Stability Analysis of Latent and Active 72-kDa Type IV Collagenase: The Role of Tissue Inhibitor of Metalloproteinases-2 (TIMP-2)†

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ABSTRACT: The degradation of extracellular matrix is an important facet of many physiological and pathological processes. The collagenases form a family of matrix degradative enzymes that have similar active site sequences and activation mechanisms and are inhibited by a specific class of proteinase inhibitors referred to as tissue inhibitors of metalloproteinases. Regulation of enzyme activity is a complex process involving control at multiple levels: message transcription and translation, activation of latent proenzymes, inhibition of activity by specific inhibitors, and degradation of activated enzymes. We have examined the role of the proteinase inhibitor tissue inhibitor of metalloproteinases-2 (TIMP-2) on two of these processes: the autoactivation and autodegradation of the human 72-kDa type IV collagenase. We compared the stability of the enzyme in these two processes using three different enzyme preparations: the enzyme–inhibitor complex as isolated from human A2058 melanoma cells, recombinant enzyme free of TIMP-2, and enzyme separated from TIMP-2 by acid denaturation. We have found little evidence to support the hypothesis that the enzyme is able to autoactivate, as no autoactivation occurs in the presence of TIMP-2 and only 20% autoactivation occurs in its absence, and then only after 24 h of incubation at 37 °C. However, TIMP-2 does appear to inhibit autodegradation, possibly by a mechanism distinct from its ability to inhibit substrate proteolysis. Enzyme isolated via chromatography involving acid mobile phases produces a mixture of cleavage products that is mostly denatured, inactive enzyme fragments. The role of TIMP-2 as an inhibitor of autodegradation suggests that the enzyme may show two physiological phenotypes: the free enzyme having a high level of activity and rapid autodegradation and enzyme–inhibitor complex having a low level of activity resistant to autodegradation.

The formation and degradation of extracellular matrix components is an important aspect of many physiologic and pathologic processes including wound healing, implantation of ova, remodeling of bone and cartilage, angiogenesis, scar formation, and tumor invasion and metastasis (Nicolson, 1991; Scher, 1987; Woessner, 1991). The regulation of enzymes involved in matrix degradation occurs at multiple levels from gene transcription to specific inhibition of active lytic enzymes (Liotta et al., 1991). Among the enzymes involved in matrix degradation are a group of zinc metalloproteinases known as the matrix metalloproteinases. The enzymes of this class share similar characteristics including a common mode of activation, conserved amino acid sequences in the putative metal-binding/active-site region, and inhibition by specific proteinase inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs)[1] (Docherty & Murphy, 1990; Murphy et al., 1991: Stetler-Stevenson, 1990). The final in vivo level of degradative activity is thought to result from the interaction of many factors which control latent enzyme expression, inhibitor expression, activation of latent enzymes, and interaction of active enzymes with inhibitors as well as inactivation and degradation of enzymes and inhibitors. After the proteins are synthesized and secreted, the latter three factors are the most important in determining the rate and extent of matrix degradation.

Activation of matrix metalloproteinases is hypothesized to occur via the disruption of an interaction between an unpaired cysteine residue in a conserved region of the amino-terminal domain and the active-site zinc atom (Nagase et al., 1990; Springman et al., 1990; Stetler-Stevenson et al., 1989b). Following activation, this amino-terminal domain is cleaved, releasing the fully activated metalloproteinase. In vivo mechanisms of activation have been proposed for some members of the enzyme family. In particular, interstitial collagenase (EC 3.4.24.7), stromelysin-1 (EC 3.4.24.17), and the 92-kDa type IV collagenase (gelatinase B, EC 3.4.24.35) can be activated by other proteases (He et al., 1989; Nagase et al., 1990; Ogata et al., 1992).

Control of enzymatic activity following activation is dependent in part on interaction with TIMPs. The majority of the matrix metalloproteinases, including interstitial collagenase, neutrophil collagenase (EC 3.4.24.34), and the stromelysins, are secreted as latent proenzymes that only interact with the TIMPs after activation has occurred. The latent forms of these enzymes do not appear to bind the TIMPs or interact with them in any significant fashion. In contrast, the latent proenzyme forms of the 72-kDa (gelatinase A, EC 3.4.24.24) and 92-kDa type IV collagenases have been isolated as a tight-binding complexes with TIMP-2 and TIMP-1, respectively (Goldberg et al., 1989; Stetler-Stevenson et al., 1989a; Wilhelm et al., 1989). These latent enzyme–inhibitor complexes may still be activated in vitro, in a way similar to that of the uncomplexed enzymes, by the use of organomercurials. Enzymatic activity is much lower than free, activated enzyme, and activity can be reduced still further with the addition of exogenous TIMP (Goldberg et al., 1989; Stetler-Stevenson et al., 1989a). The presence of inhibitor bound to

