An Internal Cysteine Plays a Role in the Maintenance of the Latency of Human Fibroblast Collagenase†

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ABSTRACT: The cDNA that encodes the proenzyme form of human fibroblast collagenase has been expressed in *Escherichia coli*. It has been shown by a number of criteria to be functionally identical with the enzyme isolated from human sources. Mutations of each of three cysteine residues found in procollagenase were constructed by site-directed mutagenesis of the cDNA. The relative activities of these mutants were compared to the wild-type enzyme. All of the mutants retained proteolytic activity, but not necessarily on collagen. Mutations that interfere with the formation of the sulfhydryl bridge in the carboxy-terminal domain in some cases increased and in other cases decreased the rate of casein cleavage. On the basis of extensive autolysis within *E. coli* of a mutant with a replacement of cysteine-73, the procollagenase molecule produced appeared to be either spontaneously active or perhaps more susceptible to autolytic activation, despite the continued presence of the propeptide. Experiments designed to capture the active forms of the mutant by use of the irreversible inhibitor α_2-macroglobulin showed that some degree of latency still persisted in the autolytic mutant. These findings suggest that the cysteine at position 73 is important for maintaining the proenzyme in an inactive state but that the maintenance of latency in MMPs may be a complex process, involving a number of interactions between the propeptide domain and the remainder of the collagenase molecule.

Matrix metalloproteinases play an important role in the degradation of extracellular matrix in higher eukaryotic organisms. In animal cells, this gene family includes both fibroblast (Welgus et al., 1981; Goldberg et al., 1986) and polymorphonuclear leukocyte (Tschesche et al., 1990) types of collagenases, two stromelysin/transins (Wilhelm et al., 1987; Nicholsen et al., 1989), two gelatinases (Goldberg et al., 1989; Wilhelm et al., 1989), and Pump-1 (Quantin et al., 1989; Woessner et al., 1989), a truncated matrix metalloproteinase (MMP)[1] which lacks a large terminal domain present in other MMPs. These proteinases are highly homologous, zinc-requiring metalloenzymes that share several unique features, including a little understood mechanism for activation which appears to be distinct from other known pathways for the conversion of inactive precursors to enzymatically active forms. Although eukaryotic MMPs share several common features with microbial MMPs, an inactive proenzyme form is observed only in the eukaryotic proteins; this precursor form provides an additional means for regulation of the activity of these enzymes.

Collagenase from human fibroblasts can be converted from a latent to an enzymatically active form by autocatalytic cleavage of at least one bond in the Gln-Phe-Val-Leu (QFVL) sequence which separates domains 1 and 2 (Figure 1; Grant et al., 1987). This cleavage can occur upon storage, but may be accelerated, either by a conformational change induced by limited proteolysis of one or more sites within domain 1, or by chaotropic ions, or by reaction with organomercurial compounds. Under certain conditions, catalytically active and competent enzyme may be generated without the loss of the propeptide by exposure to certain agents such as detergents (SDS; Birkedal-Hansen & Taylor, 1982) or certain organomercurials (Grant et al., 1987).

Recent studies have provided evidence that the autocatalytic cleavage and the subsequent removal of an amino-terminal propeptide fragment are one possible physiological mechanism in the activation of procollagenase and other members of the MMP family in eukaryotes (Grant et al., 1987, 1990; Nagase et al., 1990), but the chemical and structural basis for the maintenance of the latency of the proenzyme remains unknown. In this paper, we present evidence that a single, unpaired cysteine residue at amino acid 73 in the propeptide plays a role in maintaining the latency of the enzyme but that other features of the propeptide may also be important. This conclusion may also apply to the activation of other members of the eukaryotic matrix metalloproteinase family.

Materials and Methods

Construction of Expression Plasmid. Plasmid pCB-2 was constructed by ligation of a *Sal*I–*Bgl*II fragment from plasmid pX7 (which contains the cDNA for proCL cloned into plasmid SP 64; Angel et al., 1986; Whitham et al., 1986) to plasmid BS-SK(+) (Stratagene, San Diego, CA) cut with *Xho*I and *Bam*HI. Single-stranded DNA was prepared from this plasmid by superinfection of male *Escherichia coli* with M13KO7 helper phage (Vieira & Messing, 1987), followed by PEG precipitation and phenol extraction of phage from the culture supernatant. Site-directed mutagenesis to introduce a ribosome binding site (Shine & Delgarno, 1975) and an

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[1] Abbreviations: α2M, α_2-macroglobulin; APMA, *p*-aminophenylmercuric acetate; CL, collagenase; GuHCl, guanidine hydrochloride; MMP(s), matrix metalloproteinase(s); proCL, procollagenase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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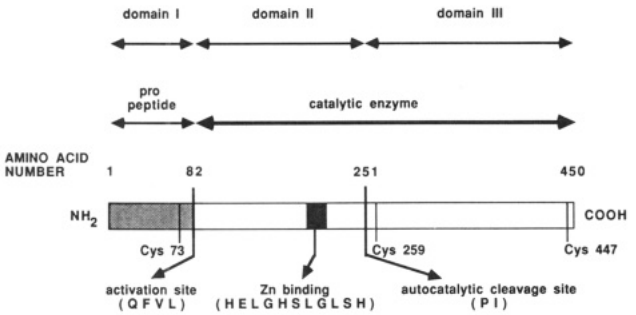


