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The Recombinant Catalytic Domain of Mouse Collagenase-3 Depolymerizes Type I Collagen by Cleaving Its Aminotelopeptides

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The sequence coding for the catalytic domain of mouse collagenase-3 (MMP-13) was amplified by polymerase chain reaction and expressed in *Escherichia coli*. The recombinant catalytic domain (CCD), mainly recovered as inclusion bodies, was renatured and purified to homogeneity by preparative SDS-PAGE. The purified CCD degraded gelatin, casein and a synthetic peptide. CCD was not able to cleave the triple-helical domain of type I collagen but conserved the specific property of full-length collagenase-3 to cleave the N-telopeptides. These results show that residues involved in the recognition and cleavage of the aminotelopeptides of type I collagen are located in the catalytic domain of mouse collagenase-3 and that the C-terminal domain is not required for this activity.

The degradation of extracellular matrix in both physiological and pathological processes is primarily dependent on matrix metalloproteinases or MMPs (1, 2). Among MMPs, collagenases are characterized by their unique ability to cleave the major fibrillar collagens (types I, II and III) at a specific site of the native triple helix, located approximately 3/4 from the N-terminal end. All collagenases share the following structural organization: a signal peptide for secretion, a propeptide responsible for latency, a catalytic domain with the conserved Zn-binding sequence HExGHxxGxxH, a short hinge region, and a C-terminal haemopexin-like domain (3).

Collagenases-3 (MMP-13 or rodent-type collagenases) form a new subfamily of MMPs. They comprise rat (4), mouse (5) and human (6) collagenases-3 which have been cloned. They differ from the other types of mammalian collagenases (interstitial collagenase or MMP-1, and neutrophil collagenase or MMP-8) by their aminoacid sequences (5) and their enzymatic properties (7). During mouse development, collagenase-3 is selectively expressed in cells involved in bone formation or remodeling (8, 9). In humans, collagenase-3 has only been found in breast carcinomas (6) and in chondrocytes from arthritic cartilage (10).

A recently described property of collagenases-3 is their ability to cleave type I collagen at an additional site, in the non-helical N-telopeptides (11, 12). This cleavage occurs between Gly and Val of the α1(I) chain, four residues preceding the beginning of the major helix and C-terminal to the putative cross-linking Lys. The N-telopeptidase activity of collagenase-3 has been postulated to be sufficient for type I collagen remodeling during mouse embryonic development (12).

Here we describe the expression in *E. coli* and purification of the recombinant catalytic domain of mouse collagenase-3 (CCD) for further crystallization studies. We also characterized the ability of CCD to degrade type I collagen and a few other substrates.

MATERIALS AND METHODS

Construction of expression vector pET-CCD. The cDNA of mouse collagenase-3 had been cloned from mouse calvaria and inserted into pBluescript KS+ (Stratagene) (5). The sequence coding for the catalytic domain (Glu 104 to Gly 268) was amplified by PCR. The first primer (5′-TGCCATGGAATACAATGTTTTCCCT-3′) contained a *Nco*I cloning site with a start codon (ATG). The second primer (5′-TCGGATCCTCAACCATAAAGAAACTG-3′) coded for a stop codon (TGA) and a *Bam*HI cloning site. PCR was performed using Perkin-Elmer Cetus Gene Amp kit and reaction mix was subjected to 30 cycles (94°C, 1 min; 60°C, 1 min; 72°C, 2 min). The amplified fragment (503 bp) was inserted into the *Nco*I and *Bam*HI cloning sites of pBluescript KS+ and sequenced (13) using TaqTrack DNA Polymerase Systems and T3/T7 primers (Promega). The sequence coding for CCD was subsequently introduced in the *Nco*I and *Bam*HI sites of pET-3d expression vector (Novagen), which contains a specific T7

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Abbreviations: CCD, catalytic domain of mouse collagenase-3; MMP, matrix metalloproteinase; IPTG, isopropyl-β-D-thiogalactoside; TIMP, tissue inhibitor of metalloproteinases.

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RNA polymerase promoter (14). This resulting plasmid was named pET-CCD (5,100 bp).

