogen bond (3.5 Å) is observed between the NZ atoms of residues Lys341 of both BPTI molecules. As shown in Fig. 2, the side chains of residues Arg339-*D* and Arg342-*B*, Arg339-*B* and Arg342-*D* provide the electrostatic compensation of the four negative charges brought about by the sulphate groups 500 and 600 present at the molecular interface. Moreover, potential hydrogen bonds to the sulphate groups can be contributed by side chain or backbone atoms of residues Arg339, Ala340, Lys341 and Arg342 of each BPTI molecule. Similarly, sulphate groups 700 and 800 are located at the inhibitor surface, not far from the local twofold axis (see Fig. 2). The 700 sulphate is at hydrogen bonding distance from the side chains of Arg320-*D* and Tyr335-*D* and close to the carbonyl oxygen of Gly337-*D*. Electrostatic compensation can further be provided by residue Lys346-*D*, such that all the interactions of this sulphate group occur with residues from the BPTI-*D* molecule. An identical arrangement is observed for the 800 sulphate group which is stabilized by interactions with residues provided by the BPTI-*B* molecule. In this respect, it should be noted that the 700 and 800 sulphate groups are located in the same site occupied by a phosphate anion in the 125 K structure of free BPTI (Parkin *et al.*, 1996). Both pairs of sulphate anions in the α-CHT:BPTI complex structure are affected by rota-

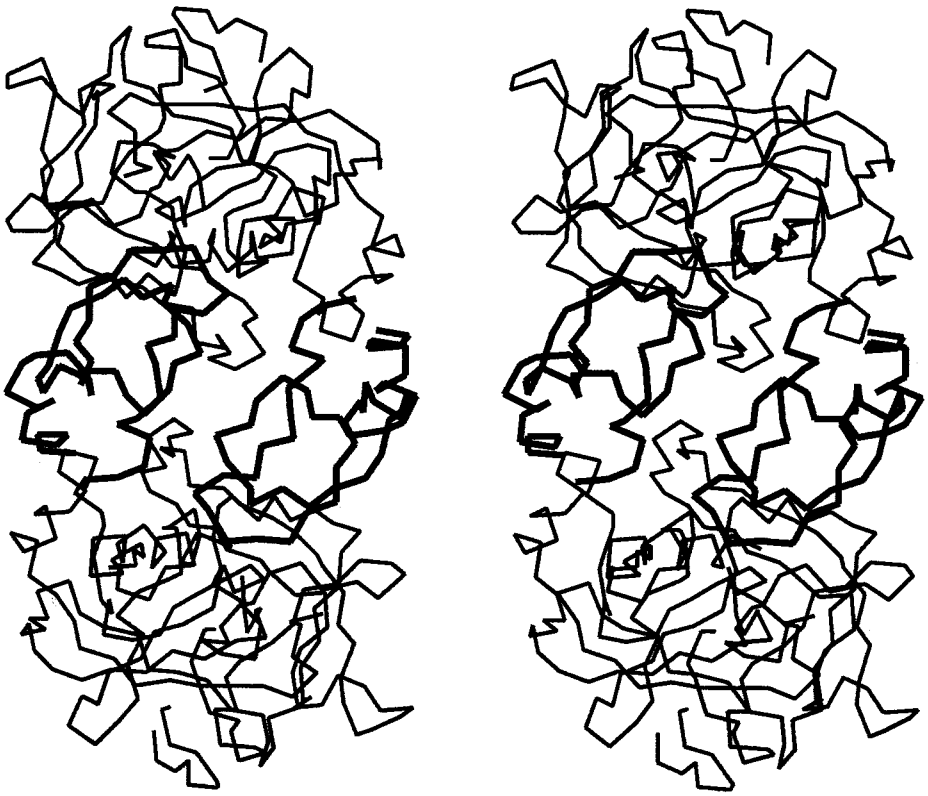

Figure 1. Stereo view of the Cα backbone trace for the two α-CHT:BPTI complex moieties present in the asymmetric unit of the $P6_1$ crystal form. BPTI molecules are drawn in heavy lines; the local two-fold axis is approximately at the centre of the picture, perpendicular to the drawing plane.

tional disorder, such that location of their oxygen atoms in the electron density is uncertain. Moreover, the 700 and 800 sulphate groups are only 50% occupied.

The *N*- and *C*-terminal regions of BPTI provide a number of polar contacts with the α-CHT molecule from the adjacent complex moiety (i.e. BPTI-*B*, from the *A*:*B* complex, contacts α-CHT-*C*, belonging to the *C*:*D* complex). These interactions (which occur identically in the other complex unit) are centred around BPTI residues Thr354-*B* and Arg342-*B* and occur with the α-CHT residues Thr174-*C* and Lys 175-*C* (see Table 2 and Fig. 3). The solvent accessible area buried by these non-inhibitory interactions is 340 Å^2, yielding 35 van der Waals contacts (<4.0 Å). In both BPTI molecules the *N*-terminal Arg301 residue displays a disordered side chain.

