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Multiple modes of activation of latent human fibroblast collagenase: Evidence for the role of a Cys^{73} active-site zinc complex in latency and a "cysteine switch" mechanism for activation

(collagenase/matrix metalloproteinase/chemical modification)

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ABSTRACT **Latent human fibroblast collagenase (HFC) can be activated by a variety of seemingly disparate means. In addition to the well-characterized activation by trypsin and organomercurial compounds, the enzyme can be activated to various extents by surfactants such as sodium dodecyl sulfate, by chaotropic ions such as SCN^-, by disulfide compounds such as oxidized glutathione, by sulfhydryl alkylating agents such as *N*-ethylmaleimide, and by oxidants such as NaOCl. The underlying basis for these activations is the modification, exposure, or proteolytic release of the Cys^{73} residue from its habitat in the latent enzyme where it is thought to be complexed to the active-site zinc atom. This residue is not accessible for reaction with small molar excesses of dithionitrobenzoate in native, latent HFC. However, on addition of EDTA, this residue becomes fully exposed and is quantitatively labeled. All modes of activation of latent HFC are believed to involve the dissociation of Cys^{73} from the active-site zinc atom and its replacement by water, with the concomitant exposure of the active site. This is thought to be the primary event that precedes the well-known autolytic cleavages that are observed following the appearance of collagenase activity. The dissociation of Cys^{73} from the zinc atom in the latent enzyme "switches" the role of the zinc from a noncatalytic to a catalytic one. This "cysteine switch" mechanism of regulation may be applicable to the entire collagenase gene family.**

Human fibroblast collagenase (HFC) is a member of a homologous class of proteinases called the matrix metalloproteinases (MMPs) that are collectively capable of catabolizing the major components of the extracellular matrix (1, 2). HFC and the collagenase from neutrophils are the only two human enzymes capable of catalyzing the breakdown of interstitial collagens in tissue at an appreciable rate, while the remaining MMPs efficiently catalyze the destruction of other matrix macromolecules. Therefore, a knowledge of the factors that control the activity of these enzymes is critical to an understanding of connective tissue breakdown *in vivo*. Although the behavior of all of the MMPs has not yet been completely characterized, these enzymes appear to share the characteristic that they are synthesized and secreted in an inactive form and are subsequently activated *in situ*. The biochemical basis for this latency and the physiological mechanisms of activation of the latent form of these enzymes have not yet been delineated. HFC is the best-characterized member of the MMP family and can now be prepared in sizable quantities (1). Thus, it is a good candidate for detailed studies of the activation of the latent form.

The sequence of latent HFC has been elucidated from a cDNA clone (3). It has a molecular mass of 52 kDa, and a variety of data suggest that the protein chain is organized into three domains, where the boundaries between these domains are defined by autolytic cleavages that accompany its activation and degradation (Fig. 1). Latent HFC can be activated by trypsin, which initially hydrolyzes the Arg^{36}-Asn^{37} bond‡ in domain 1 to generate a 46-kDa form that is still inactive (4, 5). This species subsequently activates autolytically via hydrolysis of one of the three bonds at the Gln^{80}-Phe^{81}-Val^{82}-Leu^{83} locus that marks the boundary between domains 1 and 2 to give the 42-kDa form of HFC (5). Treatment with organomercurials initially results in the activation of latent HFC without a loss in molecular mass. However, autolytic conversion to the 42-kDa form by loss of domain 1 follows as a secondary event. Both the 52- and 42-kDa forms of HFC can also undergo a poorly understood autolytic cleavage at the Pro^{250}-Ile^{251} bond that marks the boundary between domains 2 and 3 and results in a loss of collagenolytic activity (6). Domain 2 contains the putative zinc binding site (7–9), while domain 3 is weakly homologous to hemopexin, a heme-binding serum protein (10, 11).

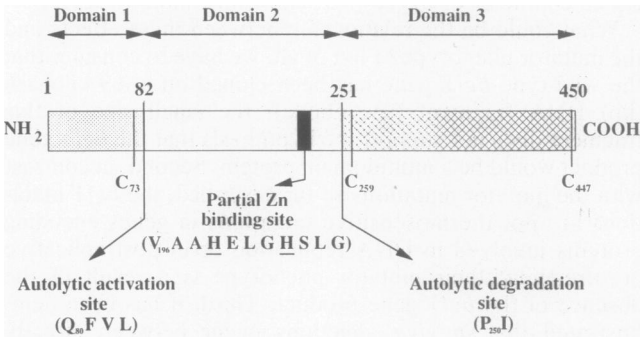

FIG. 1. Schematic representation of the three-domain structure of latent HFC. Amino acids are designated by the single-letter code.

