Role of the single cysteine residue, Cys 3, of human and bovine cystatin B (stefin B) in the inhibition of cysteine proteinases

EWA POL AND INGEMAR BJÖRK

Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, SE-751 23 Uppsala, Sweden

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Abstract

Cystatin B is unique among cysteine proteinase inhibitors of the cystatin superfamily in having a free Cys in the N-terminal segment of the proteinase binding region. The importance of this residue for inhibition of target proteinases was assessed by studies of the affinity and kinetics of interaction of human and bovine wild-type cystatin B and the Cys 3-to-Ser mutants of the inhibitors with papain and cathepsins L, H, and B. The wild-type forms from the two species had about the same affinity for each proteinase, binding tightly to papain and cathepsin L and more weakly to cathepsins H and B. In general, these affinities were appreciably higher than those reported earlier, perhaps because of irreversible oxidation of Cys 3 in previous work. The Cys-to-Ser mutation resulted in weaker binding of cystatin B to all four proteinases examined, the effect varying with both the proteinase and the species variant of the inhibitor. The affinities of the human inhibitor for papain and cathepsin H were decreased by threefold to fourfold and that for cathepsin B by ~20-fold, whereas the reductions in the affinities of the bovine inhibitor for papain and cathepsins H and B were ~14-fold, ~10-fold and ~300-fold, respectively. The decreases in affinity for cathepsin L could not be properly quantified but were greater than threefold. Increased dissociation rate constants were responsible for the weaker binding of both mutants to papain. By contrast, the reduced affinities for cathepsins H and B were due to decreased association rate constants. Cys 3 of both human and bovine cystatin B is thus of appreciable importance for inhibition of cysteine proteinases, in particular cathepsin B.

Keywords: Cathepsin; cystatin; cysteine proteinase; cysteine proteinase inhibitor; stefin

Mammalian cystatin B (also termed stefin B) belongs to cystatin family 1 (also termed stefins) of the cystatin superfamily (for reviews, see Barrett et al. 1986; Turk and Bode 1991; Abrahamson 1994; Turk et al. 1997). It is an intracellular, tight binding, and reversible inhibitor of papain-like cysteine proteinases, comprising a single 98-residue chain without disulfide bonds and carbohydrate. Other representatives of this family are mammalian cystatin A, bovine stefin C, and pig stefin D. The cystatins of family 2, represented by, for example, mammalian cystatin C and chicken cystatin, are primarily extracellular and consist of about 120 amino acids. They contain two disulfide bonds and may also have N-linked carbohydrate. Family 3 cystatins, kininogens, are blood plasma glycoproteins composed of three domains, each resembling a family 2 cystatin. Two of these domains can inhibit cysteine proteinases.

Cystatin B appears to have a general protective role in mammalian organisms, due to its regular distribution in cells and tissues (Green et al. 1984; Barrett et al. 1986;

Reprint requests to: Ingemar Björk, Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, Box 575, SE-751 23 Uppsala, Sweden; e-mail: Ingemar.Bjork@vmk.slu.se; fax: 46-18-550762.

Abbreviations: app, subscript denoting an apparent equilibrium or rate constant measured in the presence of an enzyme substrate; C3S-cystatin B, cystatin B variant in which Cys 3 is replaced with Ser; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; His-tag, 10 consecutive histidine residues fused to an expressed protein; k_{ass}, bimolecular association rate constant; K_d, dissociation equilibrium constant; k_{diss}, dissociation rate constant; K_i, inhibition constant; k_{obs}, observed pseudo-first-order rate constant; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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Lenarcic et al. 1986, 1996; Turk et al. 1992; Abrahamson 1994). It most likely prevents inappropriate proteolysis caused by the action of lysosomal cysteine proteinases, primarily cathepsins B, H, K, L, and S (Turk et al. 1997, 2000). Such a function is in agreement with findings that changes in the balance between cathepsins B and L and cystatin B in tumor tissue, resulting in excessive enzyme activity, are correlated with progression of cancer malignancy (Budihna et al. 1996; Ebert et al. 1997; Kos et al. 2000). In addition, it has been proposed that cystatin B has a specific physiological role, in particular in the brain, because congenital deficiency of the inhibitor in humans and deletion of the gene in mice give rise to a progressive myoclonus epilepsy, apparently caused by cerebellar apoptosis (Pennacchio et al. 1996, 1998). However, the underlying mechanism and the putative target proteinase of the inhibitor are still unknown.

The crystal structure of a complex between human cystatin B and papain (Stubbs et al. 1990) has greatly contributed to the understanding of the nature of cystatin inhibition of target proteinases. In this structure, a wedge formed by the N-terminal end and two hairpin loops of cystatin B fits well into the active-site cleft of papain. The contact surface is dominated by many hydrophobic interactions, with only few hydrogen bonds between the two proteins. The good fit and the large number of interactions formed are consistent with the high affinity of binding of the inhibitor to the enzyme (Green et al. 1984; Abrahamson et al. 1986; Jerala et al. 1990; Machleidt et al. 1991; Turk et al. 1992, 1993; Pol and Björk 1999). The structure of the complex is essentially identical to the docking model proposed previously for the complex between chicken cystatin and papain (Bode et al. 1988), indicating that all cystatins interact with their target proteinases in a similar manner.

Cystatin B is unique among cystatins in having a free cysteine residue, which is located in the N-terminal segment of the proteinase binding region, at position 3. In the determination of the crystal structure of the complex with papain (Stubbs et al. 1990), as well as in many other studies of the inhibitor (Jerala et al. 1990, 1994; Pol and Björk 1999), a recombinant Cys 3-to-Ser mutant was chosen, so that disulfide-linked cystatin B dimers (Lenarcic et al. 1986) were not formed. However, in the crystal structure, Ser 3 occupies the S2 substrate-binding subsite of papain (Stubbs et al. 1990; Turk et al. 1998) and makes many interactions with residues in the enzyme. In particular, it is one of four inhibitor residues that closely surround the active-site Cys of papain (Fig. 1A). The crystal structure of the complex thus suggests that Cys 3 in wild-type cystatin B is of appreciable importance for binding of proteinases. Replacement of this residue with Ser might therefore be expected to substantially affect the affinity and kinetics of proteinase inhibition.

In contrast with these deductions from the crystal structure, previous experimental work has given no clear evidence for a role of Cys 3 of cystatin B in binding of target proteinases. The affinities for proteinases reported for the human Cys 3-to-Ser cystatin B variant (Jerala et al. 1990, 1994) are not consistently different from those of the natural human inhibitor (Green et al. 1984; Abrahamson et al. 1986). On the other hand, a recombinant bovine Cys 3-to-Ser variant was found to have a 100-fold higher affinity for papain (Pol and Björk 1999) than cystatin B isolated from bovine thymus (Turk et al. 1993; Leonardi et al. 1996), although the affinity for cathepsin L was similar. In view of these inconclusive results, we have undertaken a detailed characterization of the affinity and kinetics of interaction of wild-type and Cys 3-to-Ser variants of human and bovine cystatin B with four proteinases, papain, and cathepsins L, H, and B. We find that Cys 3 indeed is of appreciable importance for the binding of cystatin B to target proteinases, in particular to cathepsin B.