α-CHT:BPTI molecular interface

As expected for serine proteinase protein inhibitors (Schechter and Berger, 1967; Laskowski and Kato, 1980; Bode and Huber, 1992), the main contact region between α-

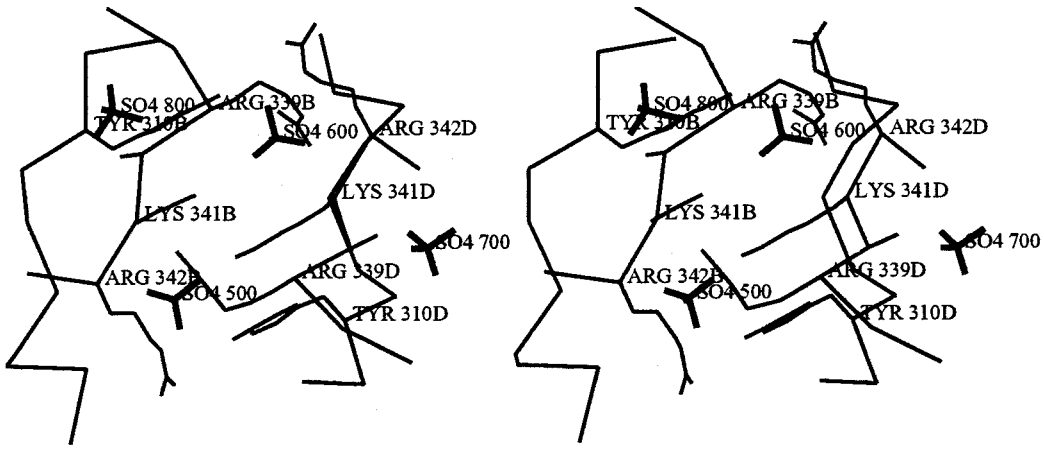

Figure 2. Details of the BPTI-B:BPTI-*D* interface, around the non-crystallographic two-fold axis, showing the location of the four sulphate ions (500, 600, 700 and 800) identified. The relevant regions of BPTI molecules are drawn in thin lines.

CHT and BPTI occurs at the inhibitor reactive site loop between residues Pro313(P_3) and Arg317(P_2'). In this region, as already reported for the crystal structures of BPTI with homologous serine proteinases (Bode and Huber, 1992), the inhibitor reactive site forms a sort of antiparallel β-structure with the active site region of α-CHT, establishing direct N and O polypeptide backbone hydrogen bonds (see Table 3, and Figs 4 and 5a). From comparison of the known three-dimensional models, it can be easily appreciated that BPTI binding to the proteinase has very little effect on its structure. The r.m.s. deviation of the BPTI Cα backbone, here reported, from that of the inhibitor in its complex with β-TRP (Rühlmann *et al.*, 1973) is 0.46 Å. As a result, the same level of BPTI intramolecular hydrogen bonding, as compared to other known structures of the inhibitor, is observed (Bode and Huber, 1992).

The intermolecular hydrogen bonding pattern at sites P_3-P_2' around the (re)active site in the α-CHT:BPTI complex is in full agreement with that observed in the β-TRP:BPTI complex (Rühlmann *et al.*, 1973) (see Table 3 and Figs 5a and 5b), and reflects both the conservation of the active site

Table 2. Hydrogen-bonded interactions occurring between α-CHT and BPTI at the non-inhibitory contact region

α-CHT atoms	BPTI atoms	Distance (Å)
N Thr174-A	OG1 Thr354-*D*	2.7
OG1 Thr174-A	OG1 Thr354-*D*	3.1
NZ Lys175-A	O Arg342-*D*	2.5
N Thr174-*C*	OG1 Thr354-*B*	2.9
OG1 Thr174-*C*	OG1 Thr354-*B*	3.2
NZ Lys175-*C*	O Arg342-*B*	2.9

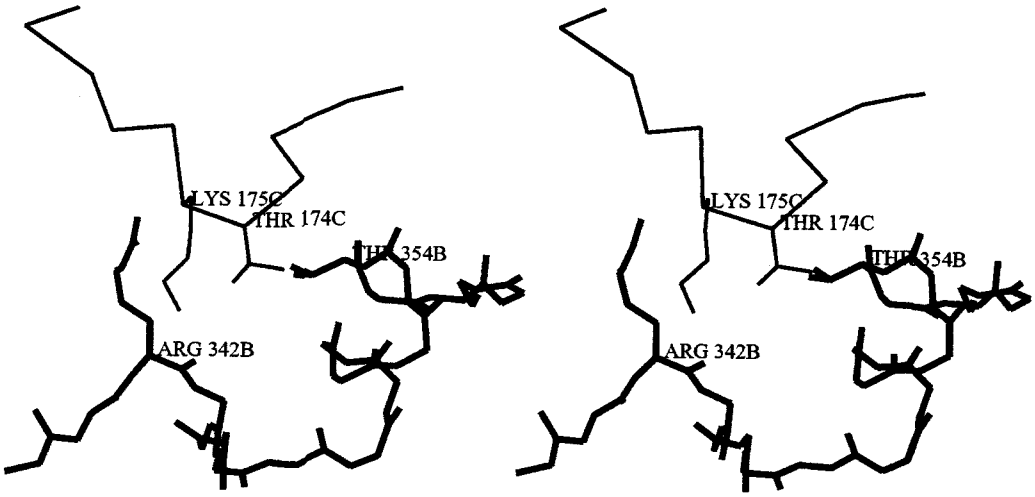

Figure 3. Stereo picture of the 174-175 loop of α-CHT (thin lines) contacting BPTI (heavy lines) at the *C*-terminal region.