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[1] Abbreviations: TIMP-2, tissue inhibitor of metalloproteinases-2; pAPMA, *para*-aminophenylmercuric acetate; SDS, sodium dodecyl sulfate.

the latent enzyme suggests that the inhibitor may play some role in addition to simple inhibition of metalloproteinase activity. Recently, there have been several studies which have attempted to define the interaction between the latent 72-kDa type IV collagenase and TIMP-2 (Fridman et al., 1992; Howard & Banda, 1991; Howard et al., 1991a,b; Kleiner et al., 1992) and the latent 92-kDa type IV collagenase and TIMP-1 (Goldberg et al., 1992).

Cross-linking studies using gelatin affinity purified latent 72-kDa type IV collagenase-TIMP-2 complex (Kleiner et al., 1992) demonstrated that TIMP-2 is bound to a site on the latent enzyme distinct from the active site. Cross-linking of inhibitor to this site on the latent enzyme does not prevent organomercurial-induced activation and subsequent autoproteolytic cleavage of the amino-terminal propeptide domain, substrate binding, or endoprotease activity. A second site of interaction, responsible for the inhibitory effects of TIMP-2, is revealed after activation. This second site is most likely to be at or near the active site of the enzyme, though this has not been definitively shown. After activation, TIMP-2 is free to interact with either one or both of these sites. However, the presence of TIMP-2 bound to the site available on the latent enzyme interferes in some fashion with binding of additional molecules of TIMP-2 to the inhibitory site, suggesting that there is an interaction between these sites that affects TIMP-2 binding.

Several studies have attempted to localize the sites of interaction between the TIMPs and the metalloproteinases using fragments of whole enzymes or inhibitors derived from proteolytic cleavage or recombinant DNA methodology (Fridman et al., 1992; Howard & Banda, 1991; Howard et al (1991a; Murphy et al., 1991, 1992). Howard et al. (1991a) used reverse-phase chromatography in 0.1% trifluoroacetic acid to separate the 72-kDa type IV collagenase from TIMP-2. On the basis of their studies of the enzyme recovered with this method, these investigators proposed that TIMP-2, when in complex with type IV collagenase, prevented the latent proenzyme from autoactivating. They also noted the presence of several autodegradative products and isolated these for subsequent binding experiments with TIMP-2 (Howard & Banda, 1991).

The suggestion that TIMP-2 plays a role in preventing autoactivation of the 72-kDa type IV collagenase is an intriguing one and does provide an explanation for why the latent enzyme and the inhibitor would be present together as a complex. However, the methods used to obtain the free enzyme were harsh. As we will show, moderately acidic pH may denature the enzyme, and this method yields a high percentage of inactive and unactivatable enzyme. In addition, Okada et al. (1990) have purified the latent 72-kDa type IV collagenase free of TIMP-2 from human synovial cell conditioned media. When this purified enzyme was incubated at 37 °C for 8 h, no autoactivation was observed. More recently, Murphy et al. (1992) briefly noted that free enzyme purified from the conditioned media of human gingival fibroblasts showed no tendency to autoactivate but did not provide experimental data or further characterization of enzyme stability. In light of these conflicting experimental results and conclusions, we set out to carefully examine the susceptibility of latent and active forms of the 72-kDa type IV collagenase with respect to autoactivation and autoproteolytic degradation. These experiments were conducted using purified preparations of enzyme–inhibitor complex from A2058 melanoma cells and recombinant enzyme isolated from baculovirus construct-infected Sf9 insect cells. We also examined the stability and specific activities of enzyme reconstituted in neutral buffer following separation of TIMP-2 under acid conditions. We feel that these results do not support the hypothesis that TIMP-2 is responsible for preventing the autoactivation of latent type IV collagenase, though it may have a role in stabilizing the complex to autodegradation following activation.

MATERIALS AND METHODS

Materials

SDS–polyacrylamide gels and 10% SDS–gelatin gels were from Novex. Molecular weight standards for SDS gel electrophoresis were from BRL. The human 72-kDa type IV collagenase–TIMP-2 complex was isolated from A2058 melanoma cell conditioned media as previously described (Stetler-Stevenson et al., 1989b). The synthetic heptapeptide substrate dinitrophenol-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 was from Peptides International, Inc. The synthetic product tetrapeptide Leu-Trp-Ala-D-Arg-NH_2 was custom synthesized by the Woods Hole Oceanographic Institute and was repurified by reverse-phase HPLC over a Beckman Ultrasphere ODS 5-mm, 4.6 × 250 mm C-18 analytical column.

