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Review

Canonical protein inhibitors of serine proteases

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Abstract. Serine proteases and their natural protein inhibitors are among the most intensively studied protein complexes. About 20 structurally diverse inhibitor families have been identified, comprising α-helical, β sheet, and α/β proteins, and different folds of small disulfide-rich proteins. Three different types of inhibitors can be distinguished based on their mechanism of action: canonical (standard mechanism) and non-canonical inhibitors, and serpins. The canonical inhibitors bind to the enzyme through an exposed convex binding loop, which is complementary to the active site of the enzyme. The mechanism of inhibition in this group is always very similar and resembles that of an ideal substrate. The non-canonical inhibitors interact through their N-terminal segment. There are also extensive secondary interactions outside the active site, contributing significantly to the strength, speed, and specificity of recognition. Serpins, similarly to the canonical inhibitors, interact with their target proteases in a substrate-like manner; however, cleavage of a single peptide bond in the binding loop leads to dramatic structural changes.

Key words. Serine protease; protein inhibitor; canonical conformation; protein-protein recognition.

Introduction to protein inhibitors of proteases

Proteases carry out an unlimited number of hydrolytic reactions both intra- and extracellularly [1]. They are found in viruses and in all living organisms from bacteria to mammals. Beside their physiological necessity, proteases are potentially hazardous to their proteinaceous environment and their activity must be precisely controlled by the respective cell or organism. When not properly controlled, proteases can be responsible for serious diseases. The basic level of control is normally achieved by regulated expression/secretion, by activation of proproteases [2], and by degradation of the mature enzymes. A second level of regulation is by inhibition of their proteolytic activity. Almost all known naturally occurring inhibitors directed toward endogenous cognate proteases are proteins; only some microorganisms secrete small non-proteinaceous compounds which block the host protease activity.

Inhibition of proteases by proteins itself sounds paradoxical. Nevertheless, there are very common examples of inhibition of proteases by structurally unrelated proteins [3–5]. In fact, inhibitor structures, modes of inhibition and the nature of enzyme-inhibitor complexes are surprisingly different (table 1). In the past, inhibitors were believed to be specific for one of the four mechanistic classes of proteases (serine, cysteine, aspartic, or metalloproteases). While this is probably true in a prevailing number of cases, there are also known examples of proteins that are able to inhibit cysteine and aspartic protease [6], a serine and metalloprotease [7, 8], a serine and aspartic protease [9, 10], or a serine protease and amylase [11, 12], employing different, non-overlapping binding sites. The latest studies, compared to older ones, include testing of a broader range of proteases and some other hydrolases. However, very few examples of cross-protease

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Table 1. Major features of protease inhibitors of serine, cysteine, metallo-, and aspartic proteases.

Protease	Inhibitor	Examples	Major features of inhibition	Size
Serine	canonical inhibitors	BPTI, OMTKY3, eglin	tight, non-covalent interaction resembling enzyme-substrate Michaelis complex, direct blockage of the active site, no conformational changes, antiparallel β sheet between enzyme and inhibitor [3], similar mode of interaction through canonical protease-binding loop despite completely different inhibitor structures [84], important role of P_1 residue, additive effects on association energy [147]	3–21 kDa per domain
	non-canonical inhibitors	hirudin, TAP, ornithodorin	extremely strong and specific interaction so far known for factor Xa and thrombin only, two-step kinetics, inhibition of the active site through N terminus of the inhibitor, two areas of interaction [150]	6–8 kDa per domain
	serpins		irreversible covalent acyl-enzyme complex, huge conformational changes in inhibitor, disruption of protease active site [17, 151]	45–55 kDa
Cysteine	cystatins	chicken cystatin, cystatin C, stefin B, kininogen	extremely tight but not specific, reversible non-covalent inhibition [26], interaction through two hairpin loops and N terminus forming a wedge, catalytic Cys25 accessible in complex, important interactions through P_2 position [29]	11–13 kDa, up to 60–120 kDa (kininogen)
	thyropins	p41, equistatin	very tight inhibition, mechanism similar to cystatins but often more specific [27, 152]	7 kDa per domain
	IAP	XIAP, cIAP1	highly specific inhibition, reversible tight binding kinetics [153], inhibition also through an interdomain flexible linker region as non-productive binding in the opposite orientation to the substrates [30–32]	9 kDa per BIR domain
		CrmA,	highly specific inhibition [34], similar to serpin mechanism of complexation [153]	38 kDa
		p35	non-specific inhibition [34], irreversible acyl-enzyme, p35 N terminus shields catalytic Cys360 from water molecules, gross conformational changes in inhibitor [33]	35 kDa
Metallo		PCI	tight enzyme-product complex, inhibition through C-terminal segment [36], key role of Val38 (P_1) [154], no conformational changes in inhibitor upon complexation [155]	4 kDa
		SMPI	rather specific inhibitor, inhibition mechanism resembling standard mechanism of canonical inhibitors of serine proteases, temporary inhibition [37], rigid protease-binding loop [37, 156, 157]	11 kDa
		Pseudomonas aeruginosa inhibitor, *Erwinia chrysanthemi* inhibitor	both tight and weak inhibition observed, major interactions through five N-terminal residues, N-terminal amino group forms a coordinative bond to catalytic Zn, in analogy to TIMPs [40, 158]	15 kDa
		TIMP1, TIMP2, TIMP3, TIMP4	tight but not highly specific non-covalent interaction [159], N terminus and five inhibitor loops form wedge contacting the active site, bidental coordination of catalytic Zn through N terminus, major interactions through P_1' residue, moderate conformational changes in inhibitor upon complexation [160]	20–22 kDa
Aspartic		IA_3	strong and highly specific [161], fully unfolded in free state, forms long helix in the complex comprising only N-terminal half of inhibitor, non-covalent complex [41].	8 kDa
		PI-3	strong but not highly specific [162], antiparallel β sheet formation between enzyme and inhibitor, no conformational changes [42]	17 kDa

BPTI, bovine pancreatic trypsin inhibitor; OMTKY3, turkey ovomucoid third domain; TAP, tick anticoagulant peptide; IAP, inhibitor of apoptosis; XIAP, X-linked IAP; cIAP1, cellular IAP protein 1; BIR, baculoviral IAP repeat; CrmA, cytokine response modifier A; PCI, potato carboxypeptidase inhibitor; SMPI, *Streptomyces* proteinaceous metalloprotease inhibitor; TIMP, tissue inhibitors of metalloproteases; IA_3, inhibitor of aspartic protease from *Saccharomyces cerevisiae*; PI-3, *Ascaris suum* pepsin inhibitor 3.

class inhibition at the same protease recognition site have been reported, but their number is growing [13].

This review is devoted to canonical protein inhibitors of serine proteases. To put serine protease inhibitors in a broader context of possible inhibition modes, a brief outline of protein inhibitors of the four mechanistic classes (no protein inhibitors of threonine proteases are known) is presented below (table 1).

From a structural point of view, blocking of the enzyme active site is almost always achieved by docking of an exposed structural element, such as a single loop or protein terminus, either independently or in combination of two or more such elements. Interestingly, antibodies, despite huge structural variability of their antigen-binding loops, cannot recognize the active site of proteases, as they only bind to flat or convex protein surfaces [14].

