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Mechanism of Interaction of the Mammalian Cysteine Protease Inhibitors, Cystatin A and B, with Target Proteases

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Abstract

Human cystatin A was shown to bind rapidly and strongly to papain and cathepsin L, with K_i of 0.2–20 pM and k_{ass} of ~3–5×10^6 $M^{-1}\cdot s^{-1}$, whereas the affinities for actinidin and cathepsins B, C and H were weaker (K_i 1–40 nM). The inhibition of cathepsin B was ~100-fold slower than that of papain, i. e. k_{ass} was ~10^4 $M^{-1}\cdot s^{-1}$. The binding to papain was consistent with a one-step binding mechanism. An N-terminally truncated cystatin A variant had an appreciably reduced affinity for papain, indicating the importance of this region for interaction with cysteine proteases.

Mutations in the second hairpin loop of cystatin B, Leu-73→Gly and His-75→Gly, decreased the affinity for papain and cathepsins L, H and B to an extent suggesting that this region contributes 20-30 % of the binding energy of cystatin B to target enzymes. A mutation in the C-terminal end of cystatin B, Tyr-97→Ala, similarly indicated that this end contributes 6-12 % of the binding energy to papain and cathepsins L and H but is of limited importance for cathepsin B binding. The increased k_{diss} for the binding of the mutants to proteases suggests that the two regions are important for complex stability.

Human and bovine wild-type cystatin B were shown to have indistinguishable inhibitory properties towards cysteine proteases, binding tightly to the endopeptidases, papain and cathepsin L, and more weakly to the exopeptidases, cathepsins H and B. Mutation of the single Cys residue, Cys-3, in cystatin B showed that this residue is involved in the binding of the inhibitor to target enzymes. Cys-3 is most important for cathepsin B binding and for the bovine inhibitor.

Sequential truncation of four residues from the N-terminal end of cystatin B resulted in progressively impaired affinities for papain and cathepsins L, H and B. The highest affinity loss was caused by removal of Cys-3, showing that this residue is most important for the binding. The decreased affinities of the truncated cystatin B mutants for papain and cathepsin H were due mainly to an increased k_{diss}, indicating that the N-terminal region keeps the inhibitor anchored to these enzymes in the complexes.

Keywords: cysteine protease, cysteine protease inhibitor, papain, cathepsin, cystatin, enzyme kinetics, inhibition, recombinant protein, stefin.

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Contents

Appendix

Papers on which this thesis is based

Papers I–IV

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Pol, E., Olsson, S-L., Estrada, S., Prasthofer, T. 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.

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

III. Pol, E. and Björk, I. 2001. Role of the single cysteine residue, Cys-3, of human and bovine cystatin B (stefin B) in the inhibition of cysteine proteinases. *Submitted to Protein Sci.*

IV. Pol. E. and Björk, I. 2001. Contribution of residues in the N-terminal region of cystatin B (stefin B) to inhibition of cysteine proteinases. *Manuscript.*

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Abbreviations

The following abbreviations are used in the text.

k_{ass}	Second-order association rate constant
K_d	Equilibrium dissociation constant
k_{diss}	Dissociation rate constant
K_i	Inhibition constant
k_{obs}	Observed pseudo-first-order rate constant
NMR	Nuclear magnetic resonance

To my parents
Moim rodzicom

Introduction

Enzymes are specialized proteins which act as biological catalysts. They catalyze biochemical reactions, i.e. increase their rate by a factor of 10^4-10^{10}, by lowering the activation energy. An enzyme catalytic site has a specific shape which facilitates binding of a substrate (or substrates), thereby stabilizing the transition state (i. e. an intermediate state of higher energy). Most enzymes have a very high specificity for the substrate and being chiral, they often act only on one enantiomer of it. Many enzymes change shape when substrates bind. This characteristic is called "induced fit", which means that the precise conformation of the enzyme required for catalytic activity can be induced by the binding of the substrate.

Enzymes are classified according to the reactions they catalyze into oxidoreductases, transferases, hydrolases, isomerases, lyases and ligases. Hydrolases catalyze the cleavage of a chemical bond with the addition of water. Hydrolysis of peptide bonds within proteins is catalyzed by a group of hydrolases called proteases, proteinases or proteolytic enzymes. Most proteases can be further divided into serine, cysteine, aspartate and metallo-proteases. This classification is mainly based on the catalytic mechanism used by the enzyme. Proteases can also be grouped according to whether they cleave peptide bonds at the N- or C-terminal end of the substrate proteins (exopeptidases), or whether the substrate is cleaved at an internal peptide sequence (endopeptidases).

Proteases are ubiquitous in every cell and occur in many extracellular fluids of every living organism. They are potentially harmful as they can quickly degrade any protein. Therefore, a careful regulation of protease activity is required. Inappropriate proteolysis is avoided in the first place by the action of various inhibitors, but also by a regulation at the level of transcription or translation of the proteases, expression of proteases as inactive zymogens and their localization in specific cell compartments.

This investigation focuses on the mechanism of inhibition of several cysteine proteases by two mammalian inhibitors, cystatins A and B.

Previous investigations

Cysteine proteases

Cysteine proteases, enzymes that utilize a cysteine residue for their catalytic activity, are divided into six clans (Rawlings and Barrett, 1993, 1994, 1999), viz. clan CA, containing papain and its relatives, clan CB, containing viral chymotrypsin-like cysteine proteases, clan CC, containing viral papain-like cysteine proteases, clan CD, containing caspases, clan CE, containing the adenovirus endopeptidases and clan CX, containing other cysteine proteases. A clan is a group of families that are thought to have a common ancestry. The members are most often recognized by similarities in tertiary structure, but evidence such as the order of catalytic residues in the sequence is also used. Each clan is identified by two capital letters, the first specifying the catalytic type, e. g. C for cysteine proteases, and the second being unique to the clan. Protease clan CA is divided into 28 families, denoted C1A to C26A, C37A and C40A. Each family member shows evolutionary relationship to at least one other member, either throughout the whole sequence or at least in the part of the sequence responsible for the catalytic activity (Rawlings and Barrett, 1993).

Three groups of cysteine proteases are especially well known because of their great biomedical importance. They are: the papain family (family C1A), containing the lysosomal cathepsins B, H, K, L, S and other proteases involved in lysosomal proteolysis and many disease processes (see below); the calpains (family C2A) (Molinari and Carafoli, 1997; Carafoli and Molinari, 1998) that mediate intracellular calcium-dependent proteolysis and are thought to contribute to neuronal cell death in stroke; and caspases (clan CD) that are currently of enormous interest because of their central role in apoptotic cell death (see below). Recently, a new family of legumains, belonging to caspases, clan CD, and denoted C13D, has been discovered. Human legumain has an important role as a key enzyme in the antigen presentation pathway (Manoury et al., 1998).

Cysteine proteases from the papain family

The papain family is the largest among cysteine proteases (Berti and Storer, 1995). Representatives of this family have been found in animals (Rawlings and Barrett, 1994; Turk et al., 1997), protozoa (Serveau et al., 1996; Chagas et al., 1997), yeast (Enenkel et al., 1993), bacteria (Sojar et al., 1993; Sugai et al., 1997; Yamanoto et al., 1997; Ellen et al., 1997; Kuramitsu et al., 1999; Genco et al., 1999; Kadowaki et al., 2000; Gregory and Liu, 2000; Orth et al., 2000) and viruses (Rawlings et al., 1992; Blight et al.,1998; Allsopp and Fazakerley,

2000). Apart from papain (described below), several other family members have been isolated from plants: actinidin (Blocklehurst et al., 1981), ficin (Englund et al., 1968), caricain (Kaarsholm and Schack, 1983; Dubois et al., 1988a,, b), bromelain (Rowan et al., 1990), aleurain (Rogers et al., 1985) and chymopapain (Baines et al., 1986).

Papain

The first discovered cysteine protease, which gave the name to whole papain family and is the prototype of all enzymes of this family, was papain from the latex of the plant, Carica papaya (Balls et al., 1937). Papain is synthesized as a zymogen, propapain (Cohen et al., 1986). The proregion, being a high-affinity papain inhibitor (Taylor et al., 1995), is responsible for proper folding and processing of the protein. The enzyme has been very well characterized (Brocklehurst et al., 1988; Brocklehurst et al., 1987; Storer and Ménard, 1994). The single polypeptide chain of papain contains 212 amino acids (Light et al., 1964), giving a molecular mass of 23 400 (Husain and Lowe 1969). The three-dimensional structure (Kamphuis et al., 1984) shows that the protein consists of two domains, which are separated by a deep active-site cleft (Fig. 1). The main feature of the left (L) domain is a ~30 residue long α-helix, and the right (R) domain is formed by an antiparallel β-barrel motif. These two domains are linked by two antiparallel β-strands at the bottom of the V-shaped cleft. Two residues of importance for the catalytic activity are located in the middle of the active-site cleft, Cys-25 from the L-domain and His-159 from the R-domain (Fig. 1).

Lysosomal cysteine proteases and regulation of their activity

In order to sustain its normal activity, a mammalian cell has to remove incorrectly synthesized proteins, proteins damaged by i.e. oxidation, cell-cycle specific proteins and other signaling proteins which are no longer necessary. A major mechanism of protein degradation is via lysosomes. Lysosomes are acidic vesicles that contain about 50 different enzymes involved in degradation: cathepsins that cleave peptide bonds, phosphatases that remove covalently bound phosphates, nucleases that cleave DNA/RNA, lipases that cleave lipid molecules and carbohydrate-cleaving enzymes that remove covalently bound sugar from glycoproteins. Protein degradation of damaged or obsolete proteins can also be carried out in a ubiquitin-dependent process by specific proteases in a large cytosolic complex called the ubiquitin/26S proteasome system.

Most lysosomal cathepsins are cysteine proteases, although serine or aspartic proteases also occur. The lysosomal cysteine proteases, being members of the papain family, share similar sequences and folds (Turk et al., 1998) (Fig. 2).

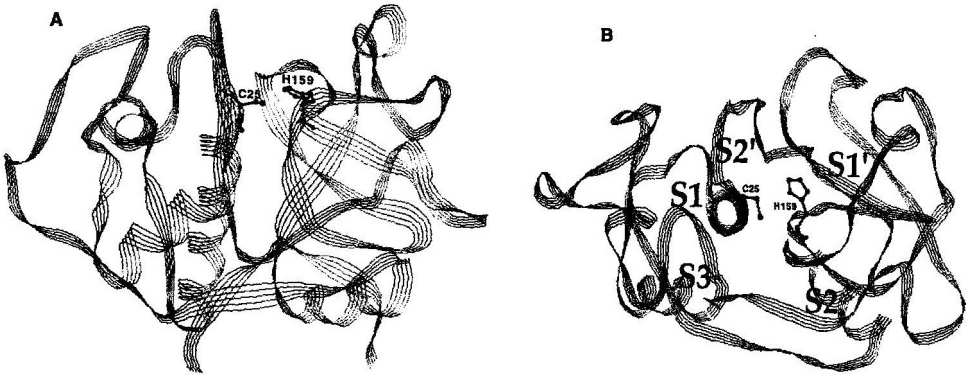

Fig. 1. The main chain of the papain structure seen in the standard view along the active-site cleft, *A*, and from the top, *B*. The side chains of the catalytically important residues, Cys-25 and His-159, are included. The positions of the substrate binding subsites, S3 to S1 and S1' to S2, are marked in the top (*B*) view of the molecule.