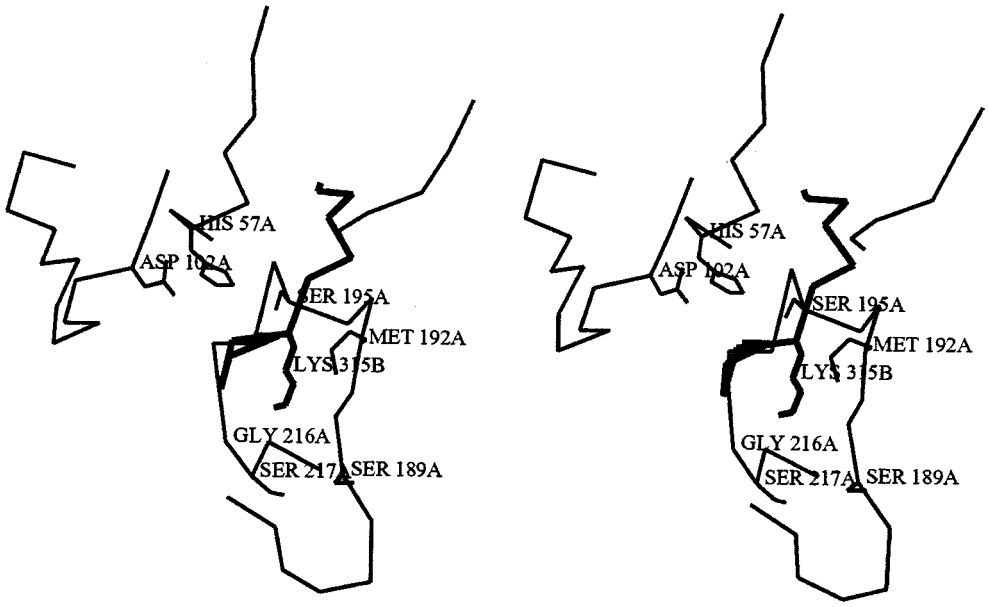

Figure 4. Schematic representation of the active site of α-CHT (thin lines) in the presence of BPTI (heavy lines). The orientation of the side chain of the BPTI Lys315 P_1 residue, outside the specificity S_1 pocket of α-CHT, can be easily appreciated.

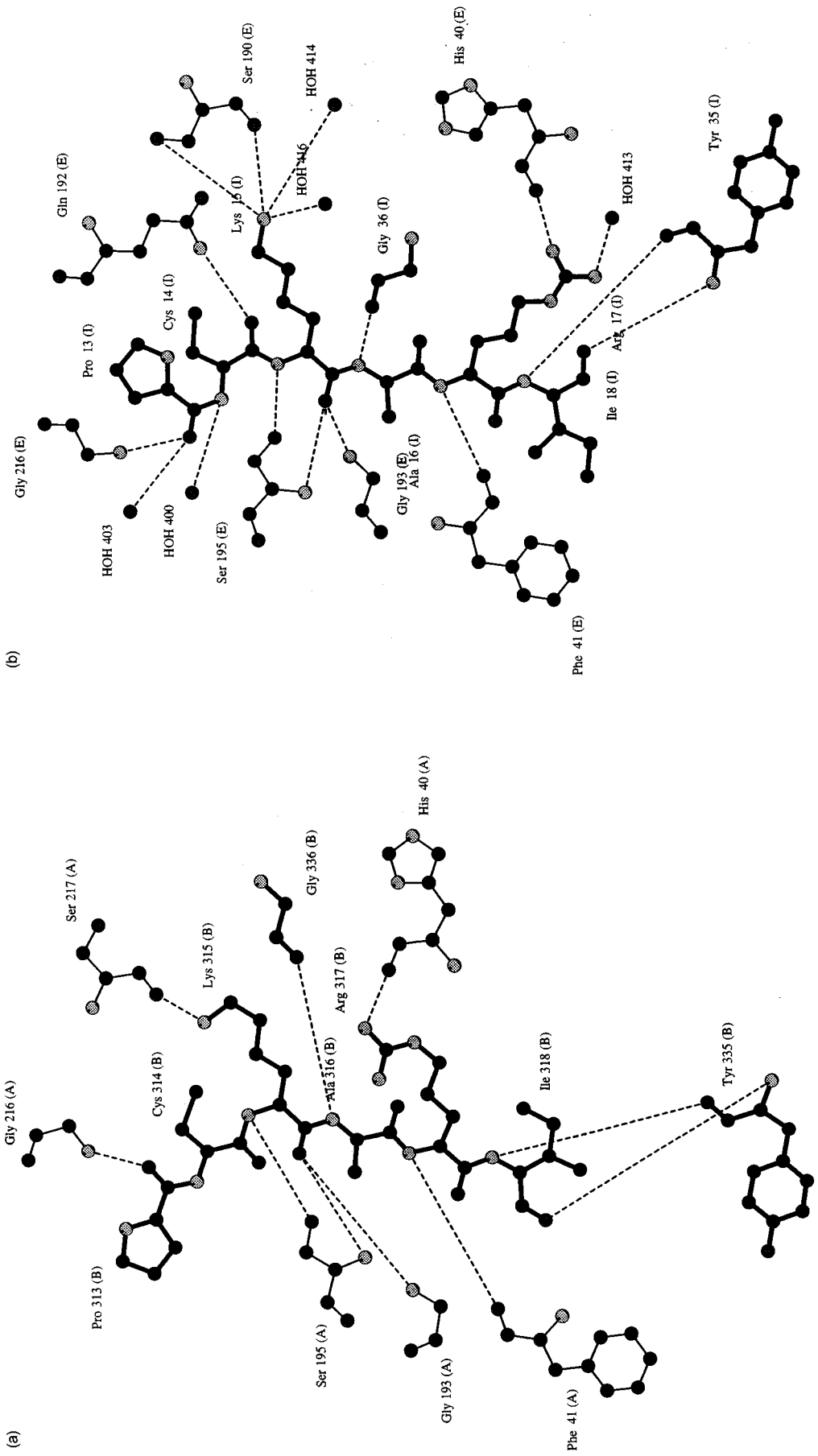

Figure 5. LIGPLOT representation (Wallace *et al.*, 1995) of the (re)active site interface in the α-CHT:BPTI complex (*A*:*B* unit; a), and in the β-TRP:BPTI complex (b), highlighting some hydrogen-bonded recognition interactions (dashed lines). The BPTI reactive site residues are drawn with heavy lines. C atoms are drawn as black spheres, N atoms are lightly shaded.