Results

Generation and properties of cystatin B variants

The previously described vector for expression of bovine C3S-cystatin B (Pol and Björk 1999) was used also for expression of human and bovine wild-type cystatin B and human C3S-cystatin B in this work. However, a new unique *Eco*RI restriction site was introduced into the vector to simplify insertion of the appropriate cDNAs in one step. The vector expresses a protein with a removable His-tag and directs the tagged protein to the periplasmic space of *Escherichia coli*, facilitating refolding and simplifying purification. All four cystatin B forms were expressed and purified with final yields of 8–12 mg/L of bacterial culture. In SDS-PAGE under reducing conditions, all purified inhibitors gave only one band with an apparent molecular mass of ~11,000 Da. However, appreciable amounts of dimers were seen in the nonreduced wild-type preparations in SDS-PAGE under nonreducing conditions. N-terminal sequence analyses of the recombinant cystatin B forms verified correct cleavages by enterokinase, as well as the presence of the desired mutations. The relative molecular masses, determined by MALDI mass spectroscopy, of all forms differed by at most 2 mass units (0.02%) from the expected values. Titrations, monitored by the decreases of tryptophan fluorescence accompanying complex formation (Pol and Björk 1999), of papain with the inhibitors under reducing conditions gave binding stoichiometries of 1.02–1.07 mol inhibitor/mol enzyme. The thiol group content of the freshly isolated or properly stored (see following) wild-type forms, measured after reduction with DTT and rapid removal of the reducing agent, was 0.9–1.1 mol/mol protein. These results show that all cystatin B forms were >99% pure, had the intended sequences as well as the correct lengths and molecular masses, and were fully active.

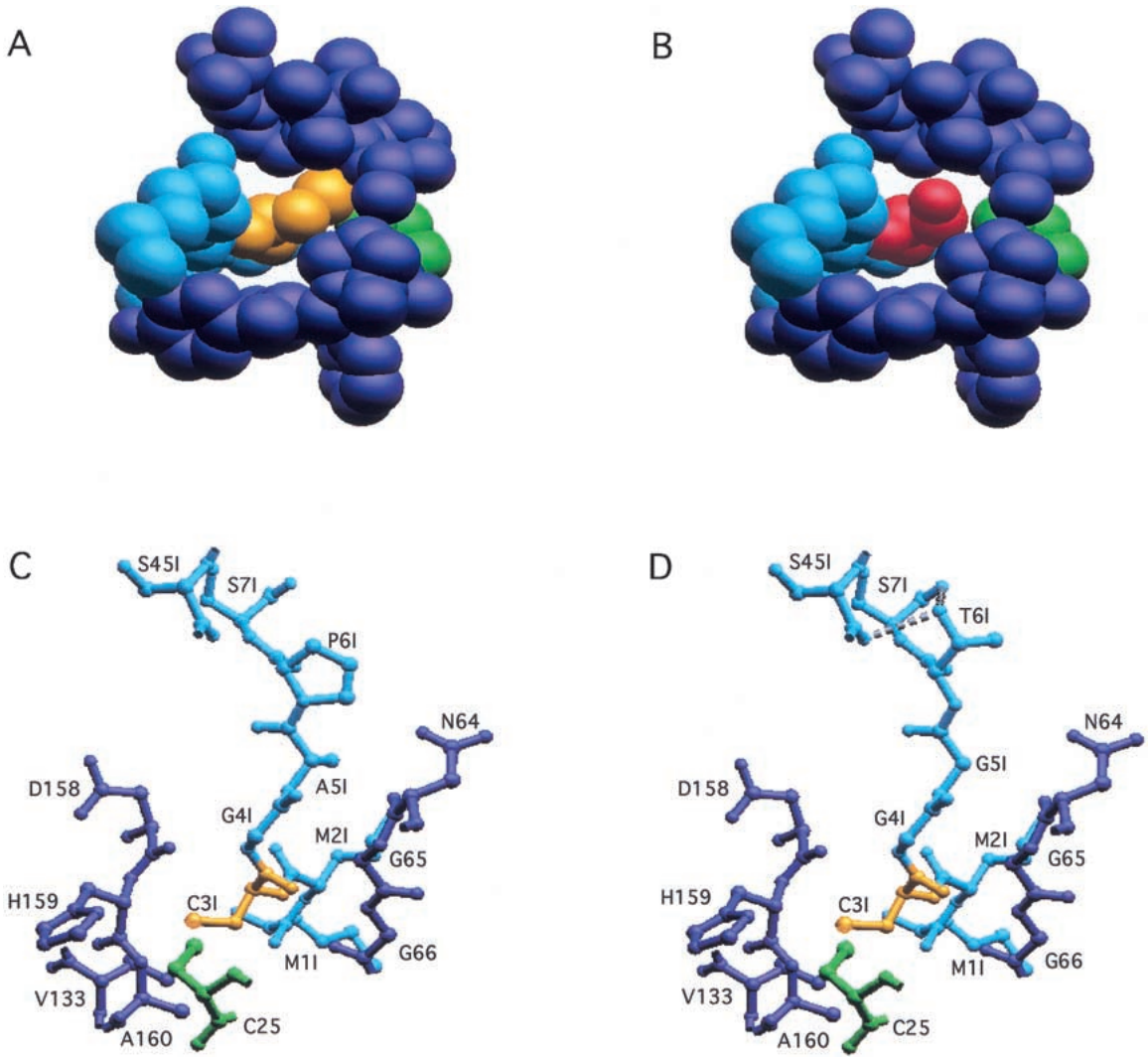

Fig. 1. Selected regions of models of complexes between wild-type or C3S forms of cystatin B and active papain. (*A*) Human wild-type cystatin B-papain. (*B*) Human C3S-cystatin B-papain. Residues in the two complexes located within 6 Å from Cys 3 or Ser 3 of the inhibitor are shown. Cys 3 is in yellow and Ser 3 in red; other cystatin B residues are in blue. The active-site Cys 25 of papain is in green; other papain residues are in violet. (*C*) Human wild-type cystatin B-papain. (*D*) Bovine wild-type cystatin B-papain. Residues in the two complexes located within 4 Å from the residues in either position 3, 5, or 6 are shown. Colors are the same as those shown in *A* and *B*. Putative hydrogen bonds are indicated by gray dotted lines. The letter I following a residue number denotes an inhibitor residue.

Storage of the wild-type cystatin B forms, whether in solution or frozen, without further treatment resulted in a slow loss of inhibitory activity, also when measured under reducing conditions. The reason for this loss was investigated by storing freshly isolated, reduced human wild-type cystatin B (pH 7.4) at 25°C after removal of the reducing agent. The content of thiol groups, measured after renewed reduction, decreased from an initial 1.0 mol/mol protein to 0.89, 0.83, and 0.80 mol/mol protein after 1, 2, and 3 weeks, respectively. This decrease in thiol group content presumably is due to irreversible oxidation of the thiol groups to, for example, sulfinic or sulfonic acid derivatives (Liu 1977). The wild-type cystatin B forms were therefore stored as their S-(methylthio) derivatives, which prevented the activity and thiol group loss. The protecting group was removed by reduction before analyses.

Possible formation of a disulfide bond between wild-type cystatin B and papain

Modeling of the complexes between human or bovine wild-type cystatin B and active papain (see following) showed that Cys 3 of the inhibitor and the active-site Cys 25 of the enzyme are located close to each other in the complexes.

