Acta Biochimica Polonica

Vol. 48 No. 2/2001

419–428

QUARTERLY

Review

Structure-function relationship of serine protease–protein inhibitor interaction★✪

Jacek Otlewski[1✉], Mariusz Jaskólski[2,3], Olga Buczek[1], Tomasz Cierpicki[1], Honorata Czapińska[1], Daniel Krowarsch[1], Arne O. Smalas[4], Damian Stachowiak[1], Agnieszka Szpineta[1] and Micha³ Dadlez[5,6]

[1]*Institute of Biochemistry and Molecular Biology, University of Wroc³aw, Wroc³aw, Poland;* [2]*Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznañ, Poland;* [3]*Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznañ, Poland;* [4]*Protein Crystallography Group, Department of Chemistry, University of Tromsø, Tromsø, Norway;* [5]*Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland;* [6]*Institute of Physics, Department of Biophysics, Warsaw University, Warszawa, Poland*

Received: 14 February, 2001; accepted: 30 April, 2001

Key words: protein–protein recognition, serine proteases, protein inhibitor, structural thermodynamics, calorimetry

We report our progress in understanding the structure-function relationship of the interaction between protein inhibitors and several serine proteases. Recently, we have determined high resolution solution structures of two inhibitors *Apis mellifera* chymotrypsin inhibitor-1 (AMCI-I) and *Linum usitatissimum* trypsin inhibitor (LUTI) in the free state and an ultra high resolution X-ray structure of BPTI. All three inhibitors, despite totally different scaffolds, contain a solvent exposed loop of similar conformation which is highly complementary to the enzyme active site. Isothermal calo-

★Presented at the International Conference on "Molecular Architecture of Evolution, Primary and Secondary Determinants" Poznañ, Poland, October 29–31, 2000.

✪Supported by grants 6 PO4B 002 from the State Committee for Scientific Research (KBN, Poland).

✉Corresponding author: Jacek Otlewski, Institute of Biochemistry & Molecular Biology, University of Wroc³aw, Tamka 2, 50-137 Wroc³aw, Poland; Tel. (48 71) 375 2824; Fax. (48 71) 375 2608; e-mail: otlewski@bf.uni.wroc.pl

Abbreviations: AMCI-1, *Apis mellifera* chymotrypsin inhibitor-1; LUTI, *Linum usitatissimum* trypsin inhibitor; BPTI, bovine pancreatic trypsin inhibitor (Kunitz); HNE, human neutrophil elastase; K_a, association constant; T_{den}, denaturation temperature.

rimetry data show that the interaction between wild type BPTI and chymotrypsin is entropy driven and that the enthalpy component opposes complex formation. Our research is focused on extensive mutagenesis of the four positions from the protease binding loop of BPTI: P_1, P_1', P_3, and P_4. We mutated these residues to different amino acids and the variants were characterized by determination of the association constants, stability parameters and crystal structures of protease–inhibitor complexes. Accommodation of the P_1 residue in the S_1 pocket of four proteases: chymotrypsin, trypsin, neutrophil elastase and cathepsin G was probed with 18 P_1 variants. High resolution X-ray structures of ten complexes between bovine trypsin and P_1 variants of BPTI have been determined and compared with the cognate P_1 Lys side chain. Mutations of the wild type Ala16 (P_1') to larger side chains always caused a drop of the association constant. According to the crystal structure of the Leu16 BPTI–trypsin complex, introduction of the larger residue at the P_1' position leads to steric conflicts in the vicinity of the mutation. Finally, mutations at the P_4 site allowed an improvement of the association with several serine proteases involved in blood clotting. Conversely, introduction of Ser, Val, and Phe in place of Gly12 (P_4) had invariably a destabilizing effect on the complex with these proteases.