Table 3. Hydrogen-bonded interactions occurring at the (re)active site of the α-CHT:BPTI complex (A:*B* and C:*D* units) and of the β-TRP:BPTI adduct[a]

BPTI atoms	α-CHT atoms	Distance (Å) *A:B* Unit	*C:D* Unit
O Pro313	N Gly216	2.8	2.9
N Lys315	OG Ser195	3.0	2.8
O Lys315	N Ser195	3.2	3.0
	N Gly193	2.7	2.9
NZ Lys315	O Gly216	3.3	3.4
	O Ser217	2.5	2.9
N Arg317	O Phe41	2.9	3.2
NE Arg317	O His40	3.4	3.5
NH2 Arg317	O His40	2.8	3.0

BPTI atoms	β-TRP atoms	Distance (Å)
O Pro13 (I)	N Gly216 (E)	3.2
O Cys14 (I)	ND2 Gln192 (E)	2.9
N Lys15 (I)	OG Ser195 (E)	3.1
O Lys15 (I)	N Ser195 (E)	2.8
	N Gly193 (E)	2.8
NZ Lys15 (I)	O Ser190 (E)	3.0
	OG Ser190 (E)	3.1
N Arg17 (I)	O Phe41 (E)	2.8
NH2 Arg17 (I)	O His40 (E)	2.8

[a] Data for the β-TRP:BPTI complex were obtained from Rühlmann *et al.* (1973).

organization in the two homologous serine proteinases, as well as the rigidity of the reactive site loops in the BPTI molecules (from the α-CHT complexes), which show an average main chain *B*-factor of 25.4 Å^2 as compared to an average *B* value of 31.4 Å^2 for all their backbone atoms (see Table 1).

As is well known (Ascenzi *et al.*, 1982), α-CHT primary specificity (for apolar and medium-sized-residues) is structurally coded by the S_1 apolar specificity pocket, which can only fit low polarity residues. A much higher pocket polarity, imposed by residue Asp189 in β-TRP (Ser189 in α-CHT), provides cationic substrate specificity in trypsin-like serine proteinases (Huber and Bode, 1978; Bode and Huber, 1992). In agreement with these observations, the BPTI polar residue Lys315(P_1) does not fill the α-CHT S_1 primary specificity pocket, but, rather, finds a hydrogen bonding compensation in the carbonyl O atoms of the enzyme surface residues Gly216 (3.3 Å) and Ser217 (2.5 Å) (see Figs 4 and 5a). Thus, at variance from the β-TRP:BPTI complex (Rühlmann *et al.*, 1973), the Lys315(P_1) side chain adopts a rather bent conformation which makes it solvent accessible for 188 Å^2. Nevertheless, Lys315(P_1) is well defined in the electron density, and displays temperature factors which are in the average for this structure (31 Å^2, in both BPTI molecules). Lys315(P_1) side chain forms 23 van der Waals contacts (<4.0 Å) with residues of the α-CHT active centre surrounding the S_1 pocket entrance. In this respect, it can be noted that, despite the close location in the complex structure, the Lys315 side chain leaves enough room on the α-CHT surface for residue Met192, whose oxidation to sulphoxide does not affect BPTI binding to the serine proteinase (Cutruzzolà *et al.*, 1993). Moreover, the location of the guanidino group of residue Arg317(P_2'), close to the side chain of the α-CHT residue His40, is in keeping with its proposed role as a stabilizing residue in the formation of the α-CHT complex with [homoserine lactone-52]-52,53-*seco*-BPTI (Oddone *et al.*, 1994).

Structural comparison

Crystal contacts in the α-CHT:BPTI complex $P6_1$ form are localized in surface regions involving α-CHT residues Cys1-Val3, Ala5-Ile6, Phe114-Gln116 and Asn204-Gly205. These loops mediate direct α-CHT-*A*: α-CHT-*C* contacts in different asymmetric units. Moreover, BPTI residues Lys326, Glu349-Gly357 provide crystal contacts with α-CHT residues Ala149, Asp35-Phe39 and Gly74-Lys79. In this respect, it should be noted that the crystal contacts affecting the *C*-terminal region of BPTI are likely to be responsible for the defined conformation of the Gly356-Gly357-Ala358 segment, whose electron density is weak or absent in other room temperature BPTI crystalline forms (Wlodawer *et al.*, 1984 and 1987). The resulting crystal packing of α-CHT:BPTI units yields a rather open super-helical molecular structure, which extends through the crystal, parallel to the *c* axis. Such an arragement defines solvent cavities of about 70 Å diameter.