The possibility that the two residues therefore may form a disulfide bond was investigated by allowing bovine cystatin B to bind to papain under reducing conditions and then inactivating the reducing agent and analyzing the complex by SDS-PAGE under nonreducing conditions. These analyses showed only two bands, migrating as free enzyme and inhibitor, and no evidence of a disulfide-linked complex (not shown). Like other cystatin-proteinase interactions, the interaction between wild-type cystatin B and papain, and presumably also other cysteine proteinases, is thus stabilized only by noncovalent bonds.

Affinity and kinetics of interaction with cysteine proteinases

Equilibrium and rate constants for the interactions of human and bovine wild-type cystatin B and human C3S-cystatin B with papain and cathepsins L, H, and B, as well as for the interaction of bovine C3S-cystatin B with cathepsin H, were determined in this work (Table 1). The data for the binding of bovine C3S-cystatin B to papain and cathepsins L and B have been reported earlier (Pol and Björk 1999) and are included in Table 1 for comparison. In addition, the effect of increasing the size of the Cys 3 side chain on binding of proteinases was evaluated by characterizing the interaction of the S-(carbamoylmethyl) derivatives of human and bovine wild-type cystatin B with papain and cathepsin B. All analyses were done in the presence of 1 mM DTT, sufficient to fully activate the target enzymes and convert the wild-type inhibitors to monomers, as shown by SDS-PAGE.

Dissociation equilibrium constants, K_d, for the binding of the human and bovine cystatin B forms to cathepsins L and B and of the bovine C3S variant to cathepsin H were determined as inhibition constants, K_i. These constants were obtained by measurements of the decrease in the equilibrium rates of cleavage of a fluorogenic substrate by the enzymes in the presence of an excess of the inhibitors (Table 1). The affinities of several interactions were too high to be accurately quantified by equilibrium methods. The K_d values for the interactions of all cystatin B forms with papain, as well as those for the interactions of human and bovine wild-type cystatin B and human C3S-cystatin B with cathepsin H, were instead calculated from separately measured values of the association rate constants, k_{ass} and dissociation rate constants, k_{diss} (Table 1). However, the K_d values for the binding of human and bovine wild-type cys-

Table 1. *Dissociation equilibrium constants (K_d), association rate constants (k_{ass}), and dissociation rate constants (k_{diss}) for the binding of human and bovine recombinant wild-type and C3S-cystatin B forms and the S-(carbamoylmethyl) derivative of the wild-type inhibitors to cysteine proteinases*

Enzyme	Cystatin B form	K_d (M)	k_{ass} ($M^{-1} \cdot s^{-1}$)	k_{diss} (s^{-1})
Papain	human, wt[a]	4.9×10^{-14}[b] [1]	$(9.7 \pm 0.3) \times 10^6$ (10) [1]	$(4.8 \pm 0.4) \times 10^{-7}$ (2) [1]
	human, C3S	1.9×10^{-13}[b] [4]	$(5.3 \pm 0.2) \times 10^6$ (10) [1.8]	$(1.0 \pm 0.1) \times 10^{-6}$ (2) [2]
	human, SCM[c]	6.0×10^{-12}[b] [120]	$(7.0 \pm 0.1) \times 10^6$ (9) [1.4]	$(4.2 \pm 0.5) \times 10^{-5}$ (2) [88]
	bovine, wt	8.7×10^{-14}[b] [1]	$(1.0 \pm 0.1) \times 10^7$ (9) [1]	$(8.8 \pm 0.6) \times 10^{-7}$ (2) [1]
	bovine, C3S	1.2×10^{-12}[b] [14]	$(6.8 \pm 0.2) \times 10^6$ (8) [1.5]	$(8.0 \pm 0.2) \times 10^{-6}$ (3) [9]
	bovine, SCM	6.7×10^{-12}[b] [70]	$(1.0 \pm 0.1) \times 10^7$ (8) [1]	$(6.7 \pm 0.5) \times 10^{-5}$ (2) [76]
Cathepsin L	human, wt	$<3 \times 10^{-12}$ (10) [1]	$(4.3 \pm 0.1) \times 10^7$ (9) [1]	$<1.3 \times 10^{-4}$[d] [1]
	human, C3S	$(1.0 \pm 0.1) \times 10^{-11}$ (9) [>3.3]	$(2.4 \pm 0.1) \times 10^7$ (10) [1.8]	2.4×10^{-4}[d] [>1.8]
	bovine, wt	$<3 \times 10^{-12}$ (14) [1]	$(8.6 \pm 0.2) \times 10^7$ (10) [1]	$<2.6 \times 10^{-4}$[d] [1]
	bovine, C3S	$(9.3 \pm 0.6) \times 10^{-12}$ (10) [>3.1]	$(1.8 \pm 0.1) \times 10^7$ (11) [4.8]	1.7×10^{-4}[d] [>0.7]
Cathepsin H	human, wt	2.9×10^{-11}[b] [1]	$(8.0 \pm 0.3) \times 10^6$ (9) [1]	$(2.3 \pm 0.2) \times 10^{-4}$ (4) [1]
	human, C3S	9.6×10^{-11}[b] [3.3]	$(2.4 \pm 0.1) \times 10^6$ (9) [3.3]	$(2.3 \pm 0.4) \times 10^{-4}$ (4) [1]
	bovine, wt	3.2×10^{-11}[b] [1]	$(1.2 \pm 0.1) \times 10^7$ (8) [1]	$(3.8 \pm 0.4) \times 10^{-4}$ (4) [1]
	bovine, C3S	$(3.3 \pm 0.1) \times 10^{-10}$ (7) [10]	$(1.5 \pm 0.1) \times 10^6$ (11) [8]	5.0×10^{-4}[d] [1.3]
Cathepsin B	human, wt	$(1.8 \pm 0.1) \times 10^{-8}$ (11) [1]	$(3.0 \pm 0.1) \times 10^5$ (9) [1]	5.4×10^{-3}[d] [1]
	human, C3S	$(3.6 \pm 0.1) \times 10^{-7}$ (9) [20]	$(2.5 \pm 0.1) \times 10^4$ (9) [12]	9.0×10^{-3}[d] [1.7]
	human, SCM	$(7.5 \pm 0.6) \times 10^{-6}$ (5) [420]	n.d.[e]	n.d.
	bovine, wt	$(2.1 \pm 0.2) \times 10^{-8}$ (11) [1]	n.d.	n.d.
	bovine, C3S	$(6.7 \pm 0.4) \times 10^{-6}$ (7) [320]	n.d.	n.d.
	bovine, SCM	$(10.7 \pm 0.2) \times 10^{-6}$ (5) [510]	n.d.	n.d.

The values for the binding of bovine C3S-cystatin B to papain and cathepsins L and B are taken from previous work (Pol and Björk 1999) and are shown for comparison. Measured values are reported as averages ± SEM with the number of measurements in parentheses. Calculated values are given without errors. Relative values, defined as $K_{d,\ variant}/K_{d,\ wild\text{-}type}$, $k_{ass,\ wild\text{-}type}/k_{ass,\ variant}$, and $k_{diss,\ variant}/k_{diss,\ wild\text{-}type}$, are given in square brackets. Relative values >1 thus indicate changes of K_d, k_{ass}, and k_{diss} expected to result in a binding affinity lower than that of the wild-type forms.