The observation that organomercurial compounds have the ability to convert the latent 52-kDa form of HFC to an active species with the same molecular mass clearly shows that activation can be achieved without proteolysis. It has also been observed that latent HFC can be activated by SDS (12), and it is shown here that it can be activated to various extents by a sulfhydryl alkylating agent, disulfide compounds, chaotropic ions, and the oxidant NaOCl. In this study, the underlying biochemical basis for these seemingly disparate means of activation has been examined. Attention has been

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Abbreviations: HFC, human fibroblast collagenase; MMP, matrix metalloproteinase; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GSSG, oxidized glutathione.

‡The numbering of the residues assumes that Phe^{11} of the preproenzyme sequenced by Goldberg and coworkers (3) is the first residue of the secreted form of the latent enzyme.

focused on the modification or exposure of the Cys^{73} residue in domain 1 of latent HFC as a central, underlying event. A model is proposed in which Cys^{73} is complexed to the catalytic zinc atom in domain 2 of latent HFC. All modes of activation share the property that they disrupt this bond, allowing a functional active site to form. This mechanism for regulating activity may also apply to the other members of the MMP family.

MATERIALS AND METHODS

Materials. Latent HFC was purified from the harvest medium of human gingival fibroblasts as described (1, 6) by a modification of the procedure of Stricklin and associates (13). This material was applied to a Sepharose-CH-Pro-Leu-Gly-NHOH column at pH 7.5 as described by Moore and Spilburg (14) to remove HFC that had spontaneously activated during the purification. The active HFC that adsorbed to the column was eluted at pH 9.0 and used for other studies. Oxidized glutathione [glutathione disulfide (GSSG)], *N*-ethylmaleimide (NEM), and *p*-chloromercuribenzoate (PCMB) were purchased from ICN; 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Na_2EDTA, and methionine were from Sigma; NaOCl was from Aldrich; NaSCN and $HgCl_2$ were from J. T. Baker; 1,10-phenanthroline was from Lancaster Synthesis; and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was from Worthington Enzymes.

Methods. Collagenolytic activity was quantitated by measuring the rate of hydrolysis of soluble ^3H-acetylated rat tendon type I collagen in 50 mM Tricine/0.2 M NaCl/10 mM $CaCl_2$, pH 7.5, at 25°C (15). For the activation studies, latent HFC (15 nM) was incubated with variable amounts of reagent in 50 mM Tris·HCl/0.2 M NaCl/10 mM $CaCl_2$/0.02% NaN_3, pH 7.5, at 30°C for 30 min in a total vol of 950 μl. The assays were initiated by addition of 50 μl of [^3H]collagen. The only exception was the activation by NaSCN that was carried out with 590 nM latent HFC and diluted 1:40 before adding it to the collagenase assay to reduce the NaSCN concentration. None of the other activating reagents affects the collagenase assay at the concentrations used. Positive (0.5 mM PCMB) and negative (no reagent) controls were included with each series of experiments. For each reagent, an activation curve was constructed that consisted of a plot of net activity relative to the positive control vs. reagent concentration. To monitor the time course of activation of latent HFC by several reagents, a continuously recording fluorometric assay based on the hydrolysis of dinitrophenyl-Pro-Leu-Ala-Leu-Trp-Ala-Arg was used as described for a similar substrate (16). The assays were carried out in the collagenase assay buffer described above at a substrate concentration of 50 μM and the fluorescence change was monitored with a Perkin–Elmer model LS-5 fluorometer.

