# Human Cystatin A Is Inactivated by Engineered Truncation. The $NH_2$-Terminal Region of the Cysteine Proteinase Inhibitor Is Essential for Expression of Its Inhibitory Activity†

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ABSTRACT: A series of $NH_2$-terminal truncated forms of human cysteine proteinase inhibitor, cystatin A, was prepared by genetic engineering using *Escherichia coli* harboring mutated genes. Each variant of cystatin A was efficiently expressed as a fused protein with porcine adenylate kinase and released by CNBr degradation after exchange of the sole inner Met to Leu. The mutant cystatin A lacking an amino-terminal Met residue (called standard variant starting from Ile2, CystA$^{2-98}$(M65L)) showed the same inhibitory activity as authentic one isolated from human epidermis. Two-residue truncation scarcely influenced the activity, but further truncations deleting Pro3 and beyond conservative Gly4 and Gly5 caused a remarkable decrease of their inhibitory activity. But little effect was observed by a substitution of Pro3 with Leu. The loss of the activity by amino-terminal truncation was compensated slightly by engineered substitution of Gly75 with His on a second loop. In the two-dimensional $^{15}N-^{1}H$ HSQC NMR spectrum, four-residue truncation was found to cause changes in the chemical shifts of Val47 and Val48, which locate on a first loop and consist of a conservative QVVAG sequence. Furthermore, the truncation led to a change in fluorescence spectroscopic behavior of Trp75, which was introduced as a probe on the second loop. Fluorescence intensity of the Trp of the truncated (5−98) form was more affected by heating than the active standard variant. Conversely, fluorescence of Trp75 in 2−98 form was more quenched by acrylamide than the 5−98 variant. Thus, the amino-terminal region of cystatin A is essential for the expression of its inhibitory activity. We concluded that the N-terminal region of cystatin A contributes to not only contact and distinguish cognate cysteine proteinases, but also maintain conformational integrity of tripartite reactive wedge of cystatin A.

Many cysteine proteinase inhibitors have been isolated from a variety of tissue and body fluid of mammals, chicken egg, and plants. Their structural analyses revealed that they belong to three subdivided classes of cystatin superfamily (Barrett et al., 1986) which comprise the inhibitors derived from an evolutionarily common ancestral gene (Müller-Esterl et al., 1985; Rawlings & Barrett, 1990) with some exceptions (Freije et al., 1991; Ritonja et al., 1989). Human cystatin A (also called stefin A) is a member of family I (stefins) of the superfamily consisting of stefin B and their counterparts in rat (α and β). The inhibitors are small, nonglycosylated protein containing about 100 amino acid residues with no disulfide linkage. Family II, called cystatins, is a group of secreted inhibitors having a molecular mass of ca. 13 kDa with disulfide bonds. Family III is composed of kininogens, large plasma proteins containing three repeats of the small cystatin domain.

One of major interests of the inhibitors is their physiological function. For intracellular cystatins of rat, cystatin β is distributed ubiquitously in many organs at a relatively constant level, but cystatin α is localized in a specific one such as epidermis (Kominami et al., 1984). Cystatin A also exists specifically in leucocytes (Brzin et al., 1983), epidermis (Järvinen, 1987), liver (Green et al., 1984), and spleen (Järvinen & Rinne, 1982). It seems likely that cystatin A plays a defensive role against disordered proteolysis by lysosomal thiol cathepsins or an external invading proteinase from virus, etc., although this is not yet clear. Another area of interest is focused on the relationship between structure and function, e.g., a mechanism of specific recognition and inhibition toward their target proteinases. Three-dimensional structures of cystatins, i.e., chicken cystatin (family II) (Bode et al., 1988; Dieckmann et al., 1993), human stefin B−papain complex (Stubbs et al., 1990), and stefin A (Martin et al., 1994, 1995), were determined by X-ray crystallography and/or NMR analyses. The structural research revealed that the amino-terminal segment and two loops of cystatins form a tripartite "wedge-shaped edge" to slot in an active site cleft of cognate cysteine proteinase (Bode et al., 1988). The interaction was illustrated directly for stefin B in a complex with papain (Stubbs et al., 1990). From this result, a

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proposed docking model for avian cystatin with papain was found to be correct in essence (Bode et al., 1988). The main contribution to the interaction with target protease was provided by the first loop, containing the conserved sequence QVVAG, and also the amino-terminal region called "trunk" (Stubbs et al., 1990). The significance of these regions was examined by site-specific replacement using genetic engineering. Mutated cystatins served to investigate the specific role of each amino acid residue of the inhibitor (Abe et al., 1988; Thiele et al., 1990; Jerala et al., 1990; Abrahamson et al., 1991; Genenger et al., 1991; Hall et al., 1993; Lindahl et al., 1994). For cystatin A, a bacterial expression system was constructed by four groups independently; three of them were using a synthetic gene (Strauss et al., 1988; Kaji et al., 1989; Nikawa et al., 1989) and the rest was a cDNA (Fong et al., 1989).

Many cystatins lost their inhibitory activity partially or completely by truncation of their amino-terminal segment (Abrahamson et al., 1987; Isemura et al., 1986). Cystatin α from rat epidermis was inactivated completely by amino-terminal truncation of 15 residues (Takeda et al., 1985). But, truncated cystatin B lacking the first 6 residues maintained its inhibitory activity (Jerala et al., 1990), and the amino-terminal 21 residues of oryzacystatin had no essential role for inhibition (Abe et al., 1988). Thus, there are some variations of contrivance to control the inhibitory activity, which might be related to a device to regulate the activity in vivo. Therefore, the importance of the amino-terminal region of cystatin A for inhibition was confirmed by successive truncation using genetic engineering in this study. In order to obtain further information on the reason for the inactivation, some lines of evidence on structural change accompanied by amino-terminal truncation around the main reactive site, the first hairpin loop, were detected as changes of the NMR spectrum and fluorescence behavior of engineered Trp.