[a] (wt) Wild type.
[b] Calculated from k_{ass} and k_{diss}.
[c] (SCM) S-(carbamoylmethyl).
[d] Calculated from K_d and k_{ass}.
[e] (n.d.) Not determined.

tatin B to cathepsin L could not be calculated in this manner, as k_{diss} could not be determined (see following). Only higher limits of K_d for these interactions were therefore estimated by equilibrium measurements (Table 1).

Values of k_{ass} for the binding of the cystatin B forms to the proteinases were determined in most cases by continuously monitoring the loss of enzyme activity against a fluorogenic substrate in a conventional fluorometer (Table 1). However, the fast reactions of human wild-type and C3S-cystatin B with cathepsin B were similarly monitored in a stopped-flow instrument (Table 1). The high enzyme concentrations that would have been required prevented the use of such analyses in the case of the interactions of the other variants with cathepsin B. For all reactions studied, the apparent pseudo-first-order association rate constants, $k_{obs,app}$, showed a linear dependence on inhibitor concentration, giving k_{ass}.

The k_{diss} values for the interactions of all cystatin B forms with papain and of human and bovine wild-type cystatin B and the human C3S variant with cathepsin H were determined by displacement experiments (Table 1). In these analyses, the association reaction was prevented by nearly irreversibly trapping enzyme or inhibitor dissociating from the complexes by an appropriate reactant. Papain liberated from cystatin B complexes was trapped by an excess of chicken cystatin, and the rates were monitored by the appearance of the newly formed chicken cystatin-papain complex, analyzed by ion-exchange chromatography. Cystatin B liberated from complexes with cathepsin H was trapped by papain, and the rates were monitored by the increase in cathepsin H activity against a fluorogenic substrate in a conventional fluorometer. In all other cases, k_{diss} was calculated from K_d and k_{ass} when both of these parameters could be measured (Table 1). k_{diss} for the interactions of the wild-type cystatin B forms with cathepsin L could not be determined by displacement experiments because of the low amounts of enzyme available, and only higher limits of k_{diss} could be estimated from k_{ass} and the higher limits of K_d (Table 1). Moreover, k_{diss} for the binding of human S-(carbamoylmethyl)-cystatin B and of all bovine cystatin B forms to cathepsin B was too high to be measured by any method available.

Modeling

Modeling of the complexes between human or bovine wild-type or C3S-cystatin B and active papain demonstrated certain structural features of apparent relevance for the observed affinities and kinetics of the interactions. Comparison between the human wild-type and C3S-cystatin B complexes with the enzyme (Fig. 1A,B) showed that the thiol group of cysteine, larger and more hydrophobic than the serine hydroxyl group, more efficiently fills the surrounding S2 substrate-binding site of papain (Turk et al. 1998). In particular, the thiol group comes closer than the hydroxyl group to Ala 160, Asp 158, and Cys 25 of the enzyme, resulting in more effective packing of the atoms in the interaction surface. The distance between the sulfur atoms of Cys 3 of cystatin B and Cys 25 of papain in the model was only about 2.6 Å. Modeling of the bovine wild-type and C3S-cystatin B complexes with papain gave the same results.

Twenty-one of 98 amino acids are different in human and bovine cystatin B. Two of these, in positions 5 and 6, are of particular interest in relation to this work, as they are located close to Cys 3 of the inhibitor. The two hydrophobic residues, Ala 5 and Pro 6, that are present in human cystatin B and all other known species variants of the inhibitor except the bovine one are replaced by the more polar residues Gly 5 and Thr 6 in bovine cystatin B (Fig. 1C,D). These residues occupy the S1′ and S2′ substrate-binding subsites of the enzyme, respectively, in the complexes of both the human and bovine inhibitors with papain. In human cystatin B, Pro 6 introduces a bend in the polypeptide chain that most likely is of importance for optimal interaction of the N-terminal region of the inhibitor with the enzyme. Moreover, Ala 5 and Pro 6 of human cystatin B also make close contacts with Asn 64 of papain, which forms parts of the S1 and S3 subsites (Fig. 1C,D). However, the modeling shows that these hydrophobic contacts cannot be made by the more polar residues of bovine cystatin B. Instead, Thr 6 is capable of forming two intramolecular hydrogen bonds to Ser 7 and Ser 45 in the bovine inhibitor. These hydrogen bonds presumably aid in stabilizing a bend in the chain comparable to but most likely less stable than that caused by Pro 6 in human cystatin B.

Discussion

The results of this work show that human and bovine wild-type cystatin B bind with about the same affinity to each of the proteinases investigated, the affinity depending on the proteinase. Wild-type cystatin B from both species thus binds tightly to papain and cathepsin L (K_d ~5×10^{-14} M and $< 3 \times 10^{-12}$ M, respectively) and more weakly to cathepsins H and B (K_d ~3×10^{-11} M and ~2×10^{-8} M, respectively), in general agreement with relative affinities reported earlier (Green et al. 1984; Abrahamson et al. 1986; Machleidt et al. 1991; Turk et al. 1993; Abrahamson 1994; Leonardi et al. 1996) and also with the ability of other cystatins to inhibit these enzymes (Barrett et al. 1986; Abrahamson 1994). However, the affinity of cystatin B for cathepsin B is appreciably weaker than those of other cystatins. The lower affinity of cystatins for the exopeptidases, cathepsins H and B, is most likely due to a restricted access of the inhibitors to the active-site clefts of these enzymes (Nycander et al. 1998; Pavlova et al. 2000), which are par-

tially blocked by the "mini-chain" and "occluding loop," respectively (Musil et al. 1991; Guncar et al. 1998).

The affinities of human wild-type cystatin B for papain, cathepsin L, and cathepsin H measured in this work are 150–2500-fold, >70-fold, and up to 20-fold higher, respectively, and the affinity of bovine wild-type cystatin B for papain ~2500-fold higher than values reported earlier (Green et al. 1984; Abrahamson et al. 1986; Popovic et al. 1988; Machleidt et al. 1991; Turk et al. 1992, 1993). However, the affinities of human wild-type cystatin B for cathepsin B and of the bovine wild-type inhibitor for cathepsin L are in agreement with earlier reports (Green et al. 1984; Abrahamson et al. 1986; Turk et al. 1992, 1993; Leonardi et al. 1996). In general, wild-type cystatin B is thus a more efficient inhibitor of cysteine proteinases than previously believed. The comparatively weak binding of the wild-type inhibitor to target proteinases reported in many previous studies may have been caused by irreversible oxidation of the cysteine residue, probably during isolation or storage, as demonstrated in this work, which would have resulted in a less potent inhibitor.

Replacement of Cys 3 of cystatin B by Ser produced a weaker inhibitor of all four proteinases examined. The decrease in affinity depended on the target proteinase and the inhibitor species, being more pronounced for cathepsin B and for the bovine inhibitor. The affinity for papain and cathepsin H, compared with that of the corresponding wild type, was thus threefold to fourfold lower for the human and 10- to 14-fold lower for the bovine Ser 3 cystatin B mutant, whereas the affinity decrease for cathepsin B was 20-fold and 300-fold for the human and bovine mutants, respectively. The loss in affinity of the Cys 3-to-Ser variants for cathepsin L could not be properly assessed, as only limits of K_i for the binding of the wild-type inhibitors to this enzyme could be estimated. Our results for the binding of human C3S-cystatin B to the four proteinases are comparable with values obtained earlier (Jerala et al. 1990, 1994), except in the case of the interaction with papain, for which a 350- to 1000-fold higher affinity was determined in this work.

