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Interaction of Cystatins with Cysteine Proteinases

Maria Nycander

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

The single tryptophan residue, Trp104, of the cysteine proteinase inhibitor, chicken cystatin, was modified with a 2-hydroxy-5-nitrobenzyl group. The change of the absorbtion spectrum on binding of the modified cystatin to papain indicated a decreased enviromental polarity of the probe. The modified inhibitor had a greatly reduced affinity for papain. These results show that Trp104 of cystatin is located in or near the proteinase-binding site.

Characterization of N-terminally truncated forms of chicken cystatin indicated that the N-terminal region contributes approximately 40% of the total free energy change for the binding of the inhibitor to both papain and actinidin. Leu7 and Leu8 account for about two-thirds of this binding energy. Also the highly conserved Gly9 residue and residues N-terminal to Leu7 contribute binding energy, but to a much smaller extent.

Cruzipain, the major cysteine proteinase from the parasite, *Trypanosoma cruzi,* was tightly and rapidly inhibited by cystatin A, B, C, chicken cystatin and kininogen. This shows that cruzipain can be effectively inhibited by host cystatins, and that cystatins may serve as starting points for the design of inhibitors as antiparasite drugs.

The affinity of Gly4 mutants of cystatin A for papain, cathepsin L and cathepsin B decreased with the size of the substituent. Even the smallest substitution, to Ala, reduced the affinity >1000-fold. For papain and cathepsin L the effect was entirely due to increased dissociation rate constants. In contrast, for cathepsin B the mutations affected both the association and dissociation rate constants, consistent with the N-terminal region of cystatin A serving as a guide in binding of the remainder of the inhibitor to cathepsin B.

Stopped-flow kinetics showed a hyperbolic concentration dependence of the observed pseudo-first-order rate constant for the binding of cystatin C to cathepsin B, indicating a two-step binding mechanism. The first step most probably involves an initial weak binding of the N-terminal region of cystatin C to cathepsin B, whereas the second step involves a conformational change due to the displacement of the occluding loop of the enzyme.

Keywords: proteinase, protease, peptidase, proteinase inhibitor, cysteine proteinase, cysteine proteinase inhibitor, papain, cathepsins, cruzipain, cystatins, stefins, inhibition, enzyme kinetics, two-step reaction mechanism

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Papers I–V

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

I. Nycander M. and Björk, I. 1990. Evidence by chemical modification that tryptophan-104 of the cysteine proteinase inhibitor chicken cystatin is located in or near the proteinase-binding site. *Biochem. J. 271*, 281-284.

II. Lindahl, P., Nycander, M., Ylinenjärvi, K., Pol, E. and Björk, I. 1992. Characterization by rapid kinetic and equilibrium methods of the interaction between *N*-terminally truncated forms of chicken cystatin and the cysteine proteinases papain and actinidin. *Biochem. J. 286*, 165-171.

III. Stoka, V., Nycander, M., Lenarcic, B., Labriola, C., Cazzulo, Björk, I. and Turk, V. 1995. Inhibition of cruzipain, the major cysteine proteinase of the proteozoan parasite, *Trypanosoma cruzi*, by proteinase inhibitors of the cystatin superfamily. *FEBS Lett. 370*, 101-104.

IV. Estrada, S., Nycander, M., Hill, N., Craven, J., Waltho, J. and Björk, I. 1998. The role of human cystatin A in the binding of target proteinases. Characterization by kinetic and equilibrium methods of the interactions of cystatin A Gly-4 mutants with papain, cathepsin B and cathepsin L. *Biochemistry,* in press.

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

Introduction

The aim of this thesis work has been to investigate structure-function relationships of cystatins and their mechanism of inhibition of cysteine proteinases.

The thesis work started by elucidation of the importance of the second hairpin loop and of different residues in the N-terminal region of chicken cystatin for proteinase binding. The inhibition of cruzipain, the major cysteine proteinase from the protozoan parasite, *Trypanosoma cruzi*, by cystatin A, B, C, chicken cystatin and kininogen was then studied. Later work concerned the N-terminal region of cystatin A and its importance for the interaction with target proteinases. Finally, the mechanism of interaction of cystatin C with the lysosomal proteinase, cathepsin B, was studied.

Previous investigations

This thesis is focused on cysteine proteinase inhibitors and their mechanism of interaction with cysteine proteinases.

Enzymes

Enzymes are catalysts, i.e. they speed up the rates of reactions without being consumed themselves. Each chemical reaction in the living cell is catalysed by its own particular enzyme, so in every cell there is a large number of enzymes. In the absence of enzymes most of these reactions would not occur even over a period of years.

Enzymes speed up the reaction rates by lowering an energy barrier. Even when a rection is termodynamically favourable, i.e. when the products have a lower free energy than the reactants, there may be an intermediate state of higher energy, the transition state, which has to be overcome. This energy barrier, the energy of activation, is lowered by enzymes and thereby the reaction rates are increased typically 10^4-10^{10} times.

In 1946 Linus Pauling introduced the concept of transition state stabilisation, which proposes that the enzyme is designed to interact specifically with the transition state in order to bind this state much stronger than the ground-state reactants. This means that *de*stabilisation of the ground state reactants, i.e. an increased free energy of the ground state, is also a fundamental concept in enzyme catalysis.

In 1966 Jencks predicted the existence of transition-state-analogue inhibitors which are compounds structurally resembling the transition state and therefore bind so strongly that the enzyme is blocked or inhibited by the compound.

The reaction mechanism varies from enzyme to enzyme. Some enzymes just provide an environment different from that of the the aqueous medium or they bring the reactants in close contact. Other enzymes add or substract a proton, or act by straining bonds in the substrate molecule or by forming transient covalent bonds between the substrate and the enzyme.

The specificity varies between different enzymes. Some enzymes, mainly degradative enzymes, have low specificities, e.g. some proteinases, phosphatases and esterases. They utilize many different substrates, provided these contain the right chemical bond, i.e. peptide, phosphate ester, and carboxylate ester bonds, respectively. But many enzymes show absolute specificity, which means that they will only catalyse the reaction with a single substrate or with highly similar substrate analogues, although the latter at a much lower rate.

The catalytic activity of enzymes can be very precisely regulated. This can be achieved by allosteric control, in which binding of a small molecule to the enzyme mediates conformational changes which alter the enzyme activity. Enzymes can also be regulated by stimulation or inhibition by low-molecular compounds or control proteins, by reversible covalent modification or by proteolytic activation, in which the enzyme is activated by hydrolysis of a peptide bond.

Today, recombinant DNA techniques and site-directed mutagenesis make it possible to alter the catalytic activity and specificity of enzymes by introducing mutations at defined positions, which leads to a greatly increased understanding of the mechanisms of enzyme catalysis.

Proteinases

Enzymes catalysing hydrolysis of peptide bonds are called proteinases, proteases, or proteolytic enzymes. They have two main functions. One is to unspecifically degrade proteins as part of the digestion of food or the metabolic cycle of the cell. For instance, lysosomal intracellular protein turnover is carried out by unspecific proteinases (Rivett, 1993; Ciechanover and Schwatz, 1994), and extracellular proteinases mediate the turnover of extracellular proteins (Woessner, 1991). The lifetime of critical molecules, cell growth (Scott, 1992) and wound healing (Hembry et al., 1993; Girard et al., 1993) are also regulated by proteinases. Moreover, malignant cell proliferation involves unspecific degradative proteolytic processes (Scott et al., 1992). The other main function of proteinases is specific cleavage to activate inactive proenzymes or other proproteins or to cleave the signal peptides of extracellular proteins (Hazuda et al., 1990). Specific proteinases, for example,

have important roles in the coagulation (Mann et al., 1988) and complement (Sim et al., 1993) systems, in which they are part of an enzymatic cascade.

The bond cleaved by a proteinase is the one that joins amino acids in proteins, the peptide bond (Fig. 1). In the synthesis of proteins, the carboxyl group of one amino acid is connected to the amino group of the next amino acid in the sequence, by the extraction of a water molecule, to form the linkage -CO-NH-. The process of hydrolysis of peptide bonds catalysed by proteinases is the reverse of this reaction. A water molecule is decomposed for each peptide bond broken, restoring the amino and carboxyl groups at the site of cleavage.

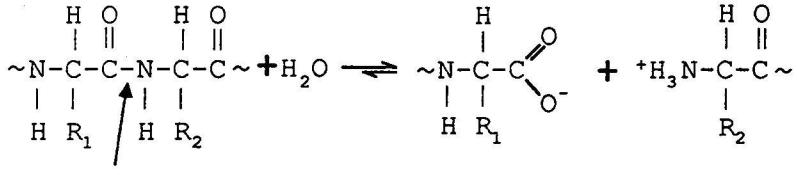

Fig. 1. The hydrolysis of a peptide-bond, the reaction catalyzed by proteolytic enzymes.

Proteinases are classified in four groups based on their active site residues. The four groups are serine, cysteine, metallo- and aspartic proteinases (Barrett 1980; Neurath 1984, 1989). Another classification of proteinases is based on where the substrate is cleaved; exoproteinases (also called exopeptidases) cleave the substrate near the amino- or carboxyterminal end, and endoproteinases (or endopeptidases) mediate cleavage internally in the substrate (Barrett and MacDonald 1985, 1986).

Three-dimensional structures of a number of proteinases have been determined by X-ray crystallography, and in all cases it has been found that the catalytic site is located in a cleft on the surface of the enzyme. The substrate polypeptide is bound along the active site cleft, and on either side of the catalytic site are subsites adapted to interacting with amino acid side chains or parts of the polypeptide backbone of the substrate. The system for reference to the different subsites of the enzyme and to the corresponding parts of the substrate molecule is illustrated in Fig. 2.

Regulation of proteinases

Careful regulation of proteinase activity is required. In addition to control of normal endogenous tissue proteinases, the control of proteinases released by microorganisms and by inflammatory and tumor cells is also needed, since such proteinases facilitate the invasion of these cells into tissues.

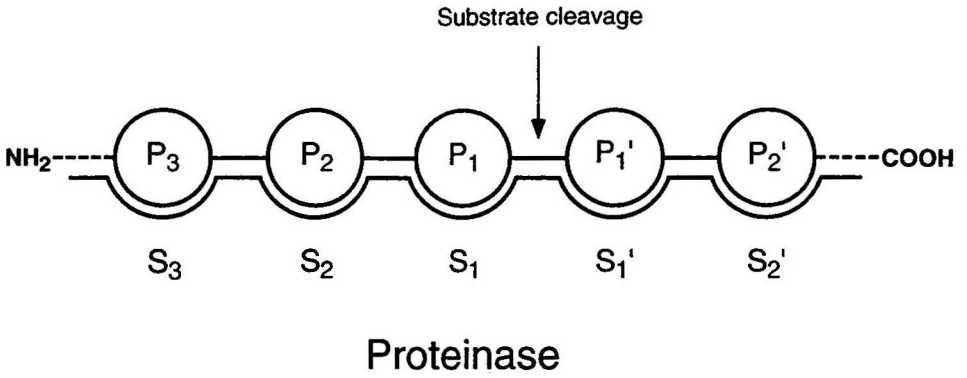

Fig. 2. Scheme for the terminology of subsites of proteinases and the corresponding amino acid side-chains or parts of the polypeptide backbone of the substrate (Schechter and Berger, 1967). The peptide bond that is cleaved, the scissile bond, is the bond between P_1 and P_1'.

The synthesis of proteinases is carefully controlled both at the level of transcription and translation. One of the most important controls of proteolytic activity is the synthesis of proteinases as inactive precursors, or zymogens. Most proteinases are transported and stored in the zymogen form. The activation of zymogens usually involves a proteolytic cleavage, which mediates a conformational change exposing the active site. Activation of zymogens often involve a cascade system, in which several zymogens are activated sequentially, finally resulting in the activation of the proteinase that plays the major role, e.g. in the coagulation cascade (Mann et al., 1988).

The activated forms of proteinases are controlled by pH, posttranslational modifications (e.g. phosphorylation, glycosylation, and oxidation), localisation (e.g. in the lysosomes, or in the corresponding organelle in plants, the vacuoles), proteolytic degradation and last but not least, by reaction with inhibitors.

Proteinase inhibitors are proteins with the specific task to bind to and block the active site of proteinases, thereby inactivating these enzymes. The physiological importance of proteinase inhibitors is seen from the fact that they constitute 10% of the plasma proteins and that a congenital or aquired deficiency of many proteinase inhibitors is associated with different diseases or defects.

Proteinase inhibitors are found inside cells and in the extracellular matrix, blood, and secreted fluids. Many inhibitors are competetive inhibitors (i.e. they bind to the active site and make binding of substrates impossible) that are specific for a given class of proteinases. These inhibitors usually form tight complexes with the proteinases.

Inhibitor synthesis like proteinase synthesis, is controlled both at the level of transcription and translation. In some cases there is a coordinated control of the synthesis of a proteinase and its inhibitor. Proteinase inhibitors are also regulated by degradation by proteinases (Abrahamson et al., 1991; Lenarcic et al., 1988a; Koj et al., 1988).