The sequences of 11 of them, cathepsins B, H, L, S, C (also referred to as dipeptidyl peptidase I or cathepsin J), K, O, F, V, X and W, are known (Kirschke et al., 1995; Turk et al., 1997; Chapman et al., 1997; Musil et al., 1991; Turk et al., 2000) The complete nucleotide sequences of the placenta-specific cathepsins M (Sol-Church et al., 2000c), R (Sol-Church et al., 2000a), Q (Sol-Church et al., 2000b) and P (Pungercar, Ivanovski, 2000; Pungercar et al., 2000) have also recently been determined. Cathepsins T and N have been described, but no sequence information is as yet available (Gohda and Pitot, 1981; Ducastaing and Etherington, 1978). The molecular weight of these enzymes varies from 20 000 to 35 000 Da, with the exception of cathepsin C, which is tetrameric and has an Mr of ~ 200 000 (Dolenc et al., 1995). Most of them are endopeptidases, except cathepsins B, H, and X, which also exhibit exopeptidase activity. Thus, cathepsin B is a dipeptidyl carboxypeptidase, cathepsin H is an aminopeptidase and cathepsin X can act as either a carboxypeptidase or a carboxydipeptidase (Turk et al., 2000). Cathepsin C, being purely a dipeptidyl aminopeptidase, is the only known strict exopeptidase of the papain family. The crystal structures of cathepsins B and H show that these enzymes differ from other papain-like proteases in having additional amino acids sequences inserted. These sequences have been proved to be responsible for the exopeptidase activity of these enzymes (Illy et al., 1997; Guncar et al., 1998). A loop of about 20 residues (the "occluding loop") partially blocks the active-site cleft of cathepsin B (Fig. 2), and in the case of cathepsin H an octapeptide (the "minichain") is attached by a

disulfide bond and binds in the active-site cleft in the substrate-binding direction.

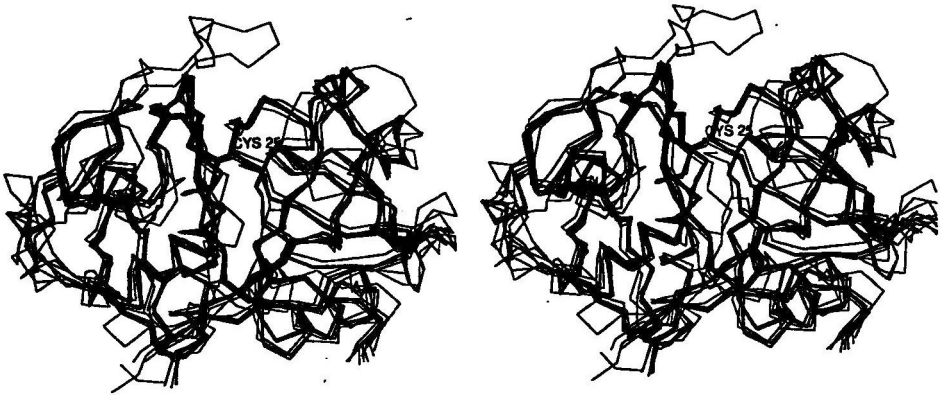

Fig. 2. Structural similarities of papain-like cysteine proteases. The backbones of nine enzymes: papain, cathepsin B, cathepsin H, actinidin, chymopapain, cruzain, interleukin 4, glycyl endopeptidase, and caracain are superimposed on each other. (Reprinted, with permission, from Turk et al., (1998). Copyright 1998, Walter de Gruyter & Co, Berlin-New York).

Lysosomal proteases are synthesized on membrane-bound ribosomes in the form of pre-proenzymes and are directed to the rough endoplasmatic reticulum (rER). The prepeptide, which acts as a signal sequence for translocation, is removed during the passage into the lumen of the rER. The proenzyme (zymogen or inactive precursor) is subjected to posttranslational glycosylation and formation of disulfides. The propeptide is responsible for the proper folding (Tao et al., 1994) and inhibition of the enzyme by restricting access to the active site (Cygler and Mort, 1997). Transfer vesicles transport the zymogen into the Golgi apparatus, where the mannose residues in the oligosaccharide side chains are phosphorylated to form mannose-6-phosphate, which enables the proenzyme to bind to mannose-6-phosphate-receptors located in the membranes of the Golgi network (Gacko et al., 1997). The receptors are responsible for the translocation to lysosomes, where the proenzyme-receptor complex dissociates due to the low pH. The proteolytic processing to the mature, active enzymes occurs in late endosomes or lysosomes under reducing and acidic conditions by the removal of the propeptide and dephosphorylation of the mannose residues (Nishimura and Kato, 1987; Kominami et al., 1988; Kirschke et al., 1995). The propeptide appears mainly to be cleaved off autocatalytically, although cleavage by other cathepsins may also occur.

The activity of lysosomal cysteine proteases outside lysosomes is regulated by physiological pH (Kirschke et al., 1989; Cole et al., 1989; Turk et al., 1993a; Twining, 1994; Turk et al., 1995; Stoka et al., 1995; Brömme et al., 1996; Wang et al., 1998;), redox potential (Chapman et al., 1997) and natural inhibitors, such as cystatins (discussed below), thyropins (Lenarcic et al., 1998) and α_2-macroglobulin (Mason, 1989). Most cathepsins are unstable and poorly active at neutral pH, and, in addition, the cysteine residue of the active site is readily oxidated. Therefore, a reducing and acidic environment is required for the optimal activity of cysteine proteases. The proteases that escape from lysosomes are inhibited by cysteine protease inhibitors, present intra- and extracellularly in high stoichiometric excess over the enzymes.

Substrate binding and mechanism of proteolysis

The substrate binds along the active-site cleft of papain-like cysteine proteases in an extended manner, interacting with both domains of the enzyme (Fig. 1). The binding site has been divided into seven subsites, since the protease was found to make contacts with seven residues of the protein substrate (Schechter and Berger, 1967; Del Nery et al., 1997). These seven residues are denoted, from the N-terminus, P4 to P1 and P1' to P3' in the direction towards the C-terminus. The cleavage occurs between the P1 and P1' residues. The corresponding enzyme subsites are called S4 – S1 and S1' – S3', respectively. However, according to the latest revision (Turk et al., 1998), only three subsites, S2, S1 and S1', which interact with the main chain as well as the side chains of the substrate, are well-defined. The S3 and S2' subsites, which are relatively large, involve solely contacts with side chains of the substrate and are therefore more uncertain (Fig. 1). However, these subsites are well-defined in cathepsin B. The existence of the S4 and S3' subsites is even more questionable.

A general mechanism of hydrolysis of peptide bonds by papain-like cysteine proteases was proposed by Storer and Ménard (1994), although it was not fully explained at the atomic level. The thiolate-imidazonium ion pair formed by Cys-25 and His-159 (papain numbering) of the enzyme (Fig. 1) is involved in this process. The mechanism can be described in the following manner (Fig. 3):

(1) The reaction starts with a nucleophilic attack of the thiolate ion of Cys-25 on the carbonyl carbon of the peptide bond. The imidazonium ion donates simultaneously a hydrogen ion to the nitrogen atom of the same peptide bond.

(2) The peptide bond is broken, a thiolester bond is formed between the N-terminal portion of the substrate (the R1 peptide) and the active-site cysteine, and the C-terminal portion of the substrate (the R2 peptide) dissociates from the active-site cleft.

Steps (1) and (2) are called acylation, since an acyl (thiolester) bond is formed.

(3) A second nucleophilic attack of a water molecule on the carbonyl carbon of the thiolester bond takes place. At the same time, the water molecule donates a hydrogen ion to the nitrogen atom of His-159.

(4) The thiolester bond is broken, the thiolate-imidazonium ion pair is regenerated and the R1 peptide dissociates from the active site-cleft of the enzyme.

Steps (3) and (4) are called deacylation.

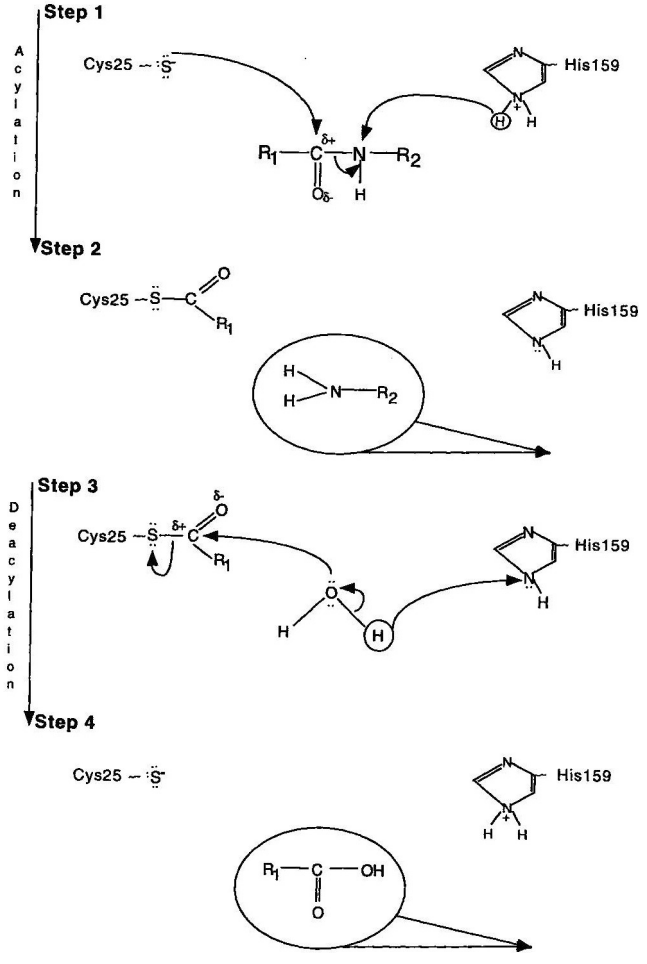

Fig. 3. A scheme of the hydrolysis of a peptide bond by papain-like cysteine proteases, consisting of the acylation (steps 1 – 2) and deacylation (steps 3 – 4) reactions.

Physiological role and involvement in diseases

Lysosomal cysteine proteases are found in a wide variety of cells, which indicates a general "housekeeping" function, consisting in a nonselective degradation of proteins inside the lysosomes (Bohley and Seglen, 1992; Chapman et al., 1994). Cathepsins B and L attain the highest, ~1 mM, lysosomal concentration (Xing et al., 1998), and cathepsin L is also the most efficient enzyme (Maciewicz et al., 1987; Mason, 1989). Apart from their function inside lysosomes, cathepsins are also involved in protein breakdown (Paris et al., 1995) and protein processing outside lysosomes, such as activation of renin (Wang et al., 1991; Neves et al., 1996; Jutras and Reudelhuber, 1999) and degradation of thyroglobulin (Yoshinari and Taurog, 1985; Dunn et al., 1996; Tepel et al., 2000).

A number of cathepsins have been shown to be tissue-specific, which provides evidence for a more specialized role of these proteases. Thus, cathepsin K is expressed in osteoclasts (Drake et al., 1996; Gelb et al., 1996; Saftig et al., 1998; Kafienah et al., 1998), cathepsin O in cancer tissue (Velasco et al., 1994), cathepsin V in thymus and testis (Brömme et al., 1999), cathepsins C, P, R, Q and M in placenta (Tisljar et al., 1999; Sol-Church et al., 2000a, b, c; Pungercar and Ivanovski, 2000; Pungercar et al., 2000), cathepsin W in CD8+ cytotoxic T lymphocytes and natural killer cells (Linnevers et al., 1997; Wex et al., 1998) and cathepsin N in lymph nodes and spleen (Maciewicz and Etherington, 1988). Moreover, cathepsin S is expressed in spleen, lung, liver, placenta, and antigen-presenting cells, including B lymphocytes and macrophages (Kirschke et al., 1989; Shi et al., 1992, 1994; Petanceska and Devi, 1992; McDonald et al., 1993; Kirschke and Wiederanders, 1994; Hall et al., 1998; Pierre and Mellman, 1998; Lecaille et al., 1999).