The concentration of free sulfhydryl groups in solutions of HFC before and after the various treatments described was determined by reaction with DTNB. In most cases, HFC (1 μM) was incubated with DTNB (10 μM) in 50 mM Tris·HCl/0.2 M NaCl/10 mM $CaCl_2$/0.02% NaN_3, pH 7.5, at 23°C and the absorbance change at 412 nm was recorded with a Varian model 210 spectrophotometer on addition of reagent [EDTA, trypsin, Hg(II), NEM]. In the activation with NaOCl, the DTNB was not added until the NaOCl had been reduced with a 4-fold excess of methionine. The direct activation by DTNB was carried out by increasing its concentration to 1 mM. A value of $\Delta\varepsilon_{412} = 14{,}150\ M^{-1}\cdot cm^{-1}$ (17) was used to calculate the concentration of reactive sulfhydryl groups. The concentration of latent HFC was measured spectrophotometrically using $\varepsilon_{280} = 6.8 \times 10^4\ M^{-1}\cdot cm^{-1}$. SDS/PAGE was performed according to the method of Laemmli (18) using the silver staining method (19) to visualize protein bands.

RESULTS

Latent HFC has been treated with a variety of reagents to assess their effectiveness as activators. In each case, activity has been measured as a function of reagent concentration to quantitate the activation and identify the optimal conditions for each treatment. In addition to the well-known activator PCMB (4, 5, 20, 21), other reagents that have been reported to activate latent MMP include NEM (22–26), $HgCl_2$ (23), GSSG (27), NaSCN (28–31), and NaOCl (32). Thus, all of these reagents, as well as DTNB, have been tested. The activation curves for these reagents are presented in Fig. 2. The mercurials Hg(II) and PCMB produce the greatest activation, with full activation achieved at a 100-fold lower concentration of the former. The other five reagents all activate to variable extents and give maximal activities that are 30–44% that obtained for the mercurials. While these reagents are less effective activators, the levels of activation measured are reproducible and catalytically significant. A very similar series of results has been obtained for the activation of latent human neutrophil collagenase (unpublished data). If these activation treatments are added to the previously reported activations produced by SDS (12), proteases (4, 5, 30, 33–36), Au(I) salts (37–39), and the autolytic activation observed on storage (6), it is clear that there are multiple modes by which latent HFC can be activated.

Six of the reagents used in this study share the property that they are capable of modifying cysteine residues in proteins by reacting with the side-chain sulfhydryl group. The reactions can be divided into classes, where Hg(II) and PCMB can complex with the sulfhydryl group to form Cys-S-Hg(II), the disulfide compounds GSSG and DTNB can undergo disulfide exchange with Cys-SH to form the mixed disulfide Cys-S-S-R (R, organic group), NEM can alkylate the sulfhydryl group to give Cys-S-R, and NaOCl can oxidize it to cysteic acid, $Cys\text{-}SO_3H$. This suggests that the modification of a cysteine residue is the basis for these activations. Latent HFC contains three cysteine residues (Fig. 1) and one of these, Cys^{73}, is present on domain 1 of the latent enzyme that is autolytically released after activation. Thus, attention has focused on the modification of Cys^{73} as a common, underlying event in these activation reactions.

To investigate the disposition of the three cysteine residues in latent HFC, an attempt has been made to react them with small molar excesses of DTNB that are normally sufficient to detect any free, exposed sulfhydryl groups of cysteine resi-

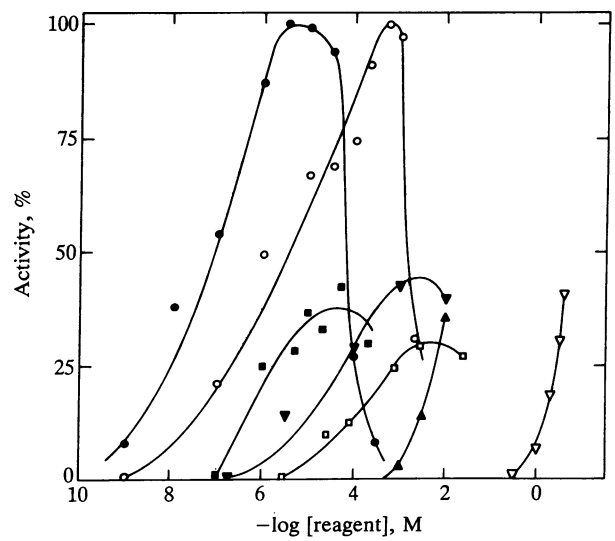

FIG. 2. Activation of latent HFC by various concentrations of PCMB (○), $HgCl_2$ (●), NaOCl (□), DTNB (■), GSSG (▲), NEM (▼), and NaSCN (▽).

dues in proteins (17). Although DTNB activates latent HFC at higher concentrations (Fig. 2), treatment of 1 μM latent HFC with 10 μM DTNB neither produces any significant activation nor labels any sulfhydryl groups. This indicates that there are no freely accessible cysteine side chains in native latent HFC, in agreement with the report of Stricklin and associates (4). The addition of 30 mM EDTA to a 1 μM solution of latent HFC, however, exposes 1.0 cysteine residue per molecule of HFC within 15 min. A likely explanation is that two of the three cysteine side chains are disulfide bonded to each other (see below) and the third is inaccessible. The release of the odd cysteine by EDTA suggests that this residue could be interacting with a metal ion in the enzyme.