The impaired affinity of the C3S-cystatin B mutants for papain was largely the consequence of an increased k_{diss}, most prominently in case of the bovine inhibitor. This behavior indicates that the major effect of Cys 3 is to aid in keeping cystatin B anchored to papain, once the complex has been formed. In contrast, a decreased k_{ass} was mainly responsible for the reduced affinity of the C3S mutants for cathepsins H and B, indicating that Cys 3 primarily contributes to increasing the rate of association of cystatin B with these enzymes. The differential effect of the Cys 3 to Ser mutation on the kinetics of papain and cathepsin B inhibition is similar to the consequence of a number of other modifications of the N-terminal regions of cystatins (Abrahamson et al. 1991; Björk et al. 1994, 1995; Estrada et al. 1999). Analogous to what has been proposed for the binding of cystatins A and C to cathepsin B (Nycander et al. 1998; Pavlova et al. 2000), the contribution of Cys 3 to increasing the rate of reaction of cystatin B with this enzyme presumably arises from the N-terminal region of cystatin B binding to the enzyme before the remainder of the inhibitor. This initial interaction facilitates displacement of the occluding loop of cathepsin B and thereby increases the overall association rate.

Covalent attachment of an S-(carbamoylmethyl) group to Cys 3 of wild-type cystatin B decreased the affinity of the inhibitor for the enzymes studied, papain and cathepsin B, by 100- to 500-fold, more than substitution by Ser. This result is in contrast to the results of previous studies, in which analogous substitutions were reported either to have no effect on the inhibitory activity of human cystatin B (Lenarcic et al. 1986) or to abolish the activity of the rat inhibitor (Wakamatsu et al. 1984). Increased k_{diss} values were responsible for the weaker binding of both the human and bovine modified inhibitors to papain, consistent with the role of Cys 3 in anchoring the inhibitor to this proteinase.

The importance of Cys 3 of cystatin B for inhibition of proteinases with open active-site clefts, such as papain and cathepsin L, is in agreement with the modeling of the complexes of the wild-type and C3S forms of the inhibitor with active papain. The models suggested that tighter hydrophobic interactions can be established between the thiol group of wild-type cystatin B and papain than can be established between the smaller and more polar hydroxyl group of the Ser 3 mutant and the enzyme. This finding is consistent with the role of Cys 3 in increasing the affinity for papain primarily by decreasing the rate of dissociation of the enzyme–inhibitor complex. The models also showed that increasing the size of the side chain on Cys 3 by covalent attachment of an S-(carbamoylmethyl) group would lead to steric interference, explaining the observed increased k_{diss} and decreased affinity. In contrast, the molecular background to the importance of Cys 3 for the interaction of cystatin B with cathepsins H and B, which both have partially blocked active sites, is difficult to assess, as no crystal structure of a complex of a cystatin with these proteinases is available, making detailed molecular modeling highly uncertain.

The greater importance of Cys 3 of bovine cystatin B than of human cystatin B for inhibition of proteinases with open active-site clefts is also consistent with the modeling of the complexes with papain. In the complex of human cystatin B with the proteinase, two hydrophobic residues of the inhibitor located close to Cys 3, viz. Ala 5 and Pro 6, form contacts with a neighboring residue of the enzyme. These contacts are absent in the model of the complex of bovine cystatin B with papain, because the two hydrophobic residues of the human inhibitor are replaced by more polar ones. The loss of these interactions presumably is largely compensated by stronger interactions of Cys 3 of bovine

cystatin B with papain, as k_{diss} of this complex is only slightly higher than that of the complex of the human inhibitor with the proteinase. The affinity therefore is only marginally lower, k_{ass} being unaffected. However, when Cys 3 of bovine cystatin B is mutated to Ser, the loss of these stronger interactions result in a much larger increase in k_{diss} of the complex of the bovine than of the human inhibitor with the proteinase and thus in a larger decrease in affinity.

In conclusion, wild-type cystatin B is a more powerful inhibitor of cysteine proteinases than indicated by many previous investigations, its efficiency against several proteinases being comparable to that of cystatins A and C. However, the weak inhibition of cathepsin B presumably disqualifies cystatin B as a physiological inhibitor of this enzyme. Cys 3 of cystatin B is important for tight binding of target proteinases, and substitution or modification of this residue, for example, by oxidation, appreciably decreases proteinase affinity. The oxidation sensitivity of Cys 3 most likely is of no major consequence for the intracellular function of cystatin B but may compromise the ability of the inhibitor to act extracellularly.

Materials and methods

Proteins

The purification and properties of papain (EC 3.4.22.2) have been reported elsewhere (Lindahl et al. 1988; Björk et al. 1994). Cathepsin L (EC 3.4.22.15) from sheep liver (Mason 1986) and cathepsin H (EC 3.4.22.16) from porcine spleen (Popovic et al. 1988; Guncar et al. 1998) were gifts from Dr. R.W. Mason (Alfred I. du Pont Institute, Wilmington, DE) and Dr. B. Turk (J. Stefan Institute, Ljubljana, Slovenia), respectively. Cathepsin B (EC 3.4.22.1) from human liver was purchased from Calbiochem. Chicken cystatin was isolated from egg white (Lindahl et al. 1988).

Expression of human and bovine cystatin B variants

A vector for expression of bovine C3S-cystatin B has been described previously (Pol and Björk 1999) and was used also for expression of the human and bovine wild-type and human C3S forms of the inhibitor. This vector contains the sequences for the leader peptide for outer membrane protein A of *E. coli*, causing the expressed protein to be transported to the periplasmic space, a His-tag and the recognition site for enterokinase, allowing the His-tag to be removed (Estrada et al. 1998). A new unique restriction site for *Eco*RI was introduced by PCR into the linker region between the sequences encoding the His-tag and the enterokinase site.

A pET-15b vector (Novagen) containing a human cystatin B cDNA (Gene Bank accession number: P04080) (Pennacchio et al. 1996) was a gift from Ms. Christina Öhrmalm (Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden). cDNAs for human wild-type cystatin B and C3S-cystatin B to be inserted into the expression vector were obtained by PCR with this vector as template. The upstream primer for generation of the wild-type form (5′-AGCGGCGAATTCGACGACGACGACAAGATGATGT**G**CGGGGCG) was complementary to the portion of the expression vector comprising (from the 5′ end) the restriction site for *Eco*RI (underlined) and the enterokinase cleavage site, and to the first 15 bases of the coding sequence of the human cystatin B cDNA. The primer for generation of the C3S variant was identical except for containing a G to C substitution in position 35 (in bold). The downstream primer (5′-TAGCAGCCGGATCCGATCACAAG) was the same for both variants and was complementary to the last bases (463–471) of the 3′-noncoding region of the human cystatin B cDNA in the pET15b vector and to the adjoining portion of the vector, including a *Bam*HI cleavage site (underlined). A cDNA for bovine wild-type cystatin B was similarly created by PCR amplification of the expression vector containing the bovine C3S variant (Pol and Björk 1999). The upstream primer (5′-AGCGGCGAATTCGACGACGACGACAACATGATGTGTGGAGGG) was complementary to the same region of the expression vector described earlier and to the first 15 bases of the coding part of the bovine wild-type cystatin B cDNA (Pol and Björk 1999). The downstream primer (5′-ATCTACGGATCCCCCCATCCGAGGACACCGCATCTG) was complementary to bases 482–505 in the 3′-noncoding region of the bovine cystatin B cDNA and contained a *Bam*HI site (underlined). The expression vector was digested with *Eco*RI and *Bam*HI, purified, and ligated with the PCR products for human wild-type or C3S-cystatin B or bovine wild-type cystatin B, all restricted with the same enzymes. Competent *E. coli* strain MC 1061 was transformed with the constructs, and individual clones were collected and sequenced.