FIGURE 1: Linear model of human fibroblast proCL deduced from the cDNA sequence. The numbering of the amino acids in the proCL molecule is taken from the published sequence (Goldberg et al., 1986), but with amino acid 1 being the first amino acid of the proCL polypeptide (a phenylalanine left after removal of the signal sequence). Human fibroblast-type proCL consists of 3 separate domains: an 81-residue domain I (propeptide); a 169-residue "catalytic" domain II, which contains the putative Zn^{2+} binding site and the active site; and a 200-residue COOH-terminal domain III which shows weak homology with hemopexin. In its active forms, human fibroblast type CL shows a relatively wide spectrum of catalytic activities toward protein substrates, including (but probably not limited to) collagen types I, II, and III, gelatin, casein, and the α-macroglobulin bait regions. In addition, the enzyme catalyzes two autocatalytic cleavages: (1) an activation-associated cleavage of the QFVL sequence at the boundary of domains I and II; (2) a cleavage of the Pro250–Ile251 bond between domains II and III. Cleavage at the second site dissociates the molecule into two major fragments: a 28-kDa fragment which consists of domains I and II and shares homology with Pump-1 (Muller et al., 1988), and a 22-kDa fragment which consists of domain III. The 28-kDa fragment is also a latent proteinase which may be activated in the same way as intact proCL to yield a residual 18-kDa active proteinase which can cleave casein and gelatin but not native triple-helical collagens. The positions of the cysteine amino acids replaced in this study are also shown in the figure.

initiation codon was performed by standard methods (Zoller & Smith, 1983), using pCB-2 single-strand DNA and the oligonucleotide 5′-GGGCGAATTGGGTAAGAAGGA-GATATACATATGTTCCCAGCGAC-3′, designed to remove a unique *Kpn*I cleavage site within the vector. The sequence of the resulting plasmid (pCB-2SD) was verified by DNA sequencing (Sanger et al., 1977; Biggin et al., 1983). After transfer into *E. coli* DE3 (Studier & Moffatt, 1986), transcription of the proCL open reading frame from the T7 promoter on the resulting plasmid was induced by addition of IPTG to activate a chromosomal copy of the T7 RNA polymerase gene.

Site-directed mutagenesis to obtain mutants at Cys73, Cys259, and Cys447 was done by similar methods. Oligonucleotides used for this mutagenesis were 5′-AAGCAGCCCAGATCTGGAGTGCCTG-3′ (Cys73 to Ser), 5′-AACCCCAAAAGCGTCTGACAGTAAGC-3′ (Cys259 to Ser), 5′-TAGCTGGTTCAACTCTAGGAA-AAATTGA-3′ (Cys447 to Ser), and 5′-AAATTT-GATCCTTGAACATTACTA-3′ (deletion of amino acids 431–450). The identity of these mutants was verified by DNA sequencing.

Western Blot Analysis of E. coli Lysates. *E. coli* cells containing plasmids expressing either normal or mutant forms of r-proCl were induced with IPTG at 37 °C for 2 h, boiled in SDS/mercaptoethanol sample buffer (Laemmli, 1970), resolved by electrophoresis on a 10% SDS/PAGE gel, and transferred to nitrocellulose by electroblotting. The resulting filter was preblocked with 0.1% BSA and then developed with a 5 μg/mL solution of a monoclonal antibody directed against either domain II (III-7) or domain I (X-2a) in 10 mM borate-buffered saline/15 mM NaN_3 (ph 8.2). Peroxidase-conjugated rabbit anti-mouse Ig antibody (DAKO Corp, Santa Barbara, CA) was used as the secondary antibody for detecting the presence of immunoreactive primary antibody on the Western blot, as described by Birkedal-Hansen (1987).

Purification of Procollagenase from E. coli. *E. coli* cells containing recombinant plasmids were induced for 2 h at 37 °C with IPTG, centrifuged and passed through a French press in the presence of 1 mM PMSF, and centrifuged to separate supernatant and pellet fractions. Recombinant proCL was extracted from the cell debris in 2 M guanidine hydrochloride, 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$; after a 20-fold dilution of this extract with 50 mM Tris-HCl (pH 7.5)/0.2 M NaCl, r-proCL was purified by passage over a monoclonal antibody affinity column (VI-3; Birkedal-Hansen et al., 1988). The enzyme was eluted with 0.1 M sodium acetate buffer (pH 4.0) containing 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ and immediately neutralized by dropwise addition of a 2 M Tris-HCl solution (pH 8.1).

Assays. Assay 1: Autocatalytic Conversion. A 10 μg/mL solution of affinity-purified recombinant ProCL was incubated with 1 mM APMA at 37 °C for 90 min in Tris–acetate buffer (pH 7.5). Samples were analyzed by SDS–PAGE in a 10% gel, transferred to nitrocellulose, and stained with a monoclonal antibody (III-7) that recognizes an epitope in domain II.