There are three distinct types of serine protease inhibitors (fig. 1). Up to the late 1980s, the majority of known protein inhibitors were those of the serine enzymes, either substrate-like-binding canonical inhibitors blocking the enzyme at the distorted Michaelis complex reaction stage [4, 15, 16], or serpins (*ser*ine *p*rotease *in*hibitors). While canonical inhibitors are small proteins, serpins are much larger, typically 350–500 amino acids in size, distributed from viruses to mammals [13, 17]. They are abundant in human plasma and mutations in serpins lead to numerous serious genetic diseases in humans [18]. Similarly to canonical inhibitors, serpins interact with their target proteases in a substrate-like manner. However, while the protease-binding loop of canonical inhibitors is kept in a well-ordered conformation, the binding loop of serpins is much longer, about 17 amino acids, and able to adopt different conformations. In the active serpin, the binding loop protrudes significantly from the serpin scaffold [19], while in a much more stable latent conformation, this segment is inserted into the middle of the central β sheet A (fig. 1) [20]. In contrast to canonical inhibitors, serpins utilize the kinetic features of a hydrolytic reaction to form a very stable acyl-enzyme intermediate. The enzyme-serpin complex is a covalent acyl-enzyme adduct and upon acylation the protease is translocated by over 70 Å from its initial recognition site [21]. Serpins are the only family of serine protease inhibitors for which complex formation with non-serine enzymes – cysteine proteases [22] and aspartyl proteases [23] – has been demonstrated.

Non-canonical inhibitors interact through their N-terminal segment which binds to the protease active site forming a short parallel β sheet. These inhibitors also form extensive secondary interactions with the target protease outside the active site, which provide additional buried area and contribute significantly to strength, speed, and specificity of recognition. The classic example is recognition of thrombin by hirudin [24]. Interestingly, such interactions are also formed by proteins possessing canonical-inhibitor-like folds or by Kazal-type inhibitors but with a distorted conformation of the binding loop. The non-canonical inhibitors are much less abundant than canonical inhibitors or serpins as they only occur in blood-sucking organisms and inhibit proteases involved in clot formation – thrombin or factor X_a. Only a few of them have been characterized in terms of structure and kinetics of interaction with the target protease.

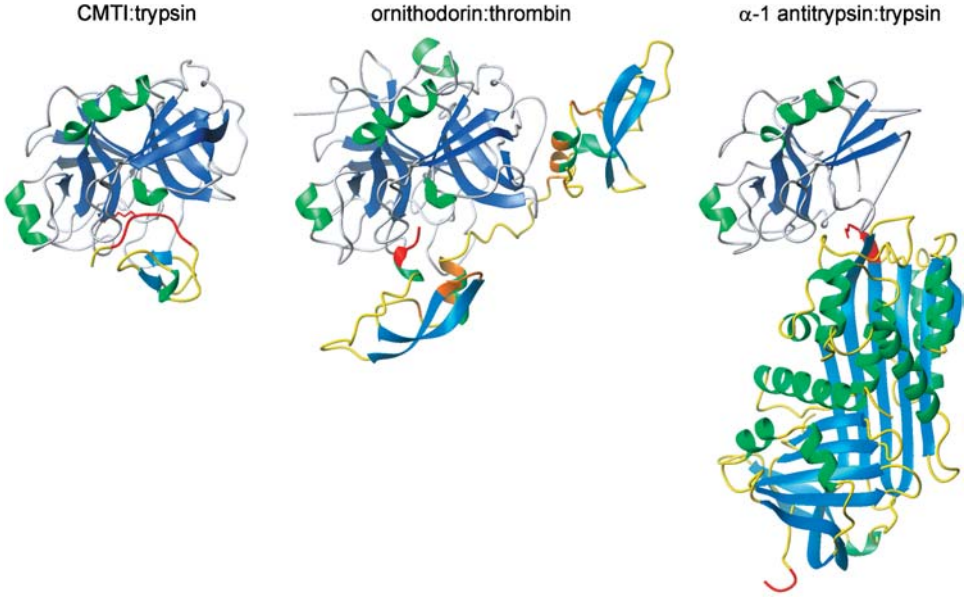

Figure 1. Examples of serine protease-inhibitor complexes: canonical, CMTI:trypsin (PDB: 1ppe); non-canonical, ornithodorin:thrombin (1toc); serpin, α-1 antitrypsin:trypsin (1ezx). The binding loop and P_1 side chain residue of CMTI, α-1 antitrypsin and the N terminus of ornithodorin are marked in red. Secondary binding sites of ornithodorin are marked in orange. Secondary-structure elements are colored in blue (β sheets) and green (α helices).

The second largest and most carefully investigated group are inhibitors of cysteine proteases (table 1). As with the serine protease inhibitors, they can be divided into several groups: cystatins, stefins, kininogen, and thyroglobulin type 1 proteins [25, 26]. While the first three groups share similar structural features and form the cystatin superfamily, the latter group is structurally different [27]. Interestingly, all these proteins, and probably also other inhibitors [28], share clear similarities in the mode of their interaction with the enzymes. Members of the cystatin superfamily are reversible, extremely fast- and tight-binding competitive inhibitors, with the inhibition constants even in a low picomolar range [26]. The crystal structures of chicken cystatin and stefin B in complex with papain show a precise steric fit of a hydrophobic wedge-shaped edge of the cystatin inserted into the active site of the protease [29]. Inhibitors of caspases, the cysteine proteases responsible for apoptosis, are surprisingly variable, structurally unrelated to the cystatin superfamily, and much more specialized to fulfill their function. One group of caspase inhibitors, called inhibitors of apoptosis (IAPs), contains one or more baculoviral IAP repeat (BIR) domain that binds to the active site in a non-productive manner [30–32]. Unexpectedly, a flexible linker connecting the BIR domains can also serve to block the active site of caspases 3 and 7. Another group of caspase inhibitors is the p35 family that has only been found in some baculoviruses. These inhibitors are able to inhibit different caspases using mechanism-based inactivation through formation of a covalent thiol ester [33]. Although the mechanism of inhibition in principle is similar to that of serpins, the molecular rearrangement of the inhibitor upon cleavage of the peptide bond is clearly different. Caspases can also be inhibited by viral serpins. The poxvirus CrmA serpin is able to block both caspases 1 and 8 and the serine protease granzyme B [34]. Although the structure of the CrmA-serpin complex remains to be determined, CrmA most probably undergoes an extensive structural transition during complex formation [35].

There are three unrelated groups of metalloprotease inhibitors, which inhibit their cognate enzymes through completely different mechanisms (table 1). The first discovered was potato carboxypeptidase A inhibitor (CPI), a small 39-residue protein, forming an enzyme-product type of complex (Gly39 is cleaved off in the crystal structure of the complex) through insertion of its C terminus into the active site of the enzyme [36]. Subsequently, a bacterial inhibitor, SMPI, was isolated from *Streptomyces nigrescens* that, surprisingly, appeared to inhibit metalloproteases of the gluzincin family by the standard mechanism of inhibition, extremely popular among canonical inhibitors of serine proteases [37]. Tissue inhibitors of metalloproteases (TIMPs) fold into a continuous wedge which blocks the active site of various matrix metalloproteases (MMPs) [38, 39]. This interaction is not highly specific and the mode of inhibition resembles the interaction between serralysins and their bacterial inhibitors from *Pseudomonas aeruginosa* and *Erwinia chrysanthemi* [40]. Although TIMPs and the two bacterial inhibitors are structurally unrelated, both groups of inhibitors form similar coordinative bonds to the catalytic Zn utilizing the N-terminal residue.