Cysteine proteinases

Structure and reaction mechanism

The facts that certain plant proteinases, such as papain and ficin, are inactivated by thiol-blocking reagents and are activated by reagents that regenerate thiols from disulfides made Hartley (1960) classify them as "thiol proteinases". Light et al. (1976) showed that the essential thiol group of papain is the side chain of Cys25, and Lowe (1976) introduced the term cysteine proteinase.

Cysteine proteinases are found in bacteria (Morihara, 1974), in eucaryotic microorganisms (North, 1982), in plants (Glaser and Smith, 1971) and in animals (Barrett and McDonald, 1980). Amino acid sequences show that cysteine proteinases of higher plants, such as papain and actinidin as well as cathepsin B, H, L, S, C and K of mammalian lysosomes, all are members of the same superfamily, the papain superfamily. These enzymes are quite small proteins with M_r of 20 000-35 000. Three amino acids are conserved in all these enzymes, Cys25 and His159, which mediate the catalytic activity, and Gln19 (all papain numbering). The enzymes are all endopeptidases, although cathepsin B also shows dipeptidyl carboxypeptidase activity, i.e. acts as an exopeptidase.

The reaction mechanism of cysteine proteinases involves a nucleophilic attack by the sulphur atom of Cys25 on the carbonyl carbon of the scissile peptide bond, with His159 functioning as the hydrogen acceptor (Polgar and Halasz, 1982), and a thioester intermediate is formed (Powers et al., 1993). The sulphydryl group of Cys25 has to be in the reduced form, and therefore a reducing and slightly acidic enviroment is needed for optimal enzyme activity (Zucker et al., 1985).

The most studied cysteine proteinase is papain from the latex of the plant Carica papaya. The amino acid sequence was determined by Light et al. (1964) and its three-dimensional structure (Fig. 3) was among the first protein structures to be determined (Drenth et al., 1968; Kamphuis et al., 1984). It consists of 212 amino acids in a single polypeptide chain with a molecular weight of 23 400. It is a bilobed protein with the catalytic site located in a cleft between the two lobes.

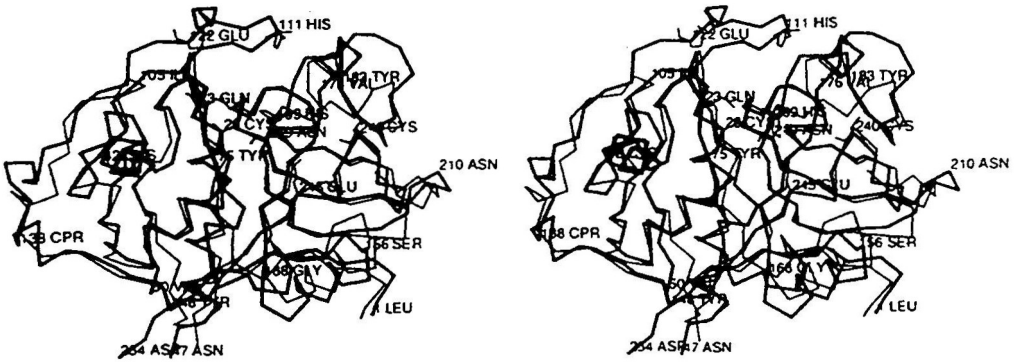

Fig. 3. Three-dimensional structure of papain (thin connections) superimposed with human cathepsin B (thick connections). The two structures has large similarities, but in papain there is no correspondence to the occluding loop of cathepsin B, containing His111. (Reprinted, with permission, from Turk and Bode (1993). Copyright 1993, Walter de Gruyter & Co, Berlin-New York.)

Papain is an endopeptidase with broad substrate specificity. The primary binding site that determines substrate specificity is, as for other similar cysteine proteinases, the S_2 subsite (Hasnain et al., 1992), which is a hydrophobic pocket that typically holds a hydrophobic P_2 residue, such as phenylalanine. Arginine or lysine is preferred in the P_1 position. The binding site contains altogether seven different subsites (Berger and Schechter, 1970).

The crystal structure of actinidin, another plant cysteine proteinase from the kiwi fruit, shows a highly similar overall fold to that of papain (Baker, 1980; Kamphuis et al., 1984), although there are some structural differences in the S_2 subsite.

Cathepsin B is the most investigated lysosomal cysteine proteinase. The three-dimensional structure of cathepsin B (Musil et al., 1991) has large similarities with that of papain (Fig 3). The overall folding pattern and the arrangement of the active site residues are very similar, but there are large insertion loops on the surface of the protein, which modify the properties of the enzyme. The most important is the "occluding loop", a 18-residue loop that blocks the S_1' and S_2' subsites of the active-site cleft. In three-dimensional structure of procathepsin B (Cygler et al., 1996) the occluding loop has an "open" conformation due to the presence of the prosegment binding at the active site. The occluding loop seems to favour binding of peptide substrates with two residues carboxy-terminal to the scissile bond, which explains the dipeptidyl carboxypeptidase activity of cathepsin B (Bond and Barrett, 1980; Marks et al., 1986). The crystal structure suggests that the two histidine residues of the occluding loop (His110 and His111) provide positively charged anchors for

the carboxyl group of the C-terminal of the substrate. This suggestion is supported by experiments showing that a mutant of cathepsin B, in which the 12 central residues of the occluding loop were deleted, totally lacked exopeptidase activity, but retained endopeptidase activity (Illy et al., 1997). A recent study (Nägler et al., 1997) showed that substitution of residues His110, Arg116, Asp22 and Asp124, which are supposed to form salt bridges that hold the occluding loop in place, result in increased endopeptidase activity, possibly due to an increased flexibility of the loop which might facilitate the binding of an endopeptidase substrate to the enzyme.

The three-dimensional structure of active cathepsin L (Fujishima et al., 1997), is highly similar to that of papain. The fold of the prosegment and the mechanism by which it inhibits the enzymatic activity of procathepsin L (Coulombe et al., 1996) are very similar to what was observed for procathepsin B (Cygler et al., 1996).

Several other lysomal cysteine proteinases are known, such as cathepsins H, C, S, K, N and T, although their properties are less well characterized (reviewed in Kirschke et al., 1995).

Several cysteine proteinases of bacterial, protozoan or viral origin have been found. One example is cruzipain, the major cysteine proteinase in Trypanosoma cruzi, which causes Chagas' disease. Cruzipain is a glycoprotein (Cazzulo, 1989) with a molecular weight of 60 kD (Bontempi et al., 1984; Martinez et al., 1989). It is located in the lysosomes (Bontempi et al., 1989) and has 65% homology with papain and cathepsin L (Cazzulo, 1989).

Occurrence and physiological or pathophysiological role

The plant proteinases papain and actinidin are mainly found in the latex and in the fruits of the plants. They are located in the vacuoles, which are the plant counterpart of lysosomes. They are suggested to have a protective role against insect predators and to promote the coagulation of the latex.

Cathepsin B, H, L, S and K are lysosomal cysteine proteinases that are found in many tissues. Their main function is the degradation of intracellular proteins. Proteins are degraded in lysosomes non-selectively, and the resulting end-products, dipeptides and amino acids, diffuse through the lysosomal membrane and are reused in protein synthesis (Brocklehurst et al., 1987; Bohley and Deglen, 1992; Kirschke et al., 1995). In addition, lysosomal cysteine proteinases have been shown to be able to specifically process other proteins, e.g. hormones (Wang et al., 1991; Dunn et al., 1991; Okazaki et al., 1992), and are probably involved in tissue resorption (Delaisse et al., 1980, 1992; Tagami et al., 1994). Cathepsin K has been shown to be expressed in high amounts in osteoclasts and to have a specific function in bone remodelling (Bossard et al., 1996). Moreover, cathepsin B was suggested to be the major cysteine proteinase involved in protein degradation for antigen

presentation (Mizuochi et al., 1994; Authier et al., 1996). The level of cathepsins varies from one tissue to another, from one cell to another (Qian et al., 1991; Nishimura et al., 1990) and between cells within the same tissue (Furuhashi et al., 1991). Cathepsin B is the most abundant of the enzymes (Kirschke and Barrett, 1981). It has been detected in every tissue examined, from macrophages to epithelial cells (Howie et al., 1985). However, cathepsin L is the most efficient lysosomal cysteine proteinase. It degrades proteins 10 times faster than for example cathepsin B (Maciewicz et al., 1987; Mason et al., 1989).

Alterations of a normal balance may lead to pathological conditions, and lysosomal cysteine proteinases have been implicated in many such cases. Lysosomal cysteine proteinases have been proposed to be involved in e.g. malignant metastasis, inflammation, muscular disorders and arthritis. More specifically, Chauan et al. (1991) demonstrated that cathepsin L and cathepsin L m-RNA was more aboundant in carcinoma of breast, ovary, colon, adrenal gland and bladder, compared with normal tissue. Moreover, overexpression of cathepsin B characterizes the malignant phenotype of tumor cells. Buck et al. (1992) thus demonstrated that increased malignancy of several types of tumors is associated with increased cathepsin B activity and gene expression. Also, cathepsin B mRNA species arising from alternative splicing can be related to tissue- and tumor-specific differences in expression. In many different cancers there is overexpression and increased activity of cathepsin B, which suggests that cathepsin B may play a functional role in malignant progression by assisting tumor cell penetration through tissue and by degrading biological barriers, such as basement membranes (Calkins and Sloane, 1995). Clinical studies provide evidence that cathepsin B expression is a prognostic indicator in colon carcinoma (Keppler and Sloane, 1996).

In recent years there has been increased interest in the role of proteolytic enzymes in parasites and in the possiblity that some of these enzymes might be appropriate targets for new terapeutic approaches, since cysteine proteinases are crucial for the proliferation of these life forms and for their penetration of host tissues. In the case of parasitic protozoa, the focus has been on their cysteine endopeptidases (North et al. 1990; North 1992; McKerrow et al. 1993). These are often the most active proteolytic enzymes present, and most protozoa produce cysteine endopeptidases during at least one stage of their life cycle. Cruzipain, the cysteine proteinase from Trypanosoma cruzi mentioned previously, is one such example. Cysteine proteinases inhibitors have been shown to be effective against a number of species of protoza in vitro (North et al. 1990; McKerrow et al. 1993).

Cysteine proteinase inhibitors

Structure, occurrence and physiological or pathophysiological role

A number of intra- and extracellular protein inhibitors protect mammalian organisms against the uncontrolled action of cysteine proteinases. Based on the amino acid sequence, these cysteine proteinase inhibitors can be divided into three families (Fig. 4; Barrett et al., 1986).

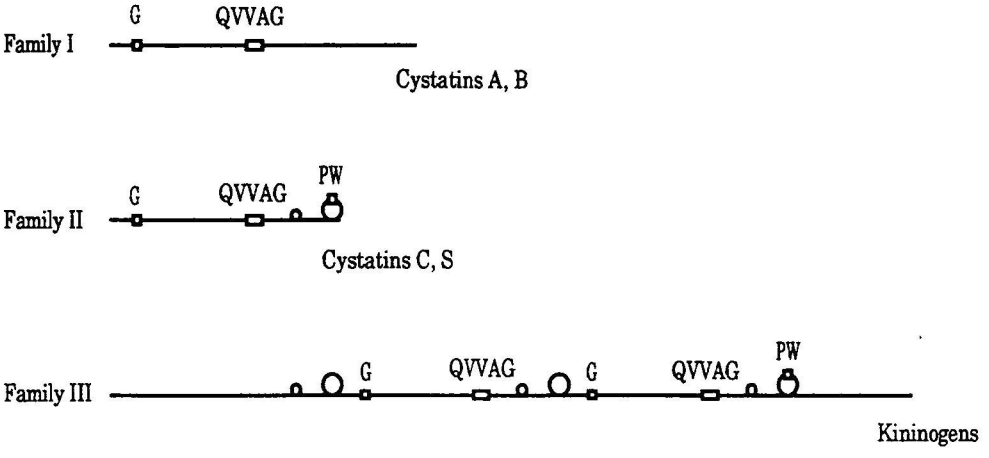

Fig. 4. Schematic representation of the polypeptide chain structures of members of the cystatin superfamily. The loops indicate internal disulphide bonds. Highly conserved regions are represented with boxes. The kininogens are represented by L-kininogen; H-kininogen has a longer carboxy-terminal extension.

Family 1

Cystatins A and B, also called stefins A and B, as well as the plant inhibitor oryzacystatin from rice, are members of this family. The cystatins belonging to family 1 are single-chain proteins consisting of about 100 amino acid residues ($M_r \approx 11\ 000$), with no disulfide bonds or carbohydrate chains. They are mainly found intracellularly. Cystatin A was first detected in epithelial cells and neutrophils, suggesting a primary defensive role against cysteine proteinases from pathogens invading the body. Cystatin B is much more widely distributed in various cells and tissues and to a much lesser extent in neutrophils (Brzin et al., 1982), indicating a protective role for cystatin B against activities of endogenous lysosomal cysteine proteinases.

Due to its epidermal origin, the role of cystatin A in inflammatory skin diseases has been studied. Hopsu-Havu et al. (1983) found that the total cystatin activity was higher in skin from psoriasis patients, and Järvinen et al. (1987) demonstrated increased amounts of cystatin A in skin from such

patients, Cystatin A may thus protect the skin from cysteine proteinases produced by inflammatory cells. Contrary to this suggestion, Othani et al. (1982) found that a cysteine proteinase inhibitor, most probably cystatin A, from psoratic skin was less stable and less active towards papain than that from normal skin.

Several independent studies have shown that different defects in the cystatin B gene cause a form of progressive myoclonus epilepsy (Pennacchio et al., 1996; Bespalova et al., 1997; Labauge et al., 1997). However, the relation between the deficiency of the inhibitor and the pathological symtoms has not been established.

Family 2

Cystatin C and its avian analogue, chicken cystatin, as well as cystatins D, E, M and S belong to this family. Members of this family are single-chain, non-glycosylated proteins consisting of 115-120 amino acid residues ($M_r \approx 13\ 000$) and with two disulphides near the carboxy-terminus. Family 2 of cystatins has been detected mainly extracellularly, such as in plasma, saliva and seminal plasma (Löfberg and Grubb, 1979; Grubb et al., 1983). Because of the widespread extracellular distribution of cystatin C, the inhibitor has been suggested to play a regulatory and defensive role against both endogenous and exogenous cysteine proteinases present in body fluids.

Icelandic hereditary cystatin C amyloid angiopathy (HCCAA) is a lethal disorder characterized by amyloid deposition of a cystatin C mutant in almost all tissues. The deposition around blood vessels in the brain leads to cerebral haemorrhages at an early age. The cystatin C mutant has a leucine in position 68 replaced by a glutamine (Ghiso et al., 1986; Palsdottir et al., 1988; Levy et al., 1989; Abrahamson et al., 1992). Abrahamson et al. (1994) showed that this mutated form of cystatin C, produced in *E. coli*, lost its activity rapidly and formed aggregates of dimers when the temperature was increased from 37 °C to 40 °C, providing an explanation for the amyloid formation. Benedikz et al. (1989) showed that patients suffering from cerebral amyloid angiopathy, present in Alzheimer's disease, had cystatin C in their senile plaques, indicating that also native cystatin C has a tendency to deposit as amyloid (Li et al., 1993; Maryama et al., 1992).

Lenarcic et al. (1988b) showed that patients suffering from rhematoid arthritis had high levels of cystatin C in their synovial fluid, suggesting a protective effect of the inhibitor in this disease.

Cleavage of viral precursor proteins in the cytoplasm of virus-infected cells is crucial for the replication and proliferation of some viruses, and therefore the effects *in vitro* of cysteine proteinase inhibitors on the growth of cultured virus-infected cells have been tested. Chicken cystatin caused a reduction of virus production in poliovirus-infected cells, and exposure of the cells to

chicken cystatin or cystatin C prior to infection resulted in no synthesis of viral proteins (Korant et al., 1985). This effect was not seen for large inhibitors, probably because they can not get into the cells (Korant et al., 1986; Björk et al., 1990). Both chicken cystatin and cystatin C have been shown to inhibit the cysteine proteinase that has been isolated from poliovirus (Korant et al., 1988). These observations indicate that family 2 cystatins have a role in the defense against invading pathogens.

Family 3

Kininogens are intravascular glycoproteins that contain three cystatin-like domains. Kininogens are much larger ($M_r \approx 100\ 000$-$120\ 000$) than the cystatins of the first two families. In mammals, three types of kininogens have been found, high molecular weight kininogen (H-kininogen), low molecular weight kininogen (L-kininogen), and T-kininogen, although the latter has been identified only in rats (Müller-Esterl et al., 1986; Müller-Esterl, 1989; Kato et al., 1981). The kininogens are synthesized in the liver and secreted into the blood plasma. The kininogens are involved in inflammation (Sharma and Mohsin, 1990; Stewart, 1993) and, together with α-2-macroglobulin, they are the major inhibitors of cysteine proteinases in blood plasma. In addition, kininogens are precursor molecules for vasoactive kinins, and H-kininogen acts as a co-factor in the contact phase of blood clotting.

Three-dimensional structure

The cystatins inhibit cysteine proteinases by forming tight complexes with the enzymes, blocking their active site. The amino acid sequences of cystatins are highly conserved in three regions, which therefore were presumed to be important for their inhibitory activity. The conserved amino acids are (in chicken cystatin numbering) Gly9 in the N-terminal region, Gln53, Val55 and Gly57 in the central QxVxG-region and Pro103 and Trp104 (which, however, in family 1 correspond to Leu73 and His75) in the C-terminal region.

The three-dimensional structure of chicken cystatin, determined by X-ray crystallography (Bode et al., 1988), showed that the molecule consists mainly of a straight five-turn α-helix, a five-stranded ß-pleated sheet, which is wrapped around the α-helix, and an appended segment of partial α-helical geometry (Figs. 5 and 6). It turned out that the highly conserved residues were clustered in one region of the molecule, dominated by the first and second hairpin loops of the ß-pleated sheet. The first hairpin loop with the QxVxG-region is flanked on either side by the N-terminal region with Gly9 and by the second hairpin loop with Trp104, creating a hydrophobic wedge-shaped edge.

The X-ray structure of chicken cystatin was determined for a truncated form starting with Gly9, so the structure and mobility of the preceding N-terminal residues remained unclear. Later, the solution stucture of chicken cystatin was determined by NMR (Dieckmann et al., 1993), and it turned out that the N-

terminal region from residue 1 to 9 is completely flexible in solution. The NMR structure differs somewhat from the crystal structure in the region of the first hairpin loop. Engh et al. (1993) ascribe the alterations of the first hairpin loop in the crystal structure to the crystal contact effect, but Tate et al. (1995) suggest that the truncation of the N-terminal region might have induced these conformational changes. A further difference between the two structures is that the appended helix seen in the crystal structure could not be identified in the NMR solution structure.

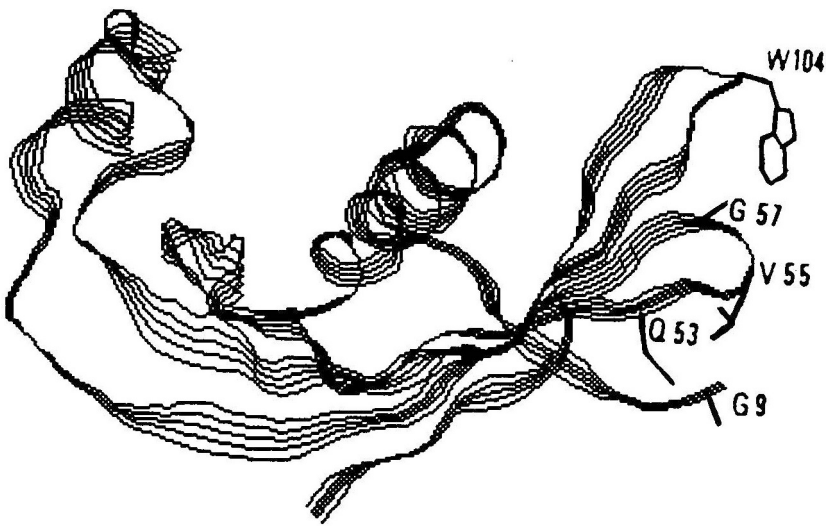

Fig. 5. Ribbon-like representation of the structure of chicken cystatin. The highly conserved residues Gly9, Gln53, Val 55, Gly57 and Trp104 are shown with side chains.

Cystatin C has 44% sequence identity, a similar inhibition profile (Anastasi et al., 1983; Schwabe et al., 1984; Lindahl et al., 1992), and no considerable differences in near-ultraviolet absorption, fluorescence or circular dichroism spectra on interaction with papain (Lindahl et al., 1988; Lindahl et al., 1992), compared with chicken cystatin. It may therefore be concluded that cystatin C has basically the same three-dimensional structure as chicken cystatin. This conclusion is supported by analyses of the secondary structure of cystatin C by NMR (Ekiel et al., 1997).

The X-ray structure of human cystatin B in complex with papain (Stubbs et al., 1990) confirmed the relationship between families 1 and 2 of the cystatins. Cystatin B has the same characteristic features as chicken cystatin; it also consists of a five stranded ß-pleated sheet which is wrapped around a five turn α-helix. However, compared with chicken cystatin there is an additional

carboxy-terminal strand which runs along one side of the ß-sheet, and the appended helix is missing. The structure of the first hairpin loop overlays better with the NMR structure than with the X-ray structure of chicken cystatin (Dieckmann et al., 1993).

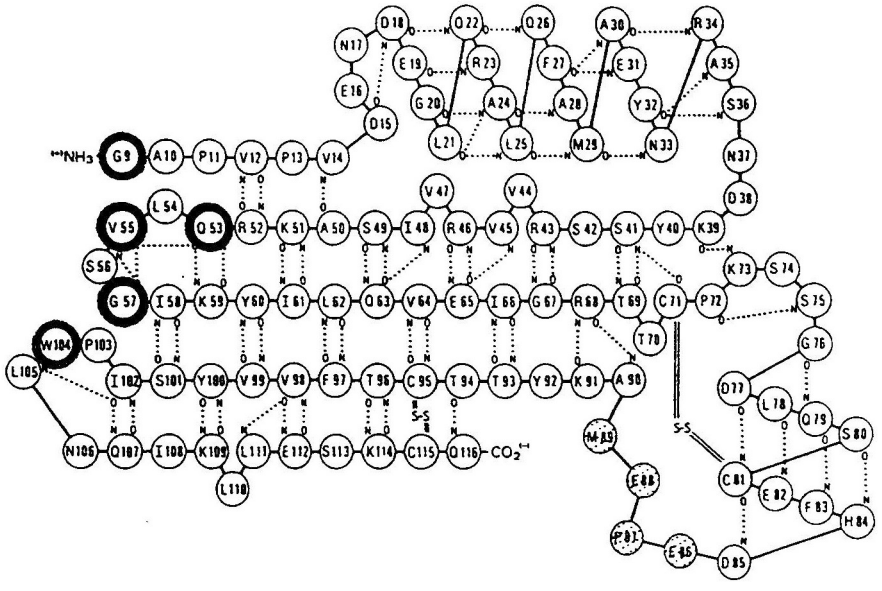

Fig. 6. Schematic representation of the structure of chicken cystatin. Main-chain hydrogen bonds are displayed by dashed lines. The two disulphide bridges are indicated by double lines. Shadowed circles represent undefined residues. The highly conserved residues Gly9, Gln53, Val55, Gly57 and Trp104 are represented by bold circles.

The solution structure of human cystatin A was determined by NMR independently by two groups. The study by Martin et al. (1995) shows large similarities between the crystal structure of cystatin B in the complex with papain (Stubbs et al., 1990) and the solution structure of cystatin A, although the second hairpin loop and the N-terminus of cystatin A have a pronounced flexibility in the solution structure. In partial conflict with this structure is that of cystatin A solved by Tate et al. (1995), which shows a break at Pro25 of the α-helix into two short but distinct helices and an ordered structure of the second hairpin loop, although appreciably different from that of cystatin B in the complex. However, the conformation of the first binding loop is very similar to that of cystatin B in complex with papain. In contrast with the Martin et al. (1995) structure, the N-terminal is oriented towards the back of the ß-pleated sheet, interacting with the residues of the C-terminal strand. The reasons for these substantial differences between the two structures are not clear, although the structure reported by Tate et al. (1995) was determined for

a Met65Leu cystatin A derivative at pH 3.8, an appreciably lower pH than that used by Martin et al. (1995).

To study the putative interaction between the N- and C-terminal regions of cystatin A, Tate et al. (1995) partially solved the NMR-structure of an N-terminally truncated cystatin A, Ala5-cystatin A. This N-terminal truncation induced conformational changes not only for the residual N-terminal region but also for the first hairpin loop and to a smaller extent also for the C-terminus. They therefore suggested that the truncation pulls the N-terminal region apart from the C-terminal region and that these specific interactions between the N- and C-termini are necessary to maintain the native conformation of the first hairpin loop of cystatin A. Tate et al. (1995) also characterized by NMR the structure of a cystatin A variant with the substitution Gly4Val, which induced a large chemical shift for Val47 of the first hairpin loop. This observation was taken as further support for the hypothesis of the importance of proper interactions between the N- and C-terminal regions to maintain the native conformation of the first hairpin loop.

Mechanism of interaction of cystatins with cysteine proteinases

The interactions between cystatins and cysteine proteinases have been characterized by a number of different methods. They have been elucidated by functional studies, which include determination of equilibrium constants as well as rate constants for the formation and dissociation of the complex of the inhibitor with the proteinase. Further information has been provided by functional studies of site-directed mutants or chemically modified forms of the inhibitors or the proteinases. Also ultraviolet absorbtion, fluorescence and circular dichroism difference spectra accompanying the interactions have been studied. Computer docking of the three-dimensional structures has been made for chicken cystatin and papain, and the three-dimensional structure of the complex between cystatin B and papain has been solved by X-ray crystallography.

Cystatins bind with 1:1 stoichiometry to papain-like cysteine proteinases (Anastasi et al., 1983; Green et al., 1984; Nicklin and Barrett, 1984; Abrahamson et al., 1987; Björk and Ylinenjärvi 1990; Lindahl et al., 1988, 1992), whereas kininogens, with two QxVxG-regions, bind with an enzyme/inhibitor stochiometry of 2:1 (Higashiyama et al., 1986; Turk et al., 1996). The binding to the target proteinases is tight and reversible (Anastasi et al., 1983; Nicklin and Barrett, 1984; Green et al., 1984). Many interactions are so tight that dissociation equilibrium constants have been difficult to determine with good accuracy by equilibrium methods. Instead, separate measurements of association and dissociation rate constants have enabled determinations of K_d-values of less than approximately 50 fM, e.g. for the interactions of chicken cystatin with papain, chymopapain A and ficin (Björk et al., 1989; Björk and Ylinenjärvi, 1990) and of cystatin C with papain (Lindahl et al.,

1992). The data obtained verify the reversibility also of these very tight interactions. In general, different cystatins show approximately the same affinity for cathepsin L as for papain, in agreement with the similar three-dimensional structures of the two proteinases. However, the affinities for cathepsin B are appreciably lower, presumably due to the presence of the occluding loop of this enzyme. Family 2 cystatins, such as cystatin C, in general show somewhat higher affinities for the different proteinases than the family 1 cystatins, A and B, and the kininogens of family 3.

Computer docking

Docking experiments based on the X-ray structures of chicken cystatin and papain (Bode et al., 1988) showed that the wedge-shaped edge of chicken cystatin, consisting of the N-terminal region and the first and second hairpin loops of the ß-pleated sheet, with the highly conserved residues Gly9, the QxVxG-region and Trp104, respectively, would sterically fit well into the active site cleft of papain, presumably without any conformational changes. (Fig. 7).

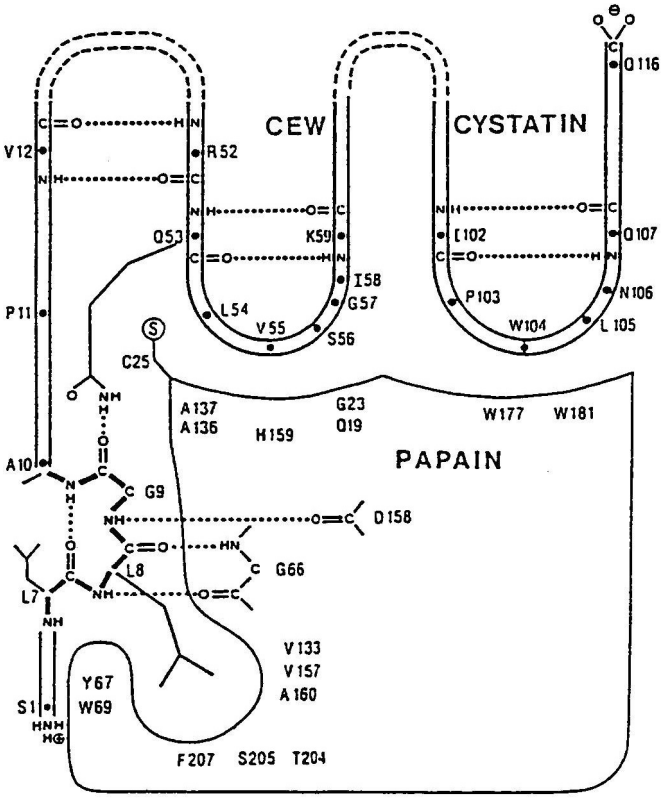

Fig. 7. A scheme of the proposed model for the interaction of chicken cystatin with papain. (Reprinted from Bode et al. (1990), with permission from the author).

The computer docking experiments also suggested that both hairpin loops of chicken cystatin make major binding interactions with the highly conserved papain residues Gly23, Gln19, Trp177 and Ala136 adjacent to the reactive site Cys25. Moreover, residues Gly9-Ala10 in the N-terminal region of the inhibitor are directed towards the S_1 subsite of papain (Fig. 7) but are in an inappropriate conformation and too far away to be attacked by the reactive site Cys25. This explains why complex formation is not substantially altered when small substituents are covalently bound to the thiol group of Cys25 of papain (Anastasi et al., 1983; Björk and Ylinenjärvi, 1989). This observation also excludes the formation of a covalent complex between the the active site Cys25 and the inhibitor, which occurs on inhibition of serine proteinases by certain inhibitors.

In the docking model, the QxVxG- and PW-regions have the appropriate size and shape to interact with the S'_1 and S'_2 subsites of papain. In the energy-minimized docked complex, the indole ring of Trp104 of chicken cystatin stacks on the side chain of Trp 177 of papain and lies edge-on with the indole-ring of Trp181. These tryptophans are totally conserved in all related cysteine proteinases (Kamphuis et al., 1985). The N-terminal segment was suggested to interact with the subsites S_1 to S_3 of papain; specifially Leu8 would interact with the S_2 subsite. To do so, this segment must form a tight turn, thereby necessitating the highly conserved Gly9 residue.

X-ray crystallography

The crystal structure of the complex between cystatin B and papain (Fig. 8; Stubbs et al., 1990), confirmed the hypothesis for the interaction of cystatins with papain proposed from the docking experiments (Bode et al., 1988). The N-terminal region and the first hairpin loop of cystatin B were found to be in direct contact with papain. In the first hairpin loop, Val55 (chicken cystatin numbering, which will be used hereafter, unless otherwise stated) was found to make the greatest number of contacts with papain. Also the second hairpin loop contributed appreciable contacts with the enzyme.

Kinetics of interaction

As discussed above, the docking model of the chicken cystatin-papain interaction (Bode et al., 1988), as well as the crystal structure of the complex between cystatin B and papain (Stubbs et al., 1990), indicate that the binding of the two proteins to each other takes place without any conformational change of either protein, which is consistent with a one-step reaction mechanism. A characteristic of one-step reactions is that the observed pseudo-first-order rate constant for the binding shows a linear dependence on the inhibitor concentration (Morrison, 1982; Fersht, 1985). Such linear dependence has been shown for the interactions of several cysteine proteinases with their inhibitors, e.g. for the reactions between chicken cystatin and

actinidin, chymopapain A and ficin, between cystatin C and papain and actinidin and between cystatin A and papain (Björk et al., 1989; Björk and Ylinenjärvi, 1990; Lindahl et al., 1992; Pol et al., 1995). The magnitude of the association rate constants, being close to 10^7 $M^{-1} \cdot s^{-1}$ for the reaction with most enzymes, i.e. approaching the value expected for a diffusion-controlled reaction, are also consistent with a one-step reaction mechanism. However, the inhibitors bind more slowly to cathepsin B, presumably because they need to displace the occluding loop of this enzyme from the active site. In general, inhibitors of family 2 bind somewhat faster than those of the other two families to their target enzymes (Björk et al., 1989; Machleidt et al., 1989; Björk and Ylinenjärvi, 1990; Abrahamson et al., 1991; Lindahl et al., 1992; Pol et al., 1995).

Fig. 8. The three-dimensional structure of the complex between human cystatin B (thick connections) and papain (thin connections).

The mechanism of cystatin interaction with target proteinases has been quite thouroughly studied, but still it remains unclear what determines the specificity of the different cystatins. For example it is obscure why chicken cystatin inhibits papain 10,000-fold stronger than actinidin (Björk et al., 1990) even though the active-site structures of papain and actinidin are highly similar., It is also not known why cystatin S has a 10,000,000-fold lower affinity for papain than the homologous inhibitor, cystatin C (Isemura et al., 1986;

Lindahl et al., 1992) and why cystatin D, unlike other family 2 cystatins, does not inhibit cathepsin B at all (Freje et al., 1993).

Importance of the different binding regions for the interaction

Machleidt et al. (1991) have estimated the relative contribition to the binding energy of the N-terminal region and the first and second hairpin loops for the complex papain-chicken cystatin by measuring K_d-values for deletion mutants. They suggested that the N-terminal region and the first and second hairpin loops contribute approximately 35%, 50% and 15% of the total binding energy, respectively.

N-terminal region

Several studies have focused on the role of the N-terminal region of the cystatins of both family 1 and 2. However the results are partly contradictory and not fully understood.

In the family 1 inhibitor, cystatin A, the amino acids corresponding to Leu8 and Gly9 of chicken cystatin are Pro3 and Gly4. The inhibitory activity of cystatin A against papain has been reported not to be affected by the deletion of the first two amino acids, but further truncations, deleting Pro3 and Gly4, caused appreciable decreases of inhibitory activity (Shibuya et al., 1995a). Substitutions of Gly4 lead to decreased affinities for papain,, the decrease in general increasing with the size of the side chain of the substituted amino acid (Shibuya et al., 1995b). For example, the cystatin A variant which had Gly4 substituted by Val showed almost no affinity for papain. However the substitution of Pro3 by Leu had no detectable effect on the affinity (Shibuya et al., 1995b).

In cystatin B, also of family 1, the amino acids corresponding to Leu8 and Gly9 of chicken cystatin are Cys3 and Gly4, of which the former is supposed to bind in the S_2 subsite. Thiele et al. (1990) reported that truncated cystatin B starting with Cys3 was equally active against papain as the full-length form, and Machleidt et al. (1991) found that substitutions of Cys3 did not appreciably change the affinity for papain of the inhibitor. The latter group reported that not even the form starting with Ser7 was affected by the truncation. These results contrast those for cystatin A and also the X-ray structure of the complex between cystatin B and papain, in which the N-terminal part of cystatin B is inserted into the active site cleft of papain.

Two studies have reported that different forms of another family 1 cystatin, oryzacystatin from rice, truncated in the N-terminal region from amino acid 21, have almost unaffected affinities for papain, compared with the intact inhibitor (Abe et al., 1988; Arai et al., 1991). These studies both conclude that the N-terminal region of oryzacystatin lacks importance for proteinase binding.

More extensive studies of the importance of the N-terminal region have been done with family 2 cystatins. Machleidt et al. (1989) found that truncated forms of chicken cystatin beginning with Gly9 or Ala10 had a 5000-fold lower affinity for papain than the unmodified inhibitor or a truncated form beginning with Leu7. They conclude that these observations demonstrate the importance of Leu8 and possibly also Leu7 for the interaction. Auerswald et al. (1994) further showed that a deletion mutant of chicken cystatin lacking amino acids 1-10 had inhibition constants for papain, actinidin and cathepsin B and L that were 1000-100,000-fold higher than those of the wild-type. These experiments are in agreement with the docking model (Bode et al., 1988), proposing that Leu7 and Leu8 are accommodated in the S_2 and S_3 subsites of the proteinase, respectively.

Machleidt et al. (1995) also demonstrated that deletions of one to five amino acids of either of the two hairpin loops of chicken cystatin resulted in cleavage of the Gly9-Ala10 bond of the N-terminal binding region of the inhibitor on reaction with papain. They suggest that distorted contacts of one of the hairpin loops in the complex with the proteinase affect the binding of the N-terminal region in a way that it can be attacked by the active site Cys25 of the enzyme and cleaved in a substrate-like manner.

In the human family 2 inhibitor, cystatin C, the amino acids corresponding to Leu8 and Gly9 of chicken cystatin are Val10 and Gly11. Evidence has been presented that the former most likely is bound in the S_2 subsite of cathepsin B (Lindahl et al., 1994), and presumably also in the analogous site of papain, as might be expected from the computer docking experiments with chicken cystatin. Abrahamson et al. (1991) showed that a truncated variant of cystatin C starting with Gly11 had more than 240-fold lower affinity than native cystatin C for papain, and the corresponding affinities for cathepsin B and L were decreased by three orders of magnitude. They also showed that a tripeptidyldiazomethane derivative of the truncated portion of the N-terminal region was a good inhibitor of cathepsins B and L and therefore concluded that the amino acid side chains of the N-terminal region bind in the substrate-binding subsites of these enzymes. Hall et al. (1993) and Björk et al. (1995) showed that substitutions of Gly11 resulted in substantially decreased affinities of cystatin C for papain and cathepsin B. Moreover, for truncated variants of cystatin C starting with Gly11, substitutions of Gly11 had no effect on the affinity, indicating that the crucial feature of Gly11 is that it will allow the N-terminal region to be highly flexible and to adopt any conformation. A contribution of the Arg8 side chain of cystatin C to the interaction with cathepsin B has also been demonstrated (Hall et al., 1995).

The decreased affinities as a consequence of N-terminal truncation of cystatin C were shown to be due to an increased dissociation rate constant for the interaction with papain, but due to a decreased association rate constant for the interaction with cathepsin B (Björk et al., 1994). The role of the N-terminal

region in the interaction of cystatin C with cathepsin B might therefore be to mediate an initial contact between the two proteins that facilitates a subsequent displacement of the occluding loop of cathepsin B, which sterically interferes with the binding. This proposal initiated the work of this thesis which provides evidence that the inhibition of cathepsin B by cystatin C is a two-step reaction (Paper 5), most probably due to an initial binding of the N-terminal region and subsequent displacement of the occluding loop.

Taken together, all these data lead to the conclusion that the N-terminal region is indispensable for effective inhibition of cysteine proteinases by cystatin A, chicken cystatin and cystatin C. In contrast, forms of cystatin B and oryzacystatin lacking the N-terminal binding region appear still to be potent inhibitors. These differences are not easily explained. Machleidt et al. (1991) suggested that the N-terminal region is needed only in family 2 cystatins to compensate for less favourable contacts made with the target enzyme by the two hairpin loops and for the missing C-terminal contact with the enzyme made by family 1 cystatins. However, at the time of this proposal it was not known that also cystatin A loses inhibitory activity when its N-terminal region is modified. It is possible, therefore, that the role of the N-terminal region may vary within the subfamilies, for reasons unknown. Tate et al. (1995) suggested that the N-terminal region must not necessarily play a direct role. Distortion of this region might lead to a distorted first hairpin loop, which could be primarily responsible for the loss of activity. NMR studies of cystatin A variants with truncated or substituted N-terminal regions thus indicated conformational changes of the first hairpin loop (Tate et al., 1995). The authors propose that those inhibitors that are not affected by modifications of the N-terminal region are those that do not need the N-terminal stabilization to maintain the native configuration of the first hairpin loop. A further, more trivial, explanation of these discrepancies are experimental errors due to the difficulties inherent in the measurements of the tight interactions involved by the equilibrium methods used.

The first hairpin loop (the QXVXG region)

Nikawa et al. (1989) reported that substitution of Gln53 of cystatin A by Lys or substitution of Val55 by Thr did not result in any significant changes in affinities for papain or cathepsin B, H and L. In agreement with this report Jerala et al. (1990) found that substitution of Val54 of cystatin B also did not cause any major differences in binding affinity. However, it was later shown that substitution of Val55 of cystatin B by Asn gave a 240-fold decrease in the affinity for papain (Machleidt et al., 1991). Moreover, Arai et al. (1991) found that mutations of Gln53 drastically reduced the affinity of oryzacystatin for papain, while mutations of Val55 resulted in no or only a moderate change of inhibitory activity. They concluded that the Gln-Val-Val-Ala-Gly (QVVAG) sequence is the most important of the three binding regions of oryzacystatin for the interaction. Again, many of these apparent discrepancies

for the family 1 inhibitors may be due to experimental difficulties in determining affinities of tight interactions.

Two studies have shown that substitutions of Gln53, Val55 or Gly57 in the first hairpin loop of chicken cystatin reduce the affinity of this inhibitor for papain, actinidin and cathepsin B by 10-1000-fold (Auerswald et al., 1992, 1995). For chicken cystatin, the importance of the first hairpin loop for the binding of target proteinases thus appears unequivocal. Unfortunately, however, no similar studies have been done with other family 2 inhibitors, such as cystatin C.

The second hairpin loop

Studies to elucidate the importance of the second hairpin loop of the cystatins have been less extensive and all concern cystatins of family 2.

Modification of Trp104 in the second hairpin loop of chicken cystatin with *N*-bromosuccinimide gave only a small reduced activity of cystatin but altered the spectroscopic changes on binding to papain drastically, compared with unmodified cystatin (Lindahl et al., 1988). This finding is consistent with the Trp104 residue interacting with papain. In agreement with this conclusion, mutants of chicken cystatin, in which residues 103-105 or 102-107 were deleted, had a 1000-1500-fold lower affinity for papain, a 50-60-fold lower affinity for cathepsin B and a 3-10-fold lower affinity for cathepsin H than the wild-type inhibitor (Auerswaldt et al., 1995). All these reduced affinities were due to increased dissociation rate constants. Björk et al. (1996) further showed that replacement of Trp 104 in cystatin C by glycine and phenylalanine resulted in a 300-900 and 30-120-fold reduced affinities, respectively, for papain, actinidin, and cathepsins B and H. These findings verify that the Trp residue in the second hairpin loop interacts with papain and demonstrate that the phenyl group of phenylalanine can partly compensate for the indole ring of tryptophan in the interaction of cystatin C with these target enzymes. Also these reduced affinities were due to increased dissociation rate constants, which together with the studies of deletion mutants referred to above (Auerswald et al., 1995) indicates that the second hairpin loop contributes to the binding by keeping the cystatin anchored to the proteinase once the complex is formed.

The C-terminal region

According to the X-ray stucture of the complex between cystatin B and papain, the carboxy-terminal end of the cystatins of family 1 constitutes an additional binding region. This region is supposed to be of minor importance for the binding and has been the subject of few studies. However, it has been shown that a mutant of oryzacystatin lacking the 11 C-terminal residues inhibited papain almost as well as the wild-type, whereas a mutant lacking the 35 C-terminal residues showed a big drop in inhibitory activity (Abe et al., 1988). A gross conformational change of the inhibitor affecting the activity

cannot be excluded for the latter, major deletion. Jerala et al. (1991) also found that the inhibitory activity of cystatin B was not changed on truncation of the C-terminal 10 amino acids. Both studies thus indicate that at least the 11 C-terminal residues are not essential for the inhibitory activity of the inhibitors studied.

Present investigation

The aim of the present investigation was to characterize the mechanism of interaction between cysteine proteinases and their inhibitors, cystatins. The different roles and the relative contributions to the binding energy of the three regions of the cystatins that interact with the proteinase, i.e. the N-terminal region and the first and second hairpin loops, were studied. The differences in binding mechanism between interactions with different proteinases were also investigated.

The importance of the highly conserved tryptophan 104 of the second hairpin loop of chicken cystatin for the binding to target enzymes (paper I)

When this work was initiated, it had been suggested from computer docking experiments based on the X-ray structures of chicken cystatin and papain (Bode et al., 1988) that the N-terminal region and two hairpin loops of the cystatin interact with target proteinases. The aim of this study was to verify the role the second hairpin loop. This loop contains the only tryptophan residue of chicken cystatin, Trp104, which therefore could be specifically modified with a 2-hydroxy-5-nitrobenzyl (HNB) group, serving as a spectral probe. Changes of the spectral properties of the probe on binding of the labelled cystatin to papain were studied. The effect of the HNB group on the affinity of the inhibitor for papain was also characterized by determination of equilibrium and rate constants.

Cystatin was purified from chicken egg white as described previously (Lindahl et al., 1988) and was modified with dimethyl-(2-hydroxy-5-nitrobenzyl)-sulphonium bromide (Koshland et al., 1964). The labelled protein was purified by affinity chromatography. Specific labelling of one tryptophan residue was confirmed by absorption measurements.

The modification of cystatin with HNB resulted in a decrease of the wavelength of the maximum of the corrected fluorescence emission spectrum, consistent with tryptophan modification. The changes of the emission spectrum that accompanied the binding of HNB-cystatin to papain were highly similar to the changes accompanying the binding of unmodified cystatin to papain. This finding indicates that the fluorescence changes arise primarily from tryptophans in papain and that the attachment of the HNB-group to Trp104 of chicken cystatin does not significantly alter the general mode of interaction of the inhibitor with papain.

The absorbtion difference spectrum between the complex of HNB-cystatin with active papain and the free proteins was measured in the 300-500 nm wavelength region, where the HNB group absorbs light and this absorbtion is

sensitive to the local environment of the probe. The difference spectrum had a minimum at approximately 412 nm and a maximum at approximately 312 nm, changes that indicate a decreased enviromental polarity of the HNB group in the complex with the enzyme (Peterson and Blackburn, 1987). The spectrum thus clearly showed that the environment of the HNB group was perturbed on binding to papain.

Dissociation equilibrium constants were measured for the interaction of HNB-cystatin with active papain and two inactivated forms of the enzyme by titrations of the enzymes with modified inhibitor. The titrations were monitored by the decrease in tryptophan fluorescence accompanying the interaction. HNB-cystatin had about $4x10^5$-fold lower affinity for active papain and $3x10^3$-fold lower affinities for the S-methylthio and S-carboxymethyl derivatives of papain than unmodified cystatin. The modification of Trp104 of chicken cystatin thus resulted in a large decrease of the affinities for both active and inactivated papains.

The kinetics of the binding of HNB-cystatin to active papain were investigated by stopped-flow fluorimetry. The second order association rate constant was only slightly decreased on HNB-modification of cystatin, showing that the lower affinity of HNB-cystatin for papain was primarily due to a higher dissociation rate constant.

The decreased environmental polarity of the HNB-group in the complex with papain, together with the markedly decreased affinity of HNB-cystatin for papain, indicate that Trp104 of chicken cystatin is located in or near the proteinase binding site of the inhibitor. This conclusion is in agreement with the model for the interaction of chicken cystatin with papain proposed from the computer docking experiments (Bode et al., 1988). In this model, Trp104 of chicken cystatin interacts primarily with two tryptophan residues in the active site cleft of papain, Trp177 and Trp181, in such a manner that the indole ring of Trp104 stacks on the side chain of Trp177 and lies edge on with the indole ring of Trp181. It is likely that the attachment of an HNB-group to Trp104 would perturb these interactions, leading to lower binding affinity, in agreement with the results of this work. However the interactions remaining in the other two binding regions, i.e. the N-terminal region and the first hairpin loop, apparently are sufficiently strong to stabilise the complex between HNB-modified cystatin and papain to an appreciable extent, as shown by the measured affinity. This affinity, which corresponds to about 60% of the total free energy for the interaction of intact cystatin with papain, may approximately reflect the binding energy contributed by these other two regions.

The importance of the N-terminal regions of chicken cystatin and human cystatin A for the binding to target enzymes (papers II and IV)

Chicken cystatin

The computer docking model, based on X-ray crystallographic structures of chicken cystatin and papain (Bode et al., 1988), suggests that the N-terminal region is part of the reactive site of the inhibitor and more specifically that Leu-8 may bind to the hydrophobic S_2 subsite of the active site cleft of papain. This proposal was supported by inhibition studies of N-terminally cleaved forms of chicken cystatin or human cystatin C with papain (Abrahamson et al., 1987; Machleidt et al., 1989). In this work we have further characterized the role of individual residues in the N-terminal region of chicken cystatin by studying the binding of five N-terminally truncated forms of the inhibitor, starting at Leu7, Leu8, Gly9, Ala10 and Asp15. Their binding to papain, actinidin and three different inactivated forms of papain was characterized by spectroscopic, kinetic and equilibrium methods.

The truncated forms of chicken cystatin were obtained by limited digestion with non-target proteolytic enzymes and were purified by gel chromatography, affinity chromatography or hydrophobic interaction chromatography. N-terminal sequence analyses verified proper cleavage of the N-terminal region and, together with SDS-PAGE, acertained the absence of internal cleavages.

The far-ultraviolet circular dichroism spectra of cystatin did not change upon truncation, indicating that the truncation did not alter the overall conformation of the protein. This finding is in agreement with the X-ray structure, in which the N-terminal region does not stabilize the tertiary structure of the inhibitor, but extends freely in the solution (Bode et al., 1988, 1990). The near-ultraviolet absorption, circular dichroism and fluorescence emission difference spectra for the binding of the truncated cystatins to papain were all similar to the corresponding spectra for intact cystatin. These results provide evidence that the changes of the interaction with proteinases observed in this work reflect the truncation of the N-terminal region, rather than an altered mode of interaction of the other binding regions of the inhibitor with target proteinases.

The dissociation equilibrium constants for the binding of the truncated forms of chicken cystatin to papain and actinidin were determined by titrations of the proteinases with the truncated cystatins, by monitoring the protein fluorescence change accompanying the interactions or by monitoring the decrease in the equilibrium rate of cleavage of a fluorogenic substrate by the proteinase. In some cases, the dissociation equilibrium constants were too low to be measured by equilibrium methods and were therefore calculated from separately determined association and dissocation rate constants. The

association rate constants for the binding of the truncated forms of cystatin to papain were measured under pseudo-first-order conditions by monitoring the fluorescence change accompanying the binding (Lindahl et al., 1988). The dissociation rate constants were determined for some complexes with papain by following the rate of the dissociation of the truncated cystatin of form 2 from the complex when it was displaced with an excess of intact cystatin of form 1. Cystatin forms 1 and 2 bind to their target enzymes in the same manner, but can easily and rapidly be separated from each other and quantified by ion-exchange chromatography.

The affinities of the truncated forms of chicken cystatin for papain decreased progressively with increasing number of residues removed from the N-terminus, from intact cystatin to Ala10-cystatin, whereas Ala10- and Asp15-cystatin bound papain with similar affinity. A comparable reduction in affinity was observed for the binding of the truncated forms of cystatin to actinidin. The affinities between the truncated cystatins and three different forms of inactivated papain, S-(methylthio)-, S-(carboxymethyl)- and S-(N-ethyl-succinimidyl)-papain, were lower than those for the active enzyme. However, Ala10- and Asp15-cystatin bound active papain and papain inactived with the smallest blocking group, S-(methylthio)-papain, with similar affinity.

The similar affinities of Ala10- and Asp15-cystatin for both active and S-(methylthio)papain show that only residues N-terminal of Ala10 contribute to the stabilization of the complex with the proteinase. The binding affinity of Ala10-cystatin corresponds to about 60% of the unitary free energy change for the binding of intact inhibitor to active and S-(methylthio)papain This free energy should represent the binding energy between the enzyme and the reminder of the binding site of the inhibitor, i.e. the first and second hairpin loops. Therefore, the contribution of the N-terminal region to the total unitary free energy change for the binding of chicken cystatin to both active and S-(methylthio)papain presumably is about 40%.

Detailed comparisons of the affinities of the truncated cystatins for papain and actinidin show that Leu7 and Leu8 together contribute about two thirds of the unitary free energy of binding of the N-terminal region of cystatin to both enzymes. Leu7 accounts for about two-thirds and about half of this energy in the interaction with papain and actinidin, respectively. This large influence of Leu7 on the interaction with papain indicates that Leu7 participates directly in the binding, presumably by binding to the S_3 subsite (Bode et al., 1988; Stubbs et al., 1990). However, the effect might also be due to Leu7 influencing the conformation of Leu8. The smaller influence of Leu7 on the interaction with actinidin is in agreement with previous studies that the N-terminal region of cystatin interacts differently with papain and actinidin (Björk and Ylinenjärvi, 1990). Apart from Leu7 and Leu8 also Gly9 and the residues N-terminal of Leu7 further stabilize the ineraction with the two enzymes, but to a much smaller extent.

The second-order association rate constants for the binding of the truncated cystatins to papain were all comparable to the association rate constant for the binding of intact cystatin, showing that the decreased affinities of the truncated cystatins for papain were due to increased dissociation rate constants. The rate of association of Leu7- and Leu8-cystatin with papain was slightly higher than the rate for intact cystatin, perhaps due to the flexibility of the N-terminal segment of the intact cystatin (Bode et al., 1988, 1990), interfering somewhat with the binding of the remainder of the inhibitor.

In the case of intact cystatin and Gly9-cystatin, the affinity for S-(methylthio)papain was 10 times lower than for active papain, whereas in the case of Ala10- and Asp15-cystatin the affinities for S-(methylthio)papain and active papain were the same. This observation indicates that Gly9 restricts the space around the reactive cysteine of papain in the complex, but that a small substituent, such as an S-methylthio group, on the reactive cysteine can be accommodated in the complex when Gly9 has been removed.

Cystatin A

The solution structure of cystatin A was recently solved by NMR spectroscopy by two independent groups (Martin et al., 1995; Tate et al., 1995) and was found to have considerable similarities with the structures of chicken cystatin and of cystatin B in complex with papain (Bode et al., 1988; Stubbs et al., 1990; Dieckman et al., 1993). These similarities suggested that the N-terminal region of cystatin A, like that of chicken cystatin, also is of importance for the interaction with target proteinases. The participation of the N-terminal region of cystatin A in binding to papain has been indicated by other studies (Shibuya et al., 1995a, b). However, the quantitative contribution of this region to papain binding is uncertain, and its importance for the interaction with other cysteine proteinases has not been investigated. The aim of this study was to further characterize the role of the highly conserved Gly4 of the N-terminal region of cystatin A in the binding to cysteine proteinases, In order to do this, we studied the interactions between five Gly4 mutants of cystatin A, *i.e.* Gly4Ala-, Gly4Ser-, Gly4Arg-, Gly4Glu-, and Gly4Trp-cystatin A, and papain, as well as the more relevant physiological target enzymes, cathepsins B and L. These interactions were investigated by both equilibrium and kinetic methods. In addition, the conformations of selected mutants were characterized by NMR.

The Gly4 mutants of cystatin A were produced by site-directed mutagenesis. N-terminal amino acid sequence analyses confirmed the correctness of the mutations, and MALDI mass spectroscopy verified the expected relative molecular masses. Titrations with papain gave activities close to 100%. Far-ultraviolet circular dichroism spectra were not changed on mutation of Gly4 of cystatin A, indicating that the substitutions did not alter the overall conformation of the protein. Comparisons of NMR spectra for Gly4Ala- and

Gly4Trp-cystatin A with the spectra for wild-type cystatin A in general showed very small differences, providing sensitive evidence for the mutations not having caused any conformational changes of the inhibitor.

Dissocation equilibrium constants as well as association and dissociation rate constants for the binding of the Gly4 cystatin A mutants to papain and cathepsins B and L were determined by methods used in previous work (Björk et al., 1994; Pol et al., 1995).

The affinities of the mutants for papain in general were found to decrease with the size of the substituent. The substitution of Gly4 by Ala or Ser thus resulted in a 1000- and an 8000-fold lowered affinity, respectively, whereas substitutions by Arg, Glu or Trp gave affinity decreases of more than $2x10^6$-fold. These effects were rationalized by computer modelling experiments, in which a tryptophan side chain was modelled onto the coordinates of the homologous inhibitor, cystatin B, in the X-ray structure of its complex with papain. This showed that the indole group of Trp would be deeply buried into the papain structure, causing severe steric hindrance and consequent weaker binding. This effect would be expected to vary with the size of the substituent, explaining the observed trend for the affinity decrease. The observed, apparent pseudo-first-order rate constants ($k_{obs,\ app}$) for the interactions of Gly4Ala, Gly4Ser and Gly4Trp cystatin A with papain showed a linear dependence on inhibitor concentration, compatible with a one-step binding mechanism. In contrast, $k_{obs,\ app}$ for the interactions of the Gly4Arg and Gly4Glu cystatin A mutants with papain showed a hyperbolic dependence on inhibitor concentration, reflecting a two-step binding mechanism. This finding indicates that these bulky and charged residues can not be accommodated in the complex with papain without conformational changes.

The affinities of the Gly4 mutants for cathepsin L were in general similar to those for papain, but the decreases in affinity compared with the wild-type were smaller for cathepsin L than for papain. This observation most likely reflects a larger tolerance of cathepsin L than of papain for a side-chain other than a hydrogen in position 4. The observed pseudo-first-order rate constants for the ineractions with cathepsin L all showed a linear dependence on inhibitor concentration, consistent with a one-step binding mechanism.

The affinities of the Gly4 mutants for cathepsin B were affected to an even larger extent than those for papain or cathepsin L. The decreases in affinity of Gly4Ala- and Gly4Ser-cystatin A (the only mutants for which values could be obtained) for cathepsin B thus were 5000- and 25000-fold, respectively, compared with the wild-type. This large effect could to some extent be due to the occluding loop interfering with the interaction of the mutant residues with the enzyme. For both Gly4Ala- and Gly4Ser-cystatin A, $k_{obs,\ app}$ showed a linear dependence on inhibitor concentration, again consistent with a one-step binding mechanism.

Gly4Ala- and Gly4Ser-cystatin A were the only mutants for which the kinetics of association could be analysed with all enzymes. In the case of papain and cathepsin L the association rate constants for the binding of the inhibitor were unaffected by the mutations. The decrease in affinity for papain and cathepsin L caused by the mutants thus is entirely due to increased dissociation rate constants, indicating that the mutations destabilize the complex but do not affect the rate at which it is formed. In contrast, in the case of cathepsin B the mutations affected both the association and dissociation rate constants. The reduced association rate constant is consistent with the intact N-terminal region of cystatin A, like that of cystatin C (Björk et al., 1994, 1996), binding first to cathepsin B, thereby serving as a guide and facilitating displacement of the occluding loop of the enzyme.

Evidence that the binding of cystatin C to cathepsin B occurs by a two-step mechanism (paper V)

As discussed above, computer docking experiments of the X-ray stuctures of chicken cystatin and papain show that a complex can be formed with negligible conformational adaptions of either protein, which is consistent with a one-step binding mechanism. Moreover, all previous kinetic studies of the interaction between cysteine proteinases and their inhibitors have been compatible with the enzyme-inhibitor complex being formed in a one-step reaction. However, in contrast with the enzymes studied so far, cathepsin B has an "occluding loop" that partially covers the active site of the enzyme. Computer docking experiments with chicken cystatin suggested that the occluding loop would interfere with the binding of the inhibitor to cathepsin B, but that this interference could be reduced by the loop being displaced during the binding (Musil et al., 1991). Björk et al. (1994) also showed that truncation of the N-terminal region of cystatin C resulted in a decreased association rate constant with cathepsin B, but with no other cysteine proteinases, These obsevations imply that the interaction between cystatins and cathepsin B is more complicated than a one-step reaction. The aim of this study was to investigate whether the binding of cystatin C to cathepsin B follows a two-step mechanism and to try to explain its nature. To do so, we characterized the kinetics of the association of cystatin C with cathepsin B or a mutated form of the enzyme, His111Trp-cathepsin B, in which a fluorescent probe had been introduced in the occluding loop by replacement of His111 with Trp.

Human cystatin C, human cathepsin B and His111Trp-cathepsin B were produced by recombinant techniques. The kinetics of binding of cystatin C to wild-type or His111Trp-cathepsin B were analysed by stopped-flow measurements, monitored by the changes of intrinsic fluorescence accompanying the binding. The reaction with wild-type cathepsin B resulted in a fluorescence decrease, whereas the reaction with His111Trp-cathepsin B

gave a fluorescence increase. The observed pseudo-first-order rate constants showed a hyperbolic dependence on inhibitor concentration for both enzyme variants, indicating a two-step binding mechanism in both cases. In the simplest such mechanism, a weak complex (PI) between proteinase (P) and inhibitor (I) is established in the first step in a rapid equilibrium. The second step is a slow isomerisation to the final, stable complex that is responsible for the fluorescence change:

$$E + I \underset{}{\overset{K_{1,app}}{\rightleftarrows}} EI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} EI^*$$

Scheme 1

The observed pseudo-first-order rate constants for both enzyme forms could be well fitted to the equation for this mechanism (Fersht, 1985), indicating that the mechanism in Scheme 1 is a satisfactory description of the binding process. The bimolecular assocation rate constant, k_{on}, which is equal to k_{+2}/K_1 (Scheme 1), was almost identical for the two enzyme variants and comparable to what has been determined previously for the interaction between cystatin C and rat cathepsin B. However, the forward rate constant of the conformational change, k_{+2}, increased appreciably on mutation of His111 in the occluding loop of cathepsin B to Trp.

These results show that the inhibition of cathepsin B by cystatin C is best described as a two-step reaction. The initial, weak interaction most likely involves the binding of the N-terminal region of cystatin C, in particular to the S_2 and S_3 subsites of cathepsin B, which thereby facilitates the binding of the rest of the inhibitor to the enzyme (Bode et al., 1988; Björk et al., 1994, 1996). The second step is presumably the conformational change comprising the displacement of the occluding loop of cathepsin B, which blocks the active site. Modelling experiments show that the steric hindrance caused by the occluding loop could be released by tilting and simultaneous rotation of the inhibitor (Musil et al., 1991). That the second step includes movement of the occluding loop is further supported by the observation that a fluorescence increase was observed for His111Trp-cathepsin B, whereas a fluorescence decrease was seen for the wild-type enzyme on binding to cystatin C. Moreover, the forward rate constant of the second step was higher for the mutated form of cathepsin B, probably due to the mutation disrupting interactions between the occluding loop and and the rest of the protein, thereby facilitating movement of the loop. Further evidence that the occluding loop is displaced in the reaction with cystatins is provided by the substantially increased affinity of cystatin C for a form of cathepsin B in which the occluding loop had been deleted by genetic engineering (Illy et al., 1997).

Inhibition of the parasite cysteine proteinase, cruzipain, by proteinase inhibitors of the cystatin superfamily (paper III)

The protozoan parasite, *Trypanosoma cruzi,* causes Chagas´ disease, which afflicts more than 24 million persons in South and Central America and is the leading cause of heart failure in many Latin American countries. The parasite produces a crucial cysteine proteinase, cruzipain, which is a glycoprotein (Cassulo et al., 1990) that is present in different stages of the life cycle of the parasite (Campetella et al., 1990). Cruzipain may be involved in the defense mechanism of the parasite against the host immune response (Bontempi et al., 1990; Souto-Padron et al., 1990). Recent studies with inhibitors have shown the importance of cruzipain in the differentiation steps of the parasite´s life cycle (Franke de Cazzulo et al., 1994). Cruzipain is composed of two domains, one of which is a catalytic domain homologous to papain-like proteinases (Berti et al., 1995). Although cystatins are well known to be strong competetive inhibitors of cysteine proteinases of this type (Rich et al., 1986), no data were available concerning their action on cruzipain. In this paper we have characterized the interaction between cruzipain and several inhibitors of the cystatin superfamily.

Cruzipain was isolated from an extract of *T. cruzi* as previously described (Cazzulo et al., 1989), and the purity of the enzyme was verified by N-terminal sequence analysis and gel chromatography. Kinetic and equilibrium constants for the interaction of cruzipain with cystatin A, B, C, chicken cystatin and kininogen were determined by methods similar to those used in papers II and IV. These data showed that all inhibitors studied are highly active in inhibiting cruzipain. They bind rapidly ($k_{on} \approx 2 - 80 \times 10^6\ M^{-1} \cdot s^{-1}$) and tightly ($K_d \approx 1 - 70$ pM) to the enzyme. Cystatin C and chicken cystatin have somewhat higher affinities and association rate constants than the other inhibitors.

The results of this study provide clear evidence that the cysteine proteinase from *T. cruzi* can be efficiently inhibited by host cystatins. Parasite proteinases are attractive targets for drug design due to their key role in the parasite life cycle, as proved by the effects of low-molecular-weight inhibitors on the parasites´ growth and and differentiation. Therefore, the data presented here, which show that cystatins are potent inhibitors of cruzipain, indicate that cystatins may serve as starting points for the design of inhibitors as antiparasite drugs.

Conclusions

The fundamental problem studied throughout this work has been structure-function relationships concerning the binding of an inhibitor to a target enzyme, specifically the interaction of cystatins with cysteine proteinases. The mechanism by which chicken cystatin, cystatin A and cystatin C inhibit target cysteine proteinases has been studied by the use of proteins modified by chemical methods, proteolytic truncation or genetic engineering. The contribution of the N-terminal region and the second hairpin loop of the cystatins to the interaction with target enzymes has been characterized. A proposed model explaining the different contributions of residues in the N-terminal region of the cystatins to the interaction mechanism with different target enzymes was verified. The equilibrium and kinetics of the inhibition of cruzipain, a protozoan cysteine proteinase, by cystatins has been characterized. The reaction mechanism between cystatin C and cathepsin B was also investigated.

The general conclusions of the papers are:

I. Trp104 of the second hairpin loop of chicken cystatin is directly involved in the interaction with the target proteinase, or alternatively, is located very close to the proteinase-binding region of the inhibitor. The second hairpin loop of chicken cystatin contributes approximately 40% of the total free energy change for the binding of chicken cystatin to papain.

II. The contribution of the N-terminal region of chicken cystatin corresponds to approximately 40% of the total free energy change for the binding of the inhibitor to both papain and actinidin. In the N-terminal region, only residues before Ala-10 participate in the interaction with the enzymes. Of these residues, Leu7 and Leu8 contribute the predominant part, about two-thirds of the binding energy of the N-terminal region, which is in agreement with the proposal that Leu7 and Leu8 bind to the S_3 and S_2 subsites, respectively, of papain. Also the highly conserved Gly9 residue and residues N-terminal of Leu7 contribute free energy of binding of chicken cystatin to papain and actinidin, but to a much smaller extent than Leu7 and Leu8.

III. The inhibition of cruzipain from the parasite, *Trypnosoma cruzi,* by cystatin A, B, C, chicken cystatin and kininogen is rapid ($k_{on} \approx 2\text{-}80 \times 10^6$ $M^{-1} \cdot s^{-1}$) and tight ($K_d \approx 1\text{-}70$ pM), providing evidence that cruzipain can be effectively inhibited by host cystatins. These results indicate the possibility that cystatins may be used as lead compounds for the synthesis of antiparasite drugs.

IV. The affinity of Gly4 mutants of cystatin A for papain decreases with the size of the substituent. Even the introduction of only a methyl group in Gly4Ala-cystatin A reduced the affinity for papain about 1000-fold compared

with the wild-type. The affinities of the Gly4 mutants of cystatin A for cathepsin L were comparable with those for papain, whereas the affinities for cathepsin B were even more effected by the substitutions, possibly due to the presence of the occluding loop in cathepsin B. In the case of papain and cathepsin L the association rate constants for the binding of Gly4Ala- and Gly4Ser-cystatin A were unaffected by the mutations, the decrease in affinity therefore being entirely due to increased dissociation rate constants. In contrast, in the case of cathepsin B the mutations affected both the association and dissociation rate constants. The reduced association rate constant is consistent with the intact N-terminal region of cystatin A binding first to cathepsin B, thereby serving as a guide and facilitating displacement of the occluding loop of the enzyme.

V. The observed pseudo-first-order rate constants for the binding of cystatin C to cathepsin B and a His111Trp mutant of cathepsin B showed a hyperbolic dependence on inhibitor concentration. This behaviour indicates a two-step binding mechanism in both cases, in contrast with the mechanism for other reactions between cystatins and proteinases. Most probably the first step involves an initial weak binding of the N-terminal region of cystatin C to the enzyme in a rapid equilibrium, and the second step involves a slow isomerization due to the displacement of the occluding loop of cathepsin B.

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References

Abe, K., Emori, Y., Kondo, H., Arai, S. and Suzuki, K. 1988. J. Biol. Chem. 263, 7655.

Abrahamson, M. and Grubb, A. 1994. Proc. Natl. Acad. Sci. USA 91, 1.

Abrahamson, M., Barrett, A.J., Salvesen, G. and Grubb, A. 1986. J. Biol. Chem. 261, 11282.

Abrahamson, M., Ritonja, A., Brown, M.A., Grubb, A., Machleidt, W. and Barrett, A.J. 1987. J. Biol. Chem. 262, 9688.

Abrahamson, M., Mason, R.W., Hansson, H., Buttle, D.J., Grubb, A. and Ohlson, K. 1991. Biochem. J. 273, 621.

Abrahamson, M., Jonsdottir, S., Olafsson, I., Jensson, O. and Grubb, A. 1992. Hum. Genet. 89, 377.

Anastasi, A., Brown, M.A., Kembhavi, A.A., Nicklin, M.J.H., Sayers, C.A., Sunter, D.C. and Barrett, A.J. 1983. Biochem. J. 211, 129.

Arai, S., Watanabe, H., Kondo, H., Emori, Y. and Abe, K. 1991. J. Biochem. 109, 294.

Auerswald, E.A., Genenger, G., Assfalg-Machleidt, I., Machleidt, W., Engh, R.A. and Fritz, H. 1992. Eur. J. Biochem. 209, 837.

Auerswald, E.A., Nägler, D.K., Schulze, A.J., Engh, R.A., Genenger, G., Machleidt, W. and Fritz, H. 1994. Eur. J. Biochem. 224, 407.

Auerswald, E.A., Nägler, D.K., Assfalg-Machleidt, I., Stubbs, M.T., Machleidt, W. and Fritz, H. 1995. FEBS Lett. 361, 179.

Baker, E.N. 1980. J. Mol. Biol. 141, 441.

Barrett, A.J. 1980. In: Protein degradation in health and disease (Ciba Foundation Symposium 75), Exerpta Medica, p.1.

Barrett, A.J. and Kirschke, H. 1981. Meth. Enzymol. 80, 535.

Barrett, A.J. and MacDonald, J.K. 1980. Mammalian Proteinases: A Glossary and Bibliography. Vol. 1. Academic Press, London.

Barrett, A.J. and MacDonald, J.K. 1985. Biochem J 231, 807.

Barrett, A.J. and MacDonald, J.K. 1986. Biochem J 237, 935.

Barrett, A.J., Nicklin, M.J.H. and Rawlings, N.D. 1984. Symp. Biol. Hung. 25, 203.

Benedikz, E., Blöndal, H., Johannesson, G., and Gudmundsson, G. 1989. In: Alzheimer's Disease and Related Disorders (Roth, M and Iversen, L.L., Eds.), p. 517.

Berger, A. and Schechter, I. 1970. Phil. Trans. R. Soc. London B 257, 249.

Berti, P.J. and Storer, A.C. 1995. J. Mol. Biol. 246, 273.

Bespalova, I.N., Adkins, S., Pranzatelli, M. and Burmeister, M. 1997. Am. J. Med. Genet. 74, 467.

Björck, L., Grubb, A. and Kjellén, L. 1990. J. Virol. 64, 941.

Björk, I. and Ylinenjärvi, K. 1989. Biochem. J. 260, 61.

Björk, I. and Ylinenjärvi, K. 1990. Biochemistry 29, 1770.

Björk, I., Alriksson, E. and Ylinenjärvi, K. 1989. Biochemistry 28, 1568.

Björk, I., Ylinenjärvi, K. and Lindahl, P. 1990. Biol. Chem. Hoppe-Seyler 371 Suppl., 119.

Björk, I., Pol, E., Raub-Segall, E., Abrahamson, M., Rowan, A.D. and Mort, J.S. 1994. Biochem. J. 299, 219.

Björk, I., Brieditis, I., Raub-Segall, E., Håkansson, K. and Abrahamson, M. 1996. Biochemistry 35, 10720.

Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J. and Turk, V. 1988. EMBO J. 7, 2593.

Bode, W., Engh, R., Musil, D., Laber, B., Stubbs, M., Huber, R. and Turk, V. 1990. Biol. Chem. Hoppe-Seyler 371 Suppl., 111.

Bohley, P. and Seglen, P.O. 1992. Experientia 48, 151.
Bond, J.S. and Barrett, A.J. 1980. Biochem J 189, 17.
Bontempi, E., Franke de Cazzulo, B.M., Ruiz, A.M. and Cazzulo, J.J. 1984. Comp. Biochem. Physiol. 77B, 599.
Bontempi, E., Martinez, J. and Cazzulo, J.J. 1989. Mol. Biochem. Parasitol. 33, 43.
Bontempi, E. and Cazzulo, J.J. 1990. FEMS Microbiol. Lett. 70, 337.
Bossard, M.J., Tomaszek, T.A., Thompson, S.K., Amegadzie, B.Y., Hanning, C.R., Jones, C., Kurdyla, J.T., McNulty, D.E., Drake, F.H., Gowen, M., and Levy, M.A. 1996. J. Biol. Chem. 271, 12517.
Brocklehurst, K., Willenbrock, F. and Salih, E. 1987. In: Hydrolytic enzymes (Neuberger, A. and Brocklehurst, K., Eds.), p. 39, Elsevier, Amsterdam-New York-Oxford.
Brzin, J., Kopitar, M., Locnikar, P. and Turk, V. 1982. FEBS Lett. 138, 193.
Buck, M.R., Karustic, D.G., Day, N.A., John, K.V. and Sloane, B.F. 1992. Biochem. J. 282, 273.
Calkins, C.C. and Sloane, B.F. 1995. Biol. Chem. Hoppe-Seyler 376, 71.
Campetella, O., Martinez, J. and Cazzulo, J.J. 1990. FEMS Microbiol. Lett. 67, 145.
Cazzulo, J.J., Couso, R., Raimondi, A., Wernstedt, C. and Hellman, U. 1989. Mol. Biochem. Parasitol. 33, 33.
Cazzulo, J.J., Hellman, U., Couso, R. and Parodi, A.J.A. 1990. Mol. Biochem. Parasitol. 38, 41.
Chauhan, S.S., Goldstein, L.J. and Gottesman, M.M. 1991. Cancer Res. 51, 1478.
Ciechanover, A. and Schwartz, A.L .1994. FASEB J. 8, 182.
Coulombe, R., Grochulski, P., Sivaraman, J., Menard, R., Mort, J.S. and Cygler, M. 1996. EMBO J. 15, 5492.
Cygler, M., Sivaraman, J., Grochulski, P., Coulombe, R., Storer, A.C. and Mort, J.S. 1996. Structure 4, 405.
Delaisse, J.M. and Vaes, G. 1992. In: Biology and Physiology of the Osteoclasts (Rifkin, B.R. and Gay, C.V., Eds.), pp. 290, CRC Press, Boca Raton.
Delaisse, J.M., Eeckhout, Y. and Vaes, G. 1980. Biochem. J. 192, 365.
Dieckmann, T., Mitschang, L., Hofmann, M., Kos, J., Turk, V., Auerswald, E.A., Jaenicke, R. and Oschkinat, H. 1993. J. Mol. Biol. 234, 1048.
Drenth, J., Jasonius, J.N., Koekoek, R., Swen, H.M. and Wolthers B.G. 1968. Nature 218, 929.
Dunn, A.D., Crutchfield, H.E. and Dunn, J.T. 1991. J. Biol. Chem. 266, 20198.
Ekiel, I., Abrahamson, M., Fulton, D.B., Lindahl, P., Storer, A.C., Levadoux, W., Lafrance, M., Labelle, S., Pomerleau, Y., Groleau, D., LeSauteur, L., and Gehring, K. 1997. J. Mol. Biol. 271, 266.
Engh, R.A., Dieckmann, T., Bode, W., Auerswald, E.A., Turk, V., Huber, R. and Oschkinat, H. 1993. J. Mol. Biol. 234, 1060.
Furuhashid, M., Nakahara, A., Fukutomi, H., Kominami, E., Grube, D. and Uchiyama, Y. 1991. Histochemistry 95, 231.
Fersht, A. 1985. Enzyme Structure and Mechanism, 2nd ed., p. 128, W.H. Freeman, New York.
Franke de Cazzulo, B.M., Martínez, J., North, M.J., Coombs, G.H. and Cazzulo, J.J. 1994. FEMS Microbiol. Lett. 124, 81.
Freije, J. P., Balbin, M., Abrahamson, M., Velasco, G., Dalbøge, H., Grubb, A. and Lopez-Otin, C. 1993. J. Biol. Chem. 268, 15737.
Fujishima, A., Imai, Y., Nomura, T., Fujisawa, Y., Yamamoto, Y. and Sugawara, T. 1997. FEBS Lett. 407, 47.
Ghiso, J., Jensson, O. and Frangione, B. 1986. Proc. Natl. Acad. Sci. USA 83, 2974.
Girard, M. T., Matsubara, M., Kublin, C., Tessier, M. J., Cintron, C.C.C. and Fini, M.E., 1993. J. Cell Sci. 104, 1101.

Glazer, A.N. and Smith, E.L. 1971. In: The Enzymes (Boyer, P.D., Ed.), Vol. 3, 3rd edn., p. 501, Academic Press, New York.
Green, G.D.J., Kembhavi, A.A., Davies, M.E. and Barrett, A.J. 1984. Biochem. J. 218, 939.
Grubb, A., Weiber, H. and Löfberg, H. 1983. Scand. J. Clin. Lab. Ivest. 43, 421.
Hall, A., Dalbøge, H., Grubb, A. and Abrahamson, M.. 1993. Biochem. J. 291, 123.
Hall, A., Håkansson, K., Mason, R.W., Grubb, A. and Abrahamson, M. 1995. J. Biol. Chem. 270, 5115.
Hartley, B.S. 1960. Annu. Rev. Biochem. 29, 45.
Hasnain, S., Huber, C. P., Muir, A., Rowan, A.D. and Mort, J.S., 1992. Biol. Chem. Hoppe-Seyler, 373, 413.
Hazuda, D. J., Strickler, J., Kueppers, F., Simon, P.L. and Young, P. R. 1990. J.Biol. Chem. 265, 6318.
Hembry, R.M., Bagga, M.R., Murphy, G., Henderson, B. and Reynolds, J.J. 1993. Am. J . Pathol. 143, 628.
Higashiyama, S., Ohkubo, I., Ishiguro, H., Kunimatsu, M., Sawaki, K. and Sasaki, M. 1986. Biochemistry 25, 1669.
Hopsu-Havu, V., Järvinen, M. and Rinne, A. 1983. Brit. J. Dermatol. 109, 77.
Howie, A.J., Brunett, D. and Crocker, J. 1985. J. Pathol. 145, 307.
Illy, C., Quraishi, O., Wang, J., Purisima, E., Vernet, T. and Mort, J.S. 1997. J. Biol. Chem. 272, 1197.
Isemura, S., Saitoh, E. and Sanada, K. 1986. FEBS Lett. 198, 145.
Jerala, R., Trstenjak-Prebanda, M., Kroon-Zitko, L., Lenarcic, B. and Turk, V. 1990. Biol. Chem. Hoppe-Seyler 371, Suppl. 157.
Jerela, R., Kroon-Zitko, L., Kopitar, M., Popovic, T. and Turk, V. 1991. Biomed. Biochim. Acta 50, 627.
Järvinen, M., Rinne, A. and Hopsu-Havu, V. 1987. Acta Histochem. 82, 5.
Kamphuis, I.G., Kalk, K.H., Swart, M.B.A. and Drenth, J. 1984. J. Mol. Biol. 179, 233.
Kamphuis, I.G., Drenth, J. and Baker, E.N. 1985. J. Mol. Biol. 182, 317.
Katao, H., Nagaswa, S. and Iwanaga, S. 1981. Meth. Enzymol. 80, 172.
Keppler, D. and Sloane, B.F. 1996. Enzyme & Protein 49, 94.
Kirschke, H., Barrett, A.J. and Rawlings, N.D. 1995. In: Protein Profile, Vol. 2, p. 1587, Academic Press, London.
Koj, A., Magielska-Zero, D., Kurdowska, A. and Bereta, J. 1988. Adv. Exp. Med. Biol. 240, 171.
Korant, B.D., Brzin, J. and Turk, V. 1985. Biochem. Biophys. Res. Commun. 127, 1072.
Korant, B.D., Towatari, T., Ivanoff, L., Petteway, S., Brzin, J., Lenarcic, B. and Turk, V. 1986. J. Cell. Biochem. 32, 91.
Korant, B.D., Towatari, T., Kelley, M., Brzin, J., Lenarc, B. and Turk, V. 1988. Biol. Chem. Hoppe-Seyler. 369, 281.
Koshland, D.E., Jr., Karkhanis, Y.D. and Latham, H.G. 1964. J. Am. Chem. Soc. 86, 162.
Lah, T. T., Babnik, J., Schiffmann, E., Turk, V. and Skaleric, U. 1993. J. Periodontol. 64, 485.
Labauge, P., Ouazzani, R., M'Rabet, A., Grid, D., Genton, P., Dravet, C., Chkili T., Beck, C. and Buresi, C. 1997. Ann. Neurol. 41, 686.
Lenarcic, B., Kos, J., Dolenc, I., Lucovnik, P., Krizaj, I., and Turk, V., 1988a. Biochem. Biophys. Res. Commun., 154, 765.
Lenarcic, B., Gabrijelcic, D., Rozman, B., Drobnic-Kosorok, M. and Turk, V. 1988b. Biol. Chem. Hoppe-Seyler. 369, 257.

Li, K., Ito, H., Kominami, E. and Hirano, A. 1993. Virchows Arch. A. Pathol. Anat. 423, 185.
Light, A., Frater, R., Kimmel, J.R. and Smith, H. 1964. Proc. Natl. Acad. Sci. USA 52, 1276.
Lindahl, P., Alriksson, E., Jörnvall, H. and Björk, I. 1988. Biochemistry 27, 5074.
Lindahl, P., Abrahamson, M. and Björk, I. 1992. Biochem. J. 281, 49.
Lowe, G. 1976. Tetrahedron 32, 291.
Löfberg, H., and Grubb, A. 1979. Scand. J. Clin. Lab. Invest. 39, 619.
Machleidt, W., Thiele, U., Laber, B., Assfalg-Machleidt, I., Esterl, A., Wiegand, G., Kos, J., Turk, V. and Bode, W. 1989. FEBS Lett. 243, 234.
Machleidt, W., Thiele, U., Assfalg-Machleidt, I., Förgler, D. and Auerswaldt, E.A. 1991. Biomed. Biochem Acta 50, 4.
Maciewicz, R.A., Etherington, D.J., Kos, J. and Turk, V. 1987. Collagen Rel. Res. 7, 295.
Mann, K.G., Jenny, R.J. and Krishnaswamy, S. 1988. Annu. Rev. Biochem. 57, 915.
Marks, N., Berg, J.M. and Benuck, M. 1986. Arch. Biochem. Biophys. 249, 489.
Martin, J.R., Craven, C.J., Jerala, R., Kroon-Zitko, L., Zerovnik, E., Turk, V. and Waltho, J. P. 1995. J. Mol. Biol. 246, 331.
Martinez, J., Silberstein, E. and Cazzulo, J.J. 1989. Anal. Assoc. Quim. Argentina 77, 113.
Maruyama, K., Kametani, F., Ikeda, S., Ishihara, T. and Yanagisawa, N. 1992. Neurosci. Lett. 144, 38.
Mason, R.V., Wilcox, D., Wikström, P and Shaw, E.N. 1989. Biochem. J. 257, 125.
McKerrow, Sun, E., Rosenthal, P.J. and Bouvier, J. 1993. Annu. Rev. Microbiol. 47, 821.
Mizuochi, T., Yee, S.T., Kasai, M., Kakiuchi, T., Muno, D. and Kominami, E. 1994. Immunol. Lett. 43, 189.
Morihara, K. 1974. Adv. Enzymol. 41, 179.
Morrison, J.F. 1982. Trends Biochem. Sci. 7, 102.
Musil, D., Zucic, D., Turk, D., Engh, R.A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N. and Bode, W. 1991. EMBO J. 10, 2321.
Müller-Esterl, W. 1989. Thromb. Haemostas. 61, 2.
Müller-Esterl, W., Iwanaga, S. and Nakanishi, S. 1986. Trends Biochem. Sci. 11, 336.
Neurath, H. 1984. Science 224, 350.
Neurath, H. 1989. In: Proteolytic enzymes; a practical approach. (Beynon, J.R. and Bond, J.S., Eds) p. 1, IRL Press, Oxford.
Nicklin, M.J.H. and Barrett, A.J. 1984. Biochem. J. 223, 245.
Nikawa, T., Towatari, T., Ike, Y. and Katunuma, N. 1989. FEBS Lett. 255, 309.
Nishimura, Y., Kawabata, T., Yano S. and Kato, K. 1990. Arch. Biochem. Biophys. 283, 458.
North, M.J. 1982. Microbiol. Rev. 46, 308.
North, M.J. 1992. Biol. Chem. Hoppe-Seyler 373, 401.
North, M.J. and Mottram, J.C. 1990. Parasitol. Today 6, 270.
Nägler, D.K., Storer, A.C., Portaro, F.C.V., Carmona, E., Juliano, L. and Ménard, R. 1997. Biochemistry 36, 12608.
Okazaki, H., Tani, C., Ando, M., Ishii, K., Ishibashi, S., Nishimura, Y. and Kato, K. 1992. J. Biochem. 112, 409.
Othani, O., Fukuyama, K. and Epstein, W.L. 1982. J. Invest. Dermatol. 82, 280.
Palsdottir, A., Thorsteinsson, L., Olafsson, I., Abrahamson, M., Arnason, A. and Grubb, A. 1988. Lancet. ii, 603.
Peterson, C.B. and Blackburn, M.N. 1987. J. Biol. Chem. 262, 7559.

Pennacchio, A., Lehesjoki, A.E., Stone, N.E., Willour, V.L., Virtaneva, K., Miao, J.M., D'Amato, E., Ramirez, L., Faham, M., Koskiniemi, M., Warrington, J.A., Norio,R., De la Chapelle, A., Cox, D.R. and Myers, R.M. 1996. Science 271, 1731.
Polgar, L. and Halasz, P. 1982. Biochem. J. 207, 1.
Powers, J.C., Odake, S., Oleksyszyn, J., Hori, H., Ueta, T., Boduszek, B. and Kam, C., 1993. Agents Act. Suppl. 42, 3.
Qian, F., Chan, S.J., Gong, Q.M., Bajkowski, A.S., Steiner, D.F. and Frankfater, A. 1991. Biomed. Biochim. Acta, 50, 531.
Rich, D.H. 1986. In: Proteinase Inhibitors (Barrett, A.J. and Salvesen, G., Eds.), p. 153, Elsevier, Amsterdam.
Rivett , A.J. 1993. Biochem. J. 291, 1.
Schwabe, C., Anastasi, A., Crow, H., McDonald, J.K. and Barrett, A.J. 1984. Biochem. J. 217, 813.
Scott, G.K. 1992. Comp. Biochem. Physiol. B. 103, 785.
Scott, G.K. and Tse, C.A. 1992. Biol. Chem. Hoppe-Seyler 373, 605.
Sharma, J.N. and Mohsin, S.S.J. 1990. Exp. Pathol. 38, 73.
Shibuya, K., Kaji, H., Itoh, T., Ohyama, Y., Tsujikami, A., Tate, S., Takeda, A., Kumagai, I., Hirao, I., Miura, K., Inagaki, F. and Samejima, T. 1995a. Biochemistry 43, 12185.
Shibuya, K., Kaji, H., Itoh, T., Ohyama, Y., Tate, S., Kainosho, M., Inagaki, F. and Samejima, T. 1995b. J. Biochem. 118, 635.
Sim, R.B., Kolble, K., McAleer, M.A., Dominguez, O. and Dee, V.M. 1993. Int. Rev. Immunol. 10, 65.
Souto-Padrón, T., Campetella, O., Cazzulo, J.J. and Souza, W. 1990. J. Cell. Sci. 96, 485.
Stewart, J.M. 1993. Agents Actions Suppl. 42, 145.
Stubbs, M.T., Laber, B., Bode, W., Huber, R., Jerala, R., Lenarcic, B. and Turk, V. 1990. EMBO J. 9, 1939.
Tagami, K., Kakegawa, H., Kamioka, H., Sumitani, K., Kawata, T., Lenarcic, B., Turk, V. and Katunuma, N. 1994. FEBS Lett. 342, 308.
Tate, S., Ushioda, T., Utsunomiya-Tate, N., Shibuya, K., Ohyama, Y., Nakano, Y., Kaji, H., Inagaki, F., Samejima, T. and Kainosho, M. 1995. Biochemistry 34, 14637.
Thiele, U., Assfalg-Machleidt, I., Machleidt, W. and Auerswald, E.A. 1990. Biol. Chem. Hoppe-Seyler 371 Suppl., 125.
Trabandt, A., Gay, R.E., Fassbender, H.G. and Gay, S. 1991. Arth. Rheum. 34, 1444.
Turk, V. and Bode, W. 1993. In: Innovations in Proteases and Their Inhibitors. (Avilés, F.X., Ed), p. 161, Walter de Gruyter, Berlin, New York.
Turk, B., Stoka, V., Turk, V., Johansson, G., Cazzulo, J.J. and Björk, I. 1996. FEBS Lett., 391, 109.
Wang, P.H., Do, Y.S., Macaulay, L., Shinagawa, T., Anderson, P.W., Baxter, J.D. and Hsueh, W.A. 1991. J. Biol. Chem. 266, 12633.
Woessner, J.F. 1991. FASEB J. 5, 2145.
Zucker, S., Buttle, D.J., Nicklin, M.J.H. and Barrett, A.J. 1985. Biochim. Biophys. Acta 828, 196.

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