ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

VETERINARIA 51

Characterisation of two Endogenous Mammalian Cysteine Proteinase Inhibitors, Bovine Cystatin C and Human Cystatin A

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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Akademisk avhandling som för vinnande av veterinärmedicine doktorsexamen kommer att offentligen försvaras i Ettans föreläsningssal, Klinikcentrum, SLU, Uppsala, tisdagen den 8 juni 1999, kl. 9.15.

Abstract

The binding of human cystatin A to papain-like proteinases was quantified with a recombinant inhibitor, expressed in *E.coli*. The interaction with papain and cathepsin L was strong, with K_i values of 10^{-11}-10^{-13} M, and rapid, with k_{ass} values of $3\text{-}5\cdot10^6$ $M^{-1}\cdot s^{-1}$. The binding to papain was consistent with a one-step reaction, occurring without detectable conformational changes of either proteinase or inhibitor.

The cDNA sequence of bovine cystatin C and analyses of the inhibitor isolated from cerebrospinal fluid showed that bovine cystatin C is synthesised as a preprotein with a signal peptide of 30 residues preceding the mature protein with 118 residues. In particular, the inhibitor has an N-terminal region similar to that of other family II cystatins.

Recombinant bovine cystatin C with a complete N-terminal region was characterised. The general properties, as well as the affinity and kinetics of inhibition of papain and cathepsins B, H and L, were comparable with those of human cystatin C. However, some differences between the bovine and human inhibitors were observed. Most importantly, bovine cystatin C bound to cathepsin L with a four-fold higher association rate constant than the human inhibitor. The full-length bovine cystatin C bound appreciably more tightly to proteinases than the shorter forms characterised previously. Digestion with elastase indicated that these forms had arisen by cleavage of a full-length inhibitor.

In-situ hybridisation with digoxigenin-labelled cRNA probes demonstrated that bovine cystatin C mRNA was heavily concentrated in the epithelial cells of the choroid plexus, in single cells speckled in lymphoid tissue and in Sertoli cells. Cystatin C mRNA was also present in occasional neurons and glial cells throughout the cerebrum and the cerebellum. In the submandibular gland, specific mRNA was found mainly in striated intralobular and interlobular ducts. The expression of cystatin C in brain tissue is of particular interest, as the inhibitor is involved in certain neurological diseases.

Keywords: cysteine proteinase, cysteine proteinase inhibitor, cystatins, bovine, cerebrospinal fluid, in-situ hybridisation, enzyme kinetics, papain, cathepsins.

Distribution:
Swedish University of Agricultural Sciences
Department of Veterinary Medical Chemistry
S-751 23 UPPSALA, Sweden

Uppsala 1999
ISSN 1401-6257
ISBN 91-576-5423-9

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 1999

Acta Universitatis Agriculturae Sueciae
Veterinaria 51

ISSN 1401-6257
ISBN 91-576-5423-9

Tryck: SLU Service/Repro, Uppsala 1999

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Olsson, S-L., 1999. *Characterisation of two endogenous mammalian cysteine proteinase inhibitors, bovine cystatin C and human cystatin A.* Doctor's dissertation.
ISSN 1401-6257, ISBN 91-576-5423-9

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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. 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, 1995

II. Olsson, S-L., Ek, B., Wilm, M., Broberg, S., Rask, L. and Björk, I. Molecular cloning and N-terminal analysis of bovine cystatin C. Identification of a full-length N-terminal region. Biochim. Biophys. Acta 1343, 203-210, 1997

III. Olsson, S-L., Ek, B., and Björk, I. The affinity and kinetics of inhibition of cysteine proteinases by intact recombinant bovine cystatin C. Biochim. Biophys. Acta 1999 In press

IV. Olsson, S-L., Pihlgren, U., Plöen, L., and Björk, I. Tissue distribution of bovine cystatin C analysed by in situ hybridisation. Manuscript 1999

Abbreviations

The following abbreviations are used in the text.

APP	β-amyloid protein precursor
CD	Circular dichroism
CNS	Central nervous system
cDNA	Complementary DNA
cRNA	Complementary RNA
E. coli	Escherichia coli
ER	Endoplasmatic reticulum
E-64	[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-4-guanidobutane
HCCAA	Hereditary cystatin C amyloid angiopathy
k_{ass}	Second order association rate constant
kb	Kilobasepairs
K_d	Dissociation equilibrium constant
KDa	KiloDalton
k_{diss}	Dissociation rate constant
K_i	Inhibition constant
k_{obs}	Observed pseudo-first-order rate constant
NMR	Nuclear magnetic resonance

Introduction

Hydrolysis of peptide bonds in proteins in living organisms is as common an event as life itself. It occurs in every organism inside every cell, as well as extracellularly. The biological catalysts of these reactions are proteolytic enzymes, called peptidases or proteases. Peptidases that cleave polypeptide chains in the interior of a protein are called endopeptidases or proteinases. Exopeptidases, on the other hand, act near the N- or C-terminal region of the polypeptide chain (Barrett, 1992). Peptidases are classified according to the functional group in their active site. Usually four classes are recognised: serine, cysteine, aspartic and metallo-peptidases. (Rawlings and Barrett, 1994).
Every living organism needs to strictly control and regulate the activity of proteases. Transcription regulation, compartmentalisation, activators and inhibitors are some frequently used solutions to achieve such control. In many pathological conditions this regulation is disturbed. The aim of this work has been to characterise two endogenous inhibitors of cysteine proteinases, human cystatin A and bovine cystatin C, with most attention given to the latter.

Previous investigations

Cysteine proteinases

General properties, synthesis and reaction mechanism

Cysteine proteinases are widely distributed in living organisms. Based on sequence homology they can be divided into several families (Rawlings and Barrett, 1993, 1994), of which the papain and calpain families are the most important. The papain-like cysteine proteinases are the most abundant and include papain and related plant proteinases, such as chymopapain, caricain, bromelain, actinidin, ficin and aleurain, and also cysteine proteinases of mammalian lysosomes (Turk et al., 1997). This family probably originated early in eukaryote evolution and may even have emerged before eukaryote and prokaryote divergence (Berti and Storer, 1995). It is possible that the first proteinase of this family existed in the feeding vacuoles of protozoa and by the route of divergent evolution developed into enzymes in plant vacuoles or mammalian lysosomes (Barrett, 1986).

In 1929 Willstätter and Bauman introduced the name cathepsin, a word originally derived from a Greek term meaning "to digest", for a proteinase that is active at a

slightly acidic pH. The term cathepsin is now used to refer to intracellular proteinases that usually, but not exclusively, are found in lysosomes. Most of these enzymes are small proteins with a molecular mass in the range of 20-35 kDa. An exception is cathepsin C, which is an oligomer of approximately 200 kDa (Rawlings and Barrett, 1994; Turk et al., 1997). Cathepsins found in the lysosomes are cathepsins A, B, C, D, E, G, H, K, L, N, S, T and W, of which cathepsins B, C, H, K, L, S and W are cysteine proteinases and the others aspartic proteinases (Turk et al., 1997). The lysosomal cysteine proteinases are optimally active at slightly acidic pH, i.e. 5.5-6.8.

As the concentration of cathepsins inside the lysosomes is high, approximately 40 mg/ml (Kirschke and Barrett, 1987; Barrett, 1992), it is not surprising that the activity of these proteinases is strictly controlled. Initially they are synthesised as preproproteins. The signal peptides are removed when the molecules pass into the lumen of the endoplasmatic reticulum, where additional posttranslational modifications like glycosylation and formation of disulphides take place. Protein disulphide isomerase, located in the ER lumen, facilitates formation of disulphide bonds (reviewed by Bassuk and Berg, 1989; Freedman et al., 1998). Mannose residues in the oligosaccharide side chains are phosphorylated in the Golgi network to form mannose-6-phosphate that enables the proenzyme to bind to mannose-6-phosphate-receptors located in the membranes of the Golgi network (Gacko et al., 1997). The receptor-proenzyme complexes are directed to lysosomes, where the receptor dissociates from the proenzyme due to the low pH. In late endosomes or in lysosomes the proenzymes are converted to active enzymes by limited proteolysis and dephosphorylation of the mannose residues (Kirschke et al., 1995). The N-terminal propeptides that are removed in this maturation step are potent inhibitors of the enzymes (Fox et al., 1992; Carmona et al., 1996). The activity of the mature proteins is controlled by endogenous inhibitors (see below), localisation, degradation (Twining, 1994; Kirschke et al., 1995) and changes in pH (Turk et al., 1993, 1994).

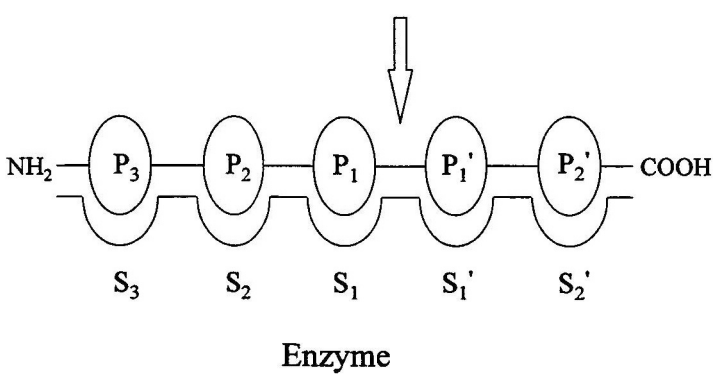

Fig. 1 The terminology of proteinase specificity according to Schechter and Berger (1967) and Berger and Schechter (1970). The scissile bond is the bond between P1 and P1'.

Most papain-like cysteine proteinases are monomers, whose structure consists of two domains separated by a cleft. They possess predominantly endopeptidase activity. Active-site sequence homologies are high among cathepsins, but overall amino acid homologies are only 20-60 % (Wiederanders et al., 1992). Two catalytic residues, Cys 25 and His 159 (papain numbering), one from each domain, are situated at the bottom of a V-shaped active-site cleft. These residues form a thiolate-imidazolium ion pair essential for the enzymatic activity.

The mechanism of cysteine proteinase hydrolysis is not fully understood, a major unresolved question being whether the Cys 25 and His 159 residues are sufficient for catalytic activity (Otto and Schirmeister, 1997). Nevertheless, the first step in hydrolysis is a nucleophilic attack by the thiolate anion on the carbonyl carbon of the scissile P1-P1' bond (Fig. 1), and an acyl-enzyme tetrahedral intermediate is produced (Otto and Schirmeister, 1997). The oxyanion in this transition state is stabilised by hydrogen bonding to the backbone NH of Cys 25 and the NH_2 group of the Gln 19 side chain (Storer and Menard, 1994). Due to the esterification of the thiol, the imidazolium ion is now acidic enough to protonate the nitrogen of the leaving group, and thereby an acyl-enzyme is formed. A water molecule that the imidazole nitrogen has polarised then attacks the carbonyl carbon in this complex. A cleaved substrate and a regenerated enzyme is finally produced through a second tetrahedral intermediate (Otto and Schirmeister, 1997).

Papain

Papain, which is found in the latex and in the papaya fruit of Carica papaya (melon tree), is the most thoroughly studied of all cysteine proteinases, and is often used as a model enzyme. Its precise physiological role is unknown, but fruit proteinases have been suggested mainly to have a protective function against invading microorganisms (Boller et al., 1986). The subcellular localisation of papain is in the vacuoles, which somewhat resemble mammalian lysosomes. Papain is formed from a zymogen, propapain (Cohen et al., 1986). The proregion is essential for correct folding and processing, but can also act as a high-affinity inhibitor of papain. (Taylor et al., 1995).