Protein inhibitors of aspartic proteases are rare. Currently, structures of only two inhibitors have been reported (table 1). A small yeast protein IA_3, composed of 68 amino acids, is able to inhibit aspartic protease A from the same organism in a very unusual way. While it shows no detectable secondary structure in solution, upon complexation with the enzyme, residues 2–32 adopt an almost perfectly helical conformation revealing that the protease body serves as a folding template [41]. The PI-3 inhibitor from the intestinal parasite *Ascaris suum* presents an unrelated mode of inhibition. Its N-terminal β strand pairs with one strand of the active-site flap, forming an extensive eight-stranded β sheet spanning both proteins [42].

Canonical inhibitors

The largest group of protein inhibitors are canonical inhibitors that act according to the standard mechanism of inhibition [15]. A huge number of canonical inhibitors have been described, isolated from various cells, tissues, and organisms; they often accumulate in high quantities especially in plant seeds, avian eggs, and various body fluids. Serine proteases and their natural protein inhibitors belong to the most intensively studied models of protein-protein recognition [14, 43]. Canonical inhibitors are widely distributed in essentially all groups of organisms and comprise proteins from 14 to about 200 amino acid residues. Canonical protein inhibitors do not form a single group but can be divided into different families. The segment responsible for protease inhibition, called the protease-binding loop, surprisingly has always a similar, canonical conformation in all known inhibitor structures [16, 44]. This convex, extended and solvent-exposed loop is highly complementary to the concave active site of the enzyme. The standard mechanism implies that inhibitors are peculiar protein substrates containing the reactive site P_1-P_1' peptide bond located in the most exposed region of the protease-binding loop (P_1, P_2 and P_1', P_2' specify inhibitor residues amino- and carboxy-terminal to the scissile peptide bond, respectively; S_1, S_2 and S_1', S_2' denote the corresponding subsites on the protease [45]). The reactive site can be selectively hydrolyzed by the enzyme, but the equilibrium value of this cleavage is often close to 1 at neutral pH, i.e., the reactive site can be cleaved to the extent of about 50%. It is usually assumed, and very often verified experimentally, that standard-

mechanism inhibitors possess canonical conformation of the binding loop. On the other hand, while loops of canonical conformations occasionally occur in various proteins, there is no evidence that they can block proteases according to the standard mechanism [44, 46].

Classification

A classification of canonical inhibitors was originally proposed by Laskowski and Kato in 1980 [15]. At that time they could distinguish eight families, based mainly on the disulfide bond topography, location of the reactive site, and sequence homology. Currently, 18 inhibitor families are recognized [5] (table 2). Crystal and/or solution structures are known for representatives of almost all families (table 2). Since the inhibitors are small, rigid, and stable, these structures have often been determined with high resolution and accuracy. Furthermore, serine proteases and enzyme-inhibitor complexes crystallize easily, often providing high-resolution data. Extensive structural information is also available for protease-inhibitor complexes. The global structures of proteins representing different inhibitor families are completely different. Most often they comprise either purely β sheet or mixed α/β proteins; they can also be α-helical or irregular proteins rich in disulfide cross-links. It is often stressed that the canonical inhibitors represent the most distinct and extensive example of convergent protein evolution, since a similar function has been implemented many times during evolution through preservation of the canonical loop conformation in many unrelated proteins [5]. Examples of different folds of inhibitor structures are shown in figure 2.

The inhibitor structure

The inhibitor scaffolds are of very different structural types. In several inhibitor families like BPTI, Kazal, potato 1 and 2, cereal, SSI, STI, and ecotin, typical secondary-structure elements together with the presence of a hydrophobic core are found. In other families, including squash, Bowman-Birk, grasshopper, hirustasin, cheloni-anin, and Ascaris there is essentially a lack of both hydrophobic core and extensive secondary structure. For these inhibitors, disulfide bonds, which are usually buried inside the molecule, are the major determinant of protein stability and/or rigidity. With respect to the cross-links, the most unusual is probably a cyclic peptide from sunflower seeds that strongly inhibits trypsin (SFTI-1). This cyclic inhibitor is homologous to the Bowman-Birk family and consists of two antiparallel β strands additionally cross-linked with a disulfde bond and stabilized by numerous hydrogen bonds [47]. Natural cyclic peptides also occur among squash inhibitors [48]. Worth mentioning is that it is possible to synthesize chemically a cyclic version of the non-cyclic protease inhibitor BPTI [49, 50].

The presence of an inhibitory domain is usually indicative of serine protease inhibition. However, examples are also known of naturally occurring inhibitors belonging to ovomucoids, e.g., with Pro at P_1, that are very poor inhibitors of all proteases tested including prolyl endopeptidase [51]. Sometimes other functions, not related to canonical inhibition, could be detected for canonical domains. For example, Kazal domains occur in follistatin [52], the squash inhibitor fold is found in the metalloprotease inhibitor PCI [53], and the BPTI domain is frequently observed in snake potassium channel blockers or linked to the phospholipase A_2 domain, Alzheimer precursor protein [54], or collagen α1 and α3 chains [55]. In one case tested of the non-inhibitory C5 domain of collagen VI, a strong protease inhibitor could be generated through multiple substitutions in the binding-loop region [56]. However, in many other cases, conversion of a non-inhibitory to inhibitory protein required more effort due to severe conformational and dynamic changes in the binding-loop region.

Two non-canonical inhibitors of coagulation proteases from the soft tick, ornithodorin and tick anticoagulant peptide (TAP), surprisingly show a scaffold of the archetypical canonical inhibitor BPTI (fig. 1). Ornithodorin contains two BPTI-like domains containing insertion/deletion in the binding-loop segments, which lead to their major distortion [57]. In fact, this binding loop does not contact the protease, but as in the hirudin-thrombin complex, the N-terminal tail of ornithodorin penetrates the thrombin active site and forms a parallel β sheet with the thrombin Ser214-Gly219 segment. Similarly, TAP, which is a strong inhibitor of factor Xa [58], interacts through the N terminus with the active site of factor X_a [59].

Inhibitors belonging to different families are generally stable or even extremely stable proteins with high denaturation temperatures and resistance to chemical denaturants. At neutral pH, BPTI shows a denaturation temperature (T_{den}) of about 100 °C and is stable in 6 M guanidinium chloride [60, 61]. Kazal inhibitors denature with a T_{den} up to 90 °C and are also stable in 6 M GdmCl [62, 63], and STI unfolds at 65 °C [64]. For small inhibitors like those from the squash family, cooperative denaturation could not be demonstrated due to a lack of secondary structure, hydrophobic core and too small size of the cooperative unit.

Inhibitors are often heavily cross-linked with conserved disulfide bonds. The topology of the disulfide bonds is usually well preserved within a single family. However, some members of the potato 1 family show either a single disulfide [CMTI-V (*Cucurbita maxima* trypsin inhibitor-V) and LUTI (*Linum usitatissimum* trypsin inhibitor)] or no disulfide (CI-2 and eglin c). Within the STI family

Table 2. Representative X-ray and NMR three-dimensional structures of protein inhibitor families of serine proteases and their enzyme complexes.