Specific protein–protein recognition is of utmost importance in many biological processes (Jones & Thornton, 1996). Formation of specific complexes between antigen and antibody, hormone and receptor, or enzyme and inhibitor are classic examples of highly complementary and specific interactions vital to living organisms. Serine proteases of the chymotrypsin and subtilisin families and their canonical (or standard mechanism) protein inhibitors are the most intensively studied group of protein–protein complexes. The inhibitors can be divided into 18 families (Laskowski & Qasim, 2000). Representatives of different families have totally different folds, but the main chain conformation of their P_3–P_3' segment (P_n, P_n' notation according to Schechter & Berger, 1967) is similar (Apostoluk & Otlewski, 1998; Otlewski *et al.*, 1999; Bode & Huber, 2000). This so called canonical conformation forms a loop of convex shape which is able to recognize the concave active site of the cognate enzyme. The central part of this extended loop contains a solvent exposed P_1–P_1' peptide bond, called the reactive site, which is recognized by the protease in a substrate-like manner. Apart from the P_3–P_3' segment, also side chains from the surrounding residues and other parts of the inhibitor make numerous van der Waals contacts and hydrogen bonds with the protease. Similar number of contacting residues – about 10–12 on the inhibitor side and 20–25 on the enzyme side and similar intermolecular contact area of about 600–900 $Å^2$ per interacting protein characterize all protease–inhibitor complexes. The interface is predominantly hydrophobic and devoid of water molecules in its central part. Nevertheless, numerous hydrogen bonds and sometimes also electrostatic interactions at the interface are formed. Of particular importance is a short antiparallel β-sheet formed through main chain–main chain hydrogen bonds between residues P_3 and P_1 and the 214–216 segment of the enzyme. Other important features are: a short 2.7 Å contact between P_1 carbonyl carbon and the catalytic Ser195 O^{γ} and two hydrogen bonds formed between carbonyl oxygen of P_1 and Gly193/ Ser195 amides (the oxyanion binding hole). Shape complementarity of interacting areas together with the above mentioned hydrogen bonds ensure very similar recognition of different proteases and inhibitors. The enzyme–inhibitor interaction is particularly rigid and resembles the lock-and-key model providing an extremely useful model to study protein–protein recognition.

In this paper we review our recent data on structure and energetics of the interaction between several serine proteases and bovine pancreatic trypsin inhibitor (BPTI). The results, taken together, provide a coherent view

of the role played by different inhibitor residues in enzyme recognition.

THREE-DIMENSIONAL STRUCTURES

Figure 1 shows some examples of three-dimensional solution structures for two canonical inhibitors which were determined in our laboratory by ^{1}H NMR spectroscopy: *Apis mellifera* chymotrypsin inhibitor-1 (AMCI-1) (Cierpicki *et al.*, 2000) and *Linum usitatissimum* trypsin inhibitor (LUTI) (Cierpicki & Otlewski, 2000). Both proteins exhibit completely different folds: AMCI-1 is a 56-residue protein and its structure consists of two approximately perpendicular β-sheets and several turns which form the scaffold bearing a long loop which contains the protease binding site. The overall tertiary structure is maintained by five disulfide bonds and no hydrophobic core is present. The organization of the secondary structure elements in the 69-residue LUTI is completely different. LUTI molecule has a clear hydrophobic core, a mixed parallel/antiparallel β-sheet, and a single α-helix.

Figure 2 shows the tertiary fold of BPTI – the protein which is the main object of this paper. Again, besides the canonical conformation of the binding loop, the structure is completely different from both LUTI and AMCI-1. BPTI has a clear hydrophobic core, three disulfide bonds, three-stranded β-sheet and two short helices – an N-terminal 3_{10} helix and a C-terminal α-helix. The presented structure has been recently determined for a Thr11Ala, Pro13Ala, Lys15Arg, Met52Leu mutant of BPTI at 0.86 Å resolution using low temperature synchrotron data (Czapińska *et al.*, 2000; and unpublished data). The first three mutations seem to play a crucial role in the crystal structure of the mutant but have practically no effect on the molecular structure in general, and on the conformation of the binding loop in particular.