Lysosomal cysteine proteinases

The tissue distribution of cathepsins varies, some of them, like cathepsins B, C, H and L being ubiquitous in lysosomes of animals, whereas others, like cathepsins S, K and W, having a more restricted localisation (Kirschke and Wiederanders, 1994; Linnevers et al., 1997; Tezuka et al., 1994; Turk et al., 1997).

Cathepsin B

Of the lysosomal cysteine proteinases, cathepsin B is the most abundant and consequently the most studied. The enzyme has been isolated from several species and tissues. Different species variants do not differ much in sequence (Otto and Schirmeister, 1997). Cathepsin B, which also has carboxydipeptidase activity, has an occluding loop of about 20 amino acid residues that partly blocks free access of substrates to the primed (S_1' to S_3'; Fig. 1) site of the cleft. Recombinant cathepsin B in which this loop was removed lost its exopeptidase activity (Illy et al., 1997), indicating that the loop directs the substrate to bind in a manner allowing this type of cleavage. It has been difficult to determine the specificity of cathepsin B due to its dipeptidyl peptidase activity (Otto and Schirmeister, 1997). However, cathepsin B is less active against substrates with Pro or Arg at P1'.

Cathepsin H

Cathepsin H has both endo- and exo-peptidase activities. The endopeptidase activity is weak, and the aminopeptidase activity is strong. Due to this aminopeptidase activity it has not yet been possible to determine the exact specificity of its endopeptidase activity (Otto and Schirmeister, 1997). It is the only lysosomal aminopeptidase characterised so far (Barrett et al., 1980; Kirschke and Wiederanders, 1987). Cathepsin H has a section of the propeptide attached by a disulphide bond, which probably fills the nonprimed (S_1 to S_3; Fig. 1) site of the cleft, thereby making the enzyme an aminopeptidase (Guncar et al., 1998).

Cathepsin L

Cathepsin L is the lysosomal enzyme that has the highest specific proteinase activity, and it has no exopeptidase activity (Barrett and Kirschke, 1981). Relatively large amounts of procathepsin L, up to 40%, are secreted (Nishimura et al., 1988). Although the proregion is a potent and selective inhibitor of cathepsin L (Fox et al., 1992; Carmona et al., 1996), procathepsin L appears to have proteolytic activity by itself on physiological surfaces. This is the first evidence that a proform of a cysteine proteinase may have a catalytic effect (Ishidoh and Kominami, 1995). Cathepsin L hydrolyses extracellular matrix proteins, such as collagen and elastin, more efficiently than collagenase and neutrophil elastase (Kirschke, 1982).

Other cathepsins

Cathepsin S has been detected in spleen, lung (Kirschke et al., 1994), liver and placenta (Linnevers et al., 1997). In contrast to other cathepsins, this enzyme is stable at pH 7 (Kirschke et al., 1994). Cathepsin S has been described as a monocyte/macrophage-specific proteinase (Chapman et al., 1994). Cathepsin N has only been found in lymph nodes and spleen (Maciewicz and Etherington, 1988). Cathepsin K is expressed selectively in osteoclasts and, due to this restricted expression, is suggested to be of importance in bone resorption (Tezuka et al., 1994). Cathepsin S, N and K show a high sequence similarity to cathepsin L (Otto and Schirmeister, 1997).

Two lysosomal cysteine proteinases similar to cathepsins B and H have been isolated from the thyroid gland, namely TP-1 and TP-2. They release thyroxine from thyroglobulin (Natagawa and Ohtaki, 1986). Cathepsin W is predominantly expressed in lymphocytes and is related to cathepsin B, H, L, K, and S, but the sequence homology is relatively low (Linnevers et al., 1997).

Legumain

Legumain was first identified as an endopeptidase in plants and parasites (Kembhavi et al., 1993; Dalton et al., 1995). The enzyme is present in the vacuoles of many leguminous and other seeds and is specific for hydrolysis of asparginyl bonds.

Mammalian legumain is a cysteine endopeptidase with predominantly lysosomal localisation (Chen et al., 1997, 1998). It has, as the plant enzyme, specificity for the hydrolysis of bonds on the carboxyl side of aspargine residues (Chen et al., 1997). Recently it was shown that legumain has a key role in processing a bacterial antigen for the MHC class II system in the lysosomal/endosomal system of antigen-presenting cells (Manoury et al., 1998).

Cytoplasmatic cysteine proteinases

Calpains

The calpains form a large family of proteinases, related to the papain family. The name refers to a neutral intracellular cysteine proteinase that requires calcium ions for activation (reviewed by Molinari and Carfoli, 1997; Ono et al., 1998; Carfoli and Molinari, 1998). Two types have been isolated which differ in calcium requirements, μM or mM calcium concentrations being needed for activation of the two forms. These forms may be inactive procalpains, since physiological calcium concentrations (< 1 μM) are insufficient for their activation. Calpains are heterodimers made up of a 80 kDa catalytic and a 30 kDa regulatory subunit. The

large subunit consists of four domains, one of which shows sequence similarities to cysteine proteinases of the papain family (Ono et al., 1998). An important route of activating calpains *in vivo* may be through membrane association, as an increasing proportion of the enzyme has been reported to become membrane-associated when intracellular Ca^{2+} increases (Carafoli and Molinari, 1998). Calpain processes proteins in a limited way, suggesting that the physiological role is to activate the substrates rather than degrade them (Carfoli and Molinari, 1998). The substrate specificity is not known, but *in vitro* experiments at non-physiological Ca^{2+} concentrations suggest that cytoskeletal proteins, some membrane proteins and cytosolic proteins targeted to membranes under certain conditions are calpain substrates (Carfoli and Molinari, 1998).

Caspases (ICE-family)

ICE, interleukin-1β-converting-enzyme, belongs to a conserved class of proteinases, that constitute the key destructive engine of programmed cell death, apoptosis, a process that has received considerable attention lately. The family shows no homology with other cysteine proteinases, but all members have a cysteine residue in their active site. They cleave their targets proteins after specific aspartic residues and are thus called caspases. In apoptosis, caspases are thought to be activated in an amplifying cascade (reviewed in Thornberry and Molineaux, 1995; Meir and Evans, 1998; Raff, 1998). Some apoptotic effectors have been identified in *Drosophila melanogaster*, but no counterparts have been found in vertebrates (Meir and Evan, 1998). Recently it was shown that a proinflammatory caspase is a substrate for cathepsin B (Schotte et al., 1998; Vancompernolle et al., 1998). However, inhibition of cathepsin B was shown not to reduce ischemic injury in mouse brain (Hara et al., 1997), nor did it prevent DNA-fragmentation in a cell culture assay (Lynch et al., 1997).

Deubiquitinating enzymes

Ubiquitin is a small (8.6 kDa), highly conserved protein. For some proteins ubiquitination leads to degradation by the 26S proteosome, for others ubiquitination results in posttranslational modifications regulating cellular targeting and enzyme activity (reviewed by Wilkinson et al., 1995).

Protein ubiquitination controls many processes such as cell cycle progression, apoptosis and growth factor-mediated signal transduction. Ubiquitination is dynamic, involving enzymes that add and enzymes that remove ubiquitin. Deubiquitinating enzymes are cysteine proteinases that specifically cleave ubiquitin from ubiquitin-conjugated protein substrates, thereby regulating the cellular pool of free monomeric ubiquitin (reviewed by Andrea and Pellman, 1998). Although many deubiquitinating enzymes show little sequence homology with lysosomal cysteine proteinases or caspases (Chinnaiyan and Dixit, 1996), the

crystal structure of one member, UCH-L3, suggests that there are mechanistic features with significant similarities to those of the papain-like cysteine proteinases (Johnston et al., 1997).

Cysteine proteinases from microorganisms

Cysteine proteinases play a vital role in the life cycle of many microorganisms, including many pathogens of specific interest in veterinary medicine. These proteinases are important chemotherapeutic targets, either for specific inhibitors or for vaccines. In particular, the possibility to develop inhibitors that are very specific for a certain proteinase, and thereby potentially non-toxic, makes the study of inhibition of cysteine proteinases an important part of a strategy to combat infectious diseases. The cysteine proteinases mentioned below are just a few examples of such proteinases from microorganisms.

Picornaviruses, of which poliovirus is the prototype, are single-stranded, positive-sense RNA-viruses. The RNA is translated into a poly-protein, which is processed by virus-encoded proteinases. These enzymes, which are cysteine proteinases (Stanway, 1990) are thus essential for virus replication. A thiol proteinase inhibitor, E-64 specifically blocked the autocatalytic cleavage of the poly-protein of foot and mouth virus, another picornavirus (Kleina and Grubman, 1992). Moreover, in experiments with foot-and-mouth virus-infected cells, the inhibitor caused a reduction in virus yield (Kleina and Grubman, 1992).

Cysteine proteinases are also found in bacteria. A cysteine proteinase from *Staphylococcus aureus* has the ability to degrade elastin and may thus have a pathological role (Shaw, 1990). Streptopain, a streptococcal cysteine proteinase with a specificity similar to that of the papain family, has been identified as a pyrogenic exotoxin (Ohara et al., 1994). It is a superantigen and is probably involved in autoimmune diseases that often are a sequel of streptococcal infections. There is a possibility that streptopain also directly modulates the host immune system (Kapur et al., 1993).

Cysteine proteinases from *Porphyromonas gingivalis*, a bacterium that plays a major role in the pathogenesis of peridontitis, have trypsin-like activity. Two enzymes with Arg-Xaa specificity, gingipain R, A and gingipain R, B, and one enzyme with Lys-Xaa specificity, gingipain K, have been purified (reviewed by Kuramitsu, 1998). These enzymes show haemaglutinin activity and can degrade complement factors, e.g. C3 and C5, and cytokines. Proteinases from *P. gingivalis* can also degrade immunoglobulins. One of the characteristic properties of peridontitis is the increased bleeding tendency of the affected tissue. This is probably due to proteinases from *P. gingivalis* being able to cleave the clotting factor, Factor X and also to degrade fibrinogen, the latter probably a result of gingipain K activity (Kuramitsu, 1998).

Cysteine proteinases have also been identified in many parasitic protozoa, trematodes and nematodes. These proteinases are involved in tissue and skin penetration, virulence, immune evasion and degradation of host proteins to make these a source of nutrients.

Malaria is still one of the most important infectious diseases in the world. The pathogens, different plasmodium species, encode cysteine proteinases that belong to the papain family. These proteinases show conservation of key amino acids in positions near the active site (Rosenthal, 1996). Falcipain, a cysteine proteinase from *Plasmodium falciparum*, the most serious human malaria parasite, is a hemoglobinase found in acid food vacuoles and required for normal parasite development (Rosenthal et al., 1988). When cultured parasites are treated with different low-molecular-weight inhibitors directed against falcipain, parasite development is halted (Rosenthal, 1996; Dominguez et al., 1997). The food vacuoles of the parasites then fill with haemoglobin, because the degradation of haemoglobin is stopped.

Cysteine proteinases from *Leishmania* species appear to be crucial for parasite survival. Cathepsin L-like cysteine proteinase genes from *L. mexicana* are found in tandem arrays of 19 copies (Mottram et al., 1997). The timing of expression of different isoenzymes may be an important survival factor for the parasite in the hostile environment of macrophages. It has been shown that expression of different cysteine proteinases in *L. donovani chagasi* is stage-specific (Omara-Opyene and Gedamu, 1997).