Family	Total number	Free inhibitor				Enzyme-inhibitor complex			
		number	representative	PDB	resolution Å	number	representative	PDB	resolution Å
BPTI	63	28	BPTI	5pti	1.0	35	BPTI: rat trypsin	1f7z	1.5
Kazal	36	16	OMSVP3	2ovo	1.5	20	OMTKY: chymotrypsin	1cho	1.8
Potato 1	27	16	CI-2	2ci2	2.0	11	eglin c: subtilisin	1cse	1.2
Squash	17	9	CMTI I	1lu0	1.03	8	CPTI II: trypsin	2btc	1.5
Ecotin	12	3	ecotin	1ifg	2.0	9	ecotin: crab collagenase	1aaz	2.3
STI	11	9	STI	1avu	2.3	2	STI: porcine trypsin	1avw	1.75
BBI	9	6	BBBI	1c2a	1.9	3	MbBBI: Ns3-protease	1df9	2.1
BBI (SFTI)	2	1	SFTI-1	1jbl	NMR	1	SFTI-1: trypsin	1sfi	1.6
Antistasin	7	3	hirustasin	1bx7	1.2	4	bdellastasin: porcine trypsin	1eja	2.7
Ascaris	7	6	AMCI	1ccv	NMR	1	C/E-1 inhibitor: porcine elastase	1eai	2.4
Grasshopper	6	4	PMP-C	1pmc	NMR	2	PMP-C: chymotrypsin	1gl1	2.1
SSI	5	1	SSI	3ssi	2.3	4	SSI: subtilisin BPN′	2sic	1.8
Potato 2	4	3	T1	1tih	NMR	1	PCI 1: SGPB	4sgb	2.1
Cereal	4	4	CHFI	1bea	1.95				
Chelonianin	2	1	R-elafin	2rel	NMR	1	Elafin: porcine elastase	1fle	1.9
Rapeseed	1	1	ATT_p	1jxc	NMR				
Arrowhead									

In the absence of X-ray structure, the representative NMR structure is indicated. Total number, total number of structures deposited in PDB (Protein Data Bank) (free structures and complexed with protease); OMSVP3, silver pheasant ovomucoid third domain; OMTKY, turkey ovomucoid; CI-2, chymotrypsin inhibitor 2; CMTI I, *Cucurbita maxima* trypsin inhibitor I; CPTI II, *Cucurbita pepo* trypsin inhibitor II; STI, soybean trypsin inhibitor; BBBI, barley Bowman-Birk inhibitor; SFTI-1, sunflower trypsin inhibitor; MbBBI, mung bean Bowman-Birk inhibitor; AMCI, *Apis mellifera* chymotrypsin inhibitor; C/E-1 inhibitor, *Ascaris* chymotrypsin/elastase inhibitor 1; PMP-C, *Pars intercerebralis* major peptide; SSI, *Streptomyces* subtilisin inhibitor; T1, trypsin inhibitor from *Nicotiana alata*; SGPB *Streptomyces griseus* protease B; CHFI, corn Hageman factor inhibitor; ATT_p, *Arabidopsis thaliana* trypsin inhibitor.

also, there is an inhibitor with a single disulfide bond instead of the two typically observed among members of this family [65]. Worthy of note is that engineering of an additional disulfide bond near the reactive site of the silver pheasant third domain (Kazal inhibitor) left intact its potent inhibitory activity toward chymotrypsin, *Streptomyces griseus* proteases A and B, but almost abolished it toward pancreatic elastase [66]. Selective reduction or elimination of disulfide bond(s) in inhibitors belonging to different families usually leads to a significant destabilization of the inhibitor molecule, to a lower association-energy and larger sensitivity to proteolysis [67–70]. The same holds for destabilizing mutation(s) introduced into the inhibitor core [71, 72].

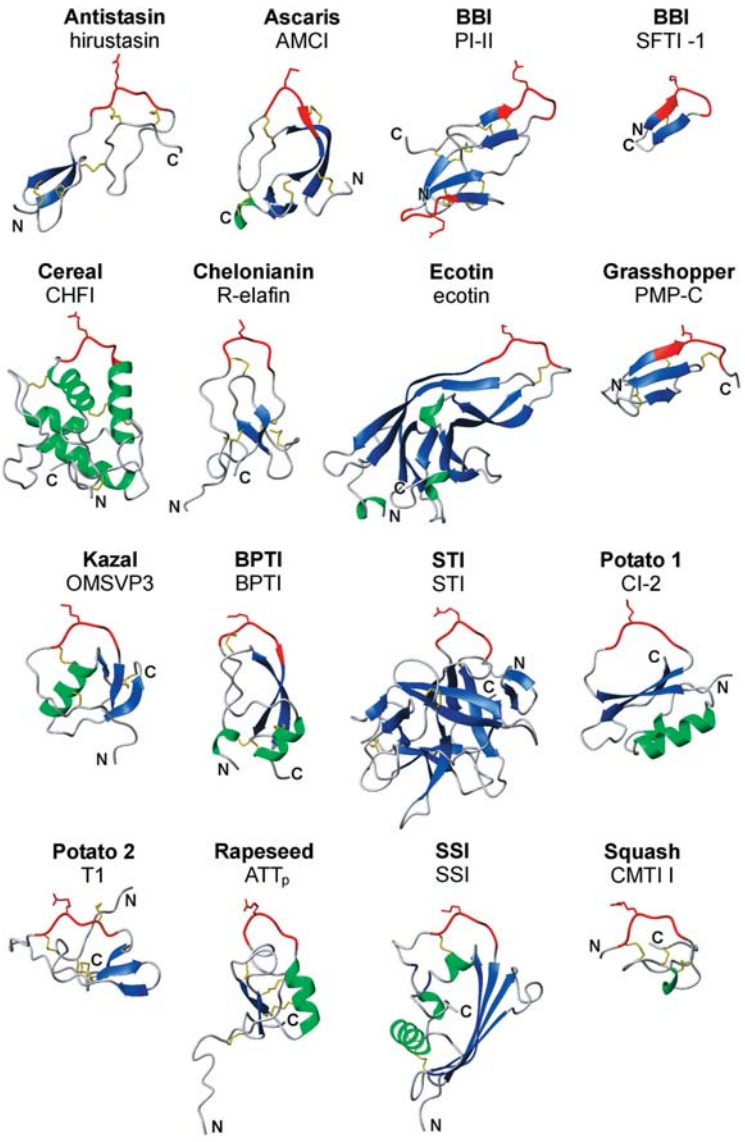

Figure 2. Representative set of canonical inhibitor structures. The following structures are shown: hirustasin (PDB: 1bx7); AMCI (1ccv); PI-II, protease inhibitor-II (1pi2); STFI-1 (1jbl); CHFI (1bea); R-elafin (2rel); ecotin (1ifg); PMP-C (1pmc); OMSVP3 (2ovo); BPTI (5pti); STI (1avu); CI-2 (2ci2); T1 (1tih); ATTp (1jxc); SSI (3ssi); CMTI I (1lu0). Binding loop and P_1 side chain residue are marked in red, secondary-structure elements are colored in blue (β sheets) and green (α helices). Name of inhibitor family (in bold) and name of representative are indicated.

Domain architecture

Many inhibitors are single-domain proteins. With a possible exception for the arrowhead protease inhibitor [73], the single inhibitory domain contains only one reactive site responsible for protease binding [74]. This is true for all members of the arrowhead, Ascaris, ecotin, STI, potato 1, cereal, rapeseed, silkworm, SSI, and squash families. In the remaining families (antistasin, Bowman-Birk, BPTI, grasshopper, Kazal, chelonianin, and potato 2) the single domain can be repeated 2, 3, 4, 5, 6, 7, 9 or even 15 times to form a multidomain, single-chain inhibitor which is able to interact independently with several protease molecules at their reactive sites belonging to separate domains.