Methods

Production of Recombinant Human 72-kDa Type IV Collagenase. The recombinant enzyme was produced using a baculovirus expression system (Summers & Smith, 1987). Briefly, full length human 72-kDa type IV collagenase cDNA was subcloned into a pVL1393 baculoviral transfer vector. After cotransfection of wild-type baculovirus and transfer vector, the recombinant virus was purified using dot blot hybridization and standard virus purification methods. Large-scale cultures of Sf9-insect cells where infected, and 72 h post-infection the culture media were collected and used for recombinant protein purification. The recombinant enzyme was purified from the culture medium using gelatin–Sepharose-affinity chromatography using 10% dimethyl sulfoxide for elution. The purified protein was stored at −70 °C.

Separation of TIMP-2 and 72-kDa Type IV Collagenase. The latent 72-kDa type IV collagenase–TIMP-2 complex was separated into purified enzyme and inhibitor fractions by gel filtration chromatography under acid conditions as previously described (Kleiner et al., 1992). The eluted enzyme fractions were lyophilized and then redissolved in 0.1% trifluoroacetic acid and dialyzed against 50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Brij-35, pH 7.5 (collagenase buffer) at 4 °C.

Activation of Type IV Collagenase. Samples of latent type IV collagenase–TIMP-2 complex were chemically activated in the presence of 1 mM *para*-aminophenylmercuric acetate (pAPMA) for 90 min at 37 °C and then dialyzed against collagenase buffer at 4 °C. Complex prepared in this manner is stable to loss of activity when stored frozen at −80 °C. Human recombinant type IV collagenase was activated in the presence of 1 mM pAPMA for 15 min at 37 °C. It was then used immediately or after removal of the pAPMA by separation on a Select-D G-50 spin column previously equilibrated with collagenase buffer (Okada et al., 1990). Activated recombinant enzyme was used as soon as possible after activation to minimize autodegradation.

Samples of latent enzyme–inhibitor complex and latent recombinant enzyme were also tested for activation following exposure to 0.1% trifluoroacetic acid. Samples of the enzymes in collagenase buffer were diluted with 2 volumes of deionized

water, 1 volume of 0.1% Brij-35, and 1 volume of 0.5% trifluoroacetic acid (final pH of a larger sample of the same buffer/acid mix was 1.95). The samples were incubated for 30 min at room temperature followed by dialysis back into collagenase buffer at 4 °C. The samples were analyzed for gelatinolytic activity and for autolytic degradative changes on SDS–polyacrylamide gels.

Enzyme Assay. Enzymatic activity was assayed by measuring degradation of [^{3}H]gelatin in a manner similar to that of Harris and Krane (1972) with the modifications as given in Kleiner et al. (1992). Degradation times were varied so that, in the majority of assays, no more than 10% of the radioactively labeled material was solubilized. Alternatively, peptidolytic activity was measured using a synthetic substrate, dinitrophenol-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, by the method of Stack and Gray (1989). The standard assay was performed in a 1-mL microcuvette using 21 mM substrate at 25 °C. The reaction was initiated by adding the enzyme, and the relative fluorescence was monitored continuously in a Perkin-Elmer LS-50B spectrofluorimeter using an excitation wavelength of 280 nm and an emission wavelength of 346 nm. The change in fluorescence was related to product formation using a standard curve based on the fluorescent product tetrapeptide Leu-Trp-Ala-D-Arg-NH_2. Protein concentrations were measured using the BCA assay system (Pierce) using bovine serum albumin as a standard and by measurement of the absorbence at 205 nm (Scopes, 1974).

Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out in a Tris/glycine Laemmli buffer system using single-percentage and gradient gels (Novex). Samples were diluted in SDS-containing sample buffer with 5–10% 2-mercaptoethanol and heated to 95–100 °C for 5 min prior to loading. High and low molecular weight standards (BRL) were used routinely for estimating molecular weight based on relative mobility. When the gel was used for Western blotting, prestained molecular weight markers (BRL) were used. Gels were stained with Coomassie Blue G-250 by the method of Neuhoff et al. (1988).

Demonstration of enzyme activity in 10% SDS gels containing gelatin (zymography) was carried out as previously described (Brown et al., 1990).

Stability of Type IV Collagenase. Samples of activated and latent type IV collagenase–TIMP-2 complex, activated and latent human recombinant type IV collagenase, and type IV collagenase reconstituted from trifluoroacetic acid were diluted in collagenase buffer to a final concentration of 0.1 mg/mL and incubated at 37 °C for 24 h. Aliquots were removed at 0, 5, 15, 30, 45, 60, 120, 360, and 1440 min and frozen immediately on dry ice. The aliquots were stored at −80 °C until they could be characterized by SDS–polyacrylamide gel electrophoretic analysis, gelatin degradative activity, and zymographic activity in SDS–gelatin gels. Aliquots of native 72-kDa type IV collagenase obtained during the dialysis of enzyme from trifluoroacetic acid into collagenase buffer were also obtained, stored, and analyzed in a similar fashion.