Comparison of the α-CHT structure from the enzyme:BPTI complex with that of the free proteinase (Tsukada and Blow, 1985) shows very contained structural differences. The r.m.s. deviation between the two Cα backbones is 0.49 Å, showing the largest average deviations (in parentheses) in the region of residues Cys1-Ile6 (0.6 Å), Asp35-Phe39 (1.6 Å) and Asn204-Gly205 (0.6 Å), all involved, together with the Phe114-Gln116 segment, in crystal lattice contacts. Both α-CHT subunits in the asymmetric unit display essentially the same r.m.s. deviations with respect to the free enzyme structure. Regions of poor electron density in both α-CHT subunits have been recognized as residues Ser11, Tyr146, Ala149 and at the calcium ion-binding loop (residues Ser75-Lys79). Moreover, from comparison of the Cα backbones of the α-CHT:BPTI and β-TRP:BPTI complexes, a r.m.s. deviation of 1.55 Å is calculated (over 229 proteinase and 56 BPTI Cα pairs considered), indicating that the different lattice packing interactions and differences in ionic strength/buffer composition have a minimal effect also on the mutual orientation of the two protein components in the enzyme:inhibitor adducts. This observation can be related to the rigidity of the reactive site loop in BPTI. In fact, additional structural overlay analyses of BPTI from the α-CHT complex show that the inhibitor's Cα backbone is essentially not altered when bound to porcine pancreatic kallikrein-A (Chen and Bode, 1983), or in its free form at 125 K (Parkin *et al.*, 1996). On the contrary, eglin *c*, a serine proteinase inhibitor devoid of intramolecular disulphide bridges, displays evident conformational adaptability according to the partner proteinase considered (Frigerio *et al.*, 1992).

Analysis of the 80 solvent molecules located in the α-CHT:BPTI complex structure shows that, besides the water molecules hydrogen bonded to the outer protein surface (36

for the four protein molecules in the asymmetric unit), 42 buried water molecules can be located. These include the solvent inaccessible water molecules in the α-CHT and BPTI core structures (18 and 3 water molecules, respectively, for each complex moiety), matching those identified in previous studies (Tsukada and Blow, 1985; Parkin *et al.*, 1996), which had been removed from the molecular replacement search model. Moreover, 14 water molecules (of various solvent accessibilities) are located at the BPTI-*B*:BPTI-*D* interface and at the α-CHT:BPTI contact area next to the inhibitor *C*-terminus.

Functional implications

From inspection of Table 4, reporting the values of the dissociation equilibrium constant for the binding of wild-type and semisynthetic BPTI variants to homologous serine proteinases, three main trends can be recognized.

The affinity of BPTI for serine proteinases endowed with Lys/Arg substrate specificity can be substantially affected by structural factors outside the catalytic centre region. Thus, an affinity decrease of four to ten orders of magnitude can be ascribed to the influence of proteinase loops which limit the inhibitor accessibility (e.g. external loops in porcine pancreatic kallikrein, and β and γ loops in human α-thrombin). Removal of such structural constraints restores, at least in part, proteinase affinity (Antonini *et al.*, 1983; Menegatti *et al.*, 1984; Ascenzi *et al.*, 1992).

In the case of β-TRP complexes, replacement of the BPTI Lys15(P_1) residue with an apolar side chain, as *nor*-Leu or Phe, forces the inhibitor P_1 hydrophobic side chain towards the proteinase S_1 specificity pocket, which hosts Asp189, causing a six to nine orders of magnitude drop in the inhibitor affinity. Although it is very speculative to comment on the basis of the K_d values listed in Table 4 in the absence of crystal structures, the data presented here (for an atypical proteinase:BPTI complex) suggest that also for the β-TRP:variant-BPTI complexes the overall geometry of the enzyme:inhibitor contact region should essentially conform to that described for other proteianse:BPTI adducts. Nevertheless, the different flexibility of P_1 *nor*-Leu or Phe residues, which may adapt differently to the structure of the β-TRP catalytic centre, may account for the large discrepancy in their affinity for β-TRP. In this respect, the very similar behaviour displayed by *nor*-Leu15- and Phe15-BPTI *versus* α-CHT is suggestive of an equally productive binding mode of the two inhibitors with respect to the apolar S_1 subsite of the proteinase. The affinity of wild-type BPTI for α-CHT is quite close to that of the two BPTI variants bearing apolar P_1 residues. Such an observation, at the light of the three-dimensional structure here described, suggests that the cost of restructuring the P_1 lysyl side chain, leaving an empty S_1 pocket (see Figs 4 and 5a and 5b) is essentially compensated by the additional interactions of this residue outside S_1, in particular through hydrogen bonds to Gly216 O and Ser217 O atoms. Nevertheless, the affinity of BPTI for α-CHT remains about five orders of magnitude lower than for β-TRP.

The present results, within the limits of a 2.8-Å resolution crystallographic study, show that the main structural features of proteinase:inhibitor recognition are essentially comparable in the α-CHT:BPTI and in the β-TRP:BPTI complexes. In fact, a molecular contact region of 697 Å^2 is observed at the α-CHT:BPTI (re)active site interface, yielding 119 van der Waals contacts (<4.0 Å). Both figures are close to those observed in the β-TRP:BPTI complex structure (709 Å^2 and 132 contacts, respectively), and should be considered together with the conserved number of proteinase:inhibitor hydrogen bonds (summarized in Table 3b, and Figs 5a and 5b). Moreover, considering the strong conservation of the interacting protein structures, the similarity and near identical extension of the enzyme:inhibitor interacting surfaces, entropic factors should play comparable roles in the formation of α-CHT:BPTI and β-TRP:BPTI complexes. Thus, it is proposed that in α-CHT dynamical effects (arising e.g. from autolysis at sites 146 and 148), as well as subtle different structuring of surface polypeptide loops or trapped water molecules, result in less efficient overall BPTI recognition and affinity. In this respect, we notice that the overall main chain B factor for the α-CHT:BPTI complex is 42.8 Å^2, whereas an average value of 22.2 Å^2 is observed for the main chain atoms of the β-TRP:BPTI complex (Rühlmann *et al.*, 1973). These effects can be compared to those observed in the bovine trypsinogen:BPTI complexes (see Table 4), for which the addition of the exogenous peptide Ile-Val, despite having negligible effects on the overall three-dimensional structure