The different organization observed in multidomain proteins is shown in figure 3. In the simplest case, represented by ovomucoids, three homologous Kazal domains, each cross-linked with three disulfide bonds, are connected by short and flexible linkers. These domains are independently able to inhibit serine proteases – for example the first domain of turkey ovomucoid inhibits Glu-specific *S. griseus* protease, the second domain inhibits trypsin and the third blocks chymotrypsin, elastase and subtilisin. Similarly, the first domain of tissue factor pathway inhibitor (TFPI) composed of three tandemly

arranged BPTI domains inhibits factor VII_a/tissue factor, the second interacts with factor X_a, and the third is without detectable inhibitory function [75]. In contrast, in the crystal structure of bikunin, composed of two BPTI domains, the binding loop of the second domain is obstructed by the first domain, thus affecting protease binding (fig. 3) [76].

A highly unique domain topology occurs in a protein inhibitor precursor from *Nicotiana alata* (NaProPI). This protein is composed of six homologous repeats; however, their sequences do not coincide with the structural domains [77]. One of these domains comprises two chain fragments from the first and last repeat, strongly suggesting that the precursor adopts a circular bracelet-like structure (fig. 3). In Bowman-Birk inhibitors, there are again two independent binding loops but the presence of seven inter- and intradomain disulfides results in a much more compact two-domain protein. Two Kazal domains of rhodniin serve to inhibit one enzyme – thrombin. While the N-terminal domain binds to the active site of thrombin through its canonical loop (interestingly with His at P_1, despite strong preference of thrombin for Arg), the binding loop of the C-terminal domain is distorted, thus excluding canonical interaction [78]. Instead, this domain recognizes the fibrinogen exosite on the thrombin surface using residues located outside the binding loop region (fig. 3). A homodimeric inhibitor from *Escherichia coli,* called ecotin, although built of a single domain is active as a dimer in which both monomers provide the protease-binding surface (fig. 3) [79]. A comparison of the inhibitory properties of dimeric ecotin with that of an engineered monomeric form reveals a complex and non-additive interplay of binding energies provided by the binding sites located on the two subunits [80]. Finally, several multidomain proteins containing combinations of WAP (whey acidic protein), Kunitz, Kazal, and thyroglobulin domains have been identified that may control multiple types of serine, aspartic, metallo and cysteine proteases [81].

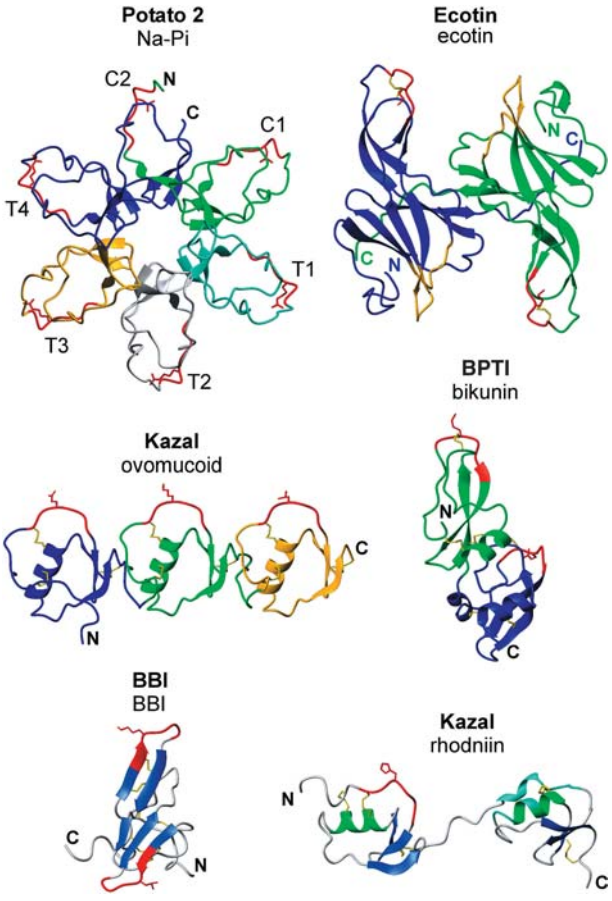

Figure 3. Examples of multidomain serine protease inhibitors: model of Na-Pi structure based on C1T1 and C2 NMR structures (PDB: 1fyb and 1qh2); ecotin dimer (1ecz); model of ovomucoid structure based on OMSVP3 crystal structure (2ovo); BBI (1bbi); bikunin (1bik); rhodniin (1tbq). Binding loop and P_1 side chain residue are marked in red, residues involved in protease dimerization by ecotin are marked in yellow, residues from the C-terminal domain of rhodniin interacting with thrombin are marked in cyan. Name of inhibitor family (in bold) and name of presented structures are indicated.

The canonical conformation of the binding loop

The convex protease-binding loop exhibits an extended conformation, which significantly protrudes from the protein scaffold and serves as a rather simple recognition motif **(**fig. 4**)**. The loop forms a sequential epitope spanning positions P_3 to P_3'. Residues that precede or follow this segment (e.g., P_6-P_4 or P_4') and residues from a sequentially remote region, called the secondary contact region, can also contact the enzyme and influence the association energy [82–84]. The central section of the loop contains a solvent-exposed P_1-P_1' peptide bond, called the reactive site. This bond is not fully inert to the proteolytic attack by the cognate enzyme – the equilibrium value of

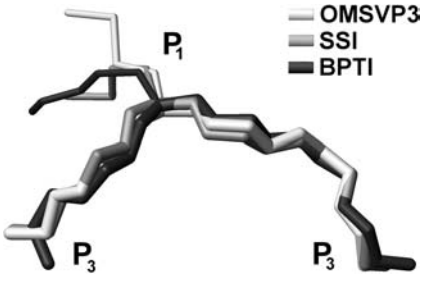

Figure 4. Superimposition of main chain P_3-P_3' segments (according to Schechter and Berger notation) of binding loops of: OMSVP3, light gray (PDB: 2ovo); SSI, gray (3ssi); BPTI, dark gray (PDB: 5pti).

the reactive-site peptide bond opening, called the hydrolysis constant, is usually not far away from unity [85–87].

The conformation of the cleaved inhibitor is very similar to the intact form with clear exceptions for the local structural changes near the P_1-P_1' peptide bond [88, 89] and increased internal mobility of the cleaved loop, but not of the inhibitor scaffold [90, 91]. Thermodynamic analysis reveals that hydrolysis of the reactive site in the native inhibitor does not lead to a significant increase in entropy [92]. The full entropy gain is realized upon denaturation of the reactive-site-cleaved inhibitor which leads to predicted values of K_{hyd} for the hydrolysis of the reactive site in the denatured inhibitor at between 100 and 1000 [64, 93]. However, in the case of CMTI-V, a large entropy increase was observed upon reactive-site cleavage of the native inhibitor [94].

The main chain conformations of the binding loops of free inhibitors representing different families are similar and become even more alike after complex formation with the enzyme **(**fig. 4**)** [44]. The canonical conformation is also presumed to be adopted by a productively bound protein substrate.

The binding loops within one family (most intensively studied for ovomucoids belonging to Kazal inhibitors [84, 95]) often show a high level of sequential variability; nevertheless, in all cases studied, the loops had canonical conformation. A lack of hypervariability has been indicated for squash [96] and potato 1 families [97].