Assay 2: Cleavage of Collagen by Recombinant CL. Tritiated acetyl rat tail type I collagen (0.6 mg/mL; 1.2 × 10^6 cpm/mg) was prepared as described (Birkedal-Hansen, 1987). This material was incubated with affinity-purified APMA-activated recombinant or wild-type CL in 0.1 M Tris–acetate (pH 7.5), 0.2 M NaCl, and 5 mM $CaCl_2$ at 22 °C for 4 h. The reaction was stopped by the addition of 1 mM 1,10-phenanthroline, and the reaction products were analyzed by using fluorography with a 7% polyacrylamide gel.

Assay 3: Zymography To Detect Cleavage of Casein by r-CL (0.3 μg) or by the Cys73 to Ser Mutant (0.5 μg). Affinity-purified enzyme was resolved by SDS–PAGE in a 10% gel copolymerized with 1 mg/mL casein. The gel was then washed repeatedly with Triton X-100, incubated at 37 °C for 4 h, and stained with Coomassie blue to visualize lytic bands.

Assay 4: Measurement of the Rate of Digestion of β-Casein by r-CL or Mutant CLs. Enzyme was activated with 1 mM APMA as described above in assay 1. Activated enzyme (1–3.5 μg) was added to a solution containing 50 mM Tris·OH (pH 7.5), 0.2 M NaCl, and 2.5 mg/mL β-casein (Sigma Chemical, St. Louis, MO); a zero time point was taken, and the remaining sample was incubated at 30 °C; time points were taken hourly up to 4 h after addition of enzyme. One-tenth volume of 10 mM 1,10-phenanthroline (Sigma) was added to each time point to stop further cleavage. The samples were mixed with an equal volume of loading dye and resolved by SDS–PAGE on a 10% gel; protein bands were visualized by staining with Coomassie blue. The amount of protein in the β-casein-specific band in each time point was determined by densitometry of the gel on a Hoeffer Model GS300 densitometer; areas under each casein peak were measured by weighing the peak cut from the tracing on a strip chart recorder.

Assay 5: Collagen Fibril Assay. The assay was performed as described by Birkedal-Hansen (1987).

Assay 6: Covalent Binding of Collagenase to α_2-Macroglobulin. A 5-μL sample of a 2 M GuHCl extract of the wild-type recombinant collagenase was incubated for 15 min in the presence or absence of 100 μg/mL α2M [isolated from outdated human plasma, as described by Sottrup-Jensen et al. (1984)] in 50 mM Tris-HCl (pH 7.5)/0.5 mg/mL BSA, in a final volume of 100 μL. For the time course reaction with

Table I: Proteolytic Activities of Wild-Type Human Fibroblast Collagenase and of Mutant Forms of the Enzyme Expressed in *E. coli*[a]

	substrate				
enzyme	collagen (units/mg)	% latent collagen	β-casein act. (mol mol^{-1} h^{-1})	domain I/II cleavage	domain II/III cleavage
fibroblast collagenase	350	>90	418 ± 14 (6)	+	+
recombinant collagenase	50	75–88	444 ± 30 (6)	+	+
Cys73-Ser	<0.5		414 ± 18 (6)	+	+
Cys259-Ser	0		917 ± 19 (4)	+	+
Cys447-Ser	0		903 ± 16 (4)	+	+
Δ(431–450)	<0.6		241 ± 29 (4)	+	+

[a] Collagenase activity was measured against reconstituted type I collagen fibrils as described (Birkedal-Hansen & Dano, 1981; Birkedal-Hansen, 1987). The rate of cleavage of β-casein in solution was determined by digestion at 30 °C, as described under Materials and Methods; the units of activity are given in moles of β-casein cleaved per mole of enzyme per hour, and the number of assays performed for each species is shown in parentheses. Ability to catalyze internal cleavages (resulting in the separation of domains I and II or of domains II and III) was inferred from Western blots of affinity-purified recombinant CL.

mutant C73S, 10 μL of a 2 M GuHCl extract was diluted to 1 mL in the same reaction buffer (with and without α2M), and 100-μL samples were taken at each time point. In both experiments, samples were diluted with an equal volume of loading dye (Laemmli, 1970) to stop the binding reaction. Twenty microliters of the diluted sample was resolved by SDS–PAGE on a 10% gel, blotted onto nitrocellulose, and reacted with 5 μg/mL monoclonal antibody III-7, as described in section B.

Results

Expression of Human Procollagenase in E. coli. The cDNA that encodes human proCL was cloned downstream of the bacteriophage T7 promoter and altered by site-directed mutagenesis to provide a ribosome binding site and an initial ATG at the position corresponding to the start of the propeptide (Figure 2). Expression of recombinant proCL in *E. coli* gave a 52-kDa polypeptide which shared the following catalytic properties with wild-type proCL isolated from cell culture media.