Cruzipain is the proteinase with the highest activity in *Trypanosoma cruzi*, the pathogen in Chagas disease (Cazzulo et al., 1989, 1990). It contains a catalytic domain homologous to cathepsin L-like cysteine proteinases from *Leishmania* species (Mottram et al., 1997). It also has an unusual C-terminal extension, which is not essential for the catalytic function of the enzyme (Eakin et al., 1992) Cruzipain is strongly inhibited by E-64 (Cazzulo et al., 1990).

Blood flukes of the genus *Schistosoma* have a diet that mainly consists of host erythrocytes. Several cysteine proteinases homologous to cathepsins B and L and legumain are involved in heamoglobin degradation (Klinkert et al., 1989; Brady et al., 1999; Dalton and Brindley, 1998). A cathepsin L-like proteinase has also been found in *Fasciola hepatica*, a trematode afflicting ruminants (Dowd et al., 1997).

A cysteine proteinase has also been identified in *Ostertagia ostertagi* (Pratt et al., 1992), a nematode causing considerable economic loss in cattle production.

Cystatins

Natural protein inhibitors of cysteine proteinases, cystatins, were first reported in mammalian tissues in 1957 (Finkenstadt 1957). The first cystatin that was isolated was from chicken egg white and was later named chicken cystatin (Fossum and Whitaker 1968; Barrett 1981). It was found to inhibit papain and cathepsins B, C, H and L (Barrett 1981). Proteins with sequence homology to chicken cystatin have been grouped into a cystatin superfamily (Barrett et al., 1986), which was divided into three subfamilies. Members of each family are present in mammals.

Family I

Members of this family are also called stefins. They are small intracellular (mainly cytoplasmic) inhibitors with about 100 amino acids and have molecular masses of approximately 11 kDa. They lack disulphides and carbohydrate residues. Members of this family that have been isolated and characterised are human, rat, mouse, cattle and pig cystatin A and B (Järvinen, 1978; Brzin et al., 1983; Green et al., 1984; Järvinen, 1976; Takio et al., 1983, 1984; Tsui et al., 1993; Turk et al., 1995; Krizaj et al., 1992), bovine stefin C and porcine stefin D. Bovine stefin C and porcine stefin D were isolated from thymus (Turk et al., 1993; Lenarcic et al., 1996). Cystatin A is found mainly in epithelial cells and neutrophilic granulocytes, while cystatin B occurs in almost all cells and tissues (Järvinen et al., 1987; Henskens et al., 1996).

Family II

Members of this family are synthesised with a signal peptide and are primarily found extracellularly. They are composed of approximately 115 amino acids and have molecular masses of 13-14 kDa. They have two disulphide bridges in their C-terminal regions, one of which can be reduced without the affinity of the inhibitor for target proteinases being appreciably altered (Björk and Ylinenjärvi, 1992). The by far most studied member, human cystatin C (reviewed by Abrahamson, 1994), has been identified in all extracellular fluids examined. In human saliva and tears, cystatins S, SA, SN and D have also been found. Some members of this family are glycosylated, e.g. cystatin C in mouse and rat (Solem et al., 1990; Esnard et al., 1988), and also the recently described cystatin E/M (Ni et al., 199;, Sotiropoulou et al., 1997) and cystatin F/leukocystatin/CMAP (cystatin-like metastasis-associated protein). (Ni et al., 1998; Halfon et al., 1998; Morita et al., 1999). The latter two inhibitors are more distantly related to other family II members. Cystatin F is expressed mainly in cells of the immune system (Ni et al., 1998), whereas cystatin E is expressed in most tissues (Ni et al., 1997).

Recently a new cystatin gene, testatin, only expressed in fetal gonads and in the adult testis, was isolated from mouse (Töhönen et al., 1998). Testatin is similar in

several ways to the previously isolated epidymal specific CRES (cystatin-related epididymal specific) gene (Cornwall et al., 1992).

Family III

This family is also called the kininogen family. The inhibitors of this family are multifunctional glycoproteins with molecular masses of 50-120 kDa and are found primarily in blood and synovial fluid. Three different types of kininogens have been identified: high- and low-molecular weight kininogen and, in the rat only, T-kininogen. The mature molecules of high- and low- molecular weight kininogen are single-chain proteins, but are converted to two-chain forms by limited proteolysis by kallikrein, which releases vasoactive kinin. The kininogen heavy chain, which is identical in the two forms, contains three tandemly repeated cystatin-like domains, D1-D3. D2 and D3 inhibit papain-like cysteine proteinases, although with different affinities (Ylinenjärvi et al., 1995; Turk et al., 1995, 1996) and D2 also inhibits calpain (Salvesen et al., 1986; Ylinenjärvi et al., 1995). An additional function of high molecular weight kininogen, residing in the light chain, is as a cofactor in the initiation of the coagulation cascade (DeLa Cadena and Colman, 1991).

Other cystatins

Several non-mammalian members of the cystatin superfamily have been characterised, including many plant cystatins, such as rice oryzacystatin (Abe et al., 1987) and a potato multicystatin that contains eight repeated cystatin domains (Waldron et al., 1993). Plant cystatins, or phytocystatins, show similarities to family I but are so distinct from family I and II inhibitors that they might be classified as an independent family (Margis et al., 1998).

Cystatins have also been found in nematodes, such as onchocystatin from Onchocerca volvolus (the pathogen in river blindness) (Lustigman et al., 1992), in snake venoms and in a horseshoe crab (Evans and Barrett, 1987; Agarwala et al., 1996; Brillard-Bourdet et al., 1998). These cystatins bear a resemblance to family II cystatins and might provisionally be classified as belonging to this family. However, as the number of members expands, the cystatin superfamily might be divided into new subfamilies.

Cystatin domains, although devoid of inhibitory activity, are also part of other functionally diverse proteins in an analogous manner as the common building blocks of many other known protein families. The cystatin superfamily thus includes also more distantly related proteins, such as fetuins, histidine-rich glycoproteins and cystatin-related proteins (reviewed by Brown and Dziegiewska, 1997).

Mechanism of inhibition

The cystatins form equimolar, tight-binding complexes with their target enzymes, blocking the active site of the enzyme. The binding is often extremely tight, with dissociation equilibrium constants for some interactions as low as 10^{-14} M (Björk et al., 1989; Lindahl et al., 1992). However, it is reversible, as demonstrated by measurements of association and dissociation rate constants (Björk et al., 1989; Björk and Ylinenjärvi, 1990; Lindahl et al., 1992). The association rate constant for most interactions is close to 10^7 $M^{-1} \cdot s^{-1}$, i.e. approaching the value expected for a diffusion-controlled reaction. Cystatins compete with the substrate, but the structure differs from that of a substrate in such a way that the enzyme does not cleave the inhibitor. Dissociation of the complex thus results in an intact inhibitor. The dissociation rate constants for many interactions are very low, corresponding to half-lives for the complexes of up to several weeks (Björk et al., 1989; Lindahl et al., 1992).

An early comparison of the primary structures of cystatins showed some regions of particular high homology (Barrett, 1987; Rawlings and Barrett, 1990). The most conserved of these regions comprises Gln 55 to Gly 59, (the numbering refers to human cystatin C), often referred to as the QVVAG region. Gln 55 and Gly 59 in this region are strictly conserved in all inhibitory cystatins. Gly 11 in the N-terminal region is also an evolutionarily highly conserved residue. These residues were therefore suggested to be of importance for cystatin inhibitory activity (Barrett, 1987; Rawlings and Barrett, 1990).

The crystal structure of chicken cystatin (Bode et al., 1988) strongly supported this suggestion. The structure showed that the molecule mainly consists of a five-stranded antiparallel β-sheet, wrapped around a five turn α-helix, and a more flexible N-terminal region. The first of the two hairpin loops that connect the strands of the β-sheet is the QVVAG region, and the second loop is known as the PW loop (Pro 105-Trp 106). These two loops and the N-terminal region form a hydrophobic wedge. Computer docking experiments showed an almost ideal fit for this wedge into the papain active-site cleft, suggesting a tight interaction involving minimal conformational changes of either protein (Bode et al., 1988). The structure of chicken cystatin was later shown by NMR to be essentially the same in solution, except for a small polypeptide chain segment in the "back" of the molecule, which formed a short helix in the X-ray structure but was disordered in solution (Dieckmann et al., 1993).

The crystal structure of the human cystatin B-papain complex showed that human cystatin B has essentially the same fold as chicken cystatin and confirmed the interaction mode suggested by the docking experiments with chicken cystatin (Stubbs et al., 1990). In the structure of the complex, the N-terminal and QVVAG regions of cystatin B are in direct contact with the enzyme. The tryptophan

residue in the second hairpin loop is not present in family I cystatins, but its role in the interaction is taken over by a Leu and a His residue in this loop. Family I cystatins also have a structurally extended carboxy-terminal region which provides an additional binding site, compared with the docking model with chicken cystatin. The reactive residue of the enzyme, Cys 25, was found not to be in close contact with the inhibitor (Stubbs et al., 1990) but positioned in the empty space between the N-terminal segment and the first hairpin-loop. This location is probably the explanation for the ability of inactivated cysteine proteinases to bind to cystatins, although the affinity decreases with size of the substituent (Björk and Ylinenjärvi, 1989; Lindahl et al., 1992). The two hairpin loops of cystatin B interact with the primed subsites and the N-terminal region interacts with the nonprimed subsites of papain in the complex (Bode et al., 1988; Stubbs et al., 1990). In particular, Gly 11 was found to be positioned close to the S1 subsite pocket of the enzyme (Bode et al., 1988). Many hydrophobic contacts explain the tight interaction between inhibitor and proteinase.

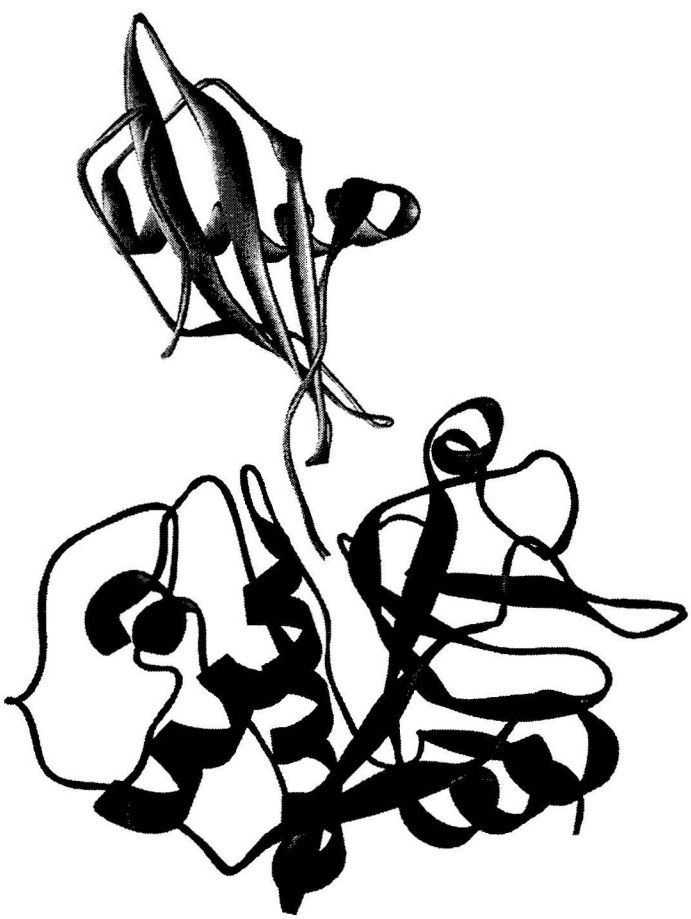

Fig. 2 The structure of the complex between human cystatin B (grey) and papain (black) according to Stubbs et al., (1990).