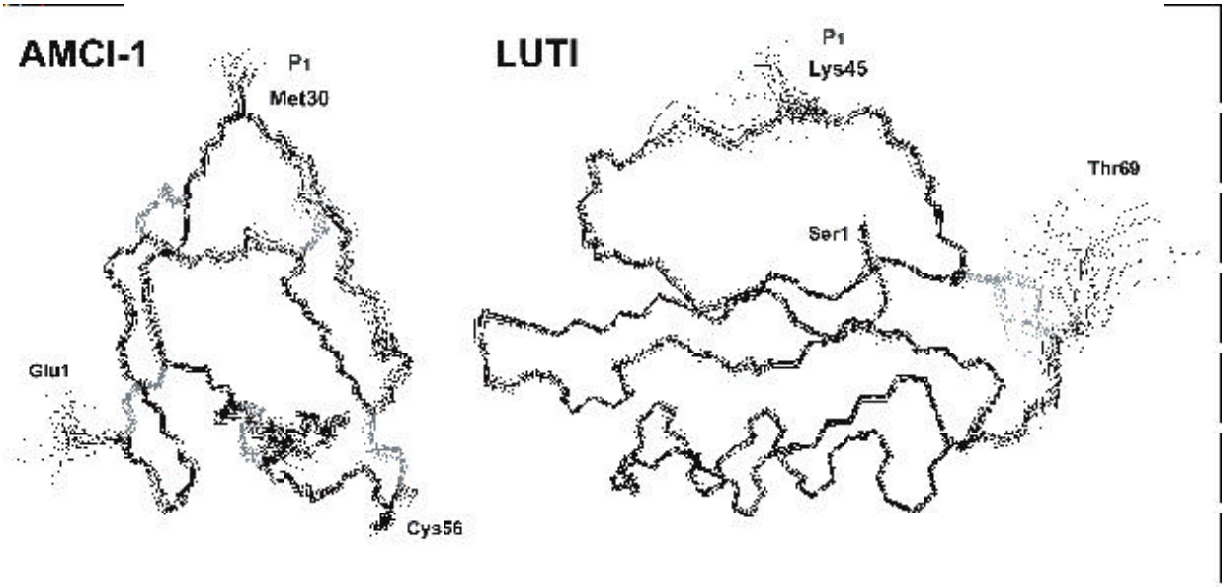

Figure 1. Polypeptide backbone atoms of the 20 lowest energy AMCI-1 (Cierpicki *et al.*, 2000) and LUTI (Cierpicki & Otlewski, 2000) structures.

The light grey lines indicate disulfide bridges.

The mutated protein forms very stable crystals of a new tetragonal form. It grows on a time scale of hours and yields diffraction data of high resolution and quality allowing precise

Figure 2. Ribbon diagram of Thr11Ala, Pro13Ala, Lys15Arg, Met52Leu BPTI, pdb code 1QLQ (Czapinska *et al.*, 2000).

Positions Ala11, Ala13 and Arg15 are indicated by black solid lines.

structural characterization of the entire molecule. The Thr11Ala and, particularly, the Pro13Ala substitutions (the latter never observed in BPTI-like proteins) contribute to stabilizing hydrophobic contacts between symmetry-related molecules. Also, the Lys15Arg substitution, through formation of electrostatic interactions between Arg15 and Cys38, is a source of intermolecular contacts, which are further stabilized by a closely located sulfate ion. The two chiralities observed for the Cys14–Cys38 disulfide seem to be a consequence of this electrostatic interaction and, on the other hand, suggest an inherent conformational instability of this bridge. The structure provides new details (double-conformation of main-chain Leu29 and Gly56–Gly57, high (20%) proportion of residues in double conformation, correlation of disorder on intermolecular scale, positions of hydrogen atoms (also in water molecules) and their involvement in C–H...O and N–H...π hydrogen bonds).

THERMODYNAMICS OF THE PROTEASE–INHIBITOR INTERACTION

In recent years with an advent of highly sensitive isothermal titration calorimetry (ITC) precise understanding of enthalpic, entropic and heat capacity factors contributing to the free energy change accompanying protein–protein complexation reaction became possible (Baker & Murphy, 1997). As an introduction to thermodynamic analysis of serine protease–protein inhibitor interaction we performed calorimetric measurements on the BPTI–bovine chymotrypsin system (Fig. 3) for which X-ray crystal structure is available (Scheidig *et al.*, 1997). $\Delta C_{p,ass}$ for the BPTI–chymotrypsin complex is in good agreement with the values calculated from the change in water accessible surface area during complex formation (Table 1). Our data show that the reaction is entropy-driven, with an enthalpy contribution opposing complex formation (Fig. 4). The entropic contribution results most probably from the release of ordered water from the hydrophobic protein–protein interface during complex formation. The large and positive value of the enthalpy change is difficult to understand at present (Table 1). It does not agree with the values calculated from well-established theoretical equations (Bardi *et al.*, 1997). The heat capacity change is negative and scales well with the extent of hydrophobic and hydrophilic surface area buried upon complex formation. Clearly, a deeper understanding of serine protease–protein inhibitor interaction in terms of structural thermodynamics will require calorimetric analysis of P_1 mutated inhibitors in a broader pH range.