The amino acid sequences of the binding loops show many clear amino acid preferences in different families. For example, half cystine is present either at P_3 (the Kazal, Bowman-Birk, grasshopper, silkworm, squash, SSI, potato 2, and Ascaris families) or at P_2 (the BPTI, antistasin, arrowhead, hirustasin, and chelonianin families). Thr is often observed at P_2 (the Kazal, potato 1, Bowman-Birk, SSI, ecotin, and Ascaris families) and Pro is conserved at P_3 in the STI family [44]. A conserved Ile is always present at the P_1' position in squash inhibitors. Its mutation to Leu leads to a severe disorder of the binding loop [98]. A similar disorder of the loop was observed upon Asp46→Ser substitution at the P_1' site of eglin c [99]. Ala and Gly are highly conserved at P_1' in the BPTI family. Introduction of larger side chains at this position leads to a huge decrease in the association constants with proteases [100]. In Bowman-Birk inhibitors, prolines are frequently observed at P_3' and P_4'. The Pro at P_3' in geometry is required for strong inhibition while that at P_4' stabilizes the P_3' configuration [101]. Thus, the canonical conformation may be achieved by many unrelated sequences.

The canonical loop conformation results from a rather extensive system of disulfide bond(s), hydrogen bonds, and/or hydrophobic interactions, which involve residues both from the loop and the inhibitor scaffold. A scheme of the loop-maintaining interactions in different families is shown in figure 5. For example, in the OMSVP3 (Kazal family) inhibitor, the carbonyl oxygens of P_2 and P_1' are involved in hydrogen bonds to the side chain of Asn33, and in CI-2 (potato 1 family), these carbonyls are hydrogen bonded to two arginines at P_6' and P_8'. The LUTI inhibitor which belongs to the same family, despite a Trp→ Arg substitution at P_8', is a strong inhibitor of trypsin and its solution structure shows that loop conformation is well preserved [102]. Interestingly, mutation of Arg at P_8' to Lys in the homologous eglin c led to a destabilization of the binding loop [99]. There is evidence based on changes in ^{15}N relaxation rates for an increased dynamics of the loop in CMTI-V mutants with eliminated side chains of Arg50 or Arg52 [103, 104]. In many inhibitor families, there is also a common hydrogen bond between the side chain or main chain of the P_2 and P_1' positions **(**fig. 5**)**. In ovomucoid third domains, the side chain-side chain hydrogen bond between Thr17 (P_2) and Glu19 (P_1') is long in the free inhibitor (and therefore not shown on fig. 5) but becomes shortened by about 0.5 Å upon complex formation, thus showing that it energetically favors the complex [105]. In Ascaris trypsin inhibitor (ATI), there is a pH-induced structural transition in the binding-loop region observed by nuclear magnetic resonance (NMR) – while the loop is rigid and canonical at pH 2.4, it becomes disordered at pH 4.75 [106]. This conformational change likely results from deprotonation of Glu32 and disruption of the hydrogen bond between side chains of Thr30 (P_2) and Glu32 (P_1'), since in a homologous inhibitor, AMCI, which has Gln instead of Glu, no pH-induced structural transition is observed [107]. Interestingly, replacing the CI-2 loop sequence with that of helix E from subtilisin Carlsberg led to a protein hybrid with a well-preserved scaffold and extended loop-like conformation of the introduced sequence [108]. This result shows that the context of the CI-2 scaffold is sufficient to impose appropriate loop conformation. Furthermore, a multiple mutant of BPTI with almost all residues in the binding loop replaced with alanines was constructed. Although this loop region was significantly less structured compared to the wild-type protein, the binding loop still could adopt the proper conformation and interact with trypsin and chymotrypsin [109]. The inhibitor scaffold, therefore, seems to play an active role in maintaining the loop conformation.

The standard mechanism

The canonical inhibitor-cognate protease interaction is preserved in all cases tested and called the standard mechanism [15]. The interaction between enzyme and inhibitor can be presented in a simplified form as a hydro-