All forms of cystatin B were expressed essentially as in previous work (Pol and Björk 1999). The content of the periplasmic space was extracted by cold osmotic shock, the fusion proteins were isolated by affinity chromatography on a Ni^{++} chelate column (Novagen), and the free inhibitors were released by enterokinase cleavage as described earlier (Estrada et al. 1998; Pol and Björk 1999).

The wild-type cystatin B forms were reduced with 1 mM DTT (pH 7.4) for 10 min immediately after preparation. After removal of excess reagent by gel chromatography on a PD-10 column (Amersham Pharmacia Biotech), the forms were converted to and stored as their S-(methylthio) derivatives (Lindahl et al. 1988) to protect the cysteine residues. The protecting group was removed by reaction with 1 mM DTT (pH 7.4) for 15 min before measurements (Lindahl et al. 1988).

Quantitative analysis, irreversible oxidation, and blocking of the thiol group in wild-type cystatin B

The thiol group content of the wild-type cystatin B forms was measured by reducing the freshly isolated proteins or their S-(methylthio) derivatives with 1 mM DTT (pH 7.4) for 15 min, removing excess reducing agent on a PD-10 column, and immediately reacting the proteins with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman 1959).

Irreversible oxidation of the thiol group in human wild-type cystatin B was investigated after reduction of the S-(methylthio) derivative with DTT and removal of the reagent as described earlier. The thiol group content was measured by reaction with 5,5′-dithiobis(2-nitrobenzoic acid), and the protein was then incubated at 25°C for 3 weeks in 0.05 M Tris-HCl (pH 7.4), containing 0.1 M NaCl and 0.1 mM EDTA. Irreversible loss of thiol groups was measured after each week by again reducing the inhibitor with DTT, removing the reducing agent on a PD-10 column, and redetermining the thiol group content.

S-(carbamoylmethyl) derivatives of human and bovine wild-type cystatin B were obtained by reducing the S-(methylthio) derivatives with 1mM DTT and reacting the protein thiol groups with 6 mM iodoacetamide (in excess of the concentration required to neutralize the DTT) (pH 8.0) for 30 min. The reagents were then removed on a PD-10 column.

Possible formation of a disulfide bond between cystatin B and papain

A complex (50 μM) between bovine wild-type cystatin B and papain was formed by mixing equimolar amounts of the two proteins, both of which had been reduced with 1 mM DTT (pH 8.0) for 10 min. Iodoacetamide was then added to a final concentration of 4 mM to inactivate the DTT and to prevent formation of a disulfide bond after denaturation of the proteins. The complex was analyzed by SDS-PAGE under nonreducing conditions as described following.

Binding stoichiometry

Stoichiometries of binding of the human and bovine cystatin B variants and S-(carbamoylmethyl) derivatives to papain were determined by titrations of 1 μM papain with the inhibitors. The titrations were monitored by the changes in tryptophan fluorescence emission accompanying the interaction (Lindahl et al. 1988, 1992).

Inhibition constants

Values of K_i for the interactions of human and bovine wild-type cystatin B and human C3S-cystatin B with cathepsins L and B, of the bovine C3S form with cathepsin H, and of the S-(carbamoylmethyl) derivatives of the wild-type cystatin B forms with cathepsin B were obtained from the equilibrium rates of cleavage of a fluorogenic substrate by the enzyme at increasing concentrations of the inhibitor, as detailed earlier (Björk et al. 1994, 1995). The substrate was carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute) for cathepsin L, L-arginine 4-methylcoumaryl-7-amide (Bachem) for cathepsin H, and carbobenzoxy-L-arginyl-L-arginine 4-methyl-coumaryl-7-amide (Peptide Institute) for cathepsin B. The substrate concentrations were 10 μM for cathepsins L and B and 50 μM for cathepsin H. Substrate hydrolysis never exceeded 5%. The inhibitor concentration was at least 10-fold higher than that of the enzyme and in most cases varied from 0.06–1 × $K_{i,app}$ (the apparent inhibition constant) to 2–10 × $K_{i,app}$. However in the analyses with cathepsin L, the lowest concentrations of wild-type cystatin B were ~2 × $K_{i,app}$ and in the analyses with cathepsin B, the highest concentrations of bovine C3S-cystatin B and the S-(carbamoylmethyl) derivatives were ~0.7 × $K_{i,app}$. Values of $K_{i,app}$ were obtained by nonlinear regression analyses of plots of the ratio between the inhibited and uninhibited rates of substrate hydrolysis against inhibitor concentration (Björk et al. 1994, 1995) and were corrected for substrate competition to give K_i with the use of K_M values reported previously (Pol et al. 1995). The K_M of L-arginine 4-methylcoumaryl-7-amide for porcine cathepsin H under the conditions of the K_i measurements was determined to 115 ± 11 μM.

Association kinetics

The rates of binding of all cystatin B variants and S-(carbamoylmethyl) derivatives to papain and cathepsins L and H were evaluated under pseudo-first-order conditions by continuous measurements of the loss of enzyme activity in the presence of a fluorogenic substrate in a conventional fluorometer (F-4000; Hitachi), essentially as in earlier work (Björk et al. 1994; Pol et al. 1995; Pol and Björk 1999). The rates of binding of the human wild-type and C3S forms to cathepsin B were determined in a similar manner but in a stopped-flow fluorometer (SX-17MV; Applied Biophysics) (Estrada et al. 1998). The substrate for papain was carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide at a concentration of 20 μM, and the substrates for cathepsins L, H, and B and their concentrations were the same as in the measurements of K_i. Maximal substrate hydrolysis never exceeded 5%. The inhibitor concentrations were at least 10-fold higher than those of the enzymes and were varied from a lower limit of 0.01–1 nM to an upper limit of 2–10 nM for reactions with papain and cathepsins L and H, whereas the range was 1 μM to 10 μM for reactions with cathepsin B. The $k_{obs,app}$ values were derived by nonlinear least-squares regression analyses of the progress curves (Björk et al. 1994; Pol and Björk 1999). Values for k_{ass} were obtained from the slopes of plots of $k_{obs,app}$ versus inhibitor concentration after correction for substrate competition as described earlier.