Expression of CCD in E. coli. Competent *E. coli* BL21(DE3) were transformed with pET-CCD or with pET-3d for control. These bacteria contain a chromosomal copy of the T7 RNA polymerase which is under the control of an inducible *lac*UV5 promoter (14). In order to express recombinant CCD, 400 μl of an overnight culture of transformed BL21(DE3) were added to 400 ml of Luria-Bertani medium (10 g/l bactotryptone, 10 g/l NaCl, 5 g/l yeast extract) containing ampicillin (100 μg/ml). Bacteria were cultured at 37°C under vigorous shaking. When the optical density at 600 nm reached 0.6, expression was induced by addition of 0.40 mM IPTG. After 4 additional hours of culture, bacteria were isolated by centrifugation (2,000 $\times$ *g*, 10 min, 4°C) and resuspended in 40 ml of TNCNZ buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 3 mM NaN_3, 1 μM $ZnCl_2$) supplemented with leupeptin and aprotinin (2 μg/ml each). Bacteria were then lysed by two passages through a French press at 14,000 psi and inclusion bodies were recovered by centrifugation at 8,000 $\times$ *g* for 10 min at 4°C.

Purification of the recombinant CCD. Inclusion bodies from 100 ml of culture were solubilized in 3 ml of 50 mM Tris-HCl, pH 7.5 containing 6 M urea. After centrifugation at 24,000 $\times$ *g* for 20 min at 4°C, an equal volume of sample buffer (250 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue) was added. The recombinant CCD was purified and renatured by preparative polyacrylamid gel electrophoresis (PrepCell 492, Bio-Rad) using a 100 ml separating gel (15% acrylamid). Proteins were eluted in 25 mM Tris, 192 mM glycine, pH 8.8 at 4°C. Fractions containing the purified CCD were pooled and dialyzed three times against 20 volumes of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 3 mM NaN_3.

SDS-PAGE and zymographies. SDS-PAGE was performed according to Laemmli (15), followed by Coomassie blue or silver staining (16). For zymographies (17), unreduced samples were electrophoresed in SDS-polyacrylamid gel (15% acrylamid) copolymerized with gelatin or casein (0.5 mg/ml). SDS was removed by rinsing with 2.5% (v/v) Triton X-100. The gels were incubated in 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 3 mM NaN_3, 1% (v/v) Triton X-100, 1 μM $ZnCl_2$ for 18 h at 35°C and stained with 0.25 % (w/v) Coomassie blue.

Assays of enzymatic activities. Hydrolysis of the synthetic substrate Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH (Bachem) was evaluated by measuring the absorbance at 365 nm after extraction with ethyl acetate as previously described (18). The substrate (0.1 mM) was incubated with enzyme in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 10 μM $ZnCl_2$, 3 mM NaN_3 for 30 min at 37°C. Collagenase activity was determined at 25°C with ^{3}H-acetylated guinea pig skin collagen in solution as described (19). EDTA (20 mM) was added to the controls.

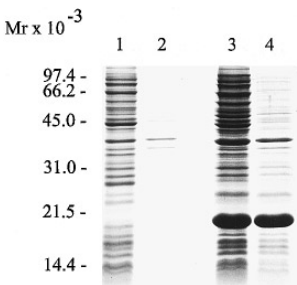

FIG. 1. SDS-PAGE analysis of the expression of CCD in *E. coli* BL21(DE3). Total (lanes 1 and 3) and insoluble (lanes 2 and 4) fractions of induced *E. coli* BL21(DE3) containing pET-3d vector alone (lanes 1 and 2) or pET-CCD (lanes 3 and 4) were analyzed by 15% SDS-PAGE. After electrophoresis, the gel was stained with Coomassie blue.

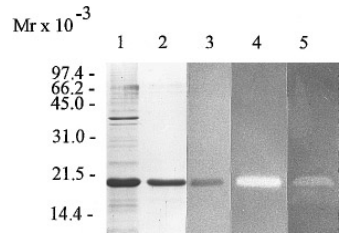

FIG. 2. Purification of CCD by preparative SDS-PAGE. Inclusion bodies were solubilized in Tris/urea buffer (lane 1) and loaded on a preparative SDS-PAGE column. The fractions containing purified CCD were pooled and analyzed by 15% SDS-PAGE (lanes 2 and 3, Coomassie blue and silver staining, respectively) and by gelatin- (lane 4) and casein- (lane 5) zymographies.

Protein assay. Protein concentrations were determined by using the bicinchoninic acid reagent (Pierce) and bovine serum albumin as standard (20).

RESULTS AND DISCUSSION

The catalytic domain of mouse collagenase-3 (CCD) consists of 164 residues, extending from Tyr 105 to Gly 268. To express CCD in *E. coli*, the sequence coding for these aminoacids plus residue Glu 104 was amplified by PCR. This last residue was added because N-terminal tyrosine is responsible for rapid degradation of proteins expressed in *E. coli* (21). The primers used for PCR allowed insertion of translation start and stop codons and new restriction sites necessary for subsequent cloning. After sequencing to check for correct amplification, the fragment was introduced downstream the T7 promoter of expression vector pET-3d.