Table 4. Values of the dissociation equilibrium constant (K_d) for the binding of native and mutant BPTI to serine (pro) enzymes

Serine (pro)enzyme	Inhibitor	K_d (M)
Bovine β-trypsin[a]	Native BPTI	6.0×10^{-14}
Bovine β-trypsin[b]	*nor*Leu15 BPTI	2.0×10^{-8}
Bovine β-trypsin[c]	Phe15 BPTI	1.0×10^{-5}
Bovine anhydrotrypsin[d]	Native BPTI	1.1×10^{-13}
Bovine trypsinogen[e]	Native BPTI	2.0×10^{-6}
Ile-Val:bovine trypsinogen[f]	Native BPTI	2.0×10^{-10}
Porcine pancreatic kallikrein[g]	Native BPTI	8.4×10^{-10}
Human urokinase[h]	Native BPTI	2.0×10^{-5}
Human α-thrombin[i]	Native BPTI	8.3×10^{-4}
Human γ-thrombin[i]	Native BPTI	1.1×10^{-4}
Bovine α-chymotrypsin[e]	Native BPTI	9.5×10^{-9}
Bovine α-chymotrypsin[b]	*nor*Leu15 BPTI	6.7×10^{-9}
Bovine α-chymotrypsin[c]	Phe15 BPTI	2.8×10^{-9}

[a] pH=8.0 and T=25°C. From Vincent and Lazdunski (1972).
[b] pH=8.0 and T=25°C. From Tschesche *et al.* (1987).
[c] pH=7.8 and T=25°C. The value of K_d was calculated from the dependence of the relative activity of β-TRP on the Phe15 BPTI concentration. Data from Jering and Tschesche (1976).
[d] pH=8.0 and T=25°C. From Vincent *et al.* (1974).
[e] pH=8.0 and T=21°C. From Antonini *et al.* (1983).
[f] pH=8.0 and T=21°C. According to linked functions (Wyman, 1964), the value of K_d for native BPTI binding to the Ile-Val:bovine trypsinogen complex was calculated by dividing the value of K_d for inhibitor binding to the zymogen in the absence of the dipeptide ($=2.0\times10^{-6}$ M) by the interaction parameter ($=1.0\times10^{4}$). Data from Antonini *et al.* (1983) and Coletta *et al.* (1990a and 1990b).
[g] pH=8.0 and T=21°C. From Menegatti *et al.* (1984).
[h] pH=8.0 and T=21°C. From Ascenzi *et al.* (1990).
[i] pH=7.5 and T=21°C. From Ascenzi *et al.* (1992).

of the (pro)enzyme (Bode and Huber, 1976; Huber and Bode, 1978; Bode *et al.*, 1984), increases the affinity for BPTI by four orders of magnitude (Coletta *et al.*, 1990a and 1990b), through an increase in rigidity (i.e. lower *B*-factors) of the polypeptide loops Ile16-Gly19, Gly142-Pro152, Gly184-Gly193 and Gly216-Asn223, building up the serine (pro)enzyme activation domain.

Acknowledgment

The authors thank Professor G. Amiconi, Professor A. Desideri and Dr F. Polticelli for helpful discussions. This work has been partially supported by grants from the Ministry of University, Scientific Research and Technology of Italy (MURST), from the National Research Council of Italy (CNR), and from the European Union Human Capital and Mobility Program (CT940690).

References

Antonini, E., Ascenzi, P., Bolognesi, M., Gatti, G., Guarneri, M. and Menegatti, E. (1983). Interaction between serine (pro)enzymes, and Kazal and Kunitz inhibitors. *J. Mol. Biol.* **165**, 543–558.

Ascenzi, P., Menegatti, E., Guarneri, M., Bortolotti, F. and Antonini, E. (1982). Catalytic properties of serine proteases: 2, comparison between human urinary kallikrein and human urokinase, bovine β-trypsin, bovine thrombin and bovine α-chymotrypsin. *Biochemistry* **21**, 2483–2490.

Ascenzi, P., Amiconi, G., Bolognesi, M., Menegatti, E. and Guarneri, M. (1990). Binding of the bovine basic pancreatic trypsin inhibitor (Kunitz) to the 33,000 M_r and 54,000 M_4 species of human urokinase: thermodynamic study. *J. Enzyme Inhibit.* **4**, 51–55.

Ascenzi, P., Aducci, P., Amiconi, G., Ballio, A., Guaragna, A., Menegatti, E., Schnebli, H. P. and Bolognesi, M. (1991). Binding of the recombinant proteinase inhibitor eglin *c* from leech *Hirudo medicinalis* to serine (pro)enzymes: a comparative thermodynamic study. *J. Mol. Recogn.* **4**, 113–119.