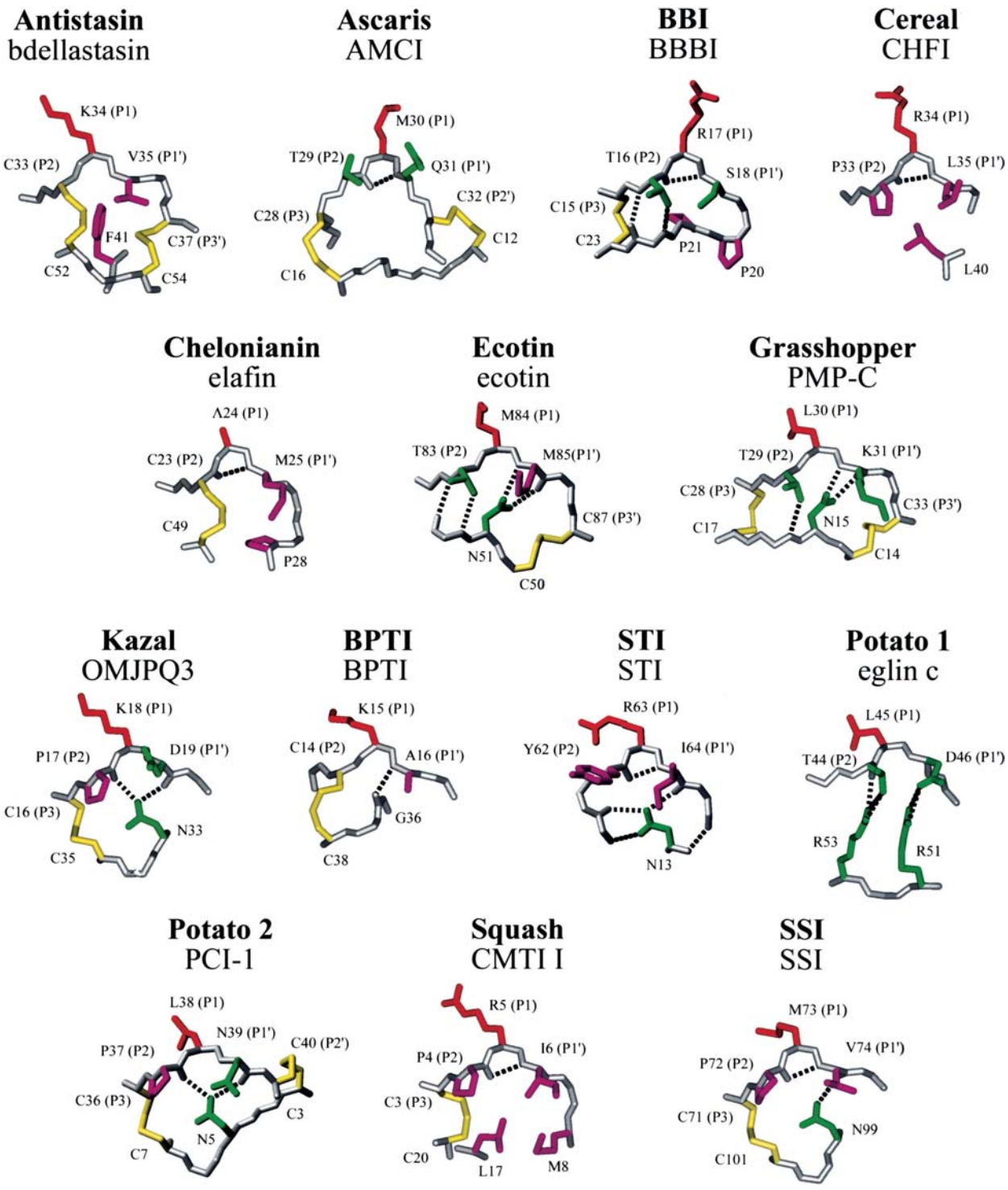

Figure 5. Binding-loop structures of representatives of inhibitor families: bdellastasin (PDB:1c9p); AMCI (1ccv); BBBI (1c2a); CHFI (1bea); elafin (1fle); ecotin (1ifg); PMP-C (1gl1); OMJPQ3, Japanese quail ovomucoid third domain (1ovo); BPTI (5pti); STI (1ba7); eglin c (1cse); PCI-1 (4sgb); CMTI I (2sta); SSI (3ssi). The fragments comprise P_3-P_2' segments with additional residues involved in binding-loop stabilization. The backbone is shown in gray, P_1 side chains in red, disulfide bridges in yellow, polar and hydrophobic side chains in green and magenta, respectively. Additionally, hydrogen bonds are shown as black dashed lines. Amino acid residues are labeled using one-letter codes including Schechter and Berger notations. Name of inhibitor family (in bold) and name of representative are indicated.

lysis/resynthesis reaction of the P_1-P_1' reactive-site peptide bond:

$$E + I \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} EI \underset{k^*_{on}}{\overset{k^*_{off}}{\Leftrightarrow}} E + I^* \quad (1)$$

where E is the protease, I is the inhibitor, I* is the reactive-site-cleaved inhibitor, EI is the stable complex, k_{on} and k^*_{on} are respective second-order association rate constants, and k_{off} and k^*_{off} are respective first-order dissociation rate constants of the complex. A recently described bacterial inhibitor of metalloproteases appears to resemble canonical inhibitors with respect to the inhibition mechanism [110].

Compared to peptide bond hydrolysis in a regular protein:

1) The complex EI is much more stable than the Michaelis ES complex. Typical inhibition constant (K_i) values are 10^6- to 10^9-fold lower than K_m values. Often, complex can be easily crystallized and shows all typical features of protein-protein recognition [14, 43].
2) The catalytic rate constant for the hydrolysis of the reactive site is extremely low at neutral pH [87, 111]. However, there are examples of hydrolysis of reactive sites by inhibited proteases, proceeding at much higher rates [112, 113].
3) The conserved mode of recognition between the protease-binding loop and the active site leads to many different serine proteases (belonging both to the chymotrypsin and subtilisin families) of different specificities being inhibited at the same reactive site in the case of the turkey ovomucoid third domain [74]. This is also true for other inhibitors. Eglin c (potato 1 family) inhibits 14 serine proteases with association constant values greater than 10^8 M^{-1} [5]. Furthermore, the three-dimensional structures of BPTI complexed with trypsin [114], chymotrypsin [115], pancreatic kallikrein [116], thrombin [117], factor VII_a [118], and trypsinogen [119] show extremely similar modes of recognition, despite one billion-fold difference in their affinities. A similar difference in the association constant exists for the interaction of trypsin with ten P_1 mutants of BPTI and, again, crystal structures of the respective complexes show virtually identical modes of recognition [120].
4) The k_{cat}/K_m index for the hydrolysis of the reactive-site peptide bond is often about 10^6 M^{-1} s^{-1}, suggesting that inhibitors are good substrates [121]. However, this parameter describes the enzyme-substrate reaction only at low substrate concentrations ($[S]<K_m$). Since the K_m values for hydrolysis are extremely low, the reaction rate is proportional to k_{cat} which is known to be extremely low in the case of reactive-site hydrolysis at neutral pH.
5) The hydrolysis reaction is reversible, i.e. the cleaved inhibitor is active and forms the same complex with the enzyme as the intact form. During complex formation, resynthesis of the reactive-site peptide bond occurs [111]. The kinetic parameters for reactive-site resynthesis are often similar to those of the hydrolysis reaction. The phenomenon of hydrolysis/resynthesis also occurs at other peptide bonds of the binding loop and can also be catalyzed by non-serine proteases [87].
6) The equilibrium value of [I*]/[I] (hydrolysis constant, K_{hyd}) is often close to unity (i.e., about 50% of the inhibitor molecules have the reactive site cleaved) at pH 6 where K_{hyd} is pH independent [86, 87, 121], but examples are known of natural ovomucoid third domain variants with K_{hyd} in the range of 0.4–35 [85]. Typical K_{hyd} values for a single peptide bond hydrolysis in a native protein containing secondary structure are in the neglibly low values of 10^{-3} to 10^{-8} [122]. Thus the values of K_{hyd} in protein inhibitors are extremely high. Substitution of the residues which maintain the binding loop conformation affects the value of K_{hyd}. However, the effect of a single mutation is relatively small and does not exceed the factor 3–5 [85].
7) While the k_{on} values for protease-inhibitor association are typically about 10^6 M^{-1} s^{-1}, the k_{off} values may differ by many orders of magnitude. The k^*_{on} values can also differ by many orders of magnitude for the interaction of an inhibitor with different proteases [74].
8) At high concentrations of enzyme and inhibitor, the existence of additional unstable loose complexes L and L* can be detected by stopped-flow methods [121]. Recent experiments revealed that the acyl-enzyme intermediate could be formed rapidly, suggesting that there is no energetic barrier to the acylation reaction [123].

The protease-inhibitor complex

The mode of recognition between different canonical inhibitors and serine proteases is always almost the same. In the stable complex, which was a subject of numerous crystallographic studies, a short antiparallel β sheet is formed between the P_3-P_1 residues and the 214–216 (Ser125-Gly127 in subtilisin) segment of the enzyme (fig. 6). The energetic contribution of one of these intermolecular main chain hydrogen bonds (donated by the H^N amide of the P_1 residue of OMTKY3) was recently found to be about 1.5 kcal/mol [124]. There is an additional antiparallel β sheet between the P_4-P_6 fragment and Tyr104-Gly102 residues in subtilisin complexes, which does not exist in chymotrypsin-like enzymes [125, 126]. Other very important features of the complex include: a short (usually about 2.7 Å) contact between the P_1 carbonyl carbon and the catalytic serine residue (significantly shorter in rhodniin-thrombin [78] and mung bean trypsin inhibitor-trypsin complexes