(1) The 52-kDa proenzyme was converted autocatalytically to a 42-kDa collagenase molecule by trypsin cleavage (data not shown) or by exposure to organomercurials (Figure 3, panel A). The conversion was quantitative, which suggested that all of the molecules were able to be cleaved and were capable of undergoing the molecular weight conversion associated with activation of proCL.

(2) The activated form of the recombinant enzyme cleaved type I collagen into 1/4 and 3/4 fragments at the characteristic collagenase sensitive site (Figure 3, panel B). In the collagen fibril assay, the activated recombinant enzyme was also active (Table I), while the precursor latent molecule was inactive in this assay (data not shown). The specific activity of the activated (stromelysin-free) recombinant enzyme was lower by 5–10-fold than that of the CL isolated from tissue culture media, as previously noted by others (Murphy et al., 1987). The recombinant enzyme was synthesized as a latent 52-kDa form and existed predominantly (greater than 75%) in this form immediately after extraction and affinity purification. It is notable that the latent rather than the active form of the enzyme is expressed in *E. coli*; this suggests either that the information required for the maintenance of the latency of the enzyme is inherent in the amino acid sequence and the polypeptide structure of the polypeptide or that *E. coli* has the necessary machinery to add any other modifications that are required for the production of the inactive proenzyme.

(3) The purified enzyme spontaneously underwent two autocatalytic internal cleavages upon storage: an activating cleavage which mimics the activation induced by organomercurials that separates domain I from domains II and III,

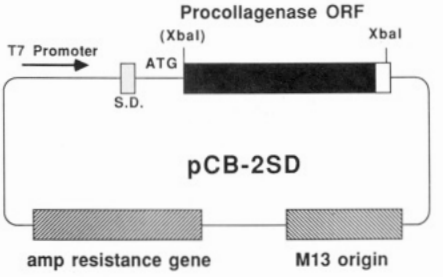


FIGURE 2: Plasmid for expression of wild-type r-CL in *E. coli*. The cDNA containing the open reading frame (ORF) that encodes human fibroblast-type proCL (solid filled rectangle) was cloned into plasmid BS-SK(+). Site-directed mutagenesis was used to replace the secretory signal encoded in the cDNA with a bacterial ribosome binding site (S.D.; Shine & Delgarno, 1975) and to position an initiating ATG in front of the Phe amino acid at position 1 in proCL (plasmid pCB-2SD). When introduced into *E. coli* strain DE3 (Studier & Moffatt, 1986) which contains a chromosomal copy of the bacteriophage T7 RNA polymerase gene under *lac* operon control, expression of proCL from the T7 promoter can be induced with isopropyl thiogalactoside (IPTG). The *Xba*I site found at nucleotide 137 (Goldberg et al., 1986) and shown in parentheses was removed by site-directed mutagenesis without changing the encoding amino acid sequence.

and a cleavage at Pro250-Ile251, which separates domain III from domains I and II, as previously described for the wild-type enzyme (Murphy et al., 1987; Birkedal-Hansen et al., 1988).

(4) The detergent-activated proenzyme was capable of cleaving casein. After exposure to SDS (which activates the enzyme without a molecular weight change; Birkedal-Hansen & Taylor, 1982), casein was cleaved in zymograms by the 52-kDa species (domains I + II + III) and by the 42-kDa (domains II + III), the 28-kDa (domains I + II), and the 18-kDa (domain II) forms of the enzyme (Figure 3, panel C). Each of the fragments with proteolytic activity on casein contains the zinc binding site which is essential for catalysis (Figure 1).

A series of mutant collagenases were constructed by site-directed mutagenesis of the proCL open reading frame and expressed in *E. coli*. Three cysteine residues at amino acids 73, 259, and 447 were individually changed to encode serine; in another mutant, the nucleotides that encode a 20 amino acid segment at the carboxy terminus of the polypeptide (residues 431–450) were deleted, in order to mimic the stromelysin-mediated cleavage proposed by He et al. (1989). The mutant polypeptides were expressed in *E. coli* by induction with IPTG and analyzed by Western blot analysis of lysates of the induced bacterial cells. When a monoclonal antibody directed against an epitope on the propeptide (X-2a) was used, the Western blot showed that all of the mutants express protein (Figure 4, panel A). The Cys73 to Ser replacement in recombinant