Ascenzi, P., Amiconi, G., Coletta, M., Lupidi, G., Menegatti, E., Onesti, S. and Bolognesi, M. (1992). Binding of hirudin to human α, β and γ-thrombin: a comparative kinetic and thermodynamic study. *J. Mol. Biol.* **225**, 177–184.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F. Jr, Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977). The protein data bank: a computer-based archival file for macromolecular structures. *J. Mol. Biol.* **112**, 535–452.

Bode, W. (1979). The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding. II. The binding of the pancreatic trypsin inhibitor and of isoleucine-valine and of sequentially related peptides to trypsinogen and to *p*-guanidinobenzoate-trypsinogen. *J. Mol. Biol.* **127**, 357–374.

Bode, W. and Huber, R. (1976). Induction of the bovine trypsinogen-trypsin transition by peptides sequentially similar to the *N*-terminus of trypsin. *FEBS Lett.* **68**, 231–236.

Bode, W. and Huber, R. (1992). Natural protein proteinase inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **204**, 433–451.

Bode, W., Walter, J., Huber, R., Wenzel, H. R. and Tschesche, H. (1984). The refined 2.2 Angstroms (0.22 nm) X-ray crystal structure of the ternary complex formed by bovine trypsinogen, valine-valine and the Arg15 analogue of bovine pancreatic trypsin inhibitor. *Eur. J. Biochem.* **144**, 185–190.

Brünger, A. T. (1992). Free *R* value: a novel statistical quantity for assessing the accuracy of crystal structures. *Nature* **355**, 472–475.

Chen, Z. and Bode, W. (1983). Refined 2.5 Å X-ray crystal structure of the complex formed by porcine kallikrein A and the bovine pancreatic trypsin inhibitor. Crystallization, Patterson search, structure determination, refinement, structure and comparison with its components and with the bovine trypsin-pancreatic trypsin inhibitor complex. *J. Mol. Biol.* **164**, 283–311.

Coletta, M., Ascenzi, P., Amiconi, G., Bolognesi, M., Guarneri, M. and Menegatti, E. (1990a). Bovine trypsinogen activation: a thermodynamic study. *Biophys. Chem.* **37**, 355–362.

Coletta, M., Ascenzi, P., Bravin, L., Amiconi, G., Bolognesi, M., Guarneri, M. and Menegatti, E. (1990b). Thermodynamic modeling of internal equilibria involved in the activation of trypsinogen. *J. Biomol. Struct. Dyn.* **7**, 959–972.

Creighton, T. E. and Darby, N. J. (1989). Functional evolutionary divergence of proteolytic enzymes and their inhibitors. *Trends Biochem. Sci.* **14**, 319–324.

Creighton, T. E., Bagley, C. J., Cooper, L., Darby, N. J., Freedman, R. B., Kemmink, J. and Sheikh, A. (1993). On the biosynthesis of bovine pancreatic trypsin inhibitor (BPTI). Structure, processing, folding and disulfide bond formation of the precursor *in vitro* and in microsomes. *J. Mol. Biol.* **232**, 1176–1196.

Cutruzzolà, F., Ascenzi, P., Barra, D., Bolognesi, M., Menegatti, E., Sarti, P., Schnebli, H. P., Tomova, S. and Amiconi, G. (1993). Selective oxidation of Met-192 in bovine α-chymotrypsin. Effect on catalytic and inhibitor binding properties. *Biochim. Biophys. Acta* **1161**, 201–208.

Engh, R. A. and Huber, R. (1991). Accurate bond and angle parameters for X-ray protein structure refinement. *Acta Crystallogr.* **A47**, 392–400.

Fioretti, E., Binotti, I., Barra, D., Citro, G., Ascoli, F. and Antonini, E. (1983). Heterogeneity of the basic pancreatic inhibitor (Kunitz) in various bovine organs. *Eur. J. Biochem.* **130**, 13–18.

Frigerio, F., Coda, A., Pugliese, L., Lionetti, C., Menegatti, E., Amiconi, G., Schnebli, H. P., Ascenzi, P. and Bolognesi, M. (1992). Crystal and molecular structure of the bovine α-chymotrypsin-eglin *c* complex at 2.0 Å resolution. *J. Mol. Biol.* **225**, 107–123.

Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M. Jr and James, M. N. G. (1987). Crystal and molecular structures of the complex of α-chymotrypsin with its inhibitor turkey ovomucoid third domain at 1.8 Å resolution. *J. Mol. Biol.* **195**, 397–418.

Greer, J. (1990). Comparative modelling methods: application to the family of the mammalian serine proteases. *Proteins* **7**, 317–334.

Hecht, H. J., Szardenings, M., Collins, J. and Schomburg, D. (1991). Three-dimensional structure of the complexes between bovine chymotrypsinogen A and two recombinant variants of human pancreatic secretory trypsin inhibitor (Kazal-type). *J. Mol. Biol.* **220**, 711–722.

Huber, R. and Bode, W. (1978). Structural basis of the activation and action of trypsin. *Acc. Chem. Res.* **11**, 114–122.

Huber, R., Kukla, D., Rühlmann, A., Epp, O. and Formanek, H. (1970). The basic trypsin inhibitor of bovine pancreas. I. Structure analysis and conformation of the polypeptide chain. *Naturwissenshaften* **57**, 339–392.

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J. and Steigemann, W. (1974). Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. II. Crystallographic refinement at 1.9 Å resolution. *J. Mol. Biol.* **89**, 73–101.

Huber, R., Bode, W., Kukla, D., Kohl, U. and Ryan, C. A. (1975). The structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. III. Structure of the anhydrotrypsin-inhibitor complex. *Biophys. Struct. Mechanism* **1**, 189–201.