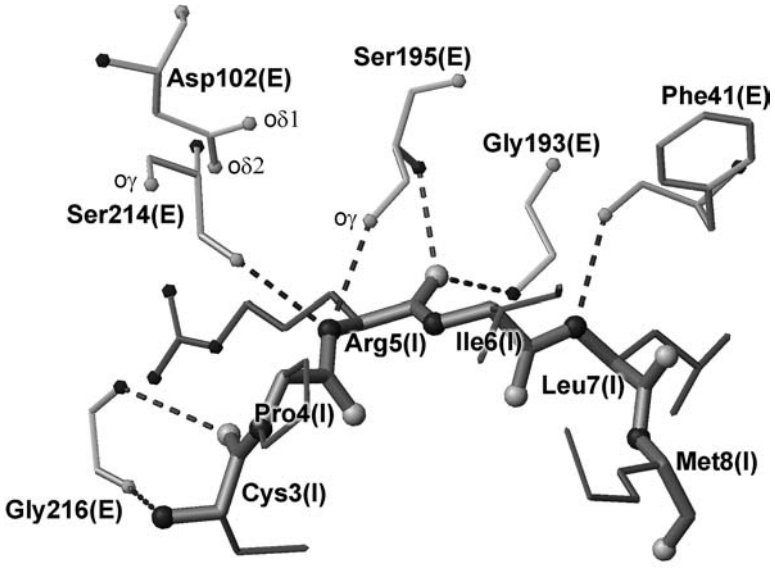

Figure 6. Schematic representation of canonical inhibition based on the structure of the CMTI I:trypsin complex (PDB: 1ppe). The inhibitor [dark gray, residues marked as (I)] binds to the protease [light gray, residues marked as (E)] in a manner similar to that of a typical substrate. Several characteristic interactions are shown: (i) antiparallel β sheet formed between residues P_1-P_3 of the inhibitor and residues 214–216 of the protease; (ii) sub-van der Waals contacts between Ser195 Oγ and P_1 carbonyl carbon, and (iii) hydrogen bonds from the oxyanion binding hole (H^N of Gly193 and Ser195) to the P_1 carbonyl oxygen. Nitrogen and oxygen atoms are shown as dark and light gray balls, respectively. Amino acid residues are labeled using three-letter codes including Schechter and Berger notation.

[127]), and two hydrogen bonds between the carbonyl oxygen of P_1 and Gly193/Ser195 amides of the oxyanion binding hole, and the hydrogen bonds between the P_1 H^N group and the side chain of Ser195 and the carbonyl of Ser214. Conversion of the P_2-P_1 amide bond to an ester bond reduces the association free energy by about 1.5 kcal/mol [124, 128]. The reactive-site peptide bond remains intact in all crystallographically studied complexes. All the above-mentioned hydrogen bonds and the shape complementarity of interacting areas ensure very similar recognition modes between different proteases and inhibitors.

In the complex, about 10–18 amino acid residues of the inhibitor and 17–30 residues of the protease make numerous interactions – mainly van der Waals (typically more than 100) and hydrogen bonds (about 8–15). The total area of the two components buried in the interface is about 1400 Å^2. According to NMR relaxation studies, the protease-binding loop, which is often poorely structured in free inhibitors [90, 91, 129], becomes significantly rigidified in the complex. There are no significant conformational changes on either the enzyme or inhibitor part accompanying complex formation, with the exception of zymogen complexes. In the trypsinogen-inhibitor complex, major structural rearrangements are observed in the activation domain comprising the active-site region [130]. The organization of the activation domain in the complex with the inhibitor is remarkably similar to one observed in the active enzyme, but fully disordered in the free zymogen. The association constant is about 10^7-fold lower than for the active enzyme [131, 132]. Despite the inherently low activity of the zymogen, the standard mechanism works also for trypsinogen, which is able to resynthesize the reactive-site peptide bond of the inhibitor [133].

The P_1 position

In canonical inhibitors, position P_1 determines to a large extent the protease-inhibitor association energy. With the exception of Trp, Ile and Cys, all amino acids have been observed at this position in inhibitors representing different families [134]. The plot of the substrate transition-state energy $\log(k_{cat}/K_m)$ versus the enzyme-inhibitor association energy $\log(K_a)$ determined for a set of P_1 oligopeptide substrates and protein inhibitors is a straight line with a slope not far from unity, suggesting that interactions within the S_1 pocket do not change as the reaction proceeds from the enzyme-inhibitor complex to the transition state [51, 135, 136].

P_1 Gly and particularly P_1 Pro are very disfavored for binding with most of the proteases tested [51, 137]. Also the charged P_1 side chains of Asp, Glu and His (but not their uncharged forms), when placed in hydrophobic S_1 pockets, strongly oppose complex formation [138]. The shifts of the pK values of these side chains placed in the hydrophobic S_1 pocket of SGPB reach 5 pH units.

The P_1 side chain is fully exposed in all free inhibitor structures (fig. 7) and becomes imbedded in the S_1 pocket

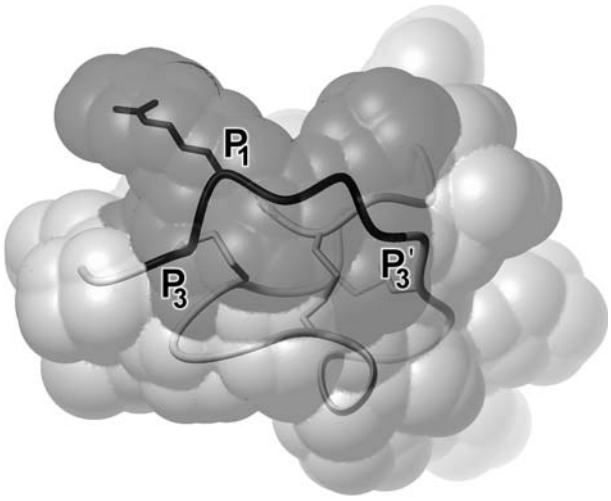

Figure 7. Solvent accessible area of CMTI I (PDB: 1lu0). Only the main chain of the inhibitor and side chain of P_1 (Arg5) are shown. The solvent-exposed protease-binding loop (residues P_3 to P_3') and corresponding surface is colored dark gray. Amino acid residues are labeled using Schechter and Berger notation.

upon complex formation. It can form about 50% of the interface contact area and provide even up to 70% of the association energy as deduced from comparisons with the P_1 Gly variant [51, 137, 139]. Cognate P_1 side chains enter the S_1 pocket preserving optimal χ angles [140, 141]. An improperly matched P_1-S_1 interaction in terms of size, shape, charge, polarity, or branching of the P_1 side chain leads to severe effects on the association energy [135, 137, 139, 141, 142]. Furthermore, alanine-scanning mutagenesis of BPTI [82] and theoretical calculations of the protease-inhibitor interaction [143] clearly reveal a dominant role for the P_1 residue. Since the P_1 residue occupies a central part of the canonical loop, its substitutions in different inhibitor families often cause very similar energetic effects on the binding to the serine protease, a phenomenon called the interscaffolding additivity [137, 139]. A lack of interscaffolding additivity was observed for the interaction of P_1 Lys variants of OMTKY3 and BPTI with chymotrypsin, and was explained based on the crystal structures of the two complexes in which completely different conformation of the P_1 side chain and its interactions were observed upon binding in the respective S_1 pockets [115, 137, 144].

The additivity

In the simplest case, additivity assumes that the energetic effects of two individual mutations sum up, within experimental error, in a mutant containing the two mutations [145]. Additivity can also be applied in systems containing a larger number of mutations. In protein chemistry, additivity is tested in various macromolecular processes almost exclusively at the level of free energy effects [146]. Free energies of protease-inhibitor interactions were extensively tested in the Laskowski laboratory for the interaction between ovomucoid third domains and six different serine proteases [147]. Depending on the experimental error analysis, the protease tested, and the number of mutations introduced, the additivity holds for from 60 to almost 100% of the analyzed cases. Additivity offers a superb possibility of creating strong (with K_a values up to 10^{17} M^{-1}) or specific inhibitors for different proteases through careful design of multiple mutants not only of ovomucoid third domains but also of inhibitors belonging to all other families [147–149]. Very recently, additivity was successfully tested in the interaction between chymotrypsin and alanine-shaved mutants of BPTI [83].

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