## MATERIALS AND METHODS

*Materials and Chemicals.* Human epidermis of heel was supplied by volunteers as the source of human cystatin A. *Escherichia coli* strain JM109 was used as a host bacterium for expression of recombinant cystatin A. The plasmid harboring human cystatin A gene was constructed as reported previously (Kaji et al., 1989, 1990). Sephadex G-75 and DEAE-Sephacel were products of Pharmacia. Proteinase-linked Sepharose affinity column was prepared with CNBr-activated Sepharose gel by the recommended method of the supplier, Pharmacia. β-Cyanoethyl-protected nucleotides (*N,N*-diisopropylphosphoramidite), reaction columns, and other reagents for oligodeoxyribonucleotide synthesis were obtained from Applied Biosystems, Inc., or MilliGen/Biosearch. Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, and DNA polymerase I large fragment (Klenow) were purchased from Nippon Gene Inc., TOYOBO, or Boehringer. [γ-$^{32}$P]ATP (220 TBq/mmol) and [$^{35}$S]-dCTP (αS) (22TBq/mmol) were obtained from Amersham Corp. The DNA sequencing kit, Sequenase ver. II, was from Stratagene. Papain Type IV and its substrate, $N^{\alpha}$-benzoyl-DL-arginine 2-naphthylamide, were from Sigma. Cathepsin B was obtained from human liver according to the method of Schwartz and Barrett (1980). (Aminomethyl)coumarin substrates, $N^{\alpha}$-(benzoxycarbonyl)phenylalanylarginine (4-methylcoumarin-7-yl)amide (Z-Phe-Arg-NMec),[1] and $N^{\alpha}$-(benzoxycarbonyl)arginylarginine (4-methylcoumarin-7-yl)-amide (Z-Arg-Arg-NMec) were products of the Peptide Institute, Inc. (Osaka, Japan).

Chart 1: Nucleotide Sequence of Synthetic Oligonucleotides Used for Site-Directed Mutagenesis[a]

| | | |
|---|---|---|
| Met65-Leu; | | 5'-AC AAA TAC cTG CAT CTG |
| Ile2-Met; | (-) | 3'-CCG GCG TAC TAc GGC CCT CCG AAT TCG C-5' |
| (EcoRI-AflII) | (+) | 5'-TGGAATTGTGAGCGGAT (M13 rev. primer) |
| Pro3-Met(Leu); | | 5'-GC ATG ATC atG GGA GGC |
| Gly4-Met; | | 5'-TG ATC CCG atg GGC TTA AG |
| Gly5-Met; | (+) | 5'-TC CCG GGA atg TTA AGC GA |
| (SmaI-KpnI) | (-) | 3'-CATGGTCCAGCTGTTCTTGTTCCTGCTGCTCAA-5' |
| des(1-8); | | 5'-G GTG ATC GAG GGC CGC / GCC AAG CCA GCT |
| Gly75-His; | | 5'- C TTA CCT cat CAG AAC G |
| Gly75-Trp; | | 5'-GC TTA CCT tGg CAG AAC GA |
| Tyr43-Trp; | | 5'-C GTT CAG Tgg AAG ACC C |
| Phe70-Trp; | | 5'-G AAG GTG Tgg AAA AGC T |

[a] Lower case(s) in the sequence shows mismatched nucleotide(s) for template DNA. Substitutions from Ile2 and Gly5 to Met were introduced by exchanging fragment produced by mismatch PCR using the listed primers and then digesting with restriction enzymes shown in parentheses. A slash (/) in the sequence for des(1-8) indicates a deletion site, looping-out from template DNA.

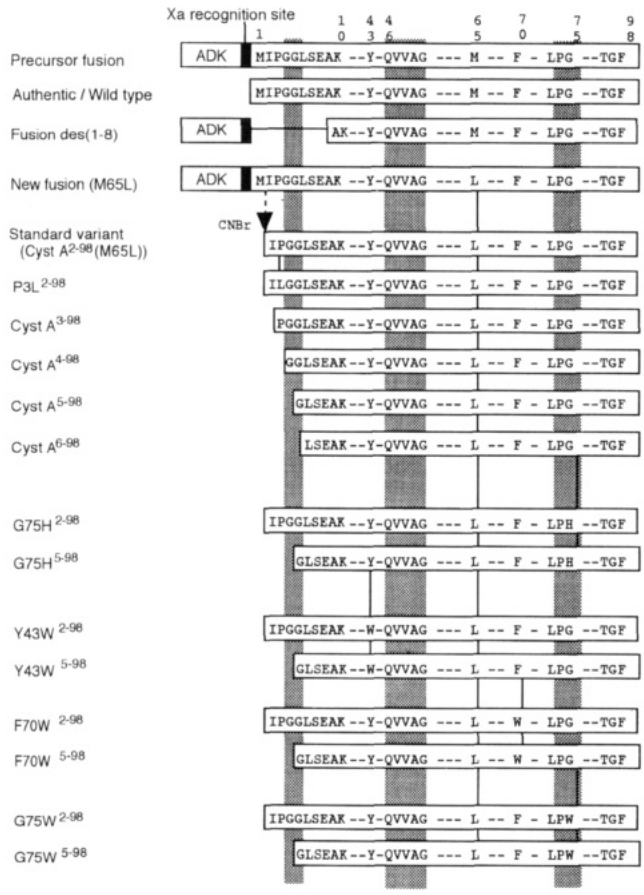
*Preparation of the Authentic Cystatin A from Human Epidermis.* Human epidermal cystatin A was extracted from cornified cells of sole (heel) and purified by the methods of Järvinen (1987) and Takeda et al. (1989). The resulting extract was separated by Sephadex G-75 column gel filtration followed by reduced and carboxymethylated papain–Sepharose affinity column chromatography. The final preparation was obtained by fast protein liquid chromatograph system (FPLC) of Pharmacia using a Mono Q ion-exchange column. Finally, homogeneity of the inhibitor was confirmed by FPLC chromatofocusing on a Mono P column.