Analysis of lysates from transformed *E. coli* BL21(DE3) after induction with IPTG (Figure 1) showed that recombinant CCD was mainly expressed as an insoluble protein of Mr 19,000 which corresponded to the calculated molecular mass of 18,630 Da. Like most recombinant proteins expressed in *E. coli* (22), CCD aggregated as insoluble inclusion bodies that were recovered by centrifugation and solubilized in 6 M urea. Recombinant CCD was efficiently renatured and purified to homogeneity by preparative SDS-PAGE, as shown in Figure 2. Zinc and calcium ions were not added to the elution buffer to prevent autolysis of CCD. Using this method, about 10 mg of purified and soluble CCD were obtained from 100 ml of culture.

Purified CCD was able to degrade both gelatin and casein, as revealed by zymography (Figure 2, lanes 4 and 5). It also cleaved the synthetic peptide Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH, a substrate for the MMPs (18), with a specific activity of 83 μg/min/nmol. However, CCD was not able to cleave native type I collagen into characteristic 3/4 N-terminal and 1/4 C-terminal fragments, as revealed by enzymatic assays

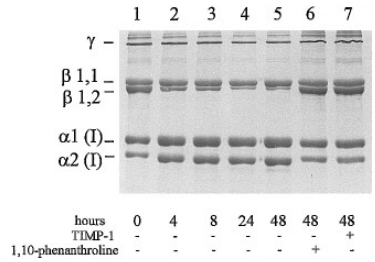

FIG. 3. Effect of purified CCD on type I collagen. One hundred μg of acid-soluble guinea pig type I collagen were incubated with 1 μg of purified CCD for 0 h (lane 1), 4 h (lane 2), 8 h (lane 3), 24 h (lane 4) and 48 h (lane 5) at 25°C. Lanes 6 and 7 : same as lane 5, plus 1,10-phenanthroline (2 mM) and purified natural bovine TIMP-1 (40 μg/ml), respectively. Incubation buffer (final volume of 200 μl) was 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 10 μM $ZnCl_2$, 3 mM NaN_3, 0.02% (v/v) Triton X-100. Reaction products were analyzed by 6% SDS-PAGE followed by Coomassie blue staining.

and migration of reaction products in SDS-PAGE (Figure 3). This result supports previous observations on the essential role of the C-terminal domain, including the proline-rich hinge region and the haemopexin-like domain, for cleavage of fibrillar collagens (23, 24). Natural human MMP-1 and MMP-8 show autoproteolytic cleavage in their hinge-regions resulting in N-terminal fragments devoid of collagenase activity despite being still active on less specific substrates such as gelatin (25, 26). Similar results were obtained with the recombinant catalytic domains of human MMP-1 and MMP-8 (27, 28). Recent crystallographic data on the catalytic domains of human MMP-1 (29) and MMP-8 (30) and on full-length porcine MMP-1 (31) showed that only one single strand of the collagen triple-helix can fit in the active site region. This finding suggested that the haemopexin-like domain is positioned over the active site, allowing partial unfolding of the bounded collagen triple helix around the cleavage site (30, 32).

After incubation of type I collagen with CCD, the amount of β dimers was diminished whereas that of α monomers was increased (Figure 3). This effect could be inhibited by 1,10-phenanthroline and TIMP-1 (a specific physiological inhibitor of MMPs), confirming that it was not due to a contaminating proteinase. This shows that CCD is still able to convert cross-linked β dimers into α chains, by cleaving the aminotelopeptides C-terminal to the lysine-derived cross-link of type I collagen. These results demonstrate that residues responsible for the recognition and the cleavage of N-telopeptides are located in the catalytic domain of collagenase-3.

Krane and coworkers (12) have produced a chimeric enzyme formed by human interstitial collagenase-1 (MMP-1) where residues extending from the middle of the catalytic domain to the Zn-binding site (Phe 138 to Leu 224) were replaced by the corresponding residues (Phe 143 to Leu 229) of mouse collagenase-3. In contrast to human MMP-1, this chimeric collagenase cleaved the N-telopeptides of type I collagen. Thus, determinants of proteolytic specificity for the aminotelopeptides are located in the exchanged portion of the catalytic domain of mouse collagenase-3. In addition, our results show that the C-terminal region of mouse collagenase-3, including the hinge region and the haemopexin-like domain, is not required for N-telopeptidase activity. Site-directed mutagenesis and crystallization studies should help determining by which mechanism the catalytic domain of collagenase-3 recognizes and cleaves the aminotelopeptides of type I collagen.

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