The stability of latent type IV collagenase–TIMP-2 complex and latent human recombinant type IV collagenase to repeated cycles of freezing and thawing was tested on enzyme samples diluted to 0.1 mg/mL in collagenase buffer. The samples were cycled for 5 min on dry ice followed by 10 min at room temperature. This was sufficient time for samples to completely freeze and thaw. Aliquots were removed for characterization after 0, 5, 10, 20, and 50 cycles of freezing and thawing.

Western Blotting. Samples of latent and activated recombinant type IV collagenase and autoproteolytic fragment obtained from type IV collagenase reconstituted from acid (40 ng each) were separated on a 4–20% Tris–glycine gel under reduced, denatured conditions and electroblotted onto Immobilon-P (Millipore). Polyclonal rabbit antibodies raised against peptide sequences in the 72-kDa type IV collagenase were use to probe the blots for immunoreactive bands. These antibodies included A1–17 and A472–490 described previously (Wacher et al., 1990) and a new antibody A371–385 prepared in an identical fashion in New Zealand white rabbits from the peptide VAAHEFGHAMGLEHSQ, which corresponds to amino acids 371–385 in the amino acid sequence of the latent 72-kDa type IV collagenase. All antibodies were affinity purified against immobilized peptide prior to use. Blots were developed using a fluorescent Western blotting detection system (Amersham).

RESULTS

Stability of the 72-kDa Type IV Collagenase at 37 °C. Samples of the native 72-kDa type IV collagenase–TIMP-2 complex, human recombinant 72-kDa type IV collagenase, and 72-kDa type IV collagenase reconstituted from the acid separation of enzyme from inhibitor (reconstituted enzyme) were examined for their susceptibility to autoactivation/autodegradation at 37 °C. Aliquots from various time points over a 24-h period were assayed for gelatinolytic activity by a radiolabeled gelatin assay and by zymography. Autodegradation was assessed on polyacrylamide gels by a Coomassie-based staining method sensitive enough to detect 10–20 ng of enzyme (Neuhoff et al., 1988). Figure 1 shows the results from the analysis of latent and pAPMA-activated enzyme–inhibitor complex. Measurement of gelatinolytic activity (Figure 1A) shows that the latent complex shows no tendency to autoactivate at 37 °C in this time period. At the end of the 24 h, the sample of latent enzyme could still be activated with pAPMA to 44% of the activity of control (enzyme–inhibitor complex removed at 0 min). The activated complex shows a slow loss of gelatinolytic activity during the 24-h incubation that has a half-time of approximately 4 h. This loss of activity is not due to the continued presence of pAPMA, since this activator was removed by dialysis prior to the beginning of the experiment. Autoproteolytic degradation was not responsible for the loss of activity since no new bands appear during the experiment on Coomassie stained protein gels (with a sensitivity limit that should have detected as little as 0.6–1.3% product formation) or on gelatin zymograms (data not shown). The possibility that TIMP-2 was interfering with the binding of gelatin to the activated enzyme was tested using the synthetic heptapeptide substrate developed by Stack and Gray (1989). In this system the initial rate of peptide cleavage also declined over 24 h with a half-time of 240 min starting from a rate of 0.061 μmol/(min·mg of enzyme–inhibitor complex). In contrast to the results seen in the solution enzyme assays, the amount of gelatinolytic activity detected by gelatin zymography did not decline significantly with time (data not shown), suggesting that the enzyme remains structurally intact over the 24-h period. No alteration in the formation of higher molecular weight complexes (Kleiner et al., 1992) was observed using either cross-linking agents or native polyacrylamide gels (data not shown), leading us to conclude that formation of high molecular weight, inactive enzyme–inhibitor aggregates does not play a role in the loss of activity. Thus, while the activated enzyme–inhibitor complex does lose activity, this loss of activity does not appear to be due to autoproteolytic