A series of experiments has been carried out in which DTNB has been used to measure the number of free sulfhydryl groups in HFC after activation by trypsin, and a member [Hg(II), NEM, NaOCl, and DTNB] from each of the four classes of reagents described above. Treatment of latent HFC with a 4-fold excess of trypsin activates the enzyme and releases 0.85 cysteine residue over a period of 20 min. If 30 mM EDTA is added at the end of the reaction, no additional cysteine residues are detected. Moreover, if the same concentration of trypsin is added to latent HFC that had already been treated with 30 mM EDTA and has 1.0 exposed cysteine, no additional cysteine residues are released. Thus, trypsin activation exposes the same cysteine residue as treatment with EDTA. The same results are obtained for the activation with 1 mM DTNB, except that the release of 1.07 cysteine residues is observed within 3 min.

In contrast, no free sulfhydryl groups can be detected after activation of latent HFC with 1 mM NEM, a reaction that is complete in 2 hr. This observation is consistent with the view that NEM activates latent HFC by alkylating this cysteine residue, since this would render it unreactive toward DTNB. No cysteine residues are detected by addition of 30 mM EDTA or 4 μM trypsin after the activation by NEM is complete, confirming that the odd cysteine residue has been alkylated. Activation by 5 μM Hg(II) in the presence of DTNB is complete in 2 hr, but no cysteine residues can be detected. This result would be expected if a Cys–Hg(II) complex were formed on activation. If 2 mM 1,10-phenanthroline is added to complex the Hg(II), 0.78 cysteine residue is detected. This concentration of 1,10-phenanthroline is not, by itself, sufficient to release any cysteine residues. Thus, this cysteine residue was apparently released during the activation but was shielded from the DTNB by the Hg(II). The addition of 4 μM trypsin to this mixture fails to release any additional cysteine residues. Finally, activation of latent HFC was carried out with 1 mM NaOCl, a reaction that is complete within 4 hr. After quenching the NaOCl with a 4-fold excess of methionine, the addition of DTNB showed that no free cysteine residues could be detected, as expected if oxidation to cysteic acid took place. The failure to detect this odd cysteine residue on addition of 30 mM EDTA or 4 μM trypsin confirms that it has been oxidized.

Several experiments on the 42-kDa active form of HFC that consists of only domains 2 and 3 were carried out for comparison with the experiments on the latent enzyme. Latent HFC that had spontaneously activated was adsorbed to a Sepharose-CH-Pro-Leu-Gly-NHOH affinity resin to separate it from latent enzyme and peptides of domain 1 that are released autolytically on activation. Its identity as the 42-kDa form was confirmed by SDS/PAGE. Treatment of this active form of HFC with DTNB revealed no free cysteine residues, even after treatment with 30 mM EDTA or 4 μM trypsin. This implies that the Cys^{259} and Cys^{447} residues in domain 3 are disulfide bonded to one another and that the cysteine residue in latent HFC that is exposed on treatment with EDTA, and modified or exposed during activation, is the Cys^{73} residue located in domain 1.