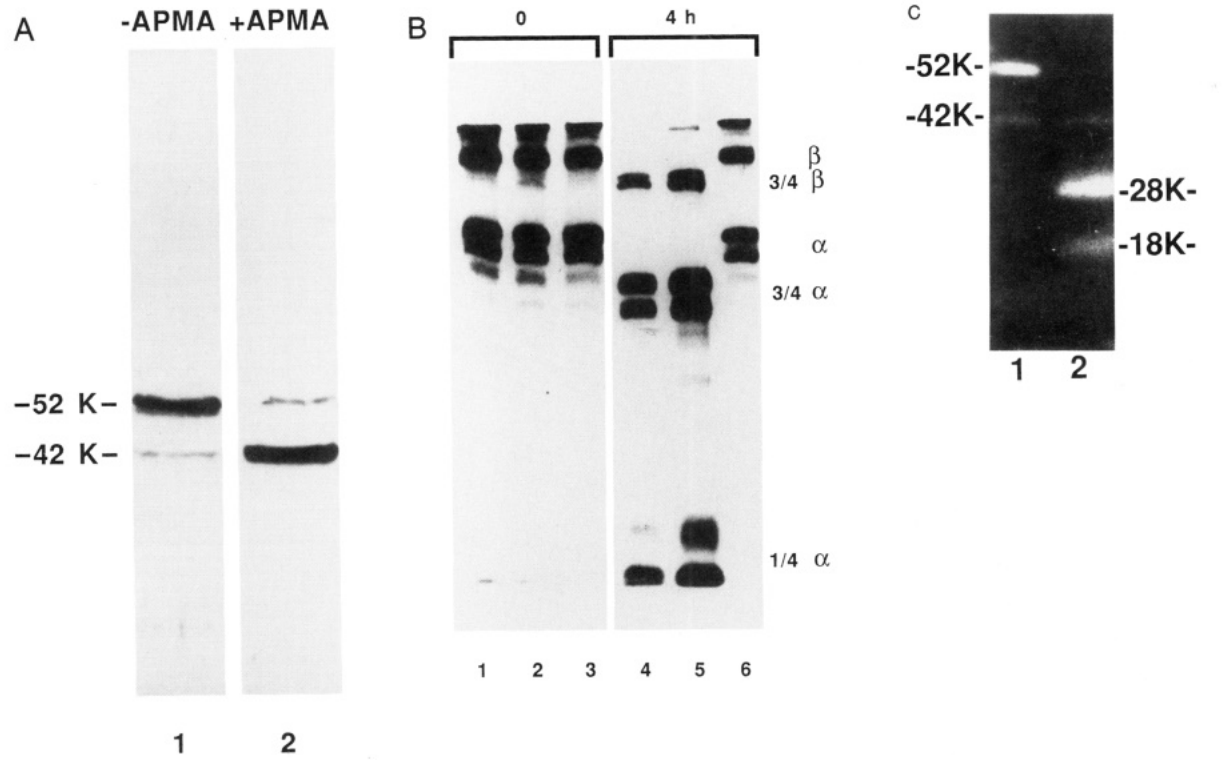


FIGURE 3: Collagenase expressed as a recombinant protein in *E. coli* shows the known activities of the human fibroblast-type enzyme. (Panel A) Autocatalytic conversion of the 52-kDa r-proCL to the 42-kDa form by treatment with APMA. After passage of IPTG-induced *E. coli* DE3 (Studier & Moffat, 1986) through a French press, r-proCL was extracted from the cell debris in 2 M guanidine hydrochloride, 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 1 mM PMSF; after a 20-fold dilution with 50 mM Tris-HCl (pH 7.5) and 0.2 M NaCl, r-proCL was purified by passage over a monoclonal antibody antibody affinity column using antibody VI-3 (Birkedal-Hansen et al., 1988). The enzyme was eluted with 0.1 M sodium acetate buffer (pH 4.0) containing 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ and immediately neutralized by dropwise addition of a 2 M Tris-HCl solution (pH 8.1). A 10 μg/mL solution of affinity-purified r-proCL was incubated with 1 mM APMA at 37 °C for 90 min in Tris–acetate buffer (pH 7.5). The samples were resolved by SDS–PAGE in a 10% gel (Laemmli, 1970), transferred to nitrocellulose, and stained with a monoclonal antibody (III-7) that recognizes an epitope in domain II (Birkedal-Hansen et al., 1988). Affinity purification of the VI-3 antibody column retained predominantly the intact 52-kDa form of the enzyme. Treatment with APMA resulted in an 8-fold increase in specific activity. (Panel B) Cleavage of collagen by r-CL. [^{3}H]Acetyl rat tail type I collagen (0.6 mg/mL; 1.2×10^6 cpm/mg) prepared as described (Birkedal-Hansen, 1987) was incubated with affinity-purified APMA-activated recombinant (r) or wild-type (w) CL in 0.1 M Tris–acetate (pH 7.5)/0.2 M NaCl at 22 °C for 4 h. The reaction was stopped by addition of 1 mM 1,10-phenanthroline, and the reaction products were analyzed by fluorography (Birkedal-Hansen & Dano, 1981) using a 7% polyacrylamide gel. Lanes 1 and 4, r-CL, 10 μg/mL; lanes 2 and 5, w-CL, 10 μg/mL; lanes 3 and 6, no enzyme. (Panel C) Cleavage of casein by r-CL. Affinity-purified r-proCL and Cys73 to Ser mutant were resolved by SDS–PAGE, using a 10% gel copolymerized with 1 mg/mL casein (Heussen & Dowdle, 1980). The gel was then washed repeatedly with Triton X-100, incubated at 37 °C for 4 h, and stained with Coomassie blue to visualize lytic bands. Lane 1, r-CL, 0.3 μg; lane 2, Cys73 to Ser mutant, 0.5 μg. The amounts of casein digested reflect the amounts of protein in each band, not the specific activity of each size species for digestion of casein.