Many studies have tried to elucidate the importance of the different binding regions of cystatins for the mechanism of inhibition of target proteinases. Several investigators have focused on the contribution of the N-terminal region. Both family I and II cystatins show a large drop in affinity for target proteinases on truncation of this region (Abrahamson et al., 1991; Lindahl et al., 1992; Björk et al., 1994; Estrada et al., 1999). Moreover, site-directed mutagenesis of the conserved Gly 11 residue, has shown that the affinity for target proteinases decreases with both size and charge of the substituting residue in both family I and family II cystatins (Björk et al., 1995; Estrada et al., 1998). Optimal interaction of the N-terminal region of cystatins with the target enzymes is thus essential for tight inhibition.

The highly conserved QVVAG region, which forms the first hairpin loop, was shown by mutagenesis to be of considerable functional importance in chicken cystatin (Auerswald et al., 1995; Machleidt et al., 1995). Deletions of residues in this region thus resulted in greatly decreased affinities of the inhibitor for target proteinases. The inhibition of cathepsin L was less affected than that of papain or cathepsin B, indicating that the first hairpin loop contributes differently to the interaction of these enzymes.

The second hairpin loop has been less well studied. However, Nycander and Björk (1990) showed by chemical modification that Trp 106 in chicken cystatin is involved in the interaction with proteinases. Moreover, mutation of this residue to Gly greatly reduced the affinity of cystatin C for papain and other proteinases (Björk et al., 1996). This observation is consistent with Trp 106 contributing appreciable binding energy by interacting with a hydrophobic region of the enzyme, in papain presumably the region around Trp 177 (Bode et al., 1988). In support of this conclusion, mutation of Trp 106 to Phe had a much smaller effect on the affinity. Deletions of the second hairpin loop in chicken cystatin has also been shown to reduce the affinity for target proteinases (Auerswald et al., 1995).

Lysosomal cysteine proteinases and their endogenous inhibitors in health and disease

Normal functions of lysosomal cysteine proteinases

Lysosomal proteinases are involved in a large number of physiological processes directed at maintaining normal cell metabolism. Some of the more important of these are intracellular catabolism of peptides and proteins (Barrett and Kirche, 1981), processing of prohormones and proenzymes (Otto and Schirmeister, 1997) and natural tissue remodeling such as bone resorption and embryo implantation (Delaissé et al., 1984; Alfonso et al., 1997). Moreover, cathepsin B has been suggested to be the major cysteine proteinase involved in protein degradation for antigen presentation (Mizuochi et al., 1994; Authier et al., 1996). Similarly, it was

recently reported that legumain initiates digestion in the processing of a protein antigen, tetanus toxin, for the MHC class II system in the lysosomal/endosomal system of antigen presenting cells (Manoury et al., 1998). Dendritic cells are potent antigen presenting cells. On maturation of these cells, i.e. when they migrate from the periphery to lymphoid organs, a dramatic alteration in the transport of MHC class II molecules occurs. It was shown that this developmental change in MHC class II trafficking is regulated by invariant chain proteolysis (Pierre and Mellman, 1998). This proteolytic event is most likely due to cathepsin S activity. Moreover, it was suggested that cathepsin S responsible for this proteolysis is not transcriptionally controlled but indirectly regulated by cystatin C (Pierre and Mellman, 1998).

The lysosomal pathway is responsible for 70-80% of the total protein degradation in the cell. Cathepsins of the cysteine proteinase class account for the main enzyme activity of this pathway, which is a nonselective degradation of proteins. The resulting products, amino acids and dipeptides, diffuse through the lysosomal membrane and are reused in protein biosynthesis (Kirschke et al., 1995).

Normal functions of cystatins

Cystatin A

Cystatin A is an intracellular inhibitor mainly found in neutrophilic granulocytes and epithelial cells, specifically the epidermal spinous cells (Järvinen et al., 1987; Brzin et al., 1983). Phosphorylated cystatin A is a natural substrate of epidermal transglutaminase and can be incorporated into skin cornified envelope (Takahashi et al., 1992). Phosphorylation seems to be important in targeting cystatin A to keratohyalin granules (Takahashi et al., 1992). Cystatin A is also known as keratolinin (Takahashi et al., 1997) and acts as a bacteriostatic barrier in skin (Takahashi et al., 1994). It is upregulated in psoriatic lesions (Järvinen et al., 1987).

Rhabdovirus-induced apoptosis was inhibited in a fish cell-line, treated with human cystatin A, and the authors speculate that this may be due to inhibition of interleukin-1β-converting enzyme (Björklund et al., 1997). Bile salt-induced apoptosis can also be inhibited by cystatin A expression (Jones et al., 1998). Furthermore, these authors claim that caspase and cathepsin B activity are linked, and that cathepsin B seems to be a downstream effector proteinase dependent on caspase activity for activation (Jones et al., 1998).

Cystatin B

Cystatin B is widely distributed and is, like other family I members, primarily located in the cytosol. It has been detected in liver, spleen, placenta, epithelial cells, lymphocytes, monocytes and to a lesser extent in polymorpohnuclear leukocytes (Brzin et al., 1982; Henskens et al., 1996). It has also been detected in one body fluid i.e. seminal plasma (Abrahamson et al., 1994). This distribution indicates a general protective role against unwanted activity of lysosomal cysteine proteinases, although further evidence for such a role is lacking.

Several tumour tissues lose their content of cystatins A and B when they become poorly differentiated (Rinne et al., 1984 a,b; Kyllönen et al., 1984; Järvinen et al., 1987; Eide et al., 1992). In view of this observation, Calkins and Sloane (1995) suggest that cystatin A and B may be important for cell differentiation. However, cystatin B-deficient mice are developmentally normal and fertile, although they develop a neurodegenerative disorder as they age (see below) (Pennacchio et al., 1998).

Cystatin C

Species variants of cystatin C have been identified in many species, including monkey (Grubb and Löfberg, 1982), sheep (Tu et al., 1990; Peloille et al., 1997), mouse (Solem et al., 1990; Håkansson et al., 1996), rat (Esnard et al., 1987; Håkansson et al., 1996), dog (Poulik et al., 1981) and rainbow trout (Li et al., 1998). Chicken cystatin (Barrett et al., 1981), which has been well studied, should also be regarded as a cystatin C homologue, as it displays very similar activity, tissue distribution and structural properties as this inhibitor. Bovine cystatin C has been isolated from colostrum (Hirado et al., 1985), skin (Turk et al., 1995), parotid gland (Cimerman et al., 1996) and cerebrospinal fluid (Present investigation: Olsson et al., 1997). The amino acid sequences of some cystatin C species variants are compared in Fig 3 (page 24).

The human cystatin C gene has been localised to chromosome 20 (Abrahamson et al., 1989). It is about 5 kb in size with three exons and has a promoter region typical of so called house-keeping genes (Abrahamson et al., 1990). Such promoters are known to direct mRNA synthesis at relatively low levels, with little tissue specificity and regulation. However, cystatin C concentrations vary considerable in different biological tissues and fluids (see below).

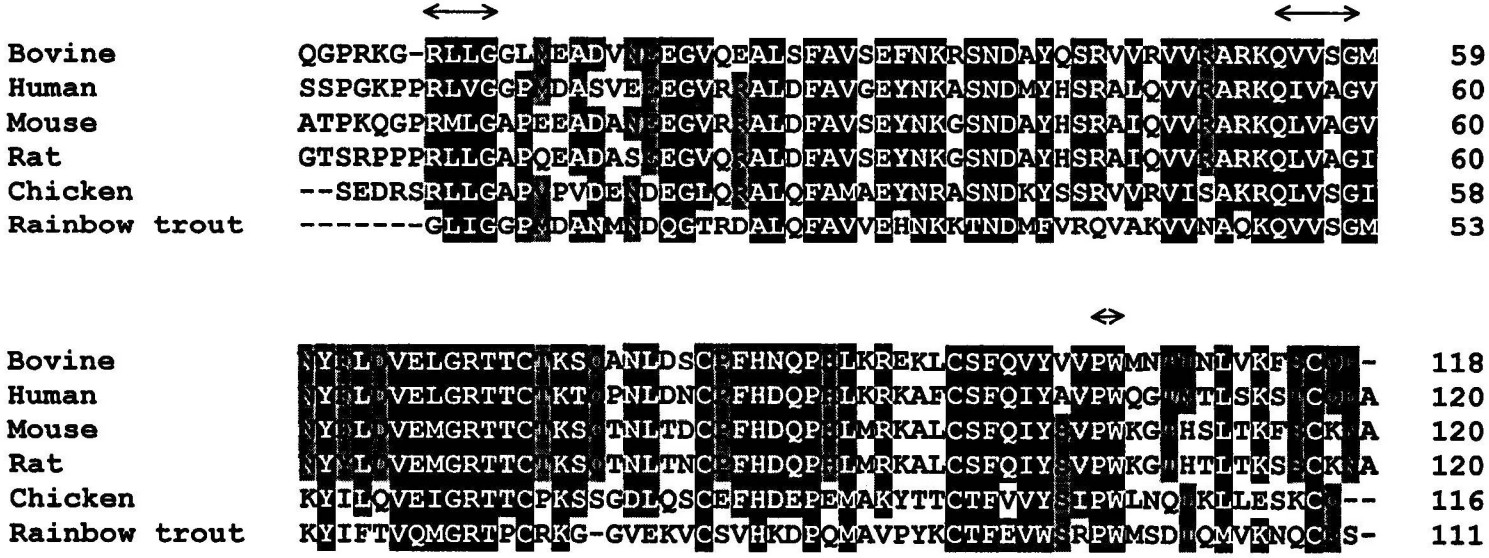

Fig. 3 Amino acid sequence alignment of bovine, human, mouse, rat, chicken and rainbow trout cystatin C. Regions of importance for interaction with target proteinases are marked by ↔. Data from Olsson et al., (1997), Abrahamson et al., (1987), Solem et al., (1990), Cole et al., (1989), Barrett et al., (1981), and Li et al., (1998).

Cystatin C is mainly found extracellularily and is widely distributed, with particularly high concentrations in cerebrospinal and seminal fluids (Löfberg and Grubb 1979; Abrahamson et al., 1986). Cystatin C is the protein with the highest ratio of its concentration to that of albumin in the cerebrospinal fluid (Aldred et al., 1995), indicating synthesis within the CNS. There is a strong conservation between species of the expression of cystatin C mRNA in choroid plexus, which is believed to be the main site of production in the CNS (Tu et al., 1992; Löfberg et al., 1980; Cole et al., 1989). Within the brain, the main production site of cystatin C is believed to be by astrocytes (Solem et al., 1990; Yasuhara et al., 1993). Immunoreactive neurons have also been found (Löfberg et al., 1981; Yasuhara et al., 1993; Lignelid et al., 1997), but appear to be associated with old age or pathological conditions, since they were found in Alzheimer's patients (Yasuhara et al., 1993) or adjacent to tumours (Löfberg et al., 1981).