THE EFFECT OF THE P_1 POSITION ON THE INTERACTION WITH PROTEASES

A large part of the enzyme–inhibitor contacts is made just by the P_1 residue, which penetrates deeply into the S_1 specificity bind-

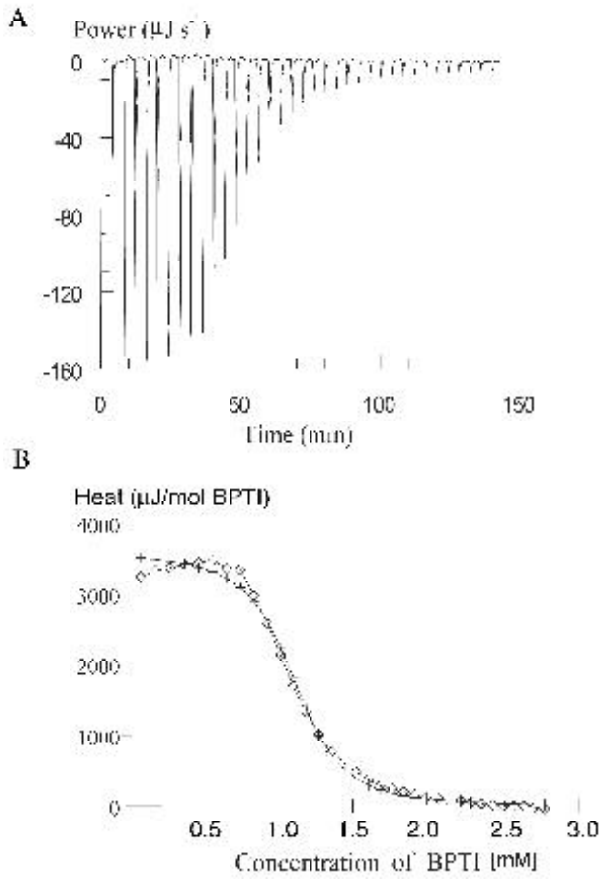

Figure 3. A typical calorimetric isothermal titration measurement for the interaction of α-chymotrypsin and BPTI.

A. Observed heat effect upon thirty injections of BPTI (1.33×10^{-4} M) to α-chymotrypsin (1.03×10^{-3} M). Both proteins were in 50 mM sodium acetate, 20 mM calcium chloride, pH 4.8, 25°C. **B.** Integration of the peaks yields a heat per injection. The solid line with (+) sign shows the best fit obtained using one-site model (determined stoichiometry value, n = 1.009). The observed injection heats were corrected for the heat of dilution.

Table 1. Comparison of isometric titration calorimetry observed and structure-based parameters for BPTI binding to α-chymotrypsin.

The measured parameters were obtained as average values from four experiments at 25°C, 50 mM sodium acetate, 20 mM calcium chloride, pH 4.8. Errors calculated as average standard deviation. Parameters were calculated according to Bardi *et al.* (1997).

Complex parameter	α-Chymotrypsin–BPTI	
	Measured value	Calculated value
ΔASA (Å^2)		
Polar		–584
Apolar		–873
Total		–1457
$\Delta C_{p,ass}$(cal K^{-1} mol^{-1})		
Polar		152
Apolar		–393
Total	–176 ± 50	–241
ΔH_{ass} (kcal mol^{-1})		
Polar		–18
Apolar		7.4
Total	12.3 ± 0.5	–2.5
ΔS_{ass} (cal K^{-1} mol^{-1})		
Solv		61.7
Conf		–25.8
Cratic		–7.9
Total	61.7 ±3.3	28
ΔG_{ass} (kcal mol^{-1})	– 6.1 ±0.07	–10.9
	– 6.5 ±0.1*	

*Value determined using classical method of association constant determination based on the measurement of residual enzyme activity.

ing pocket of the protease. Therefore, docking of the P_1 side chain inside the S_1 binding pocket of the enzyme plays a major role in the energetics of the recognition (Czapińska & Otlewski, 1999). Figure 5 presents association energy data which were determined in our laboratory with four different proteases: bovine α-chymotrypsin, bovine β-trypsin, human neutrophil elastase (HNE) and human cathepsin G (Krowarsch *et al.*, 1999; Polanowska *et al.*, 1998). What can be easily noticed is the huge dynamic range of K_a values from about 1×10^9 for trypsin to about 5×10^5 for chymotrypsin and elastase. The dynamic range for cathepsin G is much smaller due to low association constants even for the optimal Lys and Phe side chains. The above binding data correlate well with substrate specificity indexes for these proteases expressed in the form of log (k_{cat}/K_m) (Krowarsch *et al.*, 1999).

Several conclusions about the S_1 specificity can be drawn from the presented data.