Cathepsin K, which efficiently degrades type I collagen and insoluble collagen of adult bone, is responsible for bone remodeling (Drake et al., 1996; Inui et al., 1997), and its deficiency leads to severe bone abnormalities, e.g. pycnodysostosis (Gelb et al., 1996; Saftig et al., 1998, 2000). Recently, increased levels of cysteine proteases, including cathepsin K, were found in the spleen and plasma of patients with the lysosomal glycolipid storage disease, Gaucher's disease, indicating the involvement of cathepsin K in the lytic bone lesions that accompany this disease (Moran et al., 2000).

Several studies indicate that cathepsins B, D, F, L, S and V participate in the MHC (major histocompatibility complex) class II antigen presentation pathway (Mizuochi et al., 1994; Authier et al., 1996; Chapman et al., 1997; Villadangos et al., 1997; Deussing et al., 1998; Reise et al., 1998; Driessen et al., 1999; Villadangos et al., 1999; Shi et al., 2000). In this pathway, the foreign, antigenic protein is taken up in e.g. B-lymphocytes by endocytosis

and is delivered to an endosome, where it is digested by cathepsins. The resulting peptides form complexes with class II MHC proteins. These proteins consist of two chains, α and β, which together form a peptide-binding domain, the most variable region of the protein. However, immediate binding of the peptides to the α,β-dimers would give unstable and readily aggregating complexes. Moreover, undesired binding of endogenous peptides to the dimers must be prevented. Therefore, a new class II molecule, the invariant chain (Ii) (Jasanoff et al., 1998), is needed. The Ii-chain is believed to act as a chaperonin to the MHC class II α,β-heterodimers, blocking binding of peptides to the dimers as these pass through the endoplasmatic reticulum and Golgi apparatus (Teyton et al., 1994). Ii-trimers associate with three α,β-dimers, forming a nonamer. This nonamer is ultimately processed by cathepsins, cleaving the Ii-chains to yield free class II MHC protein, which then binds the foreign, antigenic peptide fragment. The resulting complex is transported to the cell surface, where the peptide can be recognized by helper T cells, leading to antibody production (Cresswell, 1998; Pierre and Mellman, 1998). Consequently, cysteine proteases are involved in two critical steps of antigen presentation, the generation of antigenic peptides and the degradation of the invariant chain, removing this chain from the binding cleft of the class II MHC protein.

An involvement of cathepsins B and C in apoptosis has been reported (Roberts et al., 1997; Guicciardi et al., 2000; Nishio et al., 2000; Ishisaka et al., 1999, 2001). Apoptosis is a form of programmed cell death in which damaged, infected and potentially dangerous cells are eliminated (Cohen, 1999). The central role in this mechanism is played by caspases, which are cysteine proteases cleaving at aspartate residues. Caspases act as the main destructive engine of apoptosis, being activated in an amplified, cascade-like manner (Thornberry, 1997). They remain in zymogen form until the apoptotic stimuli are transmitted (Salvesen, 1997), Pre-caspases can be processed by autocatalytic activation, other caspases, death receptor signalling, a mitochondrial pathway and by the action of other proteases, such as cathepsins B and C (Wolf and Green, 1999). The cathepsins are believed to be able to activate caspases either directly or indirectly through a serine protease, granzyme B (Thornberry, 1997; Salvesen, 1997; Pham and Ley, 1999), although the mechanism of this process is still unclear.

Cathepsins can be very harmful to the environment outside lysosomes, and appear for this reason to be involved in several diseases, such as inflammations and cancer (see below) rheumatoid arthritis and osteoarthritis (Mort et al., 1984, 1998; Kostoulas et al., 1999; Lang et al., 2000), Alzheimer's disease (Cataldo and Nixon, 1990; Bernstein and Wiederanders, 1994), multiple sclerosis (Bever and Garver, 1995) and muscular dystrophy (Takeda et al., 1992).

Neutrophils and mononuclear phagocytes (monocytes and macrophages) are the major kinds of inflammatory cells. Neutrophils usually predominate in the early cellular response to an inflammatory stimulus, whereas mononuclear phagocytes are referred to as the second-line defense. The major functions of these cells are phagocytosis and secretion of inflammatory substances. The purpose of phagocytosis is to destroy and remove an inflammatory stimulus. The cellular changes accompanying the phagocytic process lead to final killing and degradation of the ingested particle. Cathepsins and other hydrolytic enzymes are responsible for these effects. However, the phagocyte can be destroyed during the inflammatory reaction, causing the release of lysosomal enzymes into the environment, which results in significant damage of local tissue. The release of cathepsin B from phagocytes in association with inflammation has thus been shown to contribute to multiple organ failure in humans (Jochum, 1995, Jochum et al., 1999). Moreover, periodontal inflammatory diseases, which affect the tissue that support the teeth, have been associated with high levels of cathepsins B, H and L in gingival tissues and gingival crevical fluids (Cox and Eley, 1998; 1989; Kunimatsu et al., 1990; Eley and Cox, 1998). Accelerated collagen and elastin degradation at sites of inflammation in diseases such as atherosclerosis and emphysema may also be an effect of cathepsin activity (Chapman et al., 1997).

Cancer progression has been linked to increased activities of cathepsins B, D, H and L, which in an uncontrolled manner degrade biological barriers and the extracellular matrix and participate in processing of other proteases (Kos and Lah, 1998, Lah and Kos, 1998; Lah et al., 1989, 2000). The activities of cathepsin B and L in plasma-membrane fractions of several human tumors were shown to be considerably higher, compared with normal tissue, (Rozhin et al., 1989; Sloane et al., 1990; Sloane, 1990; Buck et al., 1992). Moreover, the levels of cathepsin B and L mRNA were highly increased in many of the cancer tissues that were examined (Chauhan et al., 1991; Buck et al., 1992). Basement membrane was shown to be degraded by cathepsins from cancer cells with simultaneous liberation of collagen IV, laminin and fibronectin (Lah et al., 1989; Guinec et al., 1992). In addition to cysteine proteases, the aspartate protease, cathepsin D, was found to be overexpressed in breast cancer cells (Rochefort et al., 2000). Since these proteases appear to be directly involved in tumor spreading, they have the potential to be prognostic markers in cancer.

Cysteine proteases from microorganisms

Many cysteine proteases have been detected in virus, bacteria and protozoa. Such proteases from poliovirus and rhinovirus type I are needed for proteolytic cleavage of precursor proteins for virus replication and for the production of new virus particles (Kay and Dunn, 1990; Stanway, 1990) (Korant et al., 1985; Korant et al., 1986). Moreover, the expression of HIV-I proteins appears to be regulated by cysteine protease-mediated proteolytic processing (Guy et al., 1991). Bacterial cysteine proteases from *Staphylococcus aureus* are believed to cause autoimmune diseases (Kapur et al., 1993; Ohara-Nemoto et al., 1994) and a pathological reaction due to their ability to degrade elastin (Potempa et al., 1988). Clostripain, a heterodimeric cysteine endopeptidase from *Clostridium histolyticum* (Dargatz et al., 1993), is thought to facilitate host invasion and participate in the digestion of host protein to give nutrients. The bacterium *Porphyromonas gingivalis* is involved in peridontitis, characterized by bleeding tendency and inflammation of gingival tissue, leading to loss of teeth. Their cysteine proteases, gingipains, are capable of cleaving clotting factor X, fibrinogen, immunoglobulins and complement factors, exacerbating the inflammation (Tokuda et al., 1998). The YopJ family members, found in Yersinia species (such as Yersinia pestis causing the Black Death in the Middle Ages), have been shown to act as cysteine proteases with ubiquitin-like molecules as substrates. They were found to alter the signalling system of the host and to prevent the host immune response by disrupting the posttranslational modification of proteins which need ubiquitin-like molecules for their activity (Orth et al., 2000). Parasites, such as Trypanosoma cruzi (Cazzulo et al., 1989, 1990), Leishmania (Mottram et al., 1997; Alves et al., 2000), Schistosoma (Klinkert et al., 1994; Dalton et al., 1996; Hola-Jamriska et al., 1998; Brady et al., 1999a, b, 2000), Ostertagia ostertagi (Pratt et al., 1992) and Plasmodium falciparum (causing malaria) (Rosenthal et al., 1988, 1994, 1996), also produce cysteine proteases, which aid in penetration of host tissue, metabolize host proteins and degrade molecules from the host immune system.

Protein inhibitors of papain-like cysteine proteases

The first discovered and sequenced inhibitor of papain-like cysteine proteases was isolated from chicken egg white and was given the name cystatin (Fossum and Whitaker, 1968; Sen and Whitaker, 1973; Keilowa and Tomasek, 1977; Barrett, 1981; Anastasi et al., 1983). A large number of related proteins have since been discovered. With respect to sequence similarity and inhibitory properties, they comprise a new group of proteins, the cystatin superfamily. On the basis of sequence homology, this superfamily has been divided into three families, stefins, cystatins and kininogens (Turk et al., 1986; Barrett et al.,

1986a, b; Turk and Bode, 1991), members of which are primarily found in mammals and higher vertebrates.

Family I (stefins)

The members of family I consist of a single polypeptide chain containing ~100 amino acid residues, giving an Mr of ~11 000. They lack disulfide bonds and carbohydrates. Stefins discovered thus far include mammalian cystatins A and B (also called stefins A and B), bovine stefin C and pig stefin D. All of them are primarily intracellular proteins, although their presence in extracellular fluids has also been reported (Abrahamson et al., 1986). Cystatin A occurs in epithelial cells and polymorphonuclear leukocytes (Brzin et al., 1983; Green et al., 1984; Järvinen et al., 1987), which indicates a defensive role against cysteine proteases which come from pathogens and parasites during invasion of the organism. By contrast, cystatin B is more evenly distributed amongst cells and tissues (Davies and Barrett, 1984; Katunuma and Kominami, 1985; Lenarcic et al., 1986; Barrett et al., 1986b; Henskens et al., 1996), which suggests a general protective function against uncontrolled activities of the host's own lysosomal cysteine proteases. Cystatin A has been isolated from human (Green et al., 1984; Abrahamson et al., 1986), bovine (Turk et al., 1995) and rat (Takeda et al., 1985), and cystatin B from bovine (Turk et al., 1992), sheep (Ritonja et al., 1996), porcine (Lenarcic et al., 1996) and rat (Hirado et al., 1981) origin. The rat forms of cystatins A and B are also called cystatins α and β, respectively. Stefin C from bovine thymus (Turk et al., 1993b) and stefin D from pig polymorphonuclear leukocytes (Lenarcic et al., 1993) exhibit a high sequence homology with bovine cystatin B and human cystatin A, respectively, and are therefore included in this family.