The expression of cystatin C seems to be age related, at least in some tissues. It was shown that the concentration in the cerebrospinal fluid is correlated with age, being 3-4 times higher in new-borns than in adults (Löfberg et al., 1980). It has also been shown that the proportion of cystatin C mRNA in total RNA from the choroid plexus of sheep embryos and foetuses increases with the increase in the weight of the brain throughout gestation, reaching adult levels at birth (Tu et al., 1990). The cerebrospinal fluid provides an appropriate extracellular environment for the developing brain. Changes of concentrations of different substances in this fluid occur in periods when brain growth is most rapid (Tu et al., 1990). These changes are species-specific, since the most rapid growth occurs before birth in some species and after birth in others. Cystatin C may play a role in this context. Cystatin C-deficient mice are born healthy, grow at normal rate and are fertile (Huh et al., 1998). Moreover, no apparent pathological differences between cystatin C-deficient mice and wild-type mice could be detected on hematoxylin/eosin-stained sections from numerous tissues, including CNS (Huh et al., 1998). However, cystatin C-deficient mice are slightly hypoactive compared with wild-type mice (Huh et al., 1998). Moreover, comparison of the weights of brains from cystatin C-deficient and wild-type mice showed that the weights of the former were lower, indicating the possibility that cystatin C has a growth-promoting effect on some cells in the brain (Håkansson, doctoral thesis, Lund 1998). The growth-promoting effect of cystatin C is supported by the previously discussed increase of cystatin C mRNA in sheep choroid plexus and by a similar increase in the rat brain, in parallel with the increase in the weight of the brain (Tu et al., 1990; Thomas et al., 1989).

Monocytes/macrophages have been reported to secrete cystatin C (Zucker-Franklin et al., 1987; Chapman et al., 1990). Moreover, primary cultures of Sertoli cells have been found to produce cystatin C (Esnard et al., 1992; Peloille et al., 1997), suggesting that the inhibitor may participate in spermatogenesis and spermiogenes. In addition, cystatin C is produced along the whole length of the mouse epididymis (Cornwell et al., 1992). Proteolytic events are essential in

spermatogenesis and spermiogenes, and strict control of such events most probably is necessary.

The tissue distribution of cystatin C seems to be highly conserved among mammals and also quite similar in birds and fish (Håkansson et al., 1996; Colella et al., 1989; Li et al., 1998). Håkansson et al. (1996) found a markedly similar tissue distribution of cystatin C in mouse, rat and man, with the highest concentrations within the brain.

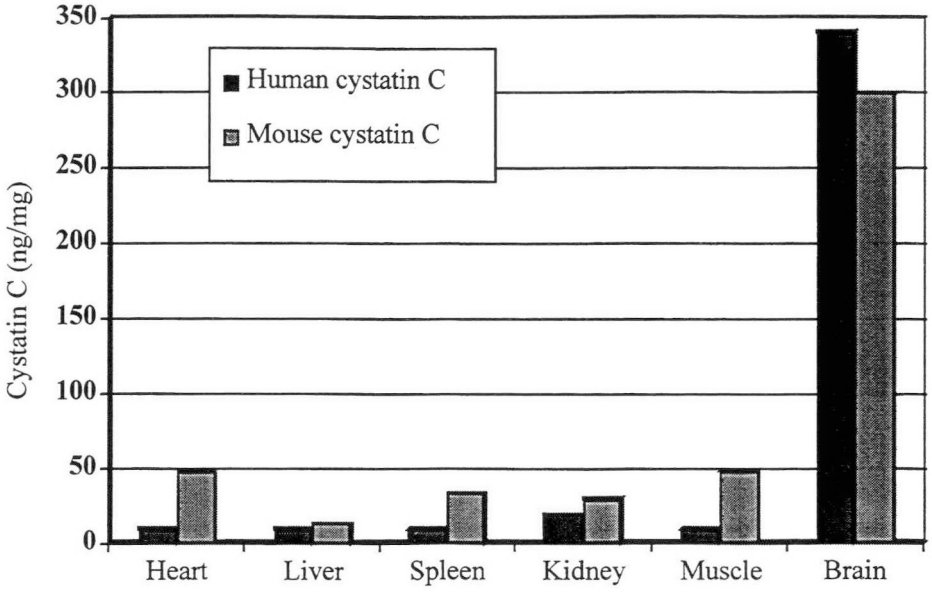

Fig. 4 Distribution of cystatin C in mouse and human tissues. Reprinted from Håkansson (Doctoral Thesis, Lund 1998) with permission from the author.

In spite of considerable knowledge of tissue distribution, the function of cystatin C is far from fully understood. Apart from maintaining protein homeostasis by inhibiting proteinases, cystatin C may be an immunomodulator in inflammatory conditions. The latter function is supported by the above-mentioned expression of cystatin C in macrophages. Furthermore, it was shown that cystatin C and a peptide (KPPR) corresponding to residues 5-8 of the inhibitor can modulate chemotaxis and phagocytosis-associated respiratory burst (Leung-Tack et al., 1990 a, b).

Cathepsins in pathological conditions

In many pathological conditions, lysosomal cysteine proteinases are released to the extracellular space, where a higher pH and inhibitors regulate their activity. However, under some conditions the equilibrium between the released lysosomal cysteine proteinases and their endogenous inhibitors is disturbed. Many factors can contribute to such an imbalance. Inflammatory processes can increase the stability of the enzymes, due to a local decrease in pH, or binding to membranes can protect the enzymes from inactivation. Also, the inhibitory activity can be reduced, due to the inhibitors being proteolytically degraded or inactivated by reactive oxygen species.

Increased extracellular levels of cysteine proteinases have been observed in a number of conditions, such a, sepsis, rheumatoid arthritis, muscular dystrophy, acute inflammation and malignant progression of tumours (Assfalg-Machleidt et al., 1990; Esser et al., 1994; Pearson and Kar, 1979; Sloane et al., 1990). Moreover, pathological relevance of cysteine proteinase activity for tissue damage is indicated by the finding that cathepsin B activity in blood plasma correlates with the extent of organ dysfunction in patients suffering septic chock and in severely traumatised patients (Assfalg-Machleidt et al., 1990). There is also evidence that undesirable cathepsin B activity is involved in the pathogenesis of rheumatoid arthritis and pulmonary emphysema (Esser et al., 1994; Chapman et al., 1994). Moreover, cathepsin L seems to be partly responsible for degradation of cartilage in arthritis (Iwata et al., 1997).

Cathepsins may also be involved in certain neurological diseases, such as Alzheimer's disease (reviewed by Ii, 1995). Abnormality and/or dysfunction of proteolytic systems in neurons or glia cells are important for amyloid formation. The β-amyloid protein precursor, APP is thought to be metabolized by at least two pathways, a secretory and an endosomal/lysosomal. In the latter pathway, cathepsins, primarily cathepsin B, are assumed to be involved in β-amyloid metabolism (Ii, 1995). A model in which calpain is partly responsible for the processing of APP has also been proposed, although very little is known about calpain in this role (Chen and Fernandez, 1998). In relation to other neurological abnormalities, an extracellular enzyme with cathepsin L-like specificity and potentially regulated by cystatin C has been shown to degrade a myelin-associated glycoprotein into a slightly smaller derivative. Loss of this myelin-associated glycoprotein has been associated with demyelinating diseases (Stebbins et al., 1998).

Cathepsins and cystatins in cancer

Degradation of the extracellular matrix in basal lamina is necessary for metastasis and invasiveness, which is characteristic of malignant tumours. Such degradation involves predominantly cathepsins B and L and collagenase.

Cathepsin B has been implicated in the progression of various human tumours. Overexpression of cathepsin B mRNA and increased cathepsin B staining have been found in several different cancers (reviewed by Sloane et al., 1990; Yan et al., 1998). For instance, increased expression of cathepsin B mRNA has been reported in glioblastoma (Rempel et al., 1994), astrocytoma (Sivaparvathi et al., 1995), colorectal carcinoma (Murnane et al., 1991) and carcinoma of the thyroid (Shuja and Murnane, 1996). In some tumours, this increase correlates with clinical progression of the tumour (Ozeki et al., 1993; Ebert et al., 1994). An indication that tumour cells secrete cathepsin B is the increased serum levels of cathepsin B in cancer patients (Leto et al., 1997 a, b; Kos et al., 1997). Moreover, Sloane et al. (1994) reported a redistribution of vesicles staining for cathepsin B from the perinuclear region towards the plasma membrane surface, occurring concomitantly with transition of cells from normal to neoplastic states. In human prostate cancer, increases in cathepsin B mRNA have been found in tumour cells at the invading margin, suggesting a role for cathepsin B in invasiveness (Sinha et al., 1993). Moreover, endothelial cells in neovessels at the infiltrating margins of prostate cancer and gliomas have increased immunostaining for cathepsin B (Mikkelsen et al., 1995; Sinha et al., 1995). The increased levels of cathepsin B in tumours have been reported to involve different isoforms of the enzyme (Krepela et al., 1995).

Altered ratios between inhibitors and proteinases may contribute to malignant progression. Because proteinases are, in general, upregulated and/or abnormally activated in metastatic tumour cells, cystatins would be expected to possess antimetastatic properties, since inhibition of cathepsins should reduce invasiveness. However, malignant tumours have been reported to have higher, similar or lower levels of cystatins compared with normal tissue (reviewed by Calkins and Sloane, 1995). This appears to be contradictory, but many different mechanisms interact in a complex way, making it difficult to evaluate the contribution of a single protein. In this context, the above-mentioned immunomodulatory role of cystatin C (Leung-Tack et al., 1990 a, b) may be of importance. Suppression of the host inflammatory response could thus protect malignant cells. Moreover, if cathepsins, as mentioned above, are involved in apoptosis, inhibition could instead promote cell survival. The importance of these mechanisms probably differs in different tumours. For example, cystatin M (E) is downregulated in breast cancer (Sotiropoulou et al., 1996), and Lignelid et al. (1997) also found a decrease in the expression of cystatin C in anaplastic astrocytomas and glioblastomas, compared with low-grade astrocytomas. Several studies also showed decreased levels of cystatin A in malignant tumours, such as

ovarian carcinoma (Lah et al., 1990) and breast carcinoma (Lah et al., 1992). Moreover, prostate adenocarcinoma cells contained no cystatin A at all, in contrast to normal prostate tissue (Söderström et al., 1995). On the other hand, cystatin CMAP (F) is upregulated in liver metastatic tumour cells (Morita et al., 1999). This observation may be related to the finding that cystatin F does not appreciably inhibit cathepsin B (Ni et al., 1998), the cathepsin that has been reported to activate a proinflammatory caspase (Scotte et al., 1998; Vancompernolle et al., 1998), and thus is a candidate for involvement in apoptosis. Furthermore, it has been reported that overexpression of cystatin C by stable transfection in a melanoma cell line inhibited motility and *in vitro* invasiveness (Sexton et al., 1997). Interestingly, a reduced number of metastases in the lungs of cystatin C-deficient mice, compared with wild-type mice, were observed following intravenous injection of the same melanoma cell line used in these transfection experiment (Huh et al., 1998). However, the reason for this reduced seeding is unknown.