Trypsin, due to the unique location and orientation of Asp189 at the bottom of a narrow

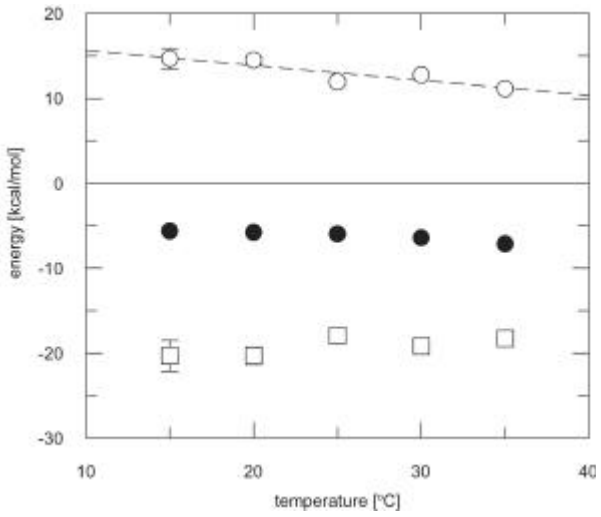

Figure 4. Temperature dependence of the observed thermodynamic parameters for the binding of BPTI to α-chymotrypsin at 25°C, pH 4.8. ( ) Enthalpy change (ΔH_{ass}), ( ) free energy change (ΔG_{ass}), ( ) entropy change ($-T\Delta S_{ass}$).

Error bars represent the average standard deviation from 4–6 experiments, the slope of broken line obtained by linear regression yields the heat capacity change ($\Delta C_{p,\,ass}$ = –176 ± 50 cal K^{-1} mol^{-1}).

and deep pocket, is particularly suited to interact with the basic side chains of Lys and Arg. Substitution of Lys15 with Gly reduces by 70% the total association energy leading to a huge nine orders of magnitude decrease in association constant. The binding of other side chains is much weaker. Phe, the next strongest binder, forms interactions that are over 1×10^5-fold weaker than those of Lys. Interaction of the hydrophobic aromatic ring of Phe with the hydrophobic walls of the S_1 pocket partially compensates for the lack of an ion pair. This is also evident in the case of Leu and Trp. The polar side chains of Ser, Asn, His and Gln also bind relatively well, probably due to weak, water-mediated hydrogen bonding interactions with Asp189. β-branched side chains (Ile, Val and Thr) bind particularly weakly to the S_1 pocket, due to steric clashes with the atoms at the narrow entrance to the pocket. As expected, binding of the negatively charged Asp is 350-fold weaker then of the isosteric Asn. Surprisingly, Asp still binds 5-fold better than Gly. From the energetic point of view, an empty pocket produces the most deleterious effect.

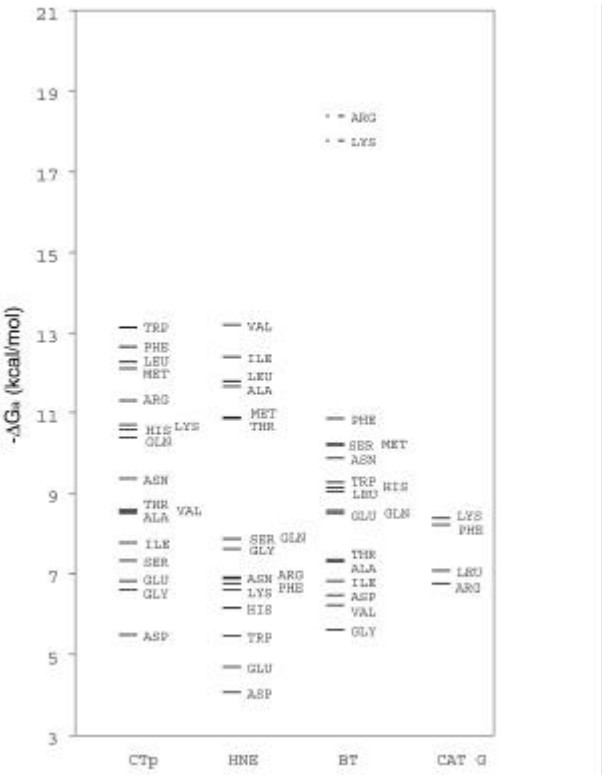

Figure 5. The $-(\Delta G_a)$ values for the association of 18 P_1 mutants of BPTI with bovine chymotrypsin (CTp), human neutrophil elastase (HNE), bovine trypsin (BT) and human cathepsin G (CAT G) at pH 8.3 and 25°C.

The estimated values for Arg and Lys side chains based on measurements at pH 5.0 are marked with a dashed line.