proCL resulted predominantly in the 28-kDa autolytic fragment (consisting of domains I and II), and only trace amounts of the expected 52-kDa form, suggesting that the normally inactive proCL was synthesized either as a spontaneously active 52-kDa form or as a more readily activated enzyme capable of catalyzing its own autocatalytic internal cleavage at positions Pro250-Ile251 when expressed inside *E. coli*. The other mutant polypeptides all showed the expected 52-kDa band on the Western blot, except for the carboxy-terminal deletion mutant which displayed a 50-kDa band. When a Western blot of the same samples was probed with a monoclonal antibody against domain II (III-7; Figure 4, panel B), the wild type and each mutant polypeptide also showed some autocatalytic self-activation (cleavage between domains I and II; Figure 1), as shown by the formation of additional 40–42-kDa bands (or 18-kDa band, in the case of the Cys73 mutant).

After purification of the recombinant proCL polypeptides on a monoclonal antibody affinity column using antibody VI-3 (which recognizes an epitope in domain II), the polypeptides were tested for proteolytic activity using collagen and casein as substrates. As shown in Table I, only the activated recombinant CL cleaved both collagen and casein; all of the mutants cleaved casein, but not collagen. In addition, all of the mutants catalyze cleavages between domains I and II and between domains II and III (Table I). The Cys73 mutant may also cleave collagen, but since the uncleaved intact form of the enzyme cannot yet be recovered from *E. coli* and purified in sufficient quantities, the activity of this substrate cannot be tested at this time. Since the enzyme can only be recovered in the 28-kDa form, it has no activity against collagen, but readily cleaves casein. It also catalyzes the cleavage of the QFVL activation sequence, as shown by the appearance of an 18-kDa band on Western blots. This 18-kDa band is devoid of the propeptide (as shown by its inability to be recognized by antibody X-2a; compare Figure 4, panels A and B) and consists only of catalytic domain II.

Substitution or deletion of the two domain III Cys residues (Cys259 and Cys447) did not interfere with proteolytic activity per se, since each mutant catalyzed the conversion of recombinant proCL to both the 42- and the 28-kDa forms and also cleaved casein (Table I). It is of particular interest to compare the catalytic rates on casein on the various native and mutant collagenases. The *recombinant* wild type and the C73S mutant both retained the same catalytic activity as the *native* wild type (414–444 mol mol^{-1} h^{-1}), which suggested that the catalytic machinery was essentially intact in both recombinant enzymes.

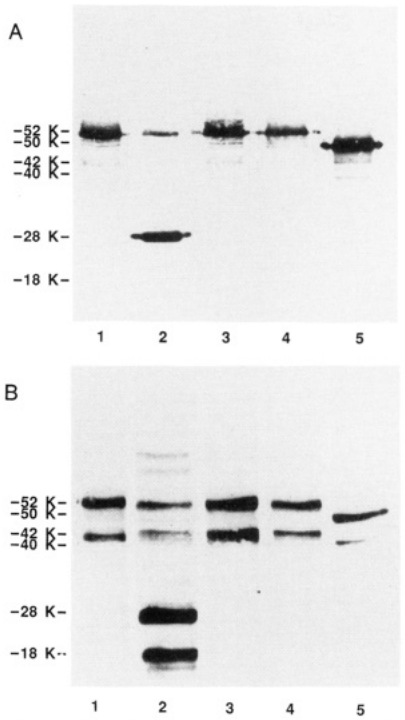


FIGURE 4: Expression of mutant forms of r-proCL analyzed by Western blots of crude *E. coli* cell lysates. *E. coli* cells containing plasmids expressing either normal or mutant forms of r-proCL were induced with IPTG at 37 °C for 2 h, boiled in SDS/mercaptoethanol sample buffer (Laemmli, 1970), resolved by SDS–PAGE, and transferred to nitrocellulose. (Panel A) Western blot stained with a monoclonal antibody directed against domain I (X-2a). (Panel B) Western blot stained with a monoclonal antibody directed against domain II (III-7). Lane 1, r-CL; lane 2, Cys73 to Ser mutant; lane 3, Cys259 to Ser mutant; lane 4, Cys447 to Ser mutant; lane 5, Δ(431–450) mutant. The Cys73 mutant was predominantly seen as an autocatalytic 28-kDa fragment, whereas each of the other mutants was expressed as full-length 50–52-kDa r-proCL. Each of the mutants showed some evidence of conversion to the "activated" 40–42-kDa (18kDa in the case of the Cys mutant) forms.

Interfering with disulfide bond formation in the COOH-terminal domain by replacement of Cys259 or Cys447 abolished collagen cleaving activity but did not reduce the catalytic efficiency against casein; rather, the two mutants cleaved casein twice as fast as the wild-type enzyme. Deletion of the last 20 amino acids (which includes Cys447) also abolished collagen cleaving activity and also reduced the activity on casein to half of that of the wild-type molecule. The absence of measurable proteolytic activity on collagen suggested that the carboxy-terminal domain III plays a role in determining the substrate specificity of the enzyme and may provide the structural basis for interaction with the collagen triple helix.