Diseases caused by mutations in cystatin genes

Human hereditary cystatin C amyloid angiopathy, HCCAA, is found in some Icelandic families. Affected individuals suffer from recurrent brain haemorrhages as young adults, and the disease usually leads to death before the age of 40. It is an autosomal dominant disorder, in which cystatin C is deposited as amyloid in the walls of the cerebral arteries (Löfberg et al., 1987; Thorsteinsson et al., 1988). A cystatin C variant having Leu 68 mutated to Gln and lacking ten N-terminal residues was found to be the main component of the amyloid deposits (Ghiso et al., 1986; Levy et al., 1989; Abrahamson et al., 1992). Amyloid deposits were also found in lymph nodes, spleen, salivary gland, seminal vesicles and skin (Löfberg et al., 1987; Thorsteinsson et al., 1988; Benedikz et al., 1990). Cystatin C in the cerebrospinal fluid of HCCAA patients has the same full-length N-terminal sequence as native cystatin C, although the levels are abnormally low, approximately one-third of the mean concentration found in healthy control subjects (Grubb et al., 1984). The serum levels of cystatin C are unaltered in affected individuals. A sporadic case of the L68Q mutation has also been reported (Graffagnino et al., 1995).

The background to the observed lesions is still unclear. Due to the low levels of cystatin C in cerebrospinal fluid, a local excess of cysteine proteinases might cause damage to blood vessels in the central nervous system, thereby causing the haemorrhages. However, the fact that cystatin C-deficient mice are born healthy, grow at normal rate and are fertile (Huh et al., 1998) does not support this explanation.

Recently it was shown that the L68Q variant dimerizes rapidly at pH 7.0-5.5, although it behaves as a stable monomer between pH 7.5 and 9.0 or at pH below 4.5 (Gerhartz et al., 1998). The tendency for L68Q cystatin C to dimerize is

highly temperature-dependent and increases considerably when the temperature is increased from 37 to 40°C (Abrahamson and Grubb, 1994). Dimerized L68Q cystatin C completely lacks inhibitory activity (Abrahamson and Grubb, 1994) It is likely that this tendency for L68Q cystatin C to dimerize is related to the deposition of the mutant in the amyloid.

In connection with the HCCAA disease it is interesting that cystatin C and β-protein colocalisation in amyloidotic arteriolar lesions has been observed in the cerebrums of patients with Alzheimer's disease, senile dementia of the Alzheimer type, leukoencephalopathy and brain haemorrhage. The occurrence of cystatin C in these lesions seems to be related to the incidence of massive cerebral haemorrhage (Vinters et al., 1990; Maruyama et al., 1990; Haan et al., 1994). Nagai et al. (1998) also recognised cystatin C in the amyloid fibril fraction from the meninx of a patient with sporadic cerebral amyloid angiopathy with cystatin C deposition. No mutation in the cystatin C gene was found and the protein was of full length. It is not clear, however, whether unmutated cystatin C can form amyloid *in vivo*, and the association of cystatin C with the amyloid might also be due to nonspecific adherence because of the high isoelectric point of the inhibitor.

Cerebral amyloid angiopathy is also found in aged dogs and some non-human primates. As in humans, the presence of cystatin C amyloid angiopathy in dogs is positively correlated with the incidence of intracerebral haemorrhage (Uchida et al., 1991). Immunoreactivity against cystatin C was found in canine amyloid brain plaques, as well as in some vascular amyloid (Uchida et al., 1991, 1997). In non-human primates, there seems to be a species variation in the localisation of the amyloid deposits. For example, the squirrel monkey is likely to get depositions in the cerebral vessels with age, whereas the rhesus monkey develops more abundant parenchymal brain amyloid. A possible explanation for this behaviour is that squirrel monkey cystatin C has an L68M amino acid substitution that is similar to the L68Q mutation found in humans with HCCAA (Wei et al., 1996; Walker, 1997).

Absence or mutation of cystatin B leads to progressive myoclonus epilepsy in humans, EPM 1, an autosomal recessive form of epilepsy (Pennacchio et al., 1996; Lalioti et al., 1997). The onset is early in life, between 6 and 13 years, and is followed by mental retardation and cerebellar ataxia (Norio and Koskiniemi, 1979). Recently, it was shown that cystatin B-deficient mice develop progressive ataxia and also myoclonic seizures during sleep, but they are otherwise developmentally normal and fertile (Pennachio et al., 1998). The gross morphology and histology of most tissues lacking cystatin B were normal, but a depletion of granule cells in the cerebellum was found. The morphology and staining for fragmented DNA suggested an apoptotic mechanism of cell death in the cerebellum to be the cause of the observed symptoms (Pennachio et al., 1998). It is not clear how cystatin B can block apoptosis, but as also a single amino acid substitution in cystatin B, G4R, which decreases the inhibitory capacity of the

molecule, can cause the disease it seems likely that a loss of inhibitory activity is involved (Lalioti et al., 1997). Maybe cystatin B inhibits caspases, although evidence for this proposal is still lacking. An alternative mechanism is that cathepsins, which cystatin B does inhibit, might be able to activate caspases. This effect of cystatin B may be related to the previously mentioned inhibition of apoptosis by cystatin A.

An additional finding for the cystatin B-deficient mice was that 35% developed ocular opacity as a sequel of corneal lesions (Pennachio et al., 1998). Normal tear fluid contains several proteinases and inhibitors. The observed lesions indicate that a minimal inflammatory response can escalate in the absence of appropriate inhibitors that can neutralise proteinases, e.g. lysosomal enzymes released by invading neutrophils. Perhaps cystatin B should be used in the treatment of injured cornea.

Some cystatins are expressed only in a few tissues, but cystatin C and B are widely distributed. It is therefore notable that deficiency or dysfunction of these cystatins results in symptoms primarily from the CNS. The reason most likely is that control of unwanted proteolysis is highly important in the CNS.

Cystatins as potential antimicrobial agents

As mentioned above, cysteine proteinases play a vital role in the life cycle of many micro-organisms. These proteinases are thus interesting chemotherapeutic targets. In a limited numbers of studies, endogenous inhibitors have shown antimicrobial properties.

Picornaviruses, of which polivirus is the prototype, encode cysteine proteinases. Bovine cystatin C showed a weak antiviral effect on polioinfected human HeLa cells (Cimerman et al., 1996). Moreover, human cystatin C has been reported to block herpes simplex virus replication (Björck et al., 1990).

It was also shown that a synthetic peptide derivative, corresponding to one part of the proteinase-binding sequence of cystatin C, inhibited growth of group A streptococci *in vitro* and *in vivo*, probably by inhibition of a bacterial cysteine proteinase (Björck et al., 1989). In contrast cysteine proteinases from *Porphyromonas gingivalis*, a bacterium associated with peridontitis, are not inhibited by cystatins. In fact, cystatin C may be a substrate. It was thus shown that a proteinase from *P. gingivalis* could cleave cystatin C at Arg 8, thereby decreasing the inhibitory capacity (Abrahamson et al., 1997).

Several inhibitors of the cystatin superfamily inhibit cruzipain, a cysteine proteinase from *Trypanosoma cruzi*, the pathogen in Chagas disease (Stoka et al., 1992).

Present investigation

The purpose of this work was to characterise two endogenous mammalian inhibitors of cysteine proteinases, human cystatin A and bovine cystatin C, with the emphasis on the latter. The main rationale for these studies was the importance of elucidating differences between related cystatins in the interaction with target enzymes. Such information might be useful for the design of inhibitors for therapeutic use, i.e. inhibitors that are very specific for a single proteinase and thereby potentially non-toxic. Comparison of cystatin C function, together with knowledge of the cellular expression pattern of the inhibitors, will also contribute to our understanding of the physiological relevance of cysteine proteinase inhibition. The expression of cystatins within the CNS is of particular interest, as some cystatins are involved in certain neurodegenerative disorders and also are implicated in other related conditions.

Characterisation of recombinant human cystatin A (paper I)

When this work was initiated, human cystatin A had been isolated from polymorphonuclear granulocytes and liver (Brzin et al., 1983; Green et al., 1984), and a cDNA sequence had been reported (Kartasova et al., 1987). Recombinant human cystatin A had also been produced in *E. coli*, in some cases from synthetic genes (Fong et al., 1989; Nikawa et al., 1989; Kaji et al., 1990; Jerala et al., 1994). In addition, homologous inhibitors had been purified from rat and bovine skin (Takeda et al., 1983; Turk et al., 1995). The overall structure of human cystatin A, analysed by NMR, had also been shown to be similar to the structure of cystatin B (Martin et al., 1995). However, no extensive characterisation of interactions with target proteinases had been reported prior to this work. Furthermore, published equilibrium constants for the binding of cystatin A to cysteine proteinases were discrepant for several proteinases.

To enable thorough analyses of the interaction of cystatin A with target proteinases by spectroscopic, kinetic and equilibrium methods, an expression system generating substantial quantities of fully active inhibitor was developed. Spectroscopic studies indicated that the features of the UV absorption and fluorescence difference spectra produced by the binding of this recombinant cystatin A to papain were caused by perturbations around tryptophan residues. As cystatin A lacks tryptophan, interactions with the papain residues Trp-177 and /or Trp 69 that are close to the active site presumably were responsible for these effects. The spectral changes induced by the binding of cystatin A and cystatin C to papain differed considerably, which most likely reflects interactions involving the single tryptophan residue of cystatin C, Trp 106, which is lacking in cystatin A.

The affinities of human cystatin A for cysteine proteinases were found to be appreciably higher than reported earlier. The inhibition constants for papain and cathepsin L were thus as much as 50-100-fold lower and that for cathepsin B five-fold lower than previous values (Green et al., 1984; Fong et al., 1989; Nikawa et al., 1989; Abrahamson et al., 1986; Kaji et al., 1989). These differences most probably are due partly to methodological difficulties inherent in the determination of inhibition constants for tight interactions by equilibrium measurements in the earlier studies. In this work, such inhibition constants were instead calculated from separately measured association and dissociation rate constants.

Of the enzymes investigated in this study, human cystatin A bound most tightly to papain and cathepsin L, with K_i of 10^{-11}-10^{-13} M. The affinity for papain was about one order of magnitude lower than those of the family II inhibitors, cystatin C and chicken cystatin (Björk et al., 1989; Lindahl et al., 1992a), but higher than that of another family I member, human cystatin B (Jerala et al., 1994). The affinity for cathepsin L was similar to that of cystatin B, but the affinities of chicken cystatin and cystatin C for this enzyme appear to be higher. The binding of cystatin A to papain and cathepsin L was rapid, with k_{ass}, of $3\text{-}5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, and comparable to values obtained for chicken cystatin and cystatins B and C. These rate constants approach those expected for a diffusion-controlled rate. Moreover, a linear increase of the observed pseudo-first-order rate constant with inhibitor concentrations was observed for the inhibition of papain, also at high cystatin A concentrations. This behaviour is consistent with a simple reversible bimolecular reaction mechanism. Cystatin A thus appears to bind to papain without major conformational changes of either protein, as also suggested for chicken cystatin and cystatin C (Bode et al., 1988; Björk et al., 1989; Lindahl et al., 1992a).

The binding of human cystatin A to the other enzymes investigated, i.e. cathepsins B, C and H and actinidin, was weaker than to papain and cathepsin L, with K_i values of 10^{-8}-10^{-9} M. The affinity of cystatin A for cathepsin B was comparable with those of chicken cystatin and cystatin C for this enzyme, but significantly higher than that of cystatin B (Green et al., 1984; Björk et al., 1994; Abrahamson et al., 1991; Jerala et al., 1994; Hall et al., 1993). The lower affinity of human cystatin A for cathepsin B than for papain or cathepsin L was due to a reduced association rate constant. In contrast, the lower affinity of chicken cystatin and human cystatin C for cathepsin B was due mainly to an increased dissociation rate constant. The occluding loop of cathepsin B restricts access to the active site cleft of the enzyme (Musil et al., 1991), and the differences in kinetic behaviour between family I and family II cystatins could reflects different mechanisms to overcome this restriction.