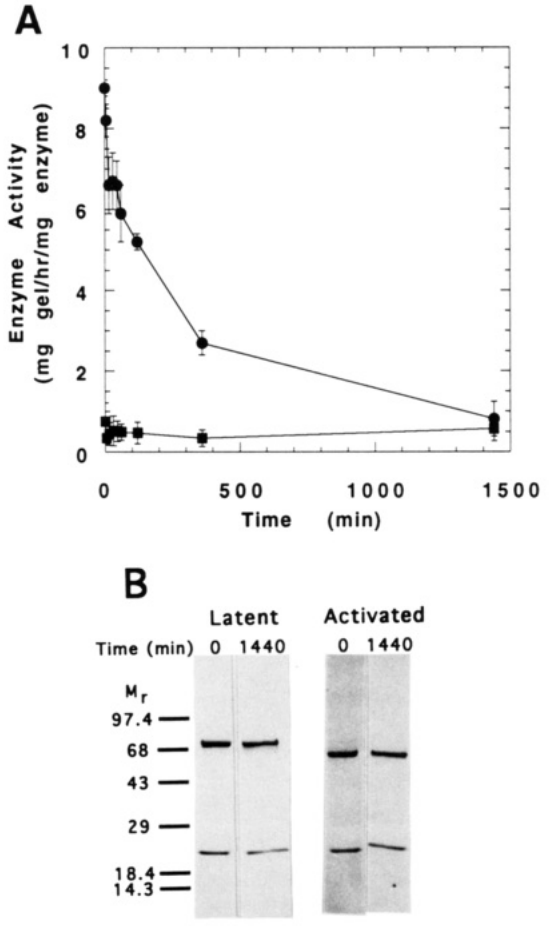

FIGURE 1: Stability of latent and activated type IV collagenase–TIMP-2 complex at 37 °C. (A) Gelatinolytic activity profile of latent and pAPMA activated type IV collagenase–TIMP-2 complex. (■) Latent complex; (●) activated complex. (B) Electrophoretic profile of the latent and activated enzyme–inhibitor complexes. Each lane was loaded with 1.5 μg of protein. Only the 0- and 24-h samples are shown; the intervening time points were identical to both the 0- and 24-h samples.

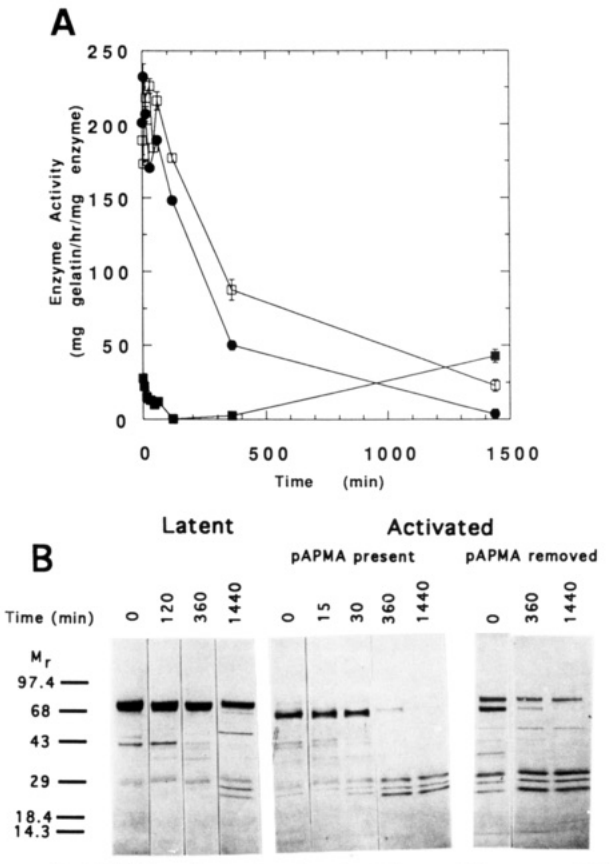

FIGURE 2: Stability of latent and activated recombinant type IV collagenase at 37 °C. (A) Gelatinolytic activity profile of latent and pAPMA activated recombinant type IV collagenase. (■) Latent enzyme; (□) activated enzyme, 1 mM pAPMA present during entire incubation; (●) activated enzyme, pAPMA removed by elution through a G-50 spin column prior to incubation at 37 °C. (B) Electrophoretic profile of the latent and activated recombinant enzyme. Each lane was loaded with 1.5 μg of protein.

degradation, aggregate formation, or denaturation. These data suggest an increase in the percentage of inhibited enzyme with time.