In order to better understand the structural features of this interaction we have determined 10 high resolution X-ray structures of the complexes between bovine β-trypsin and BPTI variants with different amino-acid residues at the P_1 position (Helland *et al.*, 1999). All P_1 mutant side chains could be accommodated at the primary binding site, but, relative to the P_1 Lys, there were small local changes within the P_1–S_1 interaction site (Fig. 6). These comprised: changes in the number and dynamics of water molecules, multiple confor-

mations and non-optimal dihedral angles for some of the P_1 side chains and Ser190 and Gln192 from the enzyme side, changes in temperature factors of the pocket walls as well as of the P_1 side chains. Only binding of the cognate Lys15 is characterized by almost optimal dihedral angles, satisfied hydrogen bonds and low temperature factors.

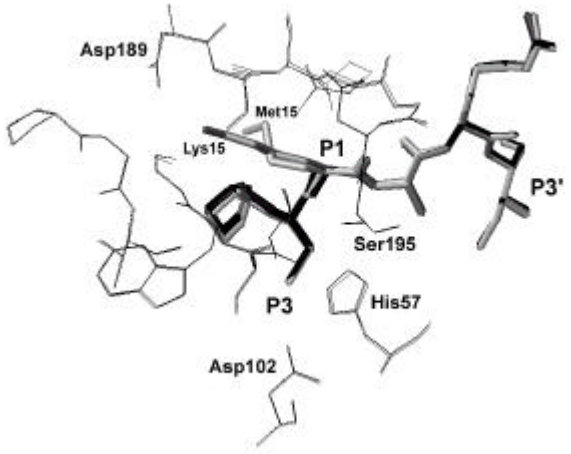

Figure 6. The conformation of P_1 Lys (dark grey), Met (light grey) and Gly (black) side chains of three BPTI mutants bound in the S_1 pocket of bovine chymotrypsin (Helland *et al.*, 1999).

The figure was made with the SYBYL software (Tripos). The chain P_3–P_3' segment of BPTI is shown as bold lines, the S_1 pocket of bovine chymotrypsin is shown as thin lines.

Interactions within the large and hydrophobic pocket of chymotrypsin are not as complicated. Energetic effects expressed in $\Delta\Delta G_{ass}$ (= $\Delta G_{ass,Xaa} - \Delta G_{ass,Gly}$, association free energy change referred to Gly) correlate well with the volume of the P_1 side chain (correlation coefficient r = 0.84) or with its area (r = 0.83). Therefore, we conclude that the hydrophobic effect is the driving force for the association reaction. Deleterious effects can be noticed in the case of polar, negatively charged (a 750-fold effect in Asp–Asn comparison) and β-branched (a 2200-fold effect in Ile–Leu comparison) side chains. Lys and Arg residues bind surprisingly well. This effect is explained by the conformation of the P_1 Lys in the recent structure of the BPTI-chymotrypsin complex (Scheidig *et al.*, 1997), where it adopts a rather unusual conformation with its charged end forming favourable interactions with carbonyl groups at the entrance to the pocket. As in the case of trypsin, an empty pocket (P_1 Gly) represents the worst case. Interestingly, if the trypsin-specific Lys/Arg amino acids are eliminated, the correlation of trypsin and chymotrypsin binding energies is high (r = 0.72) (Fig. 7). This suggests that both

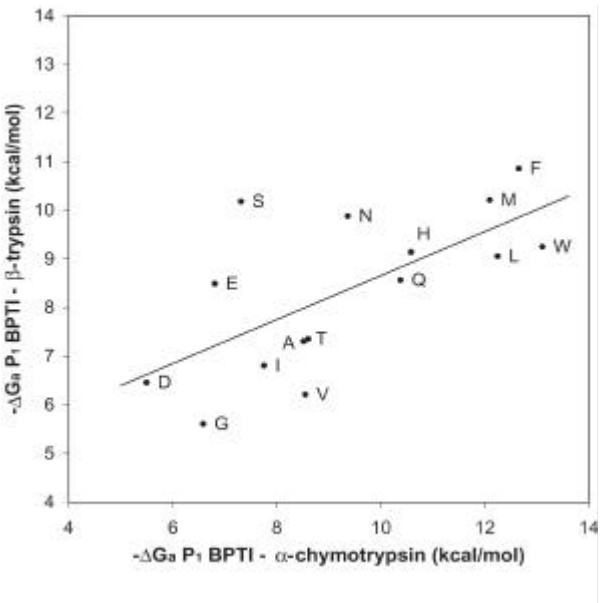

Figure 7. Correlation plot of association free energies of BPTI P_1 mutants to bovine chymotrypsin and trypsin.

enzymes recognize non-cognate substrates using similar binding interactions within their S_1 pockets.