Since replacement of Cys73 led to the expression of the normally latent proenzyme in an apparently spontaneously active form, this amino acid probably plays an important role in maintaining the latency of the proenzyme; the loss of latency in this mutant was indicated by the appearance of autocatalytic cleavage between domains II and III, a cleavage that occurred during expression inside *E. coli*. This cleavage is already known to occur in the natural enzyme upon storage (Birkedal-Hansen et al., 1988). Accelerated autoactivation of the proenzyme has also been observed in replacement mutants within the highly conserved segments surrounding this Cys residue in another matrix metalloproteinase, rat transin (Sanchez-Lopez et al., 1988).

Although the mutation of Cys73 lead to an apparently active molecule, the propeptide segment is still attached to this protein. The predominance of the 28-kDa fragment suggests that the enzyme is active inside *E. coli*; for this reason, we tested whether this molecule is fully active or rather has become more easily activatable. To this end, we used the rapid and irreversible capture of *active* enzyme by α2M (Sottrup-Jensen & Birkedal-Hansen, 1989). This reactions runs to completion within seconds when used with the active wild-type enzyme, but no complex is formed with the latent enzyme. This irreversible capture reaction is therefore a better test to discriminate between catalytic activity and latency. In Figure 5, panel A, the loss of the 42-kDa form of wild-type CL in lane 2 is due to irreversible binding of the activated, propeptide-deficient molecule. When a time course binding reaction between the Cys73 mutant and α2M is performed, only the 18-kDa form of the mutated enzyme (lacking the propeptide segment) can bind; the 52-, 28-, and the apparent 22-kDa intermediate forms (proteins which include all or part of domain I) of the mutant apparently do not bind α2M (Figure 5, panel B). This result suggests that the Cys73 mutant is not fully activated but has shifted the equilibrium between latent and active forms toward the active form. Alternatively, the Cys73 mutant may be enzymatically activated but may have adopted a less accessible conformation for α2M binding, due to the presence of the propeptide segment.

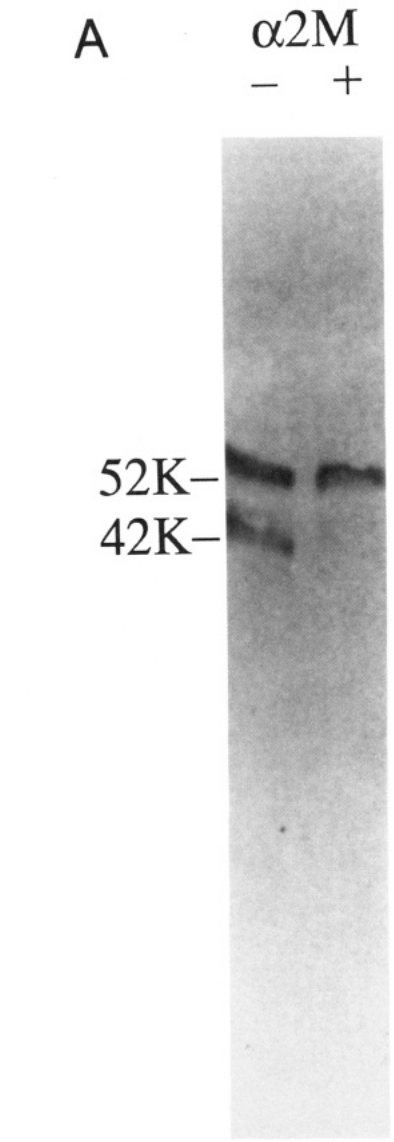
Discussion

This study has shown that the refolded recombinant enzyme attained the same catalytic rate on casein as the native enzyme but was approximately 5–10-fold slower against collagen. These findings suggest that the catalytic domain II is formed correctly in the recombinant enzymes but that some other part of the molecule, presumably domain III, also influences the substrate specificity. The observation that replacements and deletions in domain III can either increase or decrease the proteolytic activity against casein is consistent with this idea. Blockage of disulfide bond formation abolished activity against collagen completely but in some cases enhanced proteolytic activity against casein. These findings suggest that domain III plays a major role in determining substrate specificity of human collagenases, and possibly other MMPs as well.

Substitution of the single unpaired sulfhydryl group in the propeptide (Cys73) resulted in a molecule which was either spontaneously active or very rapidly activated; none of the other substitutions or replacements apparently influenced the autolytic rate of activation. Although several models might be envisioned to explain why mutation of Cys73 or adjoining residues in domain I results in the loss of latency, an attractive mechanism entails the pairing of the Cys73 residue with zinc in the active site (Figure 6; Springman et al., 1990): When Cys73 is replaced by Ser, coordination to the zinc ion is lost, and an exposed fourth zinc ligand binding site within the active site of the enzyme is now freer to interact with the substrate (Figure 6, model A); access of substrate to this site may still be partially blocked by the presence of the attached propeptide domain. This model is particularly attractive since it also explains each of the known activation pathways for the matrix metalloproteinases:

(1) Proteolytic cleavage of the propeptide, or conformational changes of the molecule caused by detergents or chaotropic agents, weaken the Cys–zinc interaction sufficiently to shift the normally latent molecule toward the enzymatically active state. Proteolytic removal of the propeptide domain further shifts the equilibrium toward activation.