Dissociation kinetics

The rates of dissociation of the complexes between all cystatin B forms and papain were analyzed by virtually irreversibly trapping the enzyme dissociated from a 5 or 10 μM complex with a large excess (60–230 μM) of chicken cystatin (form 2). Chicken cystatin binds to papain as tight as or tighter than the cystatin B forms (Björk et al. 1989), and a large excess of chicken cystatin therefore effectively prevents reassociation of the liberated cystatin B with the enzyme. The slow dissociation was monitored by the appearance of the tight complex between the displacing chicken cystatin and the released papain, analyzed by HPLC on a Mono Q column (Amersham Pharmacia Biotech) as described earlier (Björk et al. 1989; Björk and Ylinenjärvi 1990; Lindahl et al. 1992). The k_{diss} values were obtained by nonlinear least-squares regression analyses of the exponential progress curves.

The dissociation rates of the complexes between human or bovine wild-type cystatin B or human C3S-cystatin B and cathepsin H were investigated by trapping the inhibitors released from a 2–20 nM complex with 50–500 nM papain. Papain binds much stronger to the cystatin B forms than cathepsin H does (Table 1) (Green et al. 1984; Machleidt et al. 1991; Jerala et al. 1994; Pol and Björk 1999), thereby essentially blocking reversal of the dissociation reaction. The progress of the dissociation was monitored by continuous measurements in a conventional fluorometer of the fluorescence increase accompanying cleavage of the substrate, L-arginine 4-methylcoumaryl-7-amide (50 μM) by the liberated cathepsin H. The displacing papain had no activity against this substrate under the conditions used. The values of k_{diss} were obtained by nonlinear least-squares regression analyses (Björk and Ylinenjärvi 1990).

Miscellaneous procedures

SDS-PAGE was performed on 16.5% (w/v) gels with the Tricine buffer system (Pol and Björk 1999). N-terminal sequences were determined as in Björk et al. (1989). Relative molecular masses of the cystatin B variants were measured by MALDI mass spectroscopy (Pol et al. 1995).

Modeling

The complexes between human or bovine wild-type or C3S-cystatin B and active papain were modeled from the coordinates of the

human C3S-cystatin B – S-(carboxymethyl)-papain complex (PDB access code 1STF) (Stubbs et al. 1990). The modeling feature of the program `Swiss-PdbViewer` (http://www.expasy.ch/spdbv/) (Guex and Peitsch 1997) was used to replace Ser 3 with the most favorable Cys rotamer in the modeling of both the human and bovine complexes. In addition, all 21 amino acids of human cystatin B that differ between the two inhibitors were similarly replaced by the corresponding residues of bovine cystatin B in the modeling of the bovine complexes. The S-carboxymethyl group of the papain moiety of the complexes was also removed in the same manner.

Experimental conditions and protein concentrations

All analyses of inhibitor–proteinase binding were done at 25 ± 0.2°C. The buffers used were as follows: for papain 0.05 M Tris-HCl (pH 7.4), containing 0.1 M NaCl, 0.1 mM EDTA, and, in all analyses except the measurements of dissociation rate constants, 1 mM DTT and 0.01% (w/v) Brij 35; for cathepsin L 0.1 M sodium acetate (pH 5.5), containing 1 mM EDTA, 1 mM DTT, and 0.01 % (w/v) Brij 35; for cathepsin H 0.1 M sodium phosphate (pH 6.8), containing 1 mM EDTA and 1 mM DTT; for cathepsin B 0.05 M Mes-NaOH (pH 6.0), containing 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1 % (w/v) poly(ethylene glycol).

Concentrations of papain and cathepsin H were determined by absorption measurements at 280 nm from molar absorption coefficients of 55,900 $M^{-1} \cdot cm^{-1}$ (Lindahl et al. 1988) and 39,200 $M^{-1} \cdot cm^{-1}$ (Popovic et al. 1988), respectively. The weight concentration and relative molecular mass (27,500) of human cathepsin B was provided by the manufacturer. The molar concentration of cathepsin L was obtained by titration with E-64 (Mason 1986). Concentrations of cystatin B forms were determined by absorption measurements at 280 nm from a molar absorption coefficient of 4470 $M^{-1} \cdot cm^{-1}$ (Pol and Björk 1999).

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References

Abrahamson, M. 1994. Cystatins. *Methods Enzymol.* **244:** 685–700.

Abrahamson, M., Barrett, A.J., Salvesen, G., and Grubb, A. 1986. Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids. *J. Biol. Chem.* **261:** 11282–11289.

Abrahamson, M., Mason, R.W., Hansson, H., Buttle, D.J., Grubb, A., and Ohlsson, K. 1991. Human cystatin C. Role of the N-terminal segment in the inhibition of human cysteine proteinases and in its inactivation by leucocyte elastase. *Biochem. J.* **273:** 621–626.

Barrett, A.J., Rawlings, N.D., Davies, M.E., Machleidt, W., Salvesen, G., and Turk, V. 1986. Cysteine proteinase inhibitors of the cystatin superfamily. In *Proteinase inhibitors* (eds. A.J. Barrett and G. Salvesen), pp. 515–569. Elsevier, Amsterdam.

Björk, I. and Ylinenjärvi, K. 1990. Interaction between chicken cystatin and the cysteine proteinases actinidin, chymopapain A, and ficin. *Biochemistry* **29:** 1770–1776.

Björk, I., Alriksson, E., and Ylinenjärvi, K. 1989. Kinetics of binding of chicken cystatin to papain. *Biochemistry* **28:** 1568–1573.

Björk, I., Pol, E., Raub-Segall, E., Abrahamson, M., Rowan, A.D., and Mort, J.S. 1994. Differential changes in the association and dissociation rate constants for binding of cystatins to target proteinases occurring on N-terminal truncation of the inhibitors indicate that the interaction mechanism varies with different enzymes. *Biochem. J.* **299:** 219–225.

Björk, I., Brieditis, I., and Abrahamson, M. 1995. Probing the functional role of the N-terminal region of cystatins by equilibrium and kinetic studies of the binding of Gly-11 variants of recombinant human cystatin C to target proteinases. *Biochem. J.* **306:** 513–518.

Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J., and Turk, V. 1988. The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. *EMBO J.* **7:** 2593–2599.

Budihna, M., Strojan, P., Smid, L., Skrk, J., Vrhovec, I., Zupevc, A., Rudolf, Z., Zargi, M., Krasovec, M., Svetic, B., et al. 1996. Prognostic value of cathepsins B, H, L, D and their endogenous inhibitors stefins A and B in head and neck carcinoma. *Biol. Chem. Hoppe Seyler* **377:** 385–390.

Ebert, E., Werle, B., Julke, B., Kopitar-Jerala, N., Kos, J., Lah, T., Abrahamson, M., Spiess, E., and Ebert, W. 1997. Expression of cysteine protease inhibitors stefin A, stefin B, and cystatin C in human lung tumor tissue. *Adv. Exp. Med. Biol.* **421:** 259–265.

Ellman, G.L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82:** 70–77.

Estrada, S., Nycander, M., Hill, N.J., Craven, C.J., Waltho, J.P., and Björk, I. 1998. The role of Gly-4 of human cystatin A (stefin A) in the binding of target proteinases. Characterization by kinetic and equilibrium methods of the interactions of cystatin A Gly-4 mutants with papain, cathepsin B, and cathepsin L. *Biochemistry* **37:** 7551–7560.

Estrada, S., Pavlova, A., and Björk, I. 1999. The contribution of N-terminal region residues of cystatin A (stefin A) to the affinity and kinetics of inhibition of papain, cathepsin B, and cathepsin L. *Biochemistry* **38:** 7339–7345.