The major difference that distinguishes the pocket of neutrophil elastase is its clear preference for β-branched side chains of Val and Ile (Ile is favoured 3-fold over Leu, in contrast to chymotrypsin). Binding of all other side chains is much (10^4–10^5-fold) weaker. The data are in agreement with the structural organization of the neutrophil elastase pocket which exhibits a significant degree of flexibility (Bode *et al.*, 1989).

POSITION P_1'

The role of the S_1' subsite in trypsin, chymotrypsin and plasmin has been examined by measuring the association constants with seven (Gly, Ala, Ser, Val, Leu, Arg, and Trp) different P_1' mutants of BPTI (Table 2). The effects of P_1' substitutions on the association constant are very large: over five orders for chymotrypsin and seven orders of magnitude for trypsin and plasmin. All mutants showed a decrease of the association constant to the three proteases in the same order: Ala>Gly>Ser>Arg>Val>Leu>Trp. Calorimetric and circular dichroism methods showed that none of the P_1' substitutions, except for the P_1'–Val mutation, leads to a destabilization of the binding loop (Table 2). The X-ray structure of the complex formed between bovine β-trypsin and P_1'–Leu BPTI (Grzesiak *et al.*, 2000a) showed that the P_1'–Leu sterically conflicts with the side chain of P_3'–Ile, which thereby is forced to rotate by approximately 90°. Ile18 (P_3') in its new orientation is, in turn, interacting with the Tyr39 side chain of trypsin. Introduction of a large side chain at the P_1' position apparently leads to a cascade of small alterations of the trypsin–BPTI interface which seem to destabilize the complex by the less favourable packing and tilting of the BPTI molecule by up to 15° compared to the native trypsin–BPTI complex.

Table 2. Thermal denaturation parameters T_{den} for seven P_1' mutants of BPTI and the association constant values for the interaction of seven P_1' mutants of BPTI with three serine proteases.

P_1' variant	T_{den} (°C)	Bovine β-trypsin K_a [M^{-1}]	α-Chymotrypsin K_a [M^{-1}]	Human α-plasmin K_a [M^{-1}]
Ala	83.6	1.3×10^{13}	2.5×10^{8}	6.7×10^{9}
Arg	84.0	4.9×10^{9}	7.9×10^{4}	7.5×10^{7}
Gly	82.9	6.7×10^{11}	1.8×10^{7}	5.0×10^{8}
Leu	83.4	3.7×10^{7}	1.8×10^{4}	1.0×10^{4}
Ser	82.4	7.8×10^{10}	5.8×10^{6}	1.4×10^{8}
Trp	80.5	1.1×10^{6}	1.0×10^{3}	1.0×10^{3}
Val	63.8	3.8×10^{7}	2.9×10^{3}	8.3×10^{6}

The unfolding experiments were performed on a Nano 5200 calorimeter at a scan rate of 1.0 K/min and protein concentration of 100–200 μg/ml (total cell volume 323 μl). Protein scans were performed in 10 mM glycine/HCl at pH 2.0. Measurements were performed in 100 mM Tris/Cl, 20 mM $CaCl_2$, 0.05% Triton X-100, pH 8.3 at 298 K. The plasmin buffer contained additionally 150 mM NaCl.

SPECIFIC INHIBITORS – MUTATIONS AT THE P_3 AND P_4 POSITIONS

A series of 12 BPTI variants mutated in the P_3 and P_4 positions and, additionally, containing the Lys15Arg mutation were constructed to probe the role of single amino-acid substitutions on binding to the following human proteases involved in blood clotting: plasmin, plasma kallikrein, factors X_a and XII_a, thrombin, protein C, and also to bovine trypsin (Grzesiak *et al.*, 2000b). The mutants inhibited these proteases by binding with association constants ranging from 10^3 to 10^{10} M^{-1} (Table 3). The inhibition of plasma kallikrein, factors X_a and XII_a, thrombin and protein C could be improved by up to 2 orders of magnitude just by the Lys15Arg substitution. The highest increase in the association constant for P_3 mutant was measured for factor XII_a: Pro13Ser substitution increased the