*Construction of the Expression Vector of Amino-Terminally Truncated, Deleted, and Substituted Cystatin Variants Released by Chemical Excision.* For efficient preparation of recombinant cystatin A, its synthetic gene was fused with highly expressing cDNA of porcine adenylate kinase (ADK) as described previously (Kaji et al., 1990). In order to use CNBr degradation for releasing the cystatin portion from the fusion, the sole internal Met residue, Met65, was substituted to Leu by site-directed mutagenesis. The substitution was carried out by the method of Morinaga et al. (1984) using chemically synthesized oligonucleotide primer listed in Chart 1. Oligodeoxyribonucleotides used for site-directed mutagenesis were synthesized by the solid phase phosphoramidite method using an Applied Biosystems 381A or MilliGen/Biosearch Cyclone Plus DNA synthesizer.

Substitutions of Pro3 and Gly4 to Met were also carried out by the method described above. In case of replacement from Ile2 and Gly5 to Met, the mutations were introduced by exchanging the fragment produced by mismatch PCR. Mutagenesis primers used for the PCR are shown in Chart 1. The mutated PCR product for Ile2-Met mutation was digested with *Eco*RI–*Afl*II, and the resulting fragment was inserted to the corresponding position of an original vector.

[1] Abbreviations: Cyst A, cystatin A; BANA, $N^{\alpha}$-benzoyl-DL-arginine 2-naphthylamide; Z-Phe-Arg-NMec, $N^{\alpha}$-(benzoxycarbonyl)phenylalanylarginine (4-methylcoumarin-7-yl)amide; Z-Arg-Arg-NMec, $N^{\alpha}$-(benzoxycarbonyl)arginylarginine (4-methylcoumarin-7-yl)amide.

Chart 2. Ribbon Representation of Cystatin A Variants Prepared in This Study[a]




[a] The conservative glycyl bond in the amino-terminal region, the reactive first hairpin loop (QVVAG sequence), and the second loop are shaded. Positions of mutation are indicated by vertical lines.

For Gly5-Met, the same method was used exchanging *Sma*I−*Kpn*I fragment. Other mutations, such as deletion of the amino-terminal eight residues in the cystatin region of the fusion (des(1−8)) and introduction of Trp residue to the positions of Tyr43, Phe70, and Gly75 (Tyr43-Trp, Phe70-Trp, and Gly75-Trp) and of His residue to Gly75 (Gly75-His), were performed by the same method. Four-residue truncated forms of Trp mutants were constructed by substitution of Gly4 to Met by the same way. Substitution of Pro3 to Leu was accidentally found out during confirmation of the nucleotide sequence of the other mutant DNA (Pro3-Met). All recombinant cystatins prepared in this study are shown as ribbon representation in Chart 2.

*Preparation of Recombinant Cystatin Variants.* Wild-type recombinant cystatin A, its precursor fusion protein, and a fusion protein lacking the $NH_2$-terminal eight residues of cystatin portion were isolated by the method reported previously (Kaji et al., 1990). The rest of cystatin variants were purified by the same manner as described below. Transformant *E. coli* harboring the expression vector with mutated cystatin gene was cultured in 1 L of Luria broth containing ampicillin (50 mg/L, abbreviated as LB/Amp) at 37 °C for 20 h without induction with IPTG and then harvested by centrifugation. The wet bacteria, about 5−6 g, was lysed by sonication after washing, and the insoluble fraction was recovered. The precipitate was resuspended in small volume of water (typically in 6−10 mL) and solubilized by adding 4 volumes of formic acid with stirring (final concentration: 70% v/v). All methionyl bonds of solubilized proteins were cleaved by reaction with CNBr added into the solution. After 24 h of stirring, the solution was diluted with 9 volumes of water and lyophilized. Powdered protein was resolubilized with 5−10 mL of 6 M guanidine hydrochloride solution and diluted with an equivolume of 10 mM Tris-HCl, pH 8, containing 3 M urea. Cystatin variants were refolded by stepwise removal of denaturant by dialysis against the same buffer containing 3 M urea, 1 M urea, and no denaturant. Emerged precipitate, which contained little cystatin, was removed by centrifugation. Cystatin variants were isolated by DEAE-Sephacel column chromatography followed by phenyl-Sepharose hydrophobic chromatography or by HPLC using a hydrophobic interaction (phenyl-) column (HIC, Jasco). Homogeneity of the isolated proteins was confirmed by polyacrylamide gel electrophoresis (PAGE) with and without SDS. The concentration of the protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

*Characterization of Recombinant Cystatins.* For routine assay on preparation of cystatins, the inhibitory activity against papain was measured using $N^{\alpha}$-benzoyl-DL-arginine 2-naphthylamide (BANA) as a substrate of papain by the method of Barrett (1977), with some modifications in the color developing system according to the report of Matsutani et al. (1967). Amidolytic reaction was stopped by adding 0.2 N HCl (EtOH solution), and the released aminonaphthalene was determined by measuring absorbance at 540 nm after color developing with (dimethylamino)cinnamaldehyde. The inhibitory kinetics against papain and human cathepsin B were assayed fluorometrically using Z-Phe-Arg-NMec and Z-Arg-Arg-NMec as substrate, respectively, according to the method of Green et al. (1984). Inhibition constants ($K_i$ value) of cystatins for the cysteine proteinases were determined from the Dixon plot for noncompetitive inhibition (Dixon, 1953). Protein concentration of papain was determined spectrophotometrically using an extinction coefficient of 25.0 for 1% solution in a 1 cm light path. The amino acid sequence was determined by automated Edman degradation with Applied Biosystems 477A or 473A protein sequencer. Peptide maps on reverse phase HPLC for recombinant human cystatins were compared with that of an authentic one after digestion with *Achromobacter* protease I (API, otherwise called lysyl endopeptidase, Wako Pure Chemicals Inc.) at 37 °C for 6 h in the presence of 3 M urea. Circular dichroism (CD) spectra in far-UV region were measured at room temperature with a Jasco J-600 dichrograph using a quartz cuvette with 1 mm light path. All CD data were expressed in terms of mean residue ellipticity.