Green, G.D., Kembhavi, A.A., Davies, M.E., and Barrett, A.J. 1984. Cystatin-like cysteine proteinase inhibitors from human liver. *Biochem. J.* **218:** 939–946.

Guex, N. and Peitsch, M.C. 1997. Swiss-Model and Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis* **18:** 2714–2723.

Guncar, G., Podobnik, M., Pungercar, J., Strukelj, B., Turk, V., and Turk, D. 1998. Crystal structure of porcine cathepsin H determined at 2.1 Å resolution: Location of the mini-chain C-terminal carboxyl group defines cathepsin H aminopeptidase function. *Structure* **6:** 51–61.

Jerala, R., Trstenjak-Prebanda, M., Kroon-Zitko, L., Lenarcic, B., and Turk, V. 1990. Mutations in the QVVAG region of the cysteine proteinase inhibitor stefin B. *Biol. Chem. Hoppe Seyler* (Suppl.) **371:** 157–160.

Jerala, R., Kroon-Zitko, L., Popovic, T., and Turk, V. 1994. Elongation on the amino-terminal part of stefin B decreases inhibition of cathepsin H. *Eur. J. Biochem.* **224:** 797–802.

Kos, J., Krasowiec, M., Cimerman, N., Nielsen, H.J., Christensen, I.J., and Brunner, N. 2000. Cysteine proteinase inhibitors stefin A, stefin B and cystatin C in sera from patients with colorectal cancer: Relation to prognosis. *Clin. Cancer Res.* **6:** 505–511.

Lenarcic, B., Ritonja, A., Sali, A., Kotnik, M., Turk, V., and Machleidt, W. 1986. Properties and structure of human spleen stefin B—A low molecular weight protein inhibitor of cysteine proteinases. In *Cysteine proteinases and their inhibitors* (ed. V. Turk), pp. 473–487. Walter de Gruyter, Berlin.

Lenarcic, B., Krizaj, I., Zunec, P., and Turk, V. 1996. Differences in specificity for the interactions of stefins A, B and D with cysteine proteinases. *FEBS Lett.* **395:** 113–118.

Leonardi, A., Turk, B., and Turk, V. 1996. Inhibition of bovine cathepsins L and S by stefins and cystatins. *Biol. Chem. Hoppe Seyler* **377:** 319–321.

Lindahl, P., Alriksson, E., Jörnvall, H., and Björk, I. 1988. Interaction of the cysteine proteinase inhibitor chicken cystatin with papain. *Biochemistry* **27:** 5074–5082.

Lindahl, P., Abrahamson, M., and Björk, I. 1992. Interaction of recombinant human cystatin C with the cysteine proteinases papain and actinidin. *Biochem. J.* **281:** 49–55.

Liu, T.-Y. 1977. The role of sulfur in proteins. In *The proteins*, 3rd ed. (eds. H. Neurath and R.L. Hill), Vol. 3, pp. 239–402. Academic Press, New York.

Machleidt, W., Thiele, U., Assfalg-Machleidt, I., Forger, D., and Auerswald, E.A. 1991. Molecular mechanism of inhibition of cysteine proteinases by their protein inhibitors: Kinetic studies with natural and recombinant variants of cystatins and stefins. *Biomed. Biochim. Acta* **50:** 613–620.

Mason, R.W. 1986. Species variants of cathepsin L and their immunological identification. *Biochem. J.* **240:** 285–288.

Musil, D., Zucic, D., Turk, D., Engh, R.A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N., et al. 1991. The refined 2.15 Å X-ray crystal structure of human liver cathepsin B: The structural basis for its specificity. *EMBO J.* **10:** 2321–2330.

Nycander, M., Estrada, S., Mort, J.S., Abrahamson, M., and Björk, I. 1998. Two-step mechanism of inhibition of cathepsin B by cystatin C due to displacement of the proteinase occluding loop. *FEBS Lett.* **422:** 61–64.

Pavlova, A., Krupa, J.C., Mort, J.S., Abrahamson, M., and Björk, I. 2000. Cystatin inhibition of cathepsin B requires dislocation of the proteinase occluding loop. Demonstration by release of loop anchoring through mutation of His110. *FEBS Lett.* **487:** 156–160.

Pennacchio, L.A., Lehesjoki, A.E., Stone, N.E., Willour, V.L., Virtaneva, K., Miao, J.M., D'Amato, E., Ramirez, L., Faham, M., Koskiniemi, M., et al. 1996. Mutations in the gene encoding cystatin B in progressive myoclonus epilepsy (EPM1). *Science* **271:** 1731–1734.

Pennacchio, L.A., Bouley, D.M., Higgins, K.M., Scott, M.P., Noebels, J.L., and Myers, R.M. 1998. Progressive ataxia, myoclonic epilepsy and cerebellar apoptosis in cystatin B-deficient mice. *Nat. Genet.* **20:** 251–258.

Pol, E. and Björk, I. 1999. Importance of the second binding loop and the C-terminal end of cystatin B (stefin B) for inhibition of cysteine proteinases. *Biochemistry* **38:** 10519–10526.

Pol, E., Olsson, S.L., Estrada, S., Prasthofer, T.W., and Björk, I. 1995. Characterization by spectroscopic, kinetic and equilibrium methods of the interaction between recombinant human cystatin A (stefin A) and cysteine proteinases. *Biochem. J.* **311:** 275–282.

Popovic, T., Brzin, J., Kos, J., Lenarcic, B., Machleidt, W., Ritonja, A., Hanada, K., and Turk, V. 1988. A new purification procedure of human kidney cathepsin H, its properties and kinetic data. *Biol. Chem. Hoppe Seyler* (Suppl.) **369:** 175–183.

Stubbs, M.T., Laber, B., Bode, W., Huber, R., Jerala, R., Lenarcic, B., and Turk, V. 1990. The refined 2.4 Å X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: A novel type of proteinase inhibitor interaction. *EMBO J.* **9:** 1939–1947.

Turk, V. and Bode, W. 1991. The cystatins: Protein inhibitors of cysteine proteinases. *FEBS Lett.* **285:** 213–219.

Turk, B., Krizaj, I., and Turk, V. 1992. Isolation and characterization of bovine stefin B. *Biol. Chem. Hoppe Seyler* **373:** 441–446.

Turk, B., Krizaj, I., Kralj, B., Dolenc, I., Popovic, T., Bieth, J.G., and Turk, V. 1993. Bovine stefin C, a new member of the stefin family. *J. Biol. Chem.* **268:** 7323–7329.

Turk, B., Turk, V., and Turk, D. 1997. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol. Chem.* **378:** 141–150.

Turk, D., Guncar, G., Podobnik, M., and Turk, B. 1998. Revised definition of substrate binding sites of papain-like cysteine proteases. *Biol. Chem.* **379:** 137–147.

Turk, B., Turk, D., and Turk, V. 2000. Lysosomal cysteine proteases: More than scavengers. *Biochim. Biophys. Acta* **1477:** 98–111.

Wakamatsu, N., Kominami, E., Takio, K., and Katunuma, N. 1984. Three forms of thiol proteinase inhibitor from rat liver formed depending on the oxidation-reduction state of a sulfhydryl group. *J. Biol. Chem.* **259:** 13832–13838.