The results of the stability analysis for the recombinant type IV collagenase, shown in Figure 2, contrast with the results obtained with the enzyme–inhibitor complex (Figure 1). Comparison of these figures reveals that while the latent recombinant enzyme shows little autoactivation or autodegradation except after prolonged incubation, the activated recombinant enzyme undergoes rapid autodegradation. At the beginning of the experiment, the latent recombinant enzyme is not present as a single species, though the predominant band is the latent 72-kDa protein (Figure 2B, 0 min). There are two distinct bands migrating alongside and just ahead of the 43-kDa marker and fainter band that migrates slightly faster than the 29-kDa marker. These bands have calculated molecular weights of 52, 46, and 28 kDa, respectively. On the original gel the 28-kDa band appears to be composed of three distinct bands running very close together. There is a small amount of gelatinolytic activity in the preparation of latent recombinant enzyme as measured in the solution assay, about 10% of the maximum obtained upon pAPMA (activation (Figure 2A). This activity is lost over the first 6 h at 37 °C, and then between 6 and 24 h there is some autoactivation/autodegradation of the enzyme with the appearance of a small amount of a 62-kDa form as well as several low molecular weight bands on the protein gel with calculated sizes of 25 and 22 kDa. pAPMA activation of the 24-h aliquot of latent recombinant enzyme resulted in recovery of 82% of the activity of the control sample. When the latent recombinant type IV collagenase is pAPMA-activated, the majority of the enzyme is cleaved to the 62-kDa active form; however, lower molecular weight bands at 28, 25, and 22 kDa appear immediately, even with careful handling of the sample taken just after addition of pAPMA (0-min aliquot). With time the 62-kDa band slowly disappears, and the lower molecular weight bands increase in relative intensity. This occurs either when pAPMA is present for the whole time period or when it is removed by a G-50 spin column after 15 min of activation. Maximal activity appears after 15–30 min of exposure to pAPMA, after which it decays with a half-time of 4 h. Zymogram analysis reveals one active band running faster than the 62-kDa active form in the latent recombinant enzyme preparation; this increases to two or three lower molecular weight, gelatinolytically active forms in the activated preparations. The amount of gelatinolytic activity due to the 62-kDa band declines in agreement with the disappearance of this band from the Coomassie stained gels. These results are consistent with the findings of Okada et al. (1990) and Murphy et al. (1992), who noted that there was no autoactivation by the native type IV collagenase free from the inhibitor TIMP-2. We find that the latent recombinant enzyme shows no autoactivation except after 24 h at 37 °C. In contrast to the enzyme–inhibitor complex, there is considerable autodegradation of activated recombinant enzyme; new low molecular weight products appear within 5 min following the addition

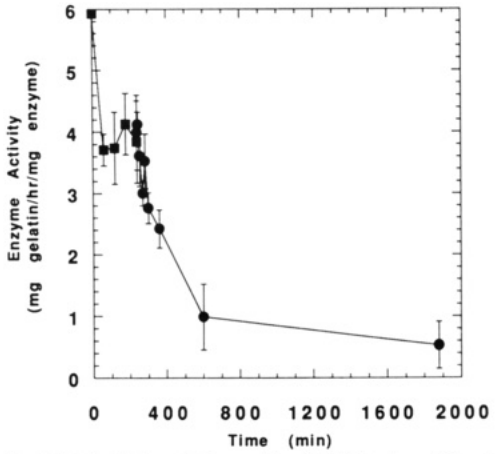

FIGURE 3: Gelatinolytic activity profile of 72-kDa type IV collagenase recovered following separation from TIMP-2 under acid conditions. The samples were not treated with pAPMA. (■) Activity during 4 h of dialysis at 4 °C; (●) activity during incubation at 37 °C.

of pAPMA. The rapidity of this process strongly suggests that TIMP-2 influences autodegradation by a mechanism distinct from simple inhibition. If the rate of autogradation was only slowed by TIMP-2 in proportion to its affect on specific activity, one would expect to see degradation products after about 2–3 h, yet even after 24 h of incubation the complex remains essentially intact.

As a final comparison to the two systems discussed above, we examined 72-kDa type IV collagenase reconstituted from trifluoroacetic acid after separation from TIMP-2 by gel permeation chromatography. The recovered enzyme was analyzed for activity before and during dialysis into neutral buffer and then during incubation at 37 °C for 24 h (Figure 3). The initial specific activity of reconstituted collagenase was only 5.9 mg of gelatin/(h·mg of enzyme). The specific activity was unchanged after exposure to 1 mM pAPMA for 15 min or 2 h, confirming previously published observations (Howard et al., 1991a). This low specific activity is in marked contrast to the reported specific activity of pAPMA-activated type IV collagenase from synovial cells (Okada et al., 1990) and also that of the activated recombinant type IV collagenase [220–230 mg of gelatin/h·mg of enzyme)]. The enzyme–inhibitor complex had an initial specific activity of 9 mg of gelatin/(h·mg of enzyme) after pAPMA activation (Figure 1). During incubation at 37 °C, the activity of the reconstituted enzyme preparation declined with a half-time of 3 h. The fragment pattern generated by autodegradation of this reconstituted preparation was also different than that seen with recombinant enzyme (Figure 4A). Prior to dialysis from acid, some lower molecular weight bands were present (data not shown). At the end of 4 h of dialysis at 4 °C into neutral buffer, there are many bands present, some which comigrate with autodegradative products from the recombinant enzyme and many which do not. The molecular weights of the most intense bands calculated from electrophoretic mobility are 50, 39, 27, 23, 17, 12, and 11 kDa. Prolonged incubation of the mixture of bands at 37 °C results in loss of higher molecular weight bands and increase in relative intensity of lower molecular weight bands, consistent with continued autodegradation. Zymography of the samples of reconstituted enzyme also shows very little digestion despite loading the gel with 10 times the amount of protein as was loaded onto adjacent lanes containing samples of enzyme–inhibitor complex and recombinant enzyme (Figure 4B). This supports the finding of much lower specific activity by the soluble gelatin assay and also suggests that the activity of the reconstituted enzyme is not