Table 3. Association constants for BPTI mutants and six human proteases

BPTI mutant				α-Plasmin	Plasma kallikrein	Factor X_a	Factor XII_a	α-Thrombin	Protein C	Bovine β-trypsin
P_4	P_3	P_2	P_1	K_a (M^{-1})	K_a (M^{-1})	K_a (M^{-1})	K_a (M^{-1})	K_a (M^{-1})	K_a (M^{-1})	K_a (M^{-1})
G	P	C	R	6.7×10^9	2.5×10^9	1.4×10^6	7.6×10^5	4.6×10^5	1.7×10^8	4.5×10^{13}
S	P	C	R	1.0×10^9 ↓	6.6×10^8 ↓	1.1×10^6 ↓	4.8×10^5 ↓	1.3×10^5 ↓	2.3×10^7 ↓	3.5×10^{10} ↓
V	P	C	R	1.3×10^7 ↓	2.3×10^6 ↓	2.3×10^5 ↓	5.1×10^4 ↓	7.0×10^4 ↓	7.4×10^5 ↓	1.0×10^{10} ↓
F	P	C	R	2.1×10^8 ↓	6.6×10^6 ↓	2.4×10^5 ↓	5.0×10^4 ↓	2.0×10^4 ↓	4.1×10^6 ↓	1.4×10^{10} ↓
G	**G**	C	R	2.3×10^9 ↓	2.8×10^8 ↓	2.2×10^5 ↓	3.5×10^6 ↑	4.1×10^4 ↓	2.7×10^8 ↑	$>8 \times 10^{11}$ -
G	**A**	C	R	3.0×10^9 ↓	8.9×10^8 ↓	1.1×10^6 ↓	8.9×10^6 ↑	5.6×10^4 ↓	2.3×10^7 ↓	$>8 \times 10^{11}$ -
G	**V**	C	R	1.2×10^9 ↓	1.4×10^9 ↓	1.1×10^7 ↑	1.5×10^6 ↑	2.1×10^6 ↑	1.0×10^9 ↑	$>8 \times 10^{11}$ -
G	**I**	C	R	6.8×10^9 -	5.4×10^9 ↑	1.7×10^6 ↓	1.7×10^5 ↓	2.6×10^6 ↑	7.5×10^8 ↑	$>8 \times 10^{11}$ -
G	**F**	C	R	6.6×10^9 -	7.5×10^8 ↓	1.1×10^7 ↑	1.0×10^7 ↑	7.0×10^5 ↑	3.4×10^8 ↑	$>8 \times 10^{11}$ -
G	**S**	C	R	4.0×10^9 ↓	3.7×10^8 ↓	4.0×10^5 ↓	4.4×10^7 ↑	2.0×10^4 ↓	3.2×10^7 ↓	$>8 \times 10^{11}$ -
G	**D**	C	R	6.5×10^9 -	7.8×10^7 ↓	2.4×10^6 ↑	1.3×10^7 ↑	3.0×10^4 ↓	4.7×10^7 ↓	$>8 \times 10^{11}$ -
G	**H**	C	R	1.0×10^{10} ↑	5.7×10^8 ↓	5.3×10^6 ↑	1.9×10^7 ↑	6.8×10^4 ↓	1.0×10^8 ↓	$>8 \times 10^{11}$ -
G	**R**	C	R	1.2×10^{10} ↑	2.1×10^9 ↓	1.4×10^6 -	7.6×10^6 ↑	3.5×10^5 ↓	5.8×10^8 ↑	$>8 \times 10^{11}$ -

Inhibition effects were compared with the pseudo wild type BPTI (K15R, M52L). Arrow pointing down indicates a decrease, and up – an increase of the association constant value upon mutation. The mutated positions in BPTI binding loop are bolded. The conditions were as follows: 100 mM Tris/Cl, 150 mM NaCl, 20 mM $CaCl_2$, 0.05% Triton X-100, pH 8.3 at 22°C.

K_α value 58-fold. Several other substitutions at P_3 resulted in an about 10-fold increase for factor X_a, thrombin and protein C.

The cumulative P_3 and P_1 effects on K_α values for the optimal mutant, compared with wild type BPTI, were in the range 2.2- (plasmin) to 4000-fold (factors XII_a and X_a). The substitutions at the P_4 site always caused negative effects (a decrease in the range from over 1000- to 1.3-fold) on the association constant with all the studied enzymes, including trypsin. All P_4 mutants showed a very large decrease of the denaturation temperature (about 22°C), suggesting that substitution of the wild type Gly12 residue leads to a significant change in the binding loop conformation (Grzesiak *et al.,* 2000b).

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