Hynes, T. R., Randal, M., Kennedy, L. A., Eigenbrot, C. and Kossiakoff, A. A. (1990). X-ray crystal structure of the protease inhibitor domain of Alzheimer's amyloid β-protein precursor. *Biochemistry* **29**, 10018–10022.

Jering, H. and Tschesche, H. (1976). Replacement of lysine by arginine, phenylalanine and tryptophan in the reactive site of the bovine trypsin-kallikrein inhibitor (Kunitz) and change of the inhibitor properties. *Eur. J. Biochem.* **61**, 453–463.

Jones, T. A. (1978). A graphics model building and refinement system for macromolecules. *J. Appl. Crystallogr.* **11**, 268–272.

Kassel, B. (1970). Bovine trypsin-kallikrein inhibitor (Kunitz inhibitor, basic pancreatic trypsin inhibitor, polyvalent inhibitor from bovine organs). *Methods Enzymol.* **19**, 844–852.

Lancelin, J.-M., Foray, M.-F., Poncin, M., Hollecker, M. and Marion, D. (1994). Proteinase inhibitor homologues as potassium channel blockers. *Nature Struct. Biol.* **1**, 246–250.

Laskowski, M. Jr and Kato, I. (1980). Protein inhibitors of proteinases. *Annu. Rev. Biochem.* **49**, 593–626.

Laskowski, R. A., Arthur, M. W. M., Moss, D. S. and Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291.

Matthews, B. W. (1968). Solvent content of protein crystals. *J. Mol. Biol.* **33**, 491–497.

Menegatti, E., Guarneri, M., Bolognesi, M., Ascenzi, P. and Amiconi, G. (1984). Binding of the bovine basic pancreatic trypsin inhibitor (Kunitz) to human urinary kallikrein and to porcine pancreatic β-kallikreins A and B. *J. Mol. Biol.* **176**, 425–430.

Navaza, J. (1994). AMoRe: an automated package for molecular replacement. *Acta Crystallogr.* **A50**, 157–163.

Oddone, R., Barra, D., Amiconi, G., Ascenzi, P., Tarricone, C., Bolognesi, M., Bortolotti, F. and Menegatti, E. (1994). Binding of native and [homoserine lactone-52]-52,53-*seco*-bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor) to porcine pancreatic β-kallikrein-B and bovine α-chymotrypsin: thermodynamic study. *J. Mol. Recogn.* **7**, 39–46.

Parkin, S., Rupp, B. and Hope, H. (1996). Structure of bovine pancreatic trypsin inhibitor at 125K: definition of carboxyl-terminal residues Gly57 and Ala58. *Acta Crystallogr.* **D52**, 18–29.

Rühlmann, A., Kukla, D., Schwager, P., Bartels, K. and Huber, R. (1973). Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. Crystal structure determination and stereochemistry of the contact region. *J. Mol. Biol.* **77**, 417–436.

Schechter, I. and Berger, A. (1967). On the size of the active site in proteases. I, Papain. *Biochem. Biophys. Res. Commun.* **27**, 157–162.

Tronrud, D., TenEyck, L. F. and Matthews, B. W. (1987). An efficient general purpose least squares refinement program for macromolecular structures. *Acta Crystallogr.* **A34**, 489–501.

Tschesche, H., Beckmann, J., Mehlich, A., Schnabel, E., Truscheit, E. and Wenzel, H. R. (1987). Semisynthetic engineering of proteinase inhibitor homologues. *Biochim. Biophys. Acta* **913**, 97–101.

Tsukada, H. and Blow, D. M. (1985). Structure of α-chymotrypsin refined at 1.68 Å resolution. *J. Mol. Biol.* **184**, 703–711.

Vincent, J.-P. and Lazdunski, M. (1972). Trypsin-pancreatic trypsin inhibitor association. Dynamics of the interaction and role of disulfide bridges. *Biochemistry* **11**, 2967–2977.

Vincent, J.-P., Peron-Renner, M., Pudles, J. and Lazdunski, M. (1974). The association of anhydrotrypsin with the pancreatic trypsin inhibitors. *Biochemistry* **13**, 4205–4211.

Wallace, A. C., Laskowski, R. A. and Thornton, J. M. (1995). LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Engineering* **8**, 127–134.

Wlodawer, A., Walter, J., Huber, R. and Sjölin, L. (1984). Structure of bovine pancreatic trypsin inhibitor. Results of joint neutron and X-ray refinement of crystal form II. *J. Mol. Biol.* **180**, 301–329.

Wlodawer, A., Nachman, J., Gilliland, G. L., Gallagher, W. and Woodward, C. (1987). Structure of form III crystals of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* **198**, 469–480.

Wüthrich, K., Wider, G., Wagner, G. and Braun, W. (1982). Sequential resonance assignments as a basis for determination of spatial protein structures by high resolution proton nuclear magnetic resonance. *J. Mol. Biol.* **155**, 311–319.

Wyman, J. (1964). Linked functions and reciprocal effects in hemoglobin: a second look. *Adv. Protein Chem.* **19**, 223–286.

Note added in Proof

While this manuscript was in press, we learnt that unpublished results quoted in Kossiakoff, A. A., Hynes, T. and de Vos, A. [Molecular recognition in biological systems. From activation to inhibition. *Biochem. Soc. Trans.* **21**, 614–618 (1993)] outline an α-CHT S_1 subsite:BPTI Lys(P_1) residue recognition mechanism in full agreement with the results here reported.