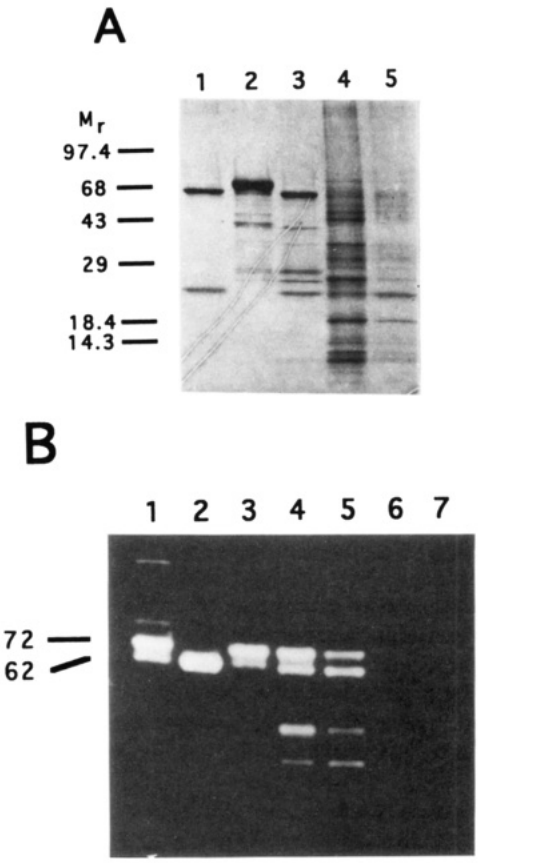

FIGURE 4: Comparison of fragmentation profiles and zymographic activity of the various enzyme preparations. (A) Fragmentation profiles on a 4–20% gradient SDS–polyacrylamide gel. Lane 1, activated enzyme–inhibitor complex, 1 μg; lane 2, latent recombinant enzyme, 1.5 μg; lane 3, activated recombinant enzyme (1 h, 37 °C), 1.5 μg; lane 4, enzyme reconstituted from acid, after 4 h of dialysis, 4.5 μg; lane 5, enzyme reconstituted from acid, dialyzed into neutral buffer, and incubated for 24 h at 37 °C, 2 μg. (B) Gelatin zymography of the various enzyme preparations. Lanes 1–5 were each loaded with 4 ng of protein, while lanes 6 and 7 were loaded with 40 ng. Lane 1, latent enzyme–inhibitor complex; lane 2, activated enzyme–inhibitor complex; lane 3, latent recombinant enzyme; lane 4, latent recombinant enzyme, 24-h incubation at 37 °C; lane 5, activated recombinant enzyme 6 h at 37 °C; lane 6, enzyme reconstituted from acid, after 4 h of dialysis; lane 7, enzyme reconstituted from acid, dialyzed into neutral buffer, and incubated for 24 h at 37 °C.

recoverable by the SDS denaturation and refolding that occurs during the processing of the zymogram. Similar quantities of enzyme–inhibitor complex and recombinant enzyme show similar amounts of gelatin digestion by this assay, despite their different specific activities by the soluble gelatin digestion assay. This is an expected result as SDS dissociates TIMP-2 from the enzyme–inhibitor complex, leaving the enzyme uninhibited during the digestion phase of the zymogram experiment. It is most important to note that enzyme recovered from the enzyme–inhibitor complex following SDS denaturation and refolding has a similar "zymographic" specific activity to recombinant enzyme also subjected to the same conditions during zymography.