DISCUSSION

The experimental observations presented here indicate that activation of latent HFC can be achieved by a variety of reagents that react with free sulfhydryl groups. It has also been shown that Cys^{73} is not freely accessible in latent HFC but is released on activation. Thus, any explanation for the latency of HFC and any proposal to explain its activation must be consistent with these facts. Many workers have noted the ability of thiol reagents to activate latent MMP (5, 23, 27, 40) and others have recognized that the Cys^{73} residue of latent HFC is conserved in other MMP (9, 11, 41), implying a possible role for this residue in activation. One model that we have considered for latent HFC has the Cys^{73} residue participating in a thiol ester bond of the type observed for the α-macroglobulin inhibitors (42) and complement components C3 and C4 (43, 44) to stabilize a conformation of the enzyme in which the active site is blocked. This would explain the inaccessibility of the Cys^{73} residue and the latency of the enzyme. Hydrolysis of this thiol ester bond could activate latent HFC by triggering a conformational change that exposes the active site. If the thiol ester were in equilibrium with a small amount of the active thiol form, the thiol reagents could activate by shifting the equilibrium to this form. While this model cannot be ruled out unequivocally, it is inconsistent with two observations. First, activation cannot be achieved with nucleophiles such as methylamine (unpublished data), which should hydrolyze this bond. Second, formation of a thiol ester bond in the other systems mentioned requires a highly conserved sequence of amino acids (Pro-Tyr/Ser-Gly-Cys-Gly-Glu-Glu-Asn/Thr-Met-Val/Ile) that is not found in latent HFC (42–44).

A model for latent HFC that is consistent with all of our observations is one in which the sulfhydryl group of Cys^{73} is coordinated to the catalytic zinc atom in a manner that covers the active site and renders the enzyme latent (Fig. 3). Since HFC has never been isolated fully (greater than ≈95%) latent, it can be viewed as existing in an equilibrium between an inactive form in which Cys^{73} is complexed to the active-site zinc atom and a small fraction of active enzyme in which Cys^{73} is dissociated from the zinc. This cysteine residue is fully dissociated on removal of the zinc atom by EDTA. It is also released by proteolytic loss of domain 1 after autolytic activation, or activation by trypsin or other proteases. This dissociation can also be brought about by chaotropic agents such as NaSCN, or surfactants such as SDS, both of which stabilize conformations of the protein in which Cys^{73} is spatially removed from the active-site zinc atom.

The reactions with the six sulfhydryl reagents studied here (Fig. 2) apparently modify this cysteine residue during moments when it is transiently dissociated from the zinc atom, thereby preventing reassociation and shifting the equilibrium to the active form by mass action. These reactions [alkylation, oxidation, Hg(II) complexation, disulfide exchange] all convert the Cys^{73} sulfhydryl group to a species that cannot serve as a ligand for the zinc atom. The variable effectiveness of these reagents most probably reflects their abilities to react with the Cys^{73} residue in its environment in HFC. Thus, the release of Cys^{73} from its bond to the zinc atom with the concomitant formation of a catalytically competent active site is thought to be the primary event that precedes the well-known autolytic cleavages that are observed following the appearance of collagenase activity. Site-directed mutagenesis studies in which mutants of HFC have been expressed in *Escherichia coli* show that the $Cys^{73} \rightarrow Ser^{73}$ substitution abolishes the ability to isolate the enzyme in a latent form and is consistent with this model (L. J. Windsor, J. A. Engler, B. Birkedal-Hansen, and H.B.-H., unpublished data). Additional studies will be necessary to confirm and/or refine details of this model.