*NMR Measurement. E. coli* JM109 harboring the expression vector for Cyst $A^{2-98}$ and Cyst $A^{5-98}$ were cultured in M9 minimal medium containing 20 amino acids (100 mg each/L) and ampicillin (50 mg/L), where only valine was labeled with $^{15}N$. At midlog phase of growth ($A_{600} = 0.4$), the expression was induced with 2 mM IPTG and cultivation was extended for 48 h at maximum. Labeled cystatins were also isolated as described above.

Labeled cystatins were dissolved at about 2 mM in 90% $H_2O$/10% $D_2O$ ($^2H_2O$) and adjusted to pH 3.8 (direct meter reading), and final protein concentration was 1.5 mM. Two-dimensional $^{15}N-^1H$ HSQC NMR spectrum was recorded on a JEOL α600 spectrometer (600 MHz for $^1H$ resonance)

Table 1: Inhibition Constants ($K_i$ Values, nM) of Amino-Terminal Variants

| cystatin A's | (proteinase, substrate) papain, Z-Phe-Arg-NMec (pH 7.0) | cathepsin B, Z-Arg-Arg-NMec (pH 6.0) |
|---|---|---|
| authentic | 0.95 | 11.2 |
| wild-type | 1.20 | 17.7 |
| fused form | 4.55 | 118 |
| standard variant (2−98, M65L) | 2.45 | 20.5 |
| Pro3-Leu mutant (2−98, M65L) | 6.35 | 293 |
| truncated (5−98, M65L) | 414 | nd[a] |

[a] nd, not determined.

at 37 °C. All signal assignment and structural analysis will be published elsewhere (Tate et al., 1995).

*Fluorescence Measurements.* Fluorescence emission spectra between 310 and 450 nm were measured by Hitachi spectrofluorometer MPF-4 at an excitation wavelength of 295 nm (slit width, 2 nm). In the heating experiment, the temperature of cuvette was set by water circulation using a temperature control system. The temperature rise was at a rate of 2 deg/min, and 10 min after it reached the desired temperature, the emission spectrum was recorded. The protein concentration used was 0.3 mg/mL of water. In quenching analysis, a small volume (10 $\mu$L) of 8 M acrylamide was added successively to 3 mL of cystatin solution (protein concentration was 0.025 mg/mL of water).

## RESULTS

*Structural Confirmation of Cystatin Variants.* The precursor fusion protein of all cystatin variants was efficiently expressed by *E. coli* JM109 in LB/Amp medium without induction with IPTG. The expressed fusions were present in the insoluble fraction of the cells. After the precipitations were suspended with water, their solubilization by formic acid was almost successful in the resulting clear solution. Solubilized fusion protein was degraded by CNBr, and then the refolding of the released cystatins was carried out as described above. Much precipitation arose in the refolding step, but little cystatin was contained in it and the protein in the solution was almost cystatin only. Based on its absorption at 230 nm (area of refolded cystatin's peak in the chromatogram on the DEAE column), the efficiency of refolding was about 40%. The efficiency was increased by repeating the denaturation-renaturation steps for the remaining partially refolded cystatin preparation. Refolding of all cystatin variants to the native state was confirmed by comparing their far-UV CD spectrum with that of authentic cystatin A. Substitution and truncation were detected by peptide mapping of their digests by API on reverse phase (ODS) chromatography and by amino-terminal amino acid sequence analyses.

*Assay for Inhibitory Activity of Cystatin Variants.* First, inhibitory activity of Cyst $A^{2-98}$ (M65L) was assayed toward a cysteine proteinase papain using Z-Phe-Arg-NMec as substrate. The inhibition constant ($K_i$) estimated for this variant (2.45 nM) was almost identical with those of authentic and wild-type cystatins (0.95 and 1.20 nM, respectively; Table 1). Though cystatins are tight-binding competitive inhibitors, the $K_i$ values were obtained using a single concentration of the substrate on the assumption that

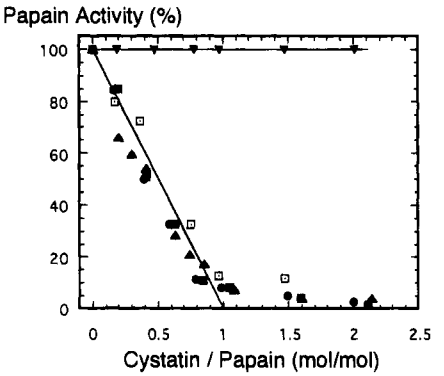



FIGURE 1: Stoichiometric inhibition assay of Cyst $A^{2-98}$(M65L) for papain and inactivation of precursor fusion by the first eight residues truncation of cystatin. The remaining amidase activity of papain in the presence of cystatin variants was measured using BANA as substrate. The detailed procedure was described in Materials and Methods. ●, authentic; ■, wild-type; ⊡, precursor fusion protein with adenylate kinase; ▲, standard variant Cyst $A^{2-98}$(M65L) obtained by the new procedure in this study; ▼, fusion protein lacking the first eight residues of the cystatin moiety. Ribbon presentation of each variant is shown in Chart 2.

cystatin A is a noncompetitive inhibitor, in order to compare the $K_i$ value of each variant for convenience. Thus, it may cause overestimation of the $K_i$ value. Based on these data, the recombinant cystatin variant, Cyst $A^{2-98}$ (M65L), prepared by CNBr degradation, was regarded as a standard to explore the role of amino-terminal region in cystatin for its inhibitory activity. We designated this variant as a standard variant of cystatin A.