The fragmentation patterns of the recombinant enzyme and the reconstituted enzyme were examined by Western blot analysis using antipeptide antibodies that recognize specific epitopes in the full length enzyme (Figure 5). These antibodies were A1–17, which recognizes the amino terminus of the latent enzyme, A371–385, which was raised against the peptide sequence of the putative metal binding domain, and A472–490, which reacts against an epitope in the C-terminal hemopexin-like domain. The significance of these antibodies

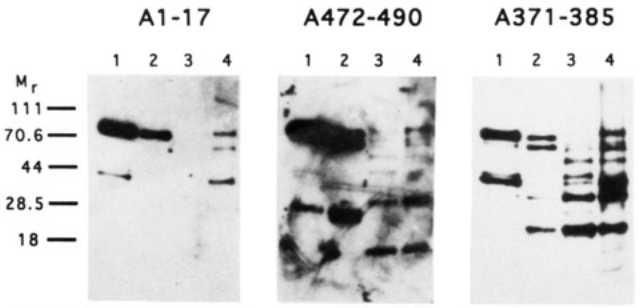

FIGURE 5: Western blotting of recombinant and acid treated type IV collagenase. Each lane was loaded with 40 ng of protein, and each blot contained the same samples in the same order. Affinity purified rabbit polyclonal antibodies were used to probe each blot as shown. Lane 1, latent recombinant enzyme; lane 2, activated recombinant enzyme; lane 3, enzyme reconstituted from acid, dialyzed into neutral buffer, and incubated for 24 h at 37 °C; lane 4, enzyme reconstituted from acid, after 4 h of dialysis.

lies in the fact that they mark different functional regions of the protein. A1–17 reacts with an epitope that is lost during the internal cleavage that follows activation. Thus, presence or absence of immunostaining for this epitope is related to activation and cleavage. The C-terminal domain has been identified as important in regulating the TIMP binding and specificity of the enzyme (Fridman et al., 1992; Howard & Banda, 1991; Murphy et al., 1992). Staining by A472–490 localizes enzyme fragments that may still contain this domain. The third antibody, A371–385, identifies fragments that may retain gelatinolytic activity and help to correlate the immunostaining pattern with the zymogram pattern. By comparing the staining pattern with the Coomassie-stained counterpart (Figure 4A), information can be obtained concerning the fragmentation pattern of the enzyme. Antibody A1–17 reacts with the 72-kDa full length enzyme, as expected (Figure 5A). It also reacts with the 50-kDa band in the reconstituted enzyme as well as a fainter intermediate band at 59 kDa. The lack of reactivity among most of the fragments generated after acid treatment is consistent with the lability of the amino-terminal propeptide. Antibody A472–490 (Figure 5B), shows reactivity with the latent and activated (62 kDa) forms of the recombinant enzyme as well as the smaller 28- and 25-kDa bands. In the lanes containing the reconstituted enzyme, antibody A472–490 reacts weakly with multiple bands, including high molecular weight latent or near full length (72, 65, and 59 kDa), intermediate molecular weight (50 and 31 kDa), and low molecular weight (17 kDa) bands.

The pattern of immunoreactivity with A371–385 is more complicated (Figure 5C), emphasizing the marked disintegration of the acid-treated enzyme. This antibody recognizes most of the recombinant enzyme fragments, with the notable exception of the 28- and the 22-kDa bands. With activation of the recombinant enzyme, the antibody recognizes the appearance of the activated 62-kDa form as well as two lower molecular weight fragments at 37 and 25 kDa. The appearance of these lower molecular weight forms correlates with the appearance of two lower molecular weight gelatinolytic bands in the zymogram analysis of activated recombinant enzyme (Figure 4B, lanes 4 and 5). Numerous immunoreactive bands are also seen in the Western blot analysis of fragments of the acid-treated enzyme, with the bands at 39 and 27 kDa reacting the most strongly. Despite the detection of this peptide epitope in numerous fragments, very little of this material was gelatinolytically active (Figure 4B, lanes 6 and 7). Interestingly, the bands smaller than 25 kDa show little immunoreactivity with this antibody in either the recombinant or acid-treated samples.

In order to try to isolate the effects of TFA treatment on the enzyme, we diluted samples of latent enzyme–inhibitor complex and latent recombinant enzyme into a TFA/Brij-35 solution so that the final concentration was 0.1% TFA and 0.2% Brij-35. The pH of the final solution was 1.95 with the amount of buffer that was present in the enzyme stock solutions. The solutions were incubated for 30 min at room temperature and then dialyzed back into neutral buffer at 4 °C. The gelatinolytic activity of the acid-treated enzyme preparations was measured both before and after exposure to pAPMA and compared to the activity of pAPMA activated preparation that had not been treated with TFA. Following acid treatment, the latent complex and the latent recombinant enzyme had 41% and 2% of the activity of pAPMA activated enzyme–inhibitor complex and recombinant enzyme, respectively. When these acid-treated preparations were pAPMA-activated, the activity of the enzyme–inhibitor complex actually fell to 16% of control, while