(2) Organomercurials may compete with zinc for binding to Cys73. Since the reaction of cysteine with organomercurials is essentially irreversible, this reaction would run to completion



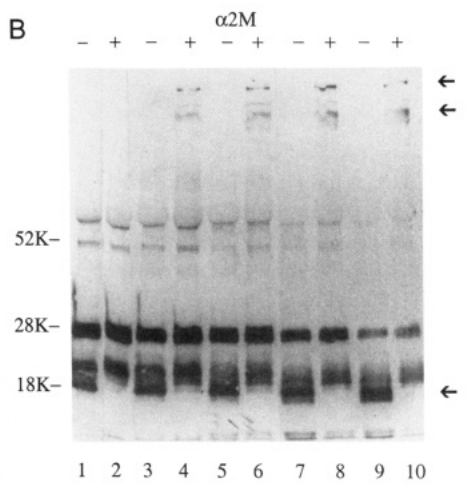


FIGURE 5: Western blot analysis of binding of recombinant or C73S mutant CL to α2M. These Western blots were stained with monoclonal antibody III_7. (Panel A) Recombinant wild-type CL without (lane 1) and with (lane 2) added α2M. (Panel B) Time course of binding of mutant C73S to α2M. Samples were incubated without (odd-numbered lanes) or with (even-numbered lanes) 100 μg/mL α2M. Incubation times were 15 min (lanes 1 and 2), 30 min (lanes 3 and 4), 1 h (lanes 5 and 6), 4 h (lanes 7 and 8), and 10 h (lanes 9 and 10).

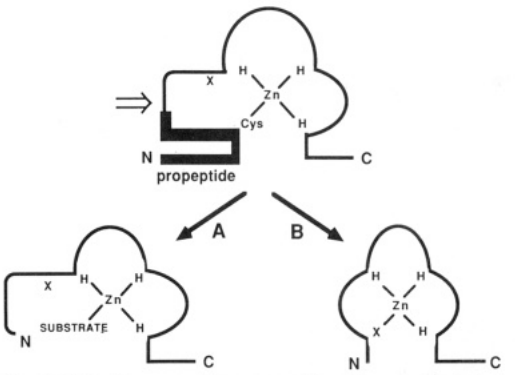


FIGURE 6: Models for procollagenase activation. In its latent form, the proCL enzyme provides four amino acids to coordinate to Zn^{2+} in the active site: Cys73, His199, His203, and His209. Upon cleavage of the propeptide domain (filled rectangle) at the QFVL sequence (arrow), the enzyme is activated. In model A, the fourth ligand binding site is left available for interaction with and selection of the collagen substrate. In model B, another amino acid within domains II and III (labeled "X") coordinates with the fourth Zn^{2+} binding site; this shift rearranges the structure of the enzyme to a catalytically active form.

and expose a fourth ligand binding site on the zinc ion in the enzyme active site. This model also suggests that the Zn ion in the active site could play both a structural role (to maintain latency) and also a catalytic role (during cleavage of collagen and other substrates). No other Zn-requiring protein is known to incorporate both structural and enzymatic roles within the same zinc binding site (Vallee & Auld, 1990).

A second model to explain the loss of latency postulates the formation of an alternate zinc binding motif: disruption of the Cys73–zinc coordination might allow rearrangement of the enzyme into an active tertiary conformation by allowing coordination to some other amino acid encoded within the CL molecule (Figure 6, model B). The latent enzyme would include Cys73 within the quaternary binding complex and hold the enzyme in an inactive conformation by blocking coordination of the alternate amino acid site within CL to the zinc ion. However, this second model cannot easily explain the activation of the proenzyme caused by SDS or chaotropic ions: one must further postulate that these treatments can disrupt the Cys–zinc interaction without disrupting subsequent conformational rearrangements or interactions.

Another alternate model involves formation of thioester bonds similar to those responsible for maintaining the latency of the α-macroglobulin inhibitors and of complement components C3 and C4. However, formation of the thioester in these proteins requires a highly conserved sequence of amino acids not found in matrix metalloproteinases. Although the last two models cannot be excluded, the first model best fits the known activation pathways for collagenase.

The active site within eukaryotic matrix metalloproteinases is highly homologous to the thermolysin class of prokaryotic metalloproteinases, except that the eukaryotic metalloproteinases also have a mechanism to ensure latency of the enzyme; this control circuit may have been added to the progenitor enzyme without modification of the structural features of the active site. That the upstream unpaired Cys residue and the zinc binding active site are highly conserved in all members of the eukaryotic MMP family suggests that this additional control circuit represents a common mechanism for helping to maintain latency in these enzymes. Addition of latency as an extra regulatory step might be required for the proper function of the enzyme, since it helps to modulate the activity of the enzyme after secretion and to limit their action to nearby areas within the matrix.

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