Human cystatin A was shown to bind to actinidin with affinities comparable with those of chicken cystatin and cystatin C (Lindahl et al., 1992a,b). However, the affinities of human cystatin A for cathepsins C and H were 10-100-fold lower

than those reported for family II cystatins (Barrett et al., 1986; Abrahamson et al., 1991; Hall et al., 1993).

Like other cystatins, human cystatin A bound also inactivated forms of papain, in which the reactive-site cysteine had been covalently linked to a blocking group. The affinity decreased as the size of the inactivating group increased, and this decrease was due to increasing dissociation rate constants, in a similar way as was shown for human cystatin C and chicken cystatin (Björk and Ylinenjärvi, 1989; Lindahl et al., 1992a). The inactivating groups had much larger effects in reducing the affinity for cystatin A than they had on the affinities for family II cystatins. Probably there is less space around the active site cysteine in the complex with cystatin A than in the corresponding complexes with family II cystatins.

The N-terminal region of cystatin A was suggested to be of importance for proteinase binding, as a proteolytically truncated form had an appreciable lower affinity for papain than the full length inhibitor. Similar results have been obtained with naturally occurring truncated variants of the rat counterpart of cystatin A, cystatin α (Takeda et al., 1986). These findings are in agreement with studies showing that the corresponding regions of human cystatin C and chicken cystatin contribute appreciable free energy to the binding of target proteinases (Abrahamson et al., 1991; Lindahl et al., 1992b; Björk et al., 1994, 1995).

Two variants of recombinant cystatin A with extended N-terminal regions, presumably produced by aberrant cleavage by the bacterial signal peptidase, were isolated. In contrast with truncation, only a minor effect on the affinity of cystatin A for papain and cathepsin H was observed by this elongation of the N-terminal region (paper I).

Molecular cloning and isolation of bovine cystatin C (paper II)

Prior to this work, no DNA-sequence was available for bovine cystatin C, but the inhibitor had been purified from colostrum (Hirado et al., 1985), skin (Turk et al., 1995) and parotid gland (Cimerman et al., 1996). In these studies, the forms that were isolated were found to lack the region corresponding to the N-terminal six to eight residues of the human inhibitor. This region had been found to be of crucial importance for tight binding of human cystatin C and chicken cystatin to target enzymes (Abrahamson et al., 1991; Lindahl et al., 1992; Björk et al., 1994). The absence of the corresponding N-terminal segment in preparations of bovine cystatin C could be due to the mature bovine inhibitor being synthesised without this region, in which case bovine cystatin C presumably would be a rather inefficient inhibitor of cysteine proteinases. Alternatively, the short forms of the bovine inhibitor that had been isolated may have resulted from proteolytic truncation of a full-length, tight binding inhibitor that could be of physiological

relevance or may have occurred during the isolation procedure. In order to resolve these alternatives, a cDNA encoding bovine precystatin C was cloned and sequenced, and the inhibitor was isolated from cerebrospinal fluid during conditions minimising proteolysis.

The cDNA encoding bovine precystatin C was found to be similar to that of human precystatin C. The regions encoding the mature proteins thus had a nucleotide identity of 70%, although the noncoding regions showed no significant identity. The predicted open reading frame from a potential initiator methionine encoded 148 amino acid residues, including a putative signal peptide, and supported the idea that bovine cystatin C has an N-terminal region homologous to the corresponding region of human cystatin C.

To allow the N-terminal sequence of mature bovine cystatin C to be unequivocally established, the protein was purified from cerebrospinal fluid by ion exchange and reverse phase chromatographies. Cerebrospinal fluid was chosen because of its low content of total protein and white blood cells, which should greatly reduce the risk of proteolytic cleavage. The small amount of cerebrospinal fluid available and the low content of protein in this fluid resulted in a low yield of the purified protein. No N-terminal amino acid could readily be identified by conventional N-terminal sequencing, and the purified inhibitor was therefore digested with endopeptidase LysC. The identity of the protein with bovine cystatin C was verified by sequencing of one purified peptide, which was found to have the sequence RSNDAYQSRVVRKVVRARK, identical to amino acids 36-53, deduced from the cDNA sequence.

To identify the putative blocked N-terminal peptide, several peptides from endopeptidase Lys-C-digested purified bovine cystatin C were analysed by mass spectrometry. One of these gave a relative molecular mass identical to that expected for the N-terminal pentapeptide QGPRK having the N-terminal blocked by cyclization to pyroglutamic acid. In additional experiments, the N-terminal sequence of bovine cystatin C was conclusively verified by tandem mass spectrometry of this peptide. The C-terminal fragment ion series obtained with a sample in which free carboxyl groups had been esterified with methanol corresponded to the peptide sequence pyro-EGPRK. This sequence was confirmed by the series of N-terminal fragments of the unmodified peptide. Thus, the N-terminal peptide was identified by three different sets of data.

These analyses unequivocally verified that the isolated bovine cystatin C indeed had an N-terminal region analogous to that of the human inhibitor and also showed that the N-terminal amino acid was blocked in the form of pyroglutamic acid. Three residues preceding Gly-10 in bovine cystatin C, an amino acid conserved in most cystatins, are highly similar to the corresponding residues of the human inhibitor, i.e. Arg-Leu-Leu vs. Arg-Leu-Val. These residues contribute considerably energy of binding of human cystatin C to target proteinases (Lindahl

et al., 1992; Björk et al., 1994; Hall et al., 1995). Their presence also in bovine cystatin C indicate a similar high affinity of the bovine inhibitor for target proteinases as has been reported for human cystatin C.

The N-terminal region of human cystatin C can be cleaved off by neutrophil elastase, which leads to decreased inhibitory activity, a process that may serve to regulate the activity of the inhibitor under inflammatory conditions (Abrahamson et al., 1991). It is possible that bovine cystatin C is cleaved in a similar manner by neutrophil elastase or other enzymes. As no full-length forms of the inhibitor had been isolated prior to this study, the bovine inhibitor seems to be more sensitive to such proteolysis.

The establishment of the N-terminus of the mature inhibitor enabled the signal peptide to be conclusively identified from the cDNA sequence. The bovine cystatin C signal peptide has characteristics typical of such peptides and is similar to that of human cystatin C. However, it is longer, 30 residues *vs.* 26 residues for human precystatin C, due to an insertion of four residues.

The pyroglutamyl residue that was identified as N-terminus may have been formed during the purification procedure. Alternatively, it might be formed *in vivo*, as enzymes catalysing this reaction have been demonstrated in many tissues, including brain (Busby et al., 1987). Whether this possible cyclisation is of physiological importance remains an open question.

Family II cystatin genes in general have three exons of characteristic sizes (Huh et al., 1995). The human cystatin C gene is about 5 kb and has a gene structure typical of family II cystatins (Abrahamson et al., 1989). A fragment of the bovine cystatin C gene was PCR-amplified from genomic DNA with primers outside but adjacent to the coding region of the cDNA. The resulting product was isolated and partially sequenced. The bovine cystatin C gene was found to be of similar length as the human gene and also to comprise three exons. Moreover, the intron/exon borders were homologous to those of the human gene (unpublished observations).

Characterisation of recombinant bovine cystatin C (paper III)

As discussed above, only the properties of forms of bovine cystatin C with an incomplete N-terminal region had been characterised before the work of this paper (Hirado et al., 1985; Turk et al., 1995; Cimerman et al., 1996). These forms bound target enzymes appreciably more weakly than human cystatin C. For instance the affinities of bovine cystatin C isolated from parotid gland, having Leu-8 as first amino acid (Cimerman et al., 1996), for papain and cathepsin B were one to three orders of magnitudes lower than the affinities of human cystatin C for these enzymes.

Bovine cystatin C with a full-length N-terminal region was expressed as a fusion protein in *E. coli*, with the expression vector pET-23(+). The inhibitor was fused to the signal peptide for OmpA, a His-tag and the recognition site for enterokinase. The purpose of the OmpA signal peptide was to target the fusion protein to the periplasmic space, where the environment is favourable for the formation of S-S-bridges and the concentration of degrading proteinases is low. The His-tag allowed facile purification, and cleavage with enterokinase released bovine cystatin C with an authentic N-terminal. The integrity of the purified protein was verified by mass spectrometry.

A heat stability test showed that the recombinant bovine cystatin C was highly stable against thermal denaturation, even somewhat more stable than human cystatin C. This result indicates that the recombinant inhibitor was folded in the correct manner. Titrations, monitored by the loss of enzyme activity, showed that different preparations of bovine cystatin C bound to papain with stoichiometries of 0.95-1.05, values experimentally indistinguishable from 1.0. The recombinant inhibitor was thus fully active in binding to the enzyme.

The general properties of bovine cystatin C, as reflected by the thermal stability, as discussed above, the isoelectric point, the far-ultraviolet circular dichroism spectrum, and the changes of tryptophan fluorescence on interaction with papain, resembled those of human cystatin C (Lindahl et al., 1992; Abrahamson et al., 1987). In particular, the latter changes indicate that the two inhibitors interact with cysteine proteinases in a highly similar way (Lindahl et al., 1992; Abrahamson et al., 1987; Abrahamson et al., 1991).

The recombinant inhibitor was shown to bind significantly more tightly to cysteine proteinases than the shorter forms characterised previously. The affinity of full-length bovine cystatin C for papain was very high, with K_d around 10^{-14} M. The bovine inhibitor also bound tightly to cathepsin L, although only a lower limit for K_d of about 10^{-11} M could be estimated. The interactions with cathepsins B and H were considerably weaker, with K_d-values around or slightly below 10^{-9} M. k_{ass} for the binding of bovine cystatin C to papain and cathepsin L was $\geq 10^7$ $M^{-1} \cdot s^{-1}$, whereas the rate of the binding to cathepsin B was about one order of magnitude lower. The high affinity for papain was mainly due to a very low k_{diss}, of about 10^{-7} s^{-1}, corresponding to a half life of the complex of about 80 days. Analogously, the lower affinity for cathepsin B was predominantly due to a higher k_{diss}, of about 10^{-4} s^{-1}.

In spite of the general similarity, some differences between bovine and human cystatin C were apparent. Bovine cystatin C bound more slowly to human cathepsin B than human cystatin C, but also dissociated more slowly, which resulted in about the same binding affinity. Moreover, the affinity of bovine cystatin C for human cathepsin H was slightly weaker than that of the human

inhibitor. Also, bovine cystatin C bound to cathepsin L with about a four-fold higher k_{ass} than that of the human inhibitor. This rate constant is comparable with the highest values observed previously for cystatin-cysteine proteinase reactions and is close to the limit expected for a diffusion-controlled reaction. These differences between bovine and human cystatin C in the interaction with target enzymes probably reflect different structures of the proteinase binding regions of the two inhibitors. One such difference could be the first hairpin loop, which in the bovine inhibitor has the sequence QVVSG, i.e. is more hydrophilic than that of the human inhibitor, QIVAG (Turk et al., 1997; Grubb and Löfberg, 1982; II, Abrahamson et al., 1987). The N-terminal region and the second hairpin loop also show sequence differences, although less prominent, which however could contribute to the observed differences in interaction with proteinases.

Digestion of the recombinant bovine cystatin C with neutrophil elastase resulted in three forms with shorter N-terminal regions, comparable with those previously isolated from various bovine tissues. As these forms could not be separated under conditions not involving protein unfolding, only an average affinity of the forms for papain was determined. However, this affinity was appreciably lower than that of the intact inhibitor and of the same order of magnitude as the values previously reported for the shorter form isolated from parotid gland (Cimerman et al., 1996). Together, the results of this work thus suggest that the forms of bovine cystatin C isolated previously have arisen by proteolytic cleavage of a mature, full-length inhibitor and that the N-terminal region of bovine cystatin C is of major importance for tight binding of cysteine proteinases.

Tissue distribution of bovine cystatin C (paper IV)

Although the mechanism of inhibition of target proteinases has been extensively studied for several species variants of cystatins, considerably less is known about the tissue distribution of the inhibitors. Human cystatin C has a promoter region typical for so called house-keeping genes (Abrahamson et al., 1990), consistent with expression in a number of tissues. However, in contrast with this suggestion, concentrations of human cystatin C vary considerably in the fluids that have been examined, with particular high concentrations in cerebrospinal fluid and seminal plasma (Abrahamson et al., 1986; Löfberg and Grubb 1979). Most of the information on tissue distribution comes from work with Northern blots of purified RNA and from measurements of concentration in body fluids, tissue extracts and cell culture supernatants (Abrahamson et al., 1986; Löfberg and Grubb, 1979; Håkansson et al., 1996; Esnard et al., 1992; Cole et al., 1989). These methods give no information regarding which sets of cells that transcribe a particular message within a heterogeneous cell population. Prior to this study, in situ hybridisation, which may answer this question, had only been used in a solitary tissue but not to analyse the tissue distribution of cystatin C expression.

Consecutive sections from several bovine tissues were hybridised to sense and antisense digoxigenin-labelled cRNA probes. Cystatin C mRNA was heavily concentrated in the epithelial cells of the choroid plexus and in single cells speckled in lymphoid tissue and mammary gland, as well as in Sertoli cells in the testis. Cystatin C mRNA was also present in occasional neurons and glial cells throughout the cerebrum and cerebellum. In the cerebellum, hybridisation to cystatin C mRNA were predominantly found in cells in the Purkinje cell layer. Specific mRNA in detectable amounts was also found in striated intralobular ducts and interlobular ducts, as well as some acinar serous cells in the submandibular gland. In contrast with this expression pattern, immunoreactive cystatin C could only be identified in the choroid plexus.

The expression of cystatin C in brain tissue is of particular interest, as the inhibitor seems be involved in certain neurologic diseases. It has been shown to accumulate within neurons in conditions such as Alzheimer's disease and old age (Yasuhara et al., 1993), in ischemia (Ishimaru et al., 1996) and after hypophysectomi (Katakai et al., 1997). Moreover, a variant of cystatin C is the major component of amyloid deposits in the human disease, hereditary cystatin C amyloid angiopathy, HCCAA (Ghisio et al., 1986). In the central nervous system, the choroid plexus is considered to be a major site for synthesis of cystatin C (Cole et al., 1989; Tu et al., 1992; Löfberg et al., 1980). Within the brain, however, the main production site of cystatin C is believed to be astrocytes (Solem et al., 1990; Yasuhara et al., 1993).

The high concentration of cystatin C mRNA and the immunoreactivity that was found in the epithelial cells of the choroid plexus is in accordance with previous findings (Cole et al., 1989; Tu et al., 1992; Lignelid et al., 1997). The observation that specific mRNA was present in the cytoplasm of occasional neurons throughout the cerebrum indicates that these cells also synthesise cystatin C, a finding consistent with previous studies showing immunoreactivity in cerebellar neurons (Löfberg et al., 1981; Yasuhara et al., 1993; Lignelid et al., 1997). However Löfberg et al. (1981) and Yasuhara et al. (1993) showed such immunoreactivity only in old individuals and in brain tumours, whereas the reactivity was minimal or absent in control cases. Moreover, the age of the individuals in the study by Lignelid et al. (1997) was uncertain. In this context it should be pointed out that aged neurons and neurons from Alzheimer's patients appear to accumulate cystatin C (Yasuhara et al., 1993), and immunoreactive neurons have also been found in HCCAA patients (Wang et al., 1997). In contrast to the immunohistochemistry analyses, in-situ hybridisation in this study suggested that also neurons from young, normal individuals express cystatin C.

The demonstration of expression of cystatin C mRNA in Sertoli cells in the testis is in agreement with a report that the supernatant of primary cultures of Sertoli cells contain cystatin C (Esnard et al., 1992). Cystatin C secreted by Sertoli cells

could be involved in the local regulation of cysteine proteinases, which may be necessary for spermatogenesis and spermiogenesis.

Weak hybridisation to cystatin C mRNA was also observed in striated intralobular ducts and interlobular ducts, as well as in some acini of the submandibular gland. Human salivary cystatin S had previously been localised by in-situ hybridisation and immunocytochemistry to the serous acinar cells in this gland (Bobeck et al., 1991). The two family II cystatins in saliva thus appear to be synthesised by different cells in the submandibular gland.

The distribution pattern of the cystatin C-positive cells observed in spleen, lymph node and mammary gland suggested that these cells were macrophages. This conclusion is supported by the reported secretion of cystatin C by monocytes/macrophages (Zucker-Franklin et al., 1987, Chapman et al., 1990). Cystatin C may be a potent down-regulator of inflammation, inhibiting the cysteine proteinases that are released in inflammatory lesions (Leung-Tack et al., 1990 a,b).

Immunoreactivity could not be demonstrated in any other tissue than the choroid plexus in this work. This observation may be due to the fact that cystatin C is a secreted protein and that thus only little is present normally within cells. Alternatively, the crossreactivity of the antibodies, which were directed against human cystatin C, with the bovine inhibitor may not have been sufficiently strong for sensitive detection.

Thorsteinsson et al., (1992) reported that more cystatin C was retained within monocytes from patients with the mutated cystatin C gene causing HCCAA than in monocytes from healthy controls. Moreover, in experiments with mouse cells transfected with a vector containing normal human cystatin C or L68Q cystatin C, i.e. the variant causing HCCAA, clones expressing the gene encoding L68Q cystatin C secreted slightly lower amounts of the protein than clones expressing wild-type cystatin C (Bjarnadottir et al., 1998). These two reports suggest that the secretion of cystatin C is impaired in HCCAA. There is a possibility that the immunoreactivity observed in neurons in other pathological conditions, such as Alzheimer's disease and various tumours (Löfberg et al., 1981; Yasuhara et al., 1993; Lignelid et al., 1997), also could be due to impaired secretion, although no mutations in the cystatin C gene have been found in such conditions. Our observation that neurons from young normal individuals expressed cystatin C mRNA but did not contain detectable amounts of the protein is consistent with such a suggestion.

General Summary

In this work, two endogenous mammalian cysteine proteinase inhibitors have been characterised, human cystatin A, a family I member, and bovine cystatin C, a family II member. The general conclusions of the work are:

I. The binding of human cystatin A to several proteinases of the papain family was quantified with a recombinant inhibitor, expressed in *E. coli*. These results showed that the affinities of cystatin A for target proteinases had been underestimated in previous work. The binding to papain and cathepsin L was tight with K_i values of 10^{-11}-10^{-13} M, and rapid, with k_{ass} values of $3\text{-}5\cdot10^6$ $M^{-1}\cdot s^{-1}$. These, k_{ass} values approach those expected for a diffusion-controlled rate. This observation, together with the linear increase in the observed pseudo-first-order rate constant for papain inhibition with inhibitor concentration, is consistent with the association of the two proteins occurring in one step, without detectable conformational changes in either protein. The binding of cystatin A to the other proteinases studied, i.e. cathepsin B, C, and H and actinidin was weaker, with K_i values of 10^{-8}-10^{-9} M. A proteolytically truncated form of cystatin A lacking the N-terminal region had an appreciably decreased affinity to papain, suggesting that this region is important for tight binding of proteinases.

II. Previously isolated forms of bovine cystatin C all had appreciably shorter N-terminal regions than the human inhibitor. Moreover, these forms inhibited target proteinases more weakly than human cystatin C. In this work, both the sequence of a bovine precystatin C cDNA and the partial amino acid sequence of bovine cystatin C purified from cerebrospinal fluid showed that the bovine inhibitor has an extended N-terminal region homologous to that of human cystatin C. The results also demonstrate that bovine cystatin C is synthesised as a preprotein with a signal peptide of 30 residues that precedes the mature protein, which contains 118 amino acid residues.

III. Recombinant bovine cystatin C with a complete N-terminal region was characterised. The general characteristics of the inhibitor, as reflected by the isoelectric point, the far–ultraviolet circular dichroism spectrum, the thermal stability and the changes of tryptophan fluorescence on interaction with papain, resembled those of human cystatin C. The affinity and kinetics of inhibition of papain and cathepsins B, H and L were also comparable for the bovine and human inhibitors. In particular, full-length bovine cystatin C bound appreciably more tightly to proteinases than the shorter forms characterised previously. However, some differences between the bovine and human inhibitors were observed. Most importantly, bovine cystatin C bound to cathepsin L with a four-fold higher association rate constant than the human inhibitor. This value is comparable with the highest values reported previously for cystatin-cysteine proteinase reactions. Digestion of the recombinant bovine cystatin C with neutrophil elastase resulted

in forms with truncated N-terminal regions and appreciably decreased affinity for papain. This result is consistent with the forms of the bovine inhibitor isolated previously having arisen by proteolytic cleavage of a mature, full-length inhibitor.

IV. In situ hybridisation with digoxigenin-labelled cRNA probes demonstrated that bovine cystatin C mRNA was heavily concentrated in the epithelial cells of the choroid plexus, in single cells speckled in lymphoid tissue and in Sertoli cells. Cystatin C mRNA was also present in occasional neurons and glial cells throughout the cerebrum and the cerebellum. In the submandibular gland, specific mRNA in detectable amounts was found mainly in striated intralobular and interlobular ducts. The expression of cystatin C in brain tissue is of particular interest, as the inhibitor is involved in certain neurological diseases. Within the brain, the main production site of cystatin C is believed to be astrocytes, but the results obtained in this work suggest that also neurons from young, normal individuals express cystatin C.

Acknowledgements

Help and encouragement from many friends and colleagues made this work possible. I wish to express my sincere gratitude to Dr. Bo Ek for scientific guidance, friendship and his never failing support, even in, for me critical situations.

I would also like to thank;
Professor Lars Rask for scientific guidance and for letting me join the Department of Cell Research, with its excellent laboratory facilities.
Dr. Eva Murén and Jonny Wernersson for numerous discussions, kind support in tough situations and many good laughs.
Bo Pontoppidan and Georg Granér for all computer-assistance.
Ulla Pihlgren not only for expertise technical assistance but also for her positive attitude towards life.
Yvonne Tillmann and Ingrid Schenning for expertise advice in the peptide lab.
Drs. Anna-Stina Höglund and Håkan Larsson for advice and support.
Margareta Gunnarsson för vänlig omtanke (och naturligtvis även för disken).
Lars-Olov Hansson, for providing numerous references from the library.
All the people at the Department of Cell Research, where most of this work was done, for a nice time. I will miss you!

This work was supported by grants from the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences and the Swedish Council for Forestry and Agricultural Research.

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ISSN 1401-6257
ISBN 91-576-5423-9