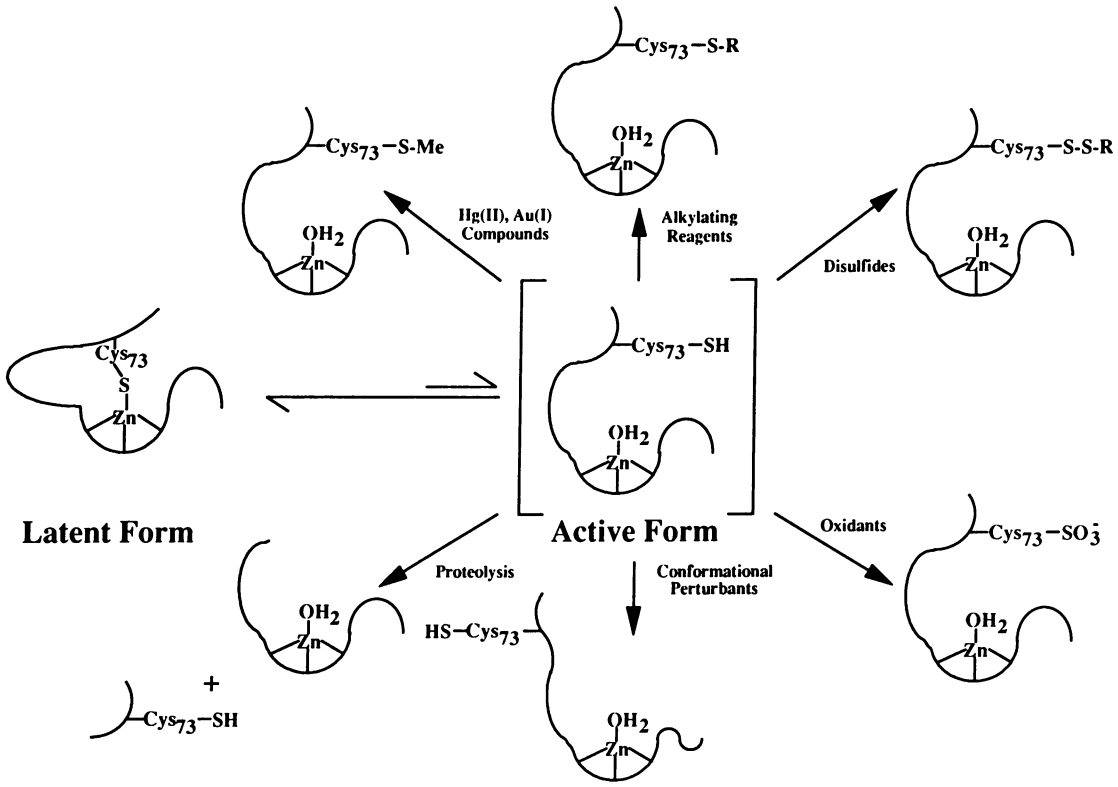

FIG. 3. Model describing the underlying biochemical basis for the multiple modes of activation of latent HFC.

It is of interest to compare the zinc binding site in latent HFC with that in other zinc metalloenzymes. The zinc atoms in enzymes can be divided functionally into catalytic versus noncatalytic or structural types. Vallee and Auld (8) have carried out a detailed analysis of the x-ray structures of 12 zinc enzymes and found that all of the catalytic zinc atoms are bound to three amino acid side chains of the protein, with a fourth coordination site occupied by water. The zinc-bound water is an essential feature of the active sites of these enzymes and is directly involved in catalysis. The x-ray structures are known for four zinc proteinases and the catalytic zinc atom is complexed to two histidine and one glutamic acid residues in *Bacillus cereus* neutral protease (45), thermolysin (46), and carboxypeptidase A (47), and to three histidine residues in DD carboxypeptidase (48). In contrast, the noncatalytic zinc atom in the zinc enzymes alcohol dehydrogenase (49) and aspartate transcarbamoylase (50) are both complexed to four cysteine residues of the protein.

The model pictured in Fig. 3 for latent HFC portrays a binding site that is unique among the zinc metalloenzymes. While the protein ligands to the zinc atom are not known, it has been recognized that HFC contains a partial amino acid sequence (residues 196–206) that is homologous to the region of thermolysin between residues 139 and 149 that contains the His^{142} and His^{146} ligands, implying that these conserved histidine residues function to bind zinc in HFC (7–9). There is no homology with HFC in the region where the third glutamic acid ligand of thermolysin is found (8). However, a third ligand (probably either glutamic acid or histidine) is presumed to bond to zinc from another region of HFC in domain 2. The catalytically active form of HFC is no doubt one in which the fourth coordination site is occupied by water with an accessible substrate binding site. The catalytic machinery is probably similar in most respects to that in carboxypeptidase A, thermolysin, and other zinc proteinases. The critical difference between these enzymes and HFC is that the latter is synthesized in a latent form in which Cys^{73} is bound at the fourth coordination site of the zinc atom. This simultaneously displaces the essential zinc-bound water and renders the active site inaccessible to substrates. Thus, the zinc atom in latent HFC can be characterized as a "latent catalytic zinc atom" that resembles a noncatalytic zinc atom with four protein ligands. The protein architecture that places Cys^{73} in the vicinity of the zinc atom is constructed in such a way that the Cys^{73}–zinc complex can be disrupted by multiple stimuli, all of which can potentially serve as a basis for activation. Thus, while the physiological mechanism of activation of latent HFC is not yet known, it must be a process that displaces the Cys^{73} residue from the zinc and "switches" it from a noncatalytic to a catalytic zinc. Since the sequences surrounding this cysteine in domain 1 and the partial zinc binding site in domain 2 of the MMPs are highly homologous, this "cysteine switch" mechanism may pertain to all of the MMPs.

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