Family II (cystatins)

Cystatins belonging to family II consist of a somewhat longer polypeptide chain, containing ~120 amino acid residues, giving an Mr ranging from 13 000 to 14 000, and possess two disulfide bonds. They are in general nonglycosylated, although rat and mouse cystatin C, human cystatin E/M and cystatin F were found to have N-linked carbohydrates (Solem et al., 1990; Esnard et al., 1992; Ni et al., 1997, 1998). Family II cystatins are primarily extracellular inhibitors present in many biological fluids at rather high concentrations (Abrahamson, 1996). They are expressed as pre-forms that include a signal peptide, which targets the proteins to the extracellular secretory system (Abrahamson et al., 1987). Human cystatin C and its avian analogue, chicken cystatin, are the most investigated members of the family (Barrett et al., 1986b; Turk and Bode, 1991; Abrahamson, 1994; Henskens et al., 1996). Other mammalian counterparts of this inhibitor have been identified in cattle (Hirado et al., 1985; Olsson et al., 1997), mouse (Solem et

al., 1990), rat (Esnard et al., 1992), dog (Poulik et al., 1981), sheep (Tu et al., 1990) and monkey (Grubb and Löfberg, 1982). Cystatin C is a widespread protein, found in blood plasma, cerebrospinal fluid, saliva, urine and even intracellularly (Grubb and Löfberg, 1982, 1985; Möller et al., 1985). Its distribution suggests a regulatory and defensive role against endogenous or exogenous cysteine proteases present in body fluids. Other mammalian proteins of family II are cystatin D present in saliva and tears (Freije et al., 1991, 1993; Balbín et al., 1994; Collins and Grubb, 1998) and cystatins S, SN and SA found in saliva, tears and seminal plasma (Isemura et al., 1984a, b, 1986, 1987, 1991). The more restricted distribution of these cystatins probably reflects a specific role in the protection of the oral cavity and the eyes from cysteine proteases coming from viruses and bacteria, as well as from inflammatory cells of the host.

Two glycosylated cystatins, E/M and F, show a very low sequence similarity with other members of the cystatin superfamily. Nevertheless, they structurally and functionally resemble cystatins of family II. Human cystatin E/M is predominantly expressed in uterus and liver (Ni et al., 1997; Sotiropoulou et al., 1997), whereas cystatin F is found in spleen and peripheral blood leukocytes (Halfon et al., 1998; Ni et al., 1998).

Family III (kininogens)

Kininogens are by far the most complex molecules among cystatins. They are single-chain glycosylated proteins, containing three cystatin-resembling domains, numbered 1 to 3 from the N-terminus. The three domains together are called the heavy chain. Domains 2 and 3 inhibit papain-like cysteine proteases and domain 2 also inhibits calpains (Salvesen et al., 1986). Kininogens are divided into three distinct types: high molecular weight kininogen (H-kininogen) with an Mr of ~120 000, low molecular kininogen (L-kininogen) with an Mr of ~68 000, and T-kininogen (found in rats and also called major acute phase protein or thiostatin) with an Mr of ~68 000 (DeLa Cadena and Colman, 1991). The region of the kininogens C-terminal to the heavy chain contains an additional short peptide sequence, kinin, which is related to neither of the cystatin families and can be released from H or L-kininogen by kallikrein cleavage (Müller-Esterl et al., 1986) and from T-kininogen (Esnard and Gauthier, 1983) by the action of cathepsin D (Sakamoto and Nishikaze, 1979; Okamoto et al., 1983). This proteolytic cleavage converts the single-chain kininogens to two-chain forms. The region adjacent to the kinin fragment on the C-terminal side is called the light chain. The light chain is significantly longer in H-kininogen than in L-kininogen, although the heavy chains of both kininogens are highly similar (Takagaki et al., 1985; Kellerman et al., 1986). Before their identification as cysteine protease inhibitors, kininogens were known as precursors of vasoactive kinin

(Müller-Esterl et al., 1986) and H-kininogen also from its involvement in the contact phase of the intrinsic blood coagulation cascade (Kato et al., 1981; Müller-Esterl et al., 1986). Other work has shown that kininogens participate in the acute phase response (Esnard and Gauthier, 1983; Cole et al., 1985), inflammation (Okamoto et al., 1983) and binding of ferritin (Torti and Torti, 1998). Kininogens are synthesized in the liver and subsequently secreted into blood plasma and synovial fluid. They are the major inhibitors of papain-like cysteine proteases in blood plasma and have an appreciably higher concentration in plasma than in tissues (Okhubo et al., 1984; Müller-Esterl et al., 1985; Seuyoshi et al., 1985).

Other cystatins

Many proteins related to the families presented above, although with varying degrees of sequence similarity, have been discovered. To the inhibitorily active cystatins belong oryzacystatins I and II from rice (Abe et al., 1988; Kondo et al., 1989, 1990, 1991) and other plant cystatins found in corn (Abe et al., 1992), potato (Waldron et al., 1993), soybean (Misaka et al., 1996), avocado (Kimura et al., 1995), papaya (Song et al., 1995), and African berry (Murzin, 1993). Many inhibitors have been discovered in lower organisms, e. g. African puff adder (bitis arietans) (Ritonja et al., 1987; Evans and Barrett, 1987), taiwan cobra (Naja naja atra) (Brillard-Bourdet et al., 1998), chum salmon (Koide and Noso, 1994), carp (Tsai et al., 1996), horseshoe crab (Agarwala et al., 1996), flesh fly larvae (Suzuki and Natori, 1985; Saito et al., 1989), Drosophila melanogaster (Delbridge and Kelly, 1990) and parasitic protozoa (Irvine et al., 1992). These findings indicate a wide distribution of cystatin-like cysteine protease inhibitors in nature. The fetuins, the histidine-rich glycoproteins (HRG) and the cystatin-related proteins (CRPs) reviewed in Brown and Dziegielewska, (1997), all have sequence homologies with cystatins, which indicates a common ancestor. However, these proteins are not inhibitors of cysteine proteases.

The role of cystatins in physiological and pathological processes

Cystatins appear to control the activity of cysteine proteases by inhibition of enzymes accidentally released within the host or coming from external aggressors, such as virus, bacteria and protozoa. An inability of cystatins to inhibit such enzymes would thus be expected to result in various pathological conditions. The physiological role of cystatins has been investigated by two experiments in which cystatin genes were knocked out in mice. Mice lacking cystatin B were shown to develop myoclonic seizures and ataxia (Pennacchio et al., 1998), most likely as result of a loss of cerebellar granule cells due to increased apoptosis. Thus, cystatin B appears to prevent cerebellar apoptosis in mice, although the target enzyme is unknown. The symptoms are similar to

those seen in the human neurological disorder known as Unverricht-Lundborg disease (see below), By contrast, cystatin C-deficient mice were born and grew normally, consistent with this inhibitor not being essential for normal life. The only abnormality detected was that injected melanoma cells formed fewer metastatic lung colonies in the deficient mice than in wild-type mice, indicating that metastatic cells in lung can be counteracted by the activity of cysteine proteases (Huh et al., 1999). In addition, the physiological importance of cystatins has been deduced indirectly from their distribution in cells, tissues and extracellular fluids, by measurements of their activity in cysteine protease-rich locations and by observations of their behavior in vitro. An essential protective role of cystatins is also indicated by studies of a number of pathological conditions in which the inhibitors appear to be involved.

Increased levels of cystatins have been demonstrated in periodontal inflammatory diseases (Cohen et al., 1989, 1990; Hall et al., 1992; Naito et al., 1992; Henskens et al., 1993; Alves et al., 1994), inflammatory skin diseases (Hopsu-Havu et al., 1983a, b; Järvinen et al., 1987) and rheumatoid arthritis (Lenarcic et al., 1988). The higher concentration of cystatins observed under these conditions appears to balance the increased activity of cysteine proteases, consistent with a protective effect. By contrast, the activity of cystatin C in inflammatory lung disease has been found to be lower than in non-inflammed tissue (Buttle et al., 1990).

Recently, attention has been paid to the role of cystatins in tumor metastasis. In general, the increased levels of cysteine proteases in malignant tissues do not appear to be balanced by cystatins (Sloane and Honn, 1984; Sloane et al., 1990a,, b; Kane and Gottesman, 1990; Ebert et al., 1997). However, the studies available to date have given contradictory results as to whether the activity of cystatins in malignant tumors is lower than, similar to or higher than in normal tissue (reviewed in Calkins and Sloane, 1995; Ebert et al., 1997; Shiraishi et al., 1998). The concentration of cystatin A in breast cancer tissue has been shown to be increased to highly variable extents, although patients with lower increases in cystatin A levels have a higher mortality risk (Lah et al., 1992; Ebert et al., 1997). Similarly, in head and neck cancer, patients with lower tumor levels of cystatin A have a significantly shorter survival time (Budihna et al., 1996; Smid et al., 1997). Lower concentrations of cystatins A and B were also found to be associated with a shorter survival time in laryngeal cancer (Smid et al., 1997) Contrary to these observations, however, a recent examination of a large number of patients with colorectal cancer and healthy reference persons showed that high levels of cystatin B and cystatin C were correlated with decreased survival of the patients, although cystatin A was not a comparable prognostic factor (Kos et al., 2000). It is clear that further studies of larger patient populations are needed to verify the initial data on the

prognostic significance of tumors levels of cystatins, as well as of cysteine proteases, for different cancer forms.

Two hereditary diseases in humans are linked to cystatins. One of these, progressive myoclonus epilepsy of the Unverricht-Lundborg type, is an autosomal, recessive disorder manifested by mental retardation and cerebellar ataxia (Norio and Koskiniemi, 1979). The age at onset of this rare disease, occurring mostly in Finland and the Mediterranean (Norio and Koskiniemi, 1979; Genton et al., 1990), is between 6 and 13 years. It has been discovered that mutation in the gene for cystatin B, located on chromosome 21q22.3, is responsible for this form of epilepsy (Pennacchio et al., 1996). One of a total of six reported nucleotide changes in the cystatin B gene or a dodecamer repeat expansion in the 5' flanking region of the gene have been found in patients with this disease (Lalioti et al., 1997a, b, c; Virtaneva et al., 1997; Lafreniere et al., 1997). These mutations give rise to either decreased levels of cystatin B mRNA, an abnormal splicing of the mRNA or expression of an inactive cystatin B. The resulting decreased levels of functional cystatin B presumably cause the disease, as suggested by the symptoms shown by the cystatin B knock-out mice.

The second cystatin-related genetic disorder is hereditary cystatin C amyloid angiopathy (HCCAA). HCCAA is an autosomal, dominantly inherited disease, which leads to amyloidosis, dementia and fatal brain haemorrhage, resulting in death of patients, generally before the age of 40 years (Gudmundsson et al., 1972; Jensson et al., 1990). The disease has been found in nine Icelandic families (Jensson et al., 1990; Jonsdottir and Palsdottir, 1993) and in an American patient of Croatic and British origin (Graffagnino et al., 1995). Cystatin C extracted from HCCAA patients' amyloid deposits lacks the first 10 amino acids residues (Cohen et al., 1983) and has an amino acid substitution, Leu-68→Gln (Grubb and Löfberg, 1982; Ghiso et al., 1986a, b; Abrahamson et al., 1987). This mutation produces a less stable protein that easily dimerizes (Abrahamson and Grubb, 1994) and is deposited as amyloid (Löfberg et al., 1987; Sveinbjörnsdóttir et al., 1996). This deposition most likely is the cause of the increased fragility of the brain vessels.

Several studies have indicated that cystatins participate in host protection against infections. The replication of herpes simplex type 1 virus was reported to be inhibited by salivary cystatins (Gu et al., 1995), cystatin C (Björck et al., 1990) and oryzacystatin (Aoki et al., 1995), whereas corona virus replication was arrested by cystatins C and D (Collins and Grubb, 1998). In addition, HIV-I virus can possibly be inhibited by salivary cystatins (Malamud and Friedman, 1993). Cystatins also appear to play a role in the inhibition of cysteine proteases from bacteria that penetrate normal tissue. Rat cystatins S and A were thus found to inhibit the growth of *Porphyromonas gingivalis*

(Naito et al., 1995) and *Staphylococcus aureus* (Takahashi et al., 1994), respectively. Moreover, cysteine proteases from parasites have also been shown to be inhibited by cystatins (Stoka et al., 1995; Chagas et al., 1997; Troeberg et al., 1996).

Mechanism of interaction of cystatins with cysteine proteases

Nature of inhibition

Early investigations revealed that cystatins form equimolar, tight and reversible complexes with cysteine proteases (Anastasi et al., 1983; Nicklin and Barrett, 1984; Green et al., 1984; Abrahamson et al., 1987; Lindahl et al., 1988). Both active and inactive proteases were shown to be inhibited by cystatins, which precludes formation of covalent bonds between inhibitor and enzyme (Anastasi et al., 1983; Björk and Ylinenjärvi, 1989; Lindahl et al., 1992). However, the reversibility and dissociation equilibrium constants of some extremely tight interactions were difficult to establish by equilibrium methods (Nicklin and Barrett, 1984; Abrahamson et al., 1986; Machleidt et al., 1986; Salvesen et al., 1986; Turk et al., 1993b). This problem was solved by the separate measurements of association and dissociation rate constants. Interactions studied by this method were those of chicken cystatin with ficin, chymopapain A or actinidin (Björk and Ylinenjärvi, 1990), of cystatin C with papain or actinidin (Lindahl et al., 1992) and of bovine cystatin B with cathepsin S (Turk et al., 1994). Dissociation equilibrium constants as low as 10 fM to 10 pM for the binding of cystatins to papain, ficin and cathepsin S were possible to determine, whereas the affinity of cystatin C or chicken cystatin for actinidin was found to be ~1000-fold lower.

Comparison of amino acid sequences of known cystatins (Barrett, 1986; Barrett et al., 1986a, b) showed that the Gly-9 residue in the N-terminal end and a centrally located sequence, Gln-Val-Val-Ala-Gly (QVVAG), were highly evolutionarily conserved, making them good candidates for protease binding regions. Several experiments confirmed this conclusion. Chicken cystatin, cystatin C, rat cystatin A and cystatin S lacking their N-terminal regions had much lower affinity for the cysteine proteases studied (Takeda et al., 1985; Isemura et al., 1986; Abrahamson et al., 1987). Additional data on the binding of a short synthetic peptide, mimicking the Gln-Val-Val-Ala-Gly segment, to papain and cathepsin B (Teno et al., 1987) provided further support for this hypothesis.

Analyses of the three-dimensional structures of cystatins have been essential in elucidating the nature of the inhibition. The crystal structures of chicken

cystatin, the first structure to be determined (Bode et al., 1988) and of cystatin B, determined subsequently in complex with papain (Stubbs et al., 1990), showed that the molecules consist of a five-stranded antiparallel β-pleated sheet and a centrally located five-turn α-helix (Fig. 4 A, C). Strands of the β-sheet are pairwise connected by hairpin loops, strands 2 and 3 being joined by the "first hairpin loop" and strands 4 and 5 by the "second hairpin loop". The evolutionarily conserved Gln-Val-Val-Ala-Gly sequence is located in the first hairpin loop. The C-terminal region of cystatin B contains a 9-residue extension, compared with chicken cystatin, which runs along the opposite side of the β-sheet than the α-helix. Another difference is an appended segment of partial α-helical structure in chicken cystatin, which is absent in cystatin B. Since the crystal structure of chicken cystatin was determined for a truncated form, starting with the ninth residue, the structure of the N-terminal region was not known. The later determined NMR solution structure of chicken cystatin (Dieckmann et al., 1993) (Fig. 4 B) revealed that this region, from residue 1 to 9, is completely flexible in solution. Moreover, in the solution structure, the appended helix was not detected, and the position of the first hairpin-loop region differed slightly from that in the crystal structure (Fig. 4 B). Conclusions from NMR studies of cystatin C (Ekiel et al., 1997), together with the observed homology in amino acid sequence and comparable circular dichroism spectra as chicken cystatin (Lindahl et al., 1992), provide evidence that cystatin C has basically the same three-dimensional structure as chicken cystatin.

The NMR solution structure of cystatin A was later determined (Martin et al., 1995) and showed a high similarity with the crystal structure of cystatin B. However, the second hairpin loop and the N-terminal part were more flexible in the solution structure of cystatin A (Fig. 4, D). The structure of a Met-65→Leu variant of cystatin A has also been determined by NMR, although at a lower pH than the wild-type (Tate et al., 1995). This structure differed somewhat from that of the wild-type protein in having two shorter helices instead of one. Moreover, the second hairpin loop of this mutant had a more ordered structure than in the wild-type, and its N-terminal region was differently oriented and was capable of interacting with the C-terminal end (Fig. 4, E). Recent revision of the cystatin A structure (Craven et al., 2000) provides evidence that the NMR structure determined by Martin et al., (1995) is more likely. In conclusion, all these structures show that cystatins have essentially the same fold, especially in the loop regions and around the conserved Gly-9 residue.

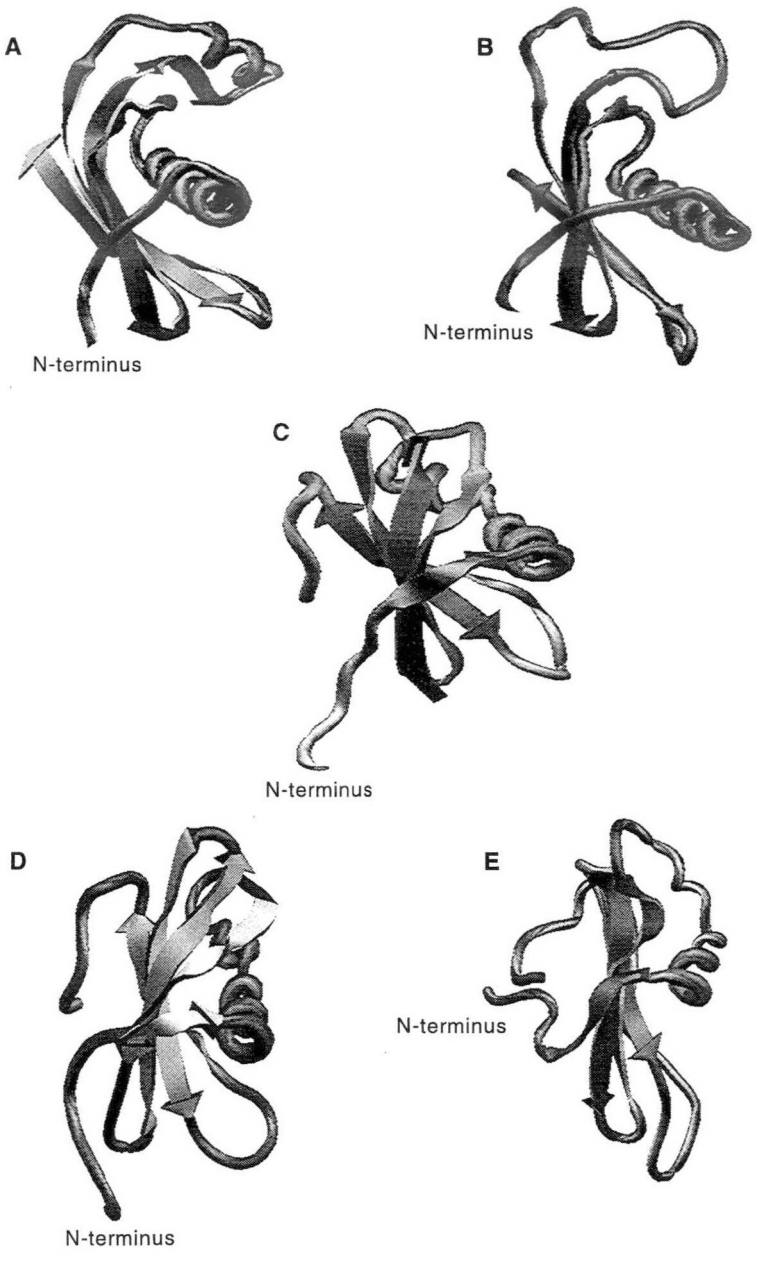

Fig. 4. Three-dimensional structures of cystatins. *A.* The crystal structure of chicken cystatin (Bode et al., 1988). *B.* The NMR structure of chicken cystatin (Dieckmann et al., 1993). *C.* The crystal structure of human cystatin B (Stubbs et al., 1990). *D.* The NMR structure of human cystatin A at pH 5.5 (Martin et al., 1995). *E.* The NMR structure of human Met-65→Leu cystatin A at pH 3.8 (Tate et al., 1995). All structures are presented as cartoons with the protease-binding regions, viz. the N-terminal region and the two hairpin loops, directed downwards.

Docking experiments based on the X-ray structures of chicken cystatin and papain (Bode et al., 1988) and the crystal structure of the cystatin B–papain complex (Stubbs et al., 1990) significantly contributed to an explanation of the mechanism of interaction at the molecular level. The structures show that three regions of the cystatin molecule form a wedge-shaped edge, which is highly complementary to the active site cleft of papain (Fig. 5). The wedge consists of the N-terminal region, the first hairpin loop, containing the Gln-Val-Val-Ala-Gly sequence, and the second hairpin loop. The involvement of the first two regions in the interaction was in agreement with previous studies (see above). The second hairpin loop is formed by Pro-103 and Trp-104 in chicken cystatin and by the Leu-73-Pro-74-His-75 sequence in cystatin B. The role of Trp-104 in chicken cystatin (corresponding to Trp-106 in cystatin C) in the interaction with proteases was verified by chemical modification by Nycander and Björk (1990). According to the three-dimensional structures, the interaction surface between cystatins and papain is dominated by hydrophobic interactions with only few hydrogen bonds. The highly similar three-dimensional structures of cystatins determined so far indicate that all cystatins interact with target enzymes in essentially the same manner.

The good sterical fit between enzyme and inhibitor demonstrated by the X-ray structures indicates that the binding proceeds in one step, without any conformational changes of either protein. A one-step reaction mechanism is characterized by a linear dependence of the observed pseudo-first-order rate constant on the inhibitor concentration (Morrison, 1982; Fersht, 1985). Several cystatins were shown to bind to cysteine proteases with such linear behavior, in accordance with a one-step mechanism, e. g. chicken cystatin to actinidin, ficin and chymopapain A, cystatin C to actinidin and papain, and bovine stefin B to cathepsin S (Björk et al., 1989; Björk and Ylinenjärvi, 1990; Lindahl et al., 1992; Turk et al., 1994). A high association rate constant of $\sim 10^7\ M^{-1} \cdot s^{-1}$, close to that for a diffusion-controlled reaction, was measured for these reactions, which is also compatible with one-step mechanism. By contrast, the binding of cystatins C and A to cathepsin B showed a hyperbolic dependence of the observed pseudo-first-order rate constant on the inhibitor concentration (Nycander et al., 1998; Pavlova et al., 2000), indicating that the reaction occurs in two steps (Morrison, 1982; Fersht, 1985). The first, fast binding step, in which a weak complex is formed, is followed by a second, slower one, in which the occluding loop of cathepsin B apparently undergoes a conformational change and is displaced by inhibitor, so that a final, tight complex is formed. Because of this two-step reaction, both the affinities of cystatins C and A for cathepsin B and the association rate constants for the reactions are considerably lower than for other cysteine proteases, being around 10^{-9} M and $10^6 - 10^4\ M^{-1} \cdot s^{-1}$, respectively.

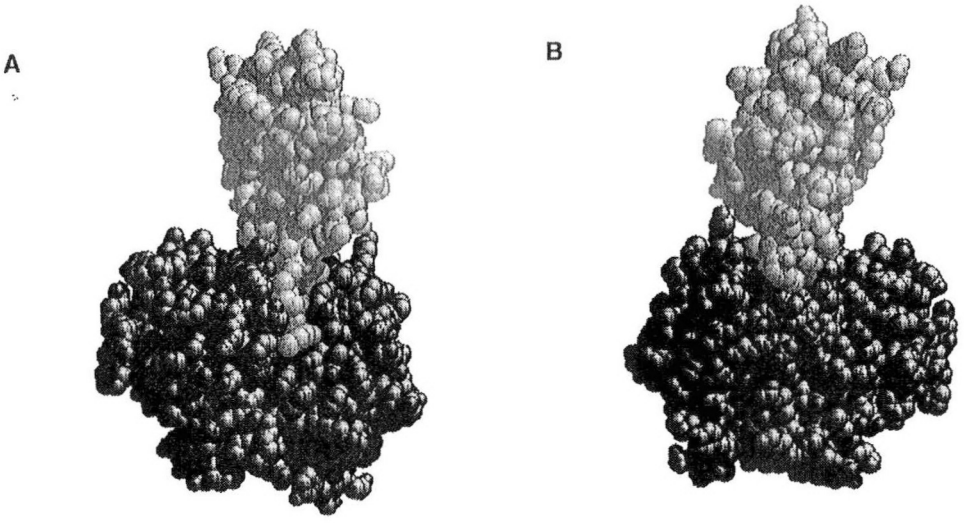

Fig. 5. Space-filling representation of the complex between human cystatin B (grey) and papain (black) according to Stubbs et al. (1990). The structure is shown from two opposite sides: the N-terminal end of cystatin B is exposed in *A*, and the second hairpin loop of the inhibitor in *B*.

Importance of individual protease-binding regions

Many investigations have focused on the roles of the individual cystatin regions involved in the interaction with cysteine proteases. In these studies, dissociation equilibrium constants, and sometimes also rate constants, for interactions of recombinantly or chemically modified cystatins with target enzymes have been determined.

The N-terminal end

The N-terminal region of the family II cystatins, chicken cystatin and cystatin C, has been studied most extensively. Native chicken cystatin and an N-terminally truncated form starting with Leu-7 were shown to bind considerably more tightly to papain than forms beginning with Gly-9 or Ala-10 (Machleidt et al., 1989), thus indicating the importance of Leu-8 and/or Leu-7 for the interaction. Further investigations confirmed this initial observation. A chicken cystatin mutant lacking the 10 first amino acid residues bound from 1000 to 100000-fold more weakly to papain, actinidin

and cathepsins B and L than the intact inhibitor (Auerswald et al., 1994). Moreover, examination of the interaction between five N-terminally truncated chicken cystatin forms (starting with Leu-7, Leu-8, Gly-9, Ala-10 or Asp-13) with papain and actinidin showed that the N-terminal end contributes ~40% of the unitary free energy of binding to these proteases (Lindahl et al., 1992). The two residues preceding the evolutionarily conserved Gly-9 residue, Leu-7 and Leu-8, turned out to together contribute approximately two-thirds of this binding energy. The interaction of the Gly-9 form of chicken cystatin with ficin and rat recombinant cathepsin B was also examined, showing a similar decrease in affinity for these enzymes (Björk et al., 1994). All these results are in agreement with the previously mentioned computer docking of chicken cystatin with papain (Bode et al., 1988), according to which Leu-7 and Leu-8 of the inhibitor occupy the S3 and S2 substrate binding subsites of the enzyme, respectively.

Truncation of human cystatin C before Gly-11 resulted in a similar decrease in affinity for papain, ficin, actinidin and cathepsins B and L as the comparable truncation of chicken cystatin (Abrahamson et al., 1991; Björk et al., 1994), confirming the importance of the N-terminal region also for the inhibitory activity of cystatin C. Leu-9, Val-10 and Gly-11 of cystatin C correspond to Leu-7, Leu-8 and Gly-9, respectively, of chicken cystatin and should thus be accommodated in the S1, S2 and S3 enzyme subsites, respectively (Bode et al., 1988). This location was considered to make them suitable candidates for mutations. Substitutions of Gly-11 of cystatin C by Ala, Glu or Trp significantly impaired the binding capacity for papain, ficin, actinidin and cathepsin B (Björk et al., 1995). This finding, and the evolutionary conservation of the corresponding Gly residue in all cystatins, is consistent with the X-ray structure of the cystatin B-papain complex, which shows that the N-terminal region backbone creates a type II turn, only allowing a Gly in this position. Further work showed that Val-10→Arg cystatin C displayed a 12 000-fold lower affinity for papain than the wild-type form, whereas the affinity for cathepsin B was only two-fold lower (Lindahl et al., 1994). Moreover, substitutions of Leu-9 and/or Val-10 of cystatin C by Gly revealed that Val-10 contributes more than Leu-9 to the binding to cathepsins L, H, and B (Hall et al., 1995). In addition, various replacements of these two residues decreased the affinities for cathepsins B, H, L and S to varying extents (Mason et al., 1998). Together, these results indicate that individual residues in the N-terminal region of cystatin C contribute differently to the affinity of interaction with different target proteases.

Examination of the association and dissociation rate constants for the interactions of N-terminally modified forms of chicken cystatin or cystatin C with various enzymes further elucidated the mechanism by which the N-terminal regions of the inhibitors participate in the binding to target enzymes

(Abrahamson et al., 1991; Björk et al., 1994; Björk et al., 1995). In the binding to endopeptidases, such as papain and cathepsin L, which have open active-site clefts, increased dissociation rate constants were found to be mainly responsible for the impaired affinity, showing that less stable complexes were formed. The N-terminal end thus contributes to the stability of the complexes of the intact cystatins with these proteases by retarding the dissociation. By contrast, the weaker binding to cathepsin B, and presumably other exopeptidases with similarly obstructed active-site clefts, was primary caused by decreased association rate constants, which is in agreement with the two-step binding mechanism of the intact cystatins to this enzyme (Nycander et al., 1998; Pavlova et al., 2000). In this interaction, the N-terminal region thus most likely is that part of the cystatin molecule which in the first, rapid binding step makes a weak complex with the enzyme. The remainder of the inhibitor then binds in the second step, displacing the enzyme residues that restrict the access to the active site. In this manner the final, tight complex is formed.

The role of the N-terminal region of cystatin A, a representative of family I cystatins, has also been examined. Sequential deletion of residues from the N-terminal end generally resulted in gradually decreased affinities for the enzymes studied (Shibuya et al., 1995a; Estrada et al., 1999). However, truncation of the first residue, Met-1, did not significantly change the inhibitory property, whereas the effect caused by removal of Pro-3, occupying the S2 subsite of the protease, was most significant. Substitutions of the evolutionarily conserved Gly-4 (corresponding to Gly-9 in chicken cystatin) by Val or Leu (Shibuya et al., 1995b) or by Ala, Ser, Arg, Glu or Trp (Estrada et al., 1998) also resulted in decreased affinities for the proteases investigated. The reduction of the binding ability was in general proportional to the size of the replacing residue. Evaluation of the kinetic data for the interactions of the N-terminally modified cystatin A forms with target proteases indicated that the mechanism of binding and the role of the N-terminal end of the inhibitor is analogous to those of cystatins from family II.

To date, investigations concerning the role of the N-terminal region of the second representative of family I, cystatin B, have indicated that this region is of minimal importance for protease binding. Both successive truncations of residues from the N-terminal end of the inhibitor (Thiele et al., 1990; Machleidt et al., 1991) and substitutions of Cys-3, corresponding to Leu-8 of chicken cystatin, (Machleidt et al., 1991) were reported not to influence the inhibitory properties of cystatin B. However, these results are in contradiction not only with observations for other cystatins, but also with the X-ray structure of the complex of this inhibitor with papain, which indicates an interaction, albeit of unknown affinity, between the N-terminal region and the protease.

The first hairpin loop

Early studies found that substitution of residues in the highly conserved Gln-Val-Val-Ala-Gly region of the first hairpin loop of cystatins A and B (Nikawa et al., 1989; Jerala et al., 1990) did not cause any significant decreases in the binding affinity for target proteases. However, subsequent work showed that replacement of Val-48 in cystatin B by Asn reduced the affinity for papain 240-fold (Machleidt et al., 1991). Mutagenesis of the first hairpin loop of chicken cystatin similarly confirmed the importance of this region for inhibition of cysteine proteases. Several single mutations, viz. Gln-53→Glu/Asn and Gly-57→Ala, as well as double mutations, viz. Arg-52-Gln53→Leu-52-Glu-53, Gln-53---Ser-56→Asn-53---Ala-56 and Leu-54---Gly-57→Met-54---Ala-57, resulted in 10- to 1000-fold lower affinities for papain, actinidin and cathepsin B (Auerswald et al., 1992). Moreover, a chicken cystatin variant lacking the first hairpin loop had a considerably lower affinity for papain and cathepsins B and L than the wild-type form (Auerswald et al., 1995). Kinetic data showed that increased dissociation rate constants were chiefly responsible for the loss of affinity of the first hairpin loop variants for cysteine proteases. Apparently, this region confers considerable stability to cystatin-protease complexes by reducing the dissociation rate.

The second hairpin loop

The importance of the second hairpin loop of cystatins for inhibition of cysteine proteases has only been examined in case of the family II cystatins, chicken cystatin and cystatin C. The Trp residue in this region, conserved in family II cystatins, has been modified chemically or by site-directed mutagenesis. Chicken cystatin, in which Trp-104 was modified with a 2-hydroxy-5-nitrobenzyl group, had a ~10^5-fold lower affinity for papain than the unmodified inhibitor (Nycander and Björk, 1990). A comparable effect was achieved by deletion of the second hairpin loop of chicken cystatin. The mutants generated in this manner exhibited ~1000-, ~60- and ~10-fold lower affinities for papain, cathepsin B and cathepsin L, respectively (Auerswald et al., 1995). Trp-106 in the second hairpin loop of cystatin C has been replaced by Gly or Phe (Hall et al., 1995; Björk et al., 1996). The Trp-106→Gly mutant was a considerably weaker inhibitor of papain, actinidin and cathepsins B, H, L and S than the wild-type inhibitor, whereas the mutation to Phe caused a much smaller decrease in affinity for these enzymes. Apparently Phe, being of similar character and size as Trp, is able to efficiently substitute for Trp in the interaction with the enzyme residues. The decrease in affinity of all these modified forms for cysteine proteases was primarily due to increased dissociation rate constants. The second hairpin loop acts thus as an anchor that keeps the inhibitor attached to the enzyme, thereby contributing to the stability of the complex.

The C-terminal end

The role of the C-terminal end of cystatins in protease binding has been studied less extensively. This region is longer in cystatins of family I (see above) and should thus be of greatest importance in these cystatins. In fact, an interaction between the C-terminal end of cystatin B and papain is indicated by the crystal structure of the complex between the two proteins (Stubbs et al., 1990). Nevertheless, cystatin B lacking its last 11 residues was reported to be as effective an inhibitor of papain as the wild-type form (Jerala et al., 1991). This finding was taken to indicate that the C-terminal region of the inhibitor is not essential for interaction with cysteine proteases.

Legumain-inhibitory region

Human cystatin C has been shown to inhibit porcine legumain (a cysteine protease from family C13D) by use of a second inhibitory site, different from that involved in inhibition of papain-like cysteine proteases (Alvarez-Fernandez et al., 1999). This site involves the loop between the α-helix and the first strand of the β-pleated sheet and is thus located on the opposite side of the molecule than the "hydrophobic wedge" discussed above. Surprisingly, cystatin C is therefore a double-headed protease inhibitor with both inhibitory sites located in the same protein domain. The legumain-inhibitory region is not present in cystatins A and B.

Present investigation

The goal of this work was to provide an extensive characterization of the interaction of cystatins A and B, two representatives of family I cystatins, with several cysteine proteases. Particularly, the roles of three of the protease binding regions of cystatin B, i. e. the N-terminal end, the second hairpin loop and the C-terminal end, in the binding to target enzymes was elucidated.

Characterization of recombinant cystatin A (Paper I)

Before this work was started, human cystatin A had been isolated from polyphonuclear granulocytes (Brzin et al., 1983) and liver (Green et al., 1984) and a recombinant human inhibitor had been expressed in Escherichia coli (Fong et al., 1989; Nikawa et al., 1989; Kaji et al., 1990; Jerala et al., 1994b). Cystatin A had also been purified from rat and cattle (Takeda et al., 1983; Turk et al., 1995). Published equilibrium constants were discrepant, and rate constants had been sparingly investigated (Green et al., 1984; Abrahamson et al., 1986; Fong et al., 1989; Nikawa et al., 1989; Kaji et al., 1990) which justified a detailed characterization of the interactions of cystatin A with several cysteine proteases.

The use of an efficient bacterial expression system enabled the production of the large quantities of recombinant, fully active cystatin A that were required for analyses of the binding of the inhibitor to the enzymes by spectroscopic, kinetic and equilibrium methods. The spectroscopic changes accompanying the binding of cystatin A to papain were characterized by near-UV absorption and fluorescence difference spectra. The observed shifts of the absorption or fluorescence maxima to shorter wavelengths most likely originate from perturbations around tryptophan residues. Since cystatin A lacks tryptophan, the most appropriate candidates responsible for this effect are Trp-177 and/or Trp-69 located close to the active site cleft of papain. Moreover, the lack of Trp in cystatin A explains why the spectral changes caused by the binding of this inhibitor to papain differed markedly from those caused by the analogous binding of cystatin C and chicken cystatin, which both have a Trp in the binding region (Lindahl et al., 1988).

The dissociation equilibrium constants determined for the interaction of human cystatin A with papain and the physiological target enzymes cathepsins L and B were appreciably lower than previously believed (Green et al., 1984; Abrahamson et al., 1986; Fong et al., 1989; Nikawa et al., 1989; Kaji et al., 1990; Pratt et al., 1992), although in accordance with the values published for

bovine cystatin A (Turk et al., 1995). The difficulties in directly measuring equilibrium constants for tight interactions may be responsible for the reported lower affinity of human cystatin A for proteases in the earlier work. By contrast, the affinity of cystatin A for cathepsins C and H were comparable with previously obtained values (Green et al., 1984; Kaji et al., 1990).

Papain and cathepsin L were most strongly inhibited by cystatin A, with K_i values of 2×10^{-13} M and 2×10^{-11} M, respectively, whereas cathepsins B, C and H and actinidin were inhibited more weakly, with K_i values in the range of 9×10^{-10} M to 4×10^{-8} M. A lower k_{ass} as well as a higher k_{diss} were responsible for the lower affinities of cystatin A for these latter enzymes. The k_{ass} values of $3\text{-}5\times10^{6}$ $M^{-1} \cdot s^{-1}$ for the binding of cystatin A to papain and cathepsin L are close to that expected for a diffusion-controlled reaction. This result, together with the observed linear increase of k_{obs} with the inhibitor concentration in the analyses of the kinetics of the binding of cystatin A to papain, are consistent with a one-step mechanism for the formation of the papain-cystatin A complex (Bode et al., 1988; Björk et al., 1989; Lindahl et al., 1992).

The affinity of cystatin A for inactivated forms of papain decreased with the size of inactivating group attached to the active-site Cys of the enzyme, due to increased dissociation rate constants. This behavior is in agreement with previous observations for the interaction between inactivated papain forms and cystatin C or chicken cystatin (Björk and Ylinenjärvi, 1989; Lindahl et al., 1992). However, the decrease in affinity of cystatin A for the papain derivatives was appreciably higher than that of chicken cystatin and cystatin C. Obviously, in the complexes formed by papain with family I cystatins, the space around the active-site cysteine is much smaller than in the complexes with cystatins from family II (Bode et al., 1988).

Truncation of the first six residues of cystatin A by proteolytic cleavage produced a less potent inhibitor of papain, showing that the N-terminal region is of importance for protease binding. The role of this region presumably is similar to that proposed for the N-terminal end of chicken cystatin and cystatin C (Abrahamson et al., 1991; Lindahl et al., 1992; Hall et al., 1993, Björk et al., 1994; Björk et al., 1995). In addition, N-terminally elongated forms of cystatin A remained as good inhibitors of papain and cathepsin H as the wild-type form.

Characterization of recombinant bovine cystatin B and examination of the role of the second hairpin loop and the C-terminal end of the inhibitor in the inhibition of cysteine proteases (Paper II)

By the time this work was started, cystatin B had been isolated from human (Green et al., 1984; Ritonja et al., 1985), bovine (Turk et al., 1992), sheep (Ritonja et al., 1996), porcine (Lenarcic et al., 1996) and rat (Hirado et al., 1981) origins, and the interaction of many of these inhibitors with cysteine proteases had been studied. Moreover, the three-dimensional structure of human cystatin B in complex with papain had been determined (Stubbs et al., 1990) which greatly contributed to the understanding of the nature of the inhibition. According to this structure, four cystatin B regions are involved in the interaction with papain: the N-terminal end, the two hairpin loops and, to some extent, the C-terminal end (which is nine residues longer in cystatin B than in family II cystatins). Of these regions, only the role of the first hairpin loop had been well established (Machleidt et al., 1991). The contribution of the second hairpin loop to the binding had not been examined at all, whereas investigations concerning the roles of the N-terminal and C-terminal ends suggested that these regions are not essential for the inhibition (Thiele et al., 1990; Machleidt et al., 1991; Jerala et al., 1991).

The importance of the second hairpin loop and the C-terminal end of cystatin B for protease binding was characterized in this work. Bovine cystatin B was chosen as a representative of mammalian forms of the inhibitor in these studies. The high sequence homology between species variants of cystatin B (Takio et al., 1983; Ritonja et al., 1985; Turk et al., 1992; Ritonja et al., 1996; Lenarcic et al., 1996) and the comparable inhibition of most enzymes by different forms of cystatin B (Green et al., 1984; Abrahamson et al., 1986; Popovic et al., 1988; Machleidt et al., 1991; Jerala et al., 1991, 1994a; Ritonja et al., 1996; Lenarcic et al., 1996; Guncar et al., 1998), indicate that the results can be extended to other species variants of the inhibitor.

Bovine cystatin B was modeled onto the crystal structure of the human inhibitor in complex with papain with minimal structural changes. Two deduced contact residues in the second hairpin loop, Leu-73 and His-75, and one contact residue in the C-terminal end, Tyr-97, were chosen for mutations. The cDNA sequence of bovine cystatin B was determined, which enabled the production of high quantities of recombinant variants of the protein by a bacterial expression system. Cystatin B and the mutants were expressed as Cys-3 to Ser variants. This replacement prevented the formation of inactive, disulfide-linked dimers during isolation and analyses.

The affinity of the C3S-cystatin B for papain determined in this work (K_d ~ 1×10^{-12} M) was about 100-fold higher than that published for the interaction of the wild-type bovine inhibitor with this enzyme (Turk et al., 1993b). This difference was due to the much higher dissociation rate constant reported in the earlier work, presumably because of difficulties in accurately measuring the very low rate constant. However, the equilibrium and rate constants for the somewhat weaker interaction of C3S-cystatin B with cathepsin L (K_d ~ 1×10^{-11} M), were comparable with those of wild-type cystatin B with this enzyme (Turk et al., 1993b; Leonardi et al., 1996). The binding of bovine C3S-cystatin B to the exopeptidases, cathepsins H and B (K_d ~ 5×10^{-10} and ~ 7×10^{-6} M, respectively), was significantly weaker than to papain and cathepsin L. This behavior is in agreement with the different structures of the active sites of the exopeptidases than of the endopeptidases, papain and cathepsin L (Musil et al., 1991; Guncar et al., 1998).

The mutations in the second hairpin loop of cystatin B, Leu-73→Gly and His-75→Gly, both resulted in decreased affinities for papain and cathepsins H and B, although the Leu mutation caused a significantly larger decrease (> 20 to ~350-fold). The affinity for cathepsin L was moderately lowered (~ 10-fold) by the Leu-73→Gly mutation, whereas the replacement of His-75 by Gly did not affect binding to this enzyme.

The mutation in the C-terminal end, Tyr-97→Ala, caused a 5 - 50-fold loss of the affinity for papain and cathepsins L and H, which is in contrast with a previous study reporting no observable effect of truncation of the C-terminal end of human cystatin B on the binding to papain. However, the affinity of the Y97A mutant for cathepsin B was the same as that of the wild-type form.

These affinity data indicate that the most important residue in the second hairpin loop of cystatin B for the interaction with papain and cathepsins L, H and B is Leu-73, His-75 playing a less prominent role. The two residues together contribute 20-30% of the free energy of binding of cystatin B to papain and cathepsins H and B. However, the second hairpin loop appears to be less essential for cystatin B binding to cathepsin L. The C-terminal end is less important than the second hairpin loop, Tyr-97 in this end contributing 6-12% of the free energy of binding of the inhibitor to papain and cathepsins L and H. Moreover, the C-terminal end appears not to participate to any significant extent in cathepsin B inhibition.

All affinity decreases were due predominantly to increased dissociation rate constants, whereas the association rate constants were only slightly affected. This behavior indicates that both the second hairpin loop and the C-terminal region are important for the stability of the complexes with proteases, keeping the inhibitor anchored to the enzyme. According to the crystal structure

(Stubbs et al., 1990), the second hairpin loop interacts with the R domain of papain in such a way that Leu-73 forms hydrophobic contacts with the highly conserved Trp-177 of papain and His-75 forms similar contacts with Gly-180, adjacent to the active cleft. A direct hydrophobic interaction of Leu-73 with Tyr-177 of papain was supported by spectroscopic studies in this work. Fluorescence emission difference spectra between complexes of bovine C3S-cystatin B or the second-loop mutants with papain and the free proteins were thus considerably lower for the mutants, the difference being most significant for the L73G mutant. The joint contribution of the two residues in the second hairpin loop of cystatin B to the binding to papain and cathepsin H is comparable to that of Trp-106 of cystatin C (Björk et al., 1996). The crystal structure further indicates that Tyr-97 in the C-terminal end of cystatin B participates in anchoring the inhibitor to the enzyme by interacting with Cys-63 and Asn-64, both located in the L domain of papain (Stubbs et al., 1990).

Importance of the residues in the N-terminal end of cystatin B for inhibition of cysteine proteases (Papers III and IV)

The crystal structure of the complex between cystatin B and papain (Stubbs et al., 1990) indicates that the N-terminal region of the inhibitor is involved in the interaction with the enzyme. Moreover, studies investigating the role of the N-terminal regions of cystatin C, chicken cystatin (Machleidt et al., 1989; Abrahamson et al., 1991; Lindahl et al., 1992; Björk et al., 1994, 1996; Hall et al., 1998) and cystatin A (Shibuya et al., 1995a, b; Estrada et al., 1998, 1999) for the binding to cysteine proteases have confirmed the importance of this region for these inhibitors. In contrast, the N-terminal end of cystatin B was reported not to contribute to the binding to cysteine proteases (Thiele et al., 1990; Machleidt et al., 1991).

In view of these contradicting results, we decided to provide unambiguous evidence regarding the role of the N-terminal end of cystatin B in protease binding. In paper III, the role of the single cysteine residue in this region is examined, whereas paper IV describes the effect of successive truncation of the residues from the N-terminal end of cystatin B on inhibition of target proteases.

Cystatin B is unique among cystatins in having a free cysteine residue in the third position. In the crystal structure of the complex between papain and cystatin (Stubbs et al., 1990), a Cys-3→Ser mutant of human cystatin B was used in order to avoid formation of disulfide-linked cystatin B dimers. For the same reason, in many studies of the interaction of the inhibitor with proteases (Jerala et al., 1990, 1994a; Pol and Björk, 1999), the Cys-3→Ser mutant of cystatin B was chosen. However, the location of the third residue of the

inhibitor in the S2 substrate binding subsite of papain in the X-ray structure of the complex (Schechter and Berger, 1967; Stubbs et al., 1990) indicates that Cys-3 in wild-type cystatin B should be of importance for binding of proteases. Thus, replacement of this residue by Ser would be expected to affect the affinity for target enzymes. In order to elucidate the role of Cys-3, we characterized the affinity and kinetics of interaction of wild-type and Cys-3→Ser mutants of human and bovine cystatin B with papain and cathepsins L, H and B.

Human and bovine wild-type cystatin B were shown to have about the same affinity for each of the proteases investigated, binding tightly (K_d ~10^{-12} – 10^{-14} M) to the endopeptidases, papain and cathepsin L, and more weakly (K_d ~10^{-8} – 10^{-11} M) to the exopeptidases, cathepsins H and B. Our data show that the affinities of the wild-type cystatin B forms for papain are 200-2500-fold higher, and those of human wild-type cystatin B for cathepsins L and H >70- and up to 20-fold higher, respectively, than the affinities reported earlier (Green et al., 1984; Abrahamson et al., 1986; Popovic et al., 1988; Machleidt et al., 1991; Turk et al., 1992, 1993b). Irreversible oxidation of the thiol group of the cysteine residue to e. g. sulfinic or sulfonic acid derivatives (Cecil, 1963; Glazer, 1970; Liu, 1977) may be responsible for the lower affinities reported previously, as suggested by experiments in this work.

Cys-3 was found indeed to be of appreciable importance for the binding of cystatin B to target enzymes. The extent of the contribution of this residue to the binding depends, however, on the enzyme and on the cystatin B species variant. The Cys-3→Ser mutation thus decreased the affinities of the human inhibitor for papain and cathepsin H by 3-4-fold and that for cathepsin B by ~20-fold. The reductions in the affinities of the bovine inhibitor for papain and cathepsins H and B were larger, ~14-, ~10- and ~300-fold, respectively. These results show that Cys-3 is more important for cathepsin B binding and for bovine cystatin B. Unfortunately, the decreases in affinity for cathepsin L could not be properly quantified but were > 3-fold. The strengths of the binding of the human Cys-3→Ser mutant to the enzymes are comparable with values obtained earlier for the interaction of this mutant with cathepsins L, H and B (Jerala et al., 1990, 1994a). However, in the case of the interaction of the mutant with papain, the affinity determined in this work was 350- to 1000-fold higher than in previous reports (Jerala et al., 1990, 1994a). Presumably this discrepancy reflects difficulties in the determination of the very low K_d -values by the method used earlier.

The Cys-3 to Ser mutation had a differential effect on the rate constants for the interaction of cystatin B with papain and cathepsins H and B. The impaired affinity of the mutants for papain was due mainly to an increased k_{diss}, whereas a decreased k_{ass} was largely responsible for the reduced affinities for

cathepsins H and B. This behavior indicates that Cys-3 contributes to the stability of the complex with papain by keeping the inhibitor attached to the enzyme, once the complex is formed. However, in the case of cathepsins H and B, both having partially blocked active-site clefts, the thiol group of Cys-3 instead probably promotes an initial interaction of the N-terminal region of cystatin B with the enzymes that increases the overall association rate. A comparable effect of the N-terminal regions of other cystatins on cathepsin B binding has been shown to be due to the initial interaction facilitating displacement of the occluding loop of the protease (Nycander et al., 1998; Pavlova et al., 2000).

As noted above, the decrease in affinity as a consequence of the Cys-3→Ser replacement in cystatin B was more pronounced for the bovine than for the human inhibitor, the bovine mutant being ~3- or ~15-fold less potent an inhibitor of papain and cathepsin H or cathepsin B than the human mutant. Modeling of the complexes between either of the two cystatin B species variants and active papain revealed that two hydrophobic residues, viz. Ala-5 and Pro-6, in the human inhibitor make many hydrophobic contacts with the enzyme. These contacts are absent in the complex with the bovine inhibitor, since Ala-5 and Pro-6 are replaced by two, more polar residues. This difference most likely is the reason why the mutation Cys-3→Ser has a smaller effect on the interaction of the human than of the bovine inhibitor with papain. The larger contribution of Cys-3 of bovine than of human cystatin B to the binding to cathepsins H and B is difficult to explain by modeling, as no crystal structures of the complexes of cystatin B with these proteases are available.

In the examination of the importance of the N-terminal region of cystatin B by sequential truncation of the first four residues, bovine cystatin B was chosen as a representative of mammalian species variants of the inhibitor, as in the studies of the role of the second hairpin loop. In general, removal of the N-terminal region residues resulted in a progressively impaired affinity for papain and cathepsins L, H and B. Truncation of the first residue, Met-1, decreased the affinity for papain and cathepsin B by ~10-fold but minimally affected the interaction with cathepsin H. The affinity for cathepsin L was also reduced, but the effect could not be quantified. Deletion of the second residue, Met-2, again decreased the affinity for papain and cathepsin B, by a further ~40-70-fold, but not that for cathepsins L and H. Removal of Cys-3 caused the largest loss of affinity, a further ~1500-fold for papain, ~700-fold for cathepsin L and ~15-fold for cathepsin H. The decrease in affinity for cathepsin B was difficult to quantify because only a lower limit of K_i could be determined. The last removed residue, the evolutionarily conserved Gly-4, turned out to be of limited importance for the interaction with the enzymes studied. An interesting point is that removal of this residue resulted in ~10-

fold higher affinity for cathepsin L, probably caused by an increased flexibility of the following residues, which were able to make contacts with the enzyme in the absence of Gly-4. These results show that the first three residues of the N-terminal region of cystatin B together contribute ~40%, >30%, ~12% and >30% of the total unitary free energy change for the binding of the inhibitor to papain and cathepsins L, H and B, respectively. The N-terminal end is thus of least importance for cathepsin H binding. Moreover, Cys-3 is responsible for most of the energy of binding of the N-terminal region to the proteases examined, the contributions of the preceding residues varying with the protease.

The decreased affinities of the N-terminally truncated cystatin B mutants for papain and cathepsin H were mainly due to increased dissociation rate constants, indicating that the N-terminal region predominantly functions as an anchor retarding the dissociation of these enzymes from the inhibitor. Deletion of the N-terminal region in cystatin B has thus a different effect than the Cys-3→Ser replacement on the kinetics of binding to cathepsin H. Difficulties in determining the rate constants for the interactions of the mutants with cathepsins L and B precluded an explanation of the mechanism of interaction with these enzymes.

The affinity data are in agreement with modeling of the complex between active papain and bovine cystatin B. The first four residues in the N-terminal end of cystatin B occupy the S4, S3, S2 and S1 substrate binding subsites of papain and make a large number of close hydrophobic contacts with papain residues. Cys-3 is involved in particularly many contacts. Successive removal of N-terminal residues of the inhibitor should thus result in a decrease of the number of interactions between the two proteins, causing the observed progressively lower affinity for target enzymes. Moreover, as shown, truncation of Cys-3 should have the largest effect.

General conclusions

The main conclusions of the studies concerning the mechanism of interaction of cystatins A and B with cysteine proteases are as follows:

- The affinities of cystatin A for several cysteine proteases are higher than previously believed. The inhibitor binds to papain and cathepsin L tightly and rapidly, with K_d of ~10^{-11}-10^{-13} M and k_{ass} of 3-5×10^6 $M^{-1} \cdot s^{-1}$. This k_{ass}, close to that expected for a diffusion-controlled reaction, and the observed linear increase of k_{obs} with the inhibitor concentration are both consistent with a one-step binding mechanism of cystatin A to papain, without any conformational changes.
- The affinities of cystatin A for cathepsins B, C and H and actinidin are lower, with K_d of 10^{-8}-10^{-9} M, due to both a lower k_{ass} and a higher k_{diss}.
- An N-terminally truncated cystatin A variant, generated by proteolytic cleavage, has an appreciably lower affinity for papain, indicating the importance of this region for interaction with cysteine proteases.

- Mutations in the second hairpin loop of cystatin B of Leu-73 to Gly and His-75 to Gly result in decreased affinities for papain and cathepsins L, H and B. The extent of the decrease suggests that the second hairpin loop contributes 20-30% of the total free energy of binding of cystatin B to target enzymes. Leu-73 contributes more to the binding than His-75.
- Mutation in the C-terminal end of cystatin B of Tyr-97 to Ala causes a loss of the affinity for papain and cathepsins L and H. This finding is in contrast to a previous study reporting no observable effect of truncation of the C-terminal region of human cystatin B on the binding to papain. The magnitude of the loss indicates that the C-terminal end contributes 6-12% of the energy of binding of cystatin B to target proteases.
- The C-terminal end of cystatin B seems to be of limited importance for the interaction with cathepsin B.
- The lower affinities of the interactions of the second hairpin loop and C-terminal end mutants of cystatin B with proteases are caused by increased k_{diss}-values. This finding suggests that the two regions are important for the stability of the complexes by keeping the inhibitor anchored to the enzymes. The structure of the complex with papain indicates that this effect is due to the second hairpin loop and the C-terminal end interacting with the R and L domains of the enzyme, respectively.
- The joint contribution of the two residues in the second hairpin loop of cystatin B, Leu-73 and His-75, to the binding to papain and cathepsin H is comparable to that of Trp-106 alone of cystatin C.

- Wild-type cystatin B of bovine and human origin have indistinguishable inhibitory properties towards cysteine proteases, binding tightly (K_d ~10^{-12}-10^{-13} M) to the endopeptidases, papain and cathepsin L, and more weakly (K_d ~10^{-8}-10^{-11} M) to the exopeptidases, cathepsins H and B.
- The affinities of the wild-type cystatin B forms for the enzymes are generally higher than previously reported, in all likelihood because of irreversible oxidation of the thiol group of the Cys-3 residue of the inhibitor to e. g. sulfinic or sulfonic acid derivatives in earlier work.
- Mutation of the single residue, Cys-3, in cystatin B to Ser shows that this residue is of appreciable importance for the binding of the inhibitor to target enzymes, although the contribution to the binding depends on the enzyme examined and on the cystatin B species variant. Cys-3 is most important for cathepsin B binding and for the bovine inhibitor.
- The impaired affinity of the Cys-3→Ser mutants for papain is mainly due to an increased k_{diss}, indicating that Cys-3 contributes to keeping cystatin B attached to this enzyme once the complex is formed. In contrast, a decreased k_{ass} is largely responsible for the reduced affinity of the mutants for cathepsins H and B. Cys-3 thus probably promotes a initial interaction of the N-terminal region of cystatin B with these enzymes that increases the overall association rate.
- The human Cys-3→Ser mutant is a better inhibitor of papain and cathepsins H and B than the corresponding bovine mutant.

- Sequential truncation of residues from the N-terminal region of cystatin B results in a progressively impaired affinity for target proteases. The decreases in affinity suggest that the first three N-terminal residues of cystatin B together contribute ~40%, >30%, ~12% and > 30% of the total energy of binding of the inhibitor to papain and cathepsins L, H and B, respectively.
- The highest loss of affinity is caused by removal of Cys-3, showing that this residue is responsible for most of the binding energy of the N-terminal region. The contributions of the preceding residues vary with the protease.
- The N-terminally truncated mutants form less stable complexes with papain and cathepsin H, as indicated by an increased k_{diss}. This finding suggests that the residues of the N-terminal region of cystatin B function as an anchor, retarding the dissociation of these enzymes from the inhibitor.

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