Figure 1 shows the effect of deletion in the amino-terminal region of the cystatin portion of its precursor fusion protein. As described above, authentic, wild-type, and standard variants of cystatin A showed the same inhibitory activity. Fused cystatin also inhibited papain with 1:1 stoichiometry. However, fused precursor lacking the N-terminal eight residues of cystatin moiety showed no inhibitory activity, although its cystatin portion has an extended N-terminal region to the fused ADK moiety. Table 1 shows $K_i$ values of several variants toward papain compared with those against cathepsin B. Fused form and Pro3-Leu mutants had relatively greater $K_i$ values toward cathepsin B than to papain. Deletion of two amino-terminal residues showed almost no effect on its activity, similar to the standard variant (Figure 2). However, truncation of Pro3 caused a drastic decrease of its inhibitory activity, yielding an almost inactive variant. As expected, both cystatins lacking successively Gly4 and Gly5 lost their activities. But substitution of Pro3 to Leu showed no appreciable loss of its activity.

*Compensation of the Second Loop for N-Terminal Truncation.* Cystatin A and the counterpart in rat, cystatin α, are devoid of aromatic amino acid residues or side chains, such as His, with ring structure on their second loop, whereas most of cystatin superfamily contains His or Trp on this loop. Therefore, a variant introducing His to position Gly75 on the loop was prepared by site-directed mutagenesis, and a change of the activity by four residues truncation was tested. This His variant had an equivalent activity to the standard variant, but the four-residue truncation from the His variant also led to a drastic decrease of the activity. However, an inhibitory activity of the truncated His variant was higher than that of truncated variant without His (Figure 3).

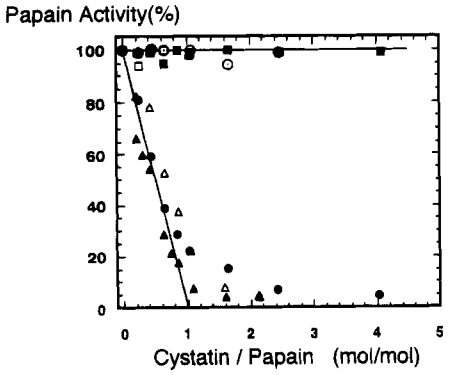



FIGURE 2: The decrease in inhibition of various cystatin variants for papain by amino-terminal successive truncation. Protocol and both axes are the same as in Figure 1. ▲, standard variant (single residue truncation starting from Ile2); ●, two-residue truncation (from Pro3); ⊙, three-residue truncation (from Gly4); ■, four-residue truncation (from Gly5); ⊡, five-residue truncation (from Leu6); △, Pro3-Leu-substituted form of the standard variant (from Ile2).

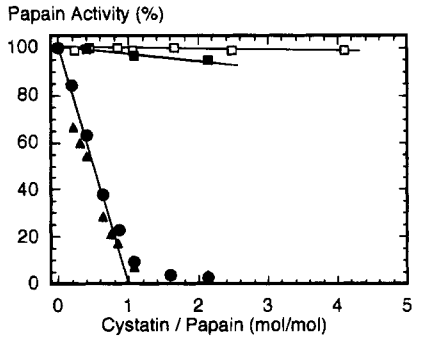



FIGURE 3: Inhibition assay of G75H variants. His residue was introduced to Gly75 on the reactive second loop to mimic cystatin B. The effect of His substitution on inactivation by N-terminal truncation was detected. Protocol and both axes are the same as in Figure 1. ▲, standard variant; □, truncated form from Gly5 without His substitution; ●, G75H variant starting from Ile2; ■, truncated G75H variant from Gly5.

*Detection of Conformational Change by Truncation Using NMR Spectroscopy.* Amino acid specific labeling of cystatin with [$^{15}$N]Val was carried out to detect conformational change accompanying truncation by NMR spectroscopy. The labeled cystatin could not be expressed efficiently by *E. coli* cultured in M9 amino acid medium without IPTG induction as in LB medium. Enough labeled cystatin (10−15 mg) was obtained from 1 L cultivation with IPTG induction (2 mM) at midlog phase ($A_{600}$ = 0.4). Figure 4 shows two-dimensional $^{15}$N−$^{1}$H HSQC spectra of the standard variant and the four-residue-truncated form. All signal assignment had been performed by a series of stable isotope-aided multidimensional NMR spectroscopy, and the data will be published elsewhere (Tate et al., 1995). In the spectrum of Cyst A$^{2-98}$, all signals of nine residues of Val and some small signals of Ala derived from cross-labeling were detected, and only two signals of Val47 and Val48 shifted in the truncated form. Chemical shifts of the other seven residues of Val did not change by the deletion.

*Change in Behavior of Trp Introduced into Cystatin A as Fluorescence Probe by Truncation.* Since cystatin A contains no Trp residue, this residue introduced artificially will be used as a fluorescence probe reflecting its microenvironment. Trp residues were introduced into the positions Tyr43, Phe70, and Gly75, and the truncated forms, Cyst A$^{5-98}$, were

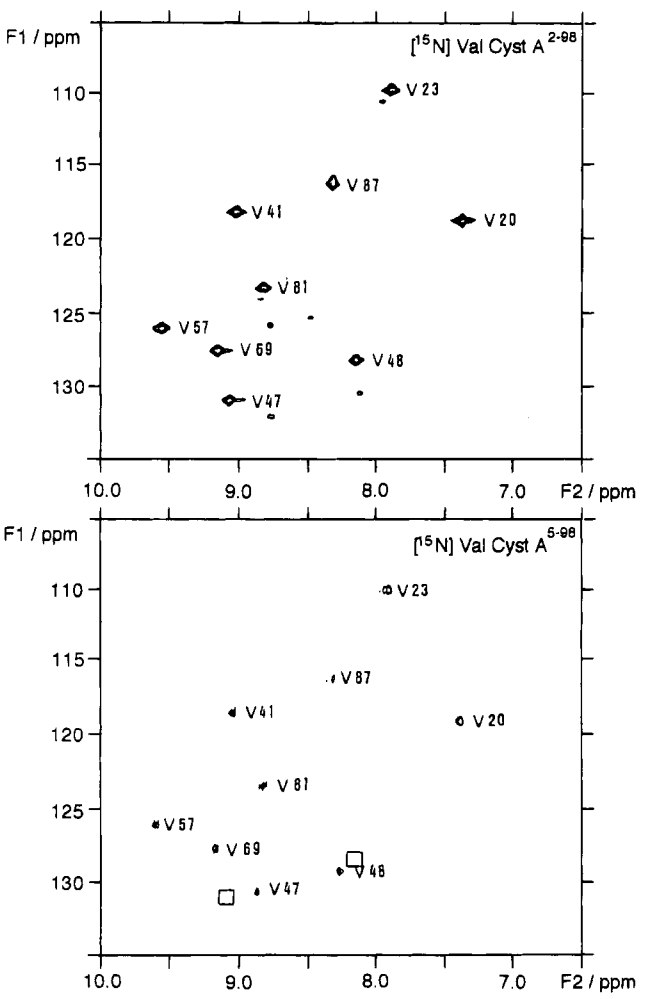



FIGURE 4: $^{1}$H−$^{15}$N HSQC NMR spectra of standard cystatin variant and truncated form starting from Gly5. [$^{15}$N]Val was incorporated into a full active standard variant and an almost inactive truncated form by growing *E. coli* in M9 minimal medium containing the labeled Val. NMR experimental conditions were described in the text. Correlation signals of all nine residues of Val were assigned (Tate et al., 1995). Weak signals of standard variant were derived from concomitantly labeled Ala residues by cross-labeling. Squares in the spectrum of the truncated form show the original position of shifted signals of Val47 and Val48.

prepared by the same way as for other mutants. G75W mutant had the same activity as a standard variant like the G75H mutant, but inhibitory activities of the Y43W and F70W mutants were somewhat decreased. Four-residue truncation made all these mutants almost inactive. Fluorescence emission spectra of these three Trp mutants and their truncated forms between 310 and 450 nm were recorded with an excitation wavelength of 295 nm at 25 °C. The maximum emission wavelength and its intensity for each variant differed depending upon the position of Trp introduced. Four-residue-truncated (5−98) forms showed somewhat larger intensity than those of the 2−98 form without any shift of the maximal wavelength (data not shown). The effects of truncation on quenching by addition of quencher (acrylamide) and heating were compared between active and inactive forms. Changes in the relative intensity at 352 nm, maximal emission of F70W and G75W, and shoulder of Y43W, by addition of quencher, were compared between the 2−98 form and the 5−98 form (Figure 5). Y43W and F70W mutants showed no difference by the truncation. But Trp75 in the 2−98 form was more quenched than in the 5−98 form. By

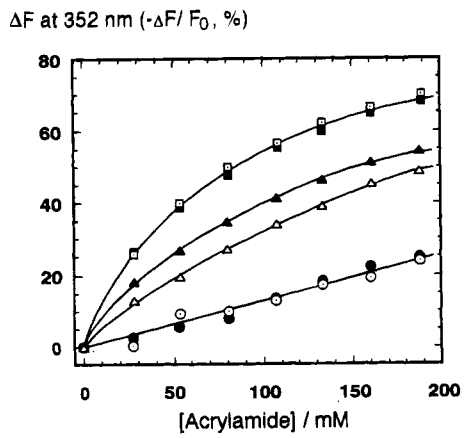



FIGURE 5: Effect of quencher (acrylamide) on fluorescence intensity of Trp variants. The susceptibilities of three Trp's against fluorescence quencher were compared between the 2−98 form and the 5−98 form of Trp variants. The differences of relative fluorescence intensities ($-\Delta F/F_0$, %) at 352 nm at various concentrations of acrylamide are plotted (excitation wavelength was 295 nm). ● ⊙, Tyr43-Trp; ■ ⊡, Phe70-Trp; ▲ △, Gly75-Trp. Solid symbols and open symbols represent the 2−98 form and the 5−98 form of Trp variants, respectively.

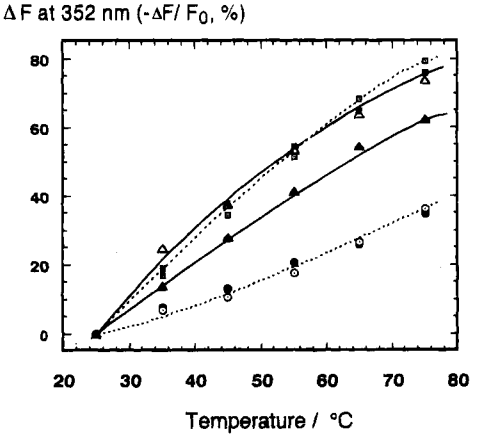



FIGURE 6: Change in fluorescence intensity of Trp at various temperatures. The differences of relative fluorescence intensities ($-\Delta F/F_0$, %) at various temperatures are plotted. ● ⊙, Tyr43-Trp; ■ ⊡, Phe70-Trp; ▲ △, Gly75-Trp. Solid symbols and open symbols represent the 2−98 form and the 5−98 form of Trp variants, respectively.

varying the temperature ranging from 25 to 75 °C, the intensities of 352 nm were also plotted in terms of the difference in relative intensity to that of 25 °C (Figure 6). Quenching of the 5−98 form of G75W was remarkable in contrast to that of the 2−98 form, though Y43W and F70W mutants showed almost the same changes.

## DISCUSSION

We had previously isolated a full-length form and two derivatives of cystatin α from rat epidermal extract and determined the amino acid sequence of the two derivatives (Takeda et al., 1985). Then, it was found that the two derivatives are amino-terminal truncated forms lacking the first six and fifteen residues. Six-residue truncation caused a decrease of inhibitory activity to one-eighth; meanwhile fifteen-residue truncation inactivated the inhibitor completely. Amino-terminal clipping of the cystatin superfamily had been reported in some cases such as chicken cystatin (Abrahamson et al., 1987), saliva cystatins (Isemura et al., 1986), human cystatin B (Thiele et al., 1988), and oryzacystatin (Abe et al., 1988). The inhibitory activities of chicken cystatin and saliva cystatins were decreased by N-terminal truncation, whereas cystatin B maintained its activity against six-residue deletion, and the amino-terminal twenty-one residues of oryzacystatin were not essential for the inhibition. Thus, it seems likely that the essentiality of the amino-terminal region of cystatins would be dependent upon the molecular species. Therefore, engineered truncation was carried out for cystatin A to test the necessity of its amino-terminal region. Expressed precursor fusion of wild-type cystatin A showed the same inhibitory activity as authentic inhibitor. But, deletion of first eight residues of cystatin from the fusion led to complete inactivation (Figure 1). These results indicated that the amino-terminal region of cystatin A is essential for inhibition of the cysteine proteinase. Then, to obtain more detailed information on the role of this region, a series of truncated forms was prepared in this study.

For efficient expression of recombinant cystatin A, the gene was fused with highly expressing cDNA of porcine adenylate kinase (ADK) as reported previously (Kaji et al., 1990). Thus far, blood coagulation factor $X_a$ digestion was used to release the cystatin region from the fusion, but the enzyme was costly and the digestive condition of complete cleavage was difficult to set. Therefore, in the present study, we constructed a new vector overexpressing the fusion cleavable by chemical reaction, BrCN degradation, instead of the enzymatic digestion. Cystatin A contained two residues of Met at position 1 (amino terminus) and 65. Met65 is not conserved over the cystatin superfamily and is substituted to Leu in the counterpart in rat, cystatin α. And this exchange does not seem to accompany a large change of its hydrophobicity and bulkiness. If this is the case, the presence of the remaining sole Met in its amino terminus will be useful in preparing a series of amino-terminal truncated variants by introducing Met into any position of cystatin's amino-terminal region of the precursor fusion and cleaving it by BrCN degradation. The chemical excision is performed in 70% formic acid for 24 h at room temperature, which may cause most proteins to lose their biological activity. Since cystatin A had great stability and reversibility in a wide range of pHs and at high temperature such as boiling water, the cystatin variant having a replacement of Met65 to Leu and lacking amino-terminal Met showed, as expected, the same inhibitory activity toward papain as authentic cystatin A (Figure 1). Results of far-UV CD spectroscopy indicated that the variant could be refolded into an intact state (data not shown). Furthermore, in two-dimensional NMR spectroscopy analyses of DQF-COSY, especially in the fingerprint region (HN−Hα), most of the chemical shifts did not change except for a few signals around Leu65 (data not shown).

A loss of amino-terminal Met seemed to have no effect on the inhibition, and clipping of Ile2 also showed little influence on the activity. But truncation up to Pro3 led to an abrupt decrease of the activity, and the inhibitor became almost inactive. Further truncation beyond Gly4 and Gly5 also caused drastic loss of the activity (Figure 2). Gly4 is a conservative residue over the cystatin superfamily, and the corresponding residue of cystatin B comprised a trunk region which invaginates to the unprimed side of subsite of the active site cleft (Stubbs et al., 1990). In cystatin C, the conserved residue (Gly11) seems to allow the N-terminal

segment to adopt a conformation suitable for interaction with the substrate-binding pockets of cysteine endopeptidases from the results of site-directed mutagenesis for the Gly residue (Hall et al., 1993). As mentioned above, the deletion of Pro3 of cystatin A led an abrupt decrease of the inhibitory activity. In cystatin B, a major contribution of the trunk region on contact with papain (two direct hydrogen bonds) is provided by Ser3 (Ser8I, chicken cystatin numbering) corresponding to Pro3 of cystatin A. The results of a computer model building of cystatin C with human cathepsin B and a site-specific replacement study revealed that Val10 (cystatin C numbering, corresponding to Pro3 in cystatin A) interacts with the S2 subsite in cathepsin B (Lindahl et al., 1994). However, from the result on substitution of Pro3 to Leu of cystatin A (Figure 2), the Pro3 residue seems unlikely to have a specific role such as fixation of main chain rotation or turn formation. Therefore, Pro3 of cystatin A is presumed to have some important contribution, which differs from the role in contact with proteinase. Both cystatin A and cystatin B belong to the same class I (stefin family of the cystatin superfamily) and were composed of 98 residues. The identity of residues between them is 53%. Although the significance of the amino-terminal region of cystatin B was deduced from the above data, cystatin B lacking the first six residues was still active and was present as a tightly binding inhibitor. But cystatin A became almost inactive by three-residue truncation. Therefore, the amino-terminal region, especially Pro3 and Gly4, must have a different responsibility to expression of the inhibitory activity from cystatin B.

Cystatin A and rat cystatin α have no residues, such as His, with ring structure (cystatin B and β have) or Trp (chicken cystatin and others) on the second loop, which also contributes to interaction with proteinase. To test the hypothesis that a lack of such residues on the second loop might affect indirectly the response of the amino-terminal region in cystatin A, His was introduced to residue 75 instead of Gly, corresponding to His104I position (chicken cystatin munbering) in cystatin B. The substituted form G75H inhibited the papain activity with 1:1 stoichiometry, but four-residue truncation of the variant also decreased its activity substantially (Figure 3). Though the truncated G75H variant showed a weak but greater inhibitory activity than the 5−98 form without the His substitution, the compensation by His substitution was not so eminent but was significant. From comparison of amino acid sequence between cystatin A and B, two possible reasons were suggested for the different nature between them. One is the presence of Pro25 in cystatin A, which corresponds to the position of two-thirds of a five-turn α-helix in cystatin B. This region in cystatin A is predicted to form α-helix as cystatin B using the Chou-Fasman method, but Pro25 is presumed to terminate α-helix or interrupt it into two short helices. Such deformation of the central helix might change the overall conformation of cystatin A. The second one is the lack of carboxy-terminal Tyr97 (Y124I, egg cystatin numbering) in place of Gly97 in cystatin A, while in cystatin B, Y124I forms five solvent-mediated interactions with papain, constructing an additional binding site. The role of the two residues mentioned above will be investigated by engineered modification.

Structural analyses on X-ray crystallography revealed an interesting problem about the difference of peptide orientation between Val55I and Ala56I on the first loop of human cystatin B (Stubbs et al., 1990). The authors mentioned that the difference between them seemed unlikely to be derived from their inherent structure. The difference would be due to complex formation, with papain having an additional carboxymethyl group on active site Cys, or crystal packaging. But, we presumed that the difference may result from the fact that chicken cystatin analyzed was truncated form with reduced activity, but cystatin B was fully active. Therefore, structural change caused by the amino-terminal truncation in cystatin A was detected by 2D NMR and fluorescence spectroscopies.

The presumed deformation would be detected as a change in chemical shift in the two-dimensional NMR spectrum. To detect local conformational change, especially in the reactive site, specific amino acid was labeled with [$^{15}$N], which was introduced in the peptide backbone. Cystatin A contains nine Val residues, and two of them were located in the reactive first loop, QVVAG, while a central α-helix and four β-strands flanking both first and second loops contained two and five residues, respectively. Nine separated signals were observed on 2D $^{15}$N−$^{1}$H HSQC spectrum, and they were assigned as reported elsewhere in detail (Tate et al., 1995). By comparison of the spectra between Cyst A$^{2-98}$ and 5−98 forms, only two signals corresponding to Val47 and Val48 were shifted (Figure 4). As the two Val residues were located just on the first loop, this result suggested that the deletion of three amino-terminal residues, Ile2-Pro3-Gly4, caused a conformational change in the reactive first loop. No change of chemical shift was observed for the other seven signals of Val, indicating that the truncated 5−98 form of cystatin A still retained a rigid conformation and the conformational change by the truncation was limited to a local place. If the conformational change took place in the first loop, the neighboring second loop may be affected by this change. In order to detect this influence, a Trp residue was introduced to the position of Gly75 on the second loop as fluorescence probe and also to other two positions, Phe70 and Tyr43, as control. Authentic cystatin A does not contain Trp residue originally, and Trp75 is conserved in the cystatin superfamily mentioned above. All Trp mutants showed their drastically decreased activities by the truncation. Only Trp75 of 2−98 form was more affected by fluorescence quencher than the 5−98 form, suggesting that Trp75 in the 2−98 form was more exposed to solvent than truncated 5−98 form, whereas the other two Trp residues as control were quenched to the same extent (Figure 5). These results indicated that the local environment of the Trp residue on the second loop was altered by the truncation, probably via deformation of the first loop.

Combination of NMR and fluorescence spectroscopy, reflecting the change of the local environment in protein molecules with an engineered truncation or substitution, revealed the role of the amino-terminal region of cystatin A. From the three-dimensional structure data of cystatin A obtained by NMR studies (Martin et al., 1995; Tate et al., 1995), the amino-terminal region of the inhibitor is not able to interact directly with the second loop, and the change of fluorescence behavior did not always reflect the conformational change of the loop. But the changes of NMR signals indicate a conformational change of the first loop. So, the N-terminal truncation caused a conformational change in the reactive first loop, which also affected the second loop. The deformation of reactive site by the truncation would not be

tolerated to maintain the inhibitory activity of cystatin A. The importance of the amino-terminal region in cystatin A was shown by other results. $K_i$ values of fused cystatin and P3L mutant toward cathepsin B were relatively greater than those to papain (Table 1). These results indicated that the amino-terminal region of cystatin A participates in recognition of cognate proteinase. Similar results were reported on cystatin C; i.e., substitutions of Gly11 showed a different effect on endopeptidases (papain or cathepsin B) and exopeptidase (dipeptidyl peptidase I) as well as amino-terminal ten-residue truncation (Hall et al., 1993). Furthermore, the results of fluorescence quenching showed that the truncated 5−98 form was more affected by heating than the 2−98 form (Figure 6). This fact suggested that the deletion of several amino-terminal residues led to the reduction of thermostability of the inhibitor.

Thus, the amino-terminal region, especially the two residues Pro3-Gly4, is presumed not only to contribute to significant contact with proteinase recognizing a cognate proteinase, but also to maintain a conformation of tripartite reactive wedge favorable to interacting with proteinase as "a conformational stabilizer". The different responsibility on the amino-terminal region between cystatins A and B might be related to their distinctive physiological function and its control mechanism. Analysis of detailed spatial structure of cystatin A will provide further information on the role of the amino-terminal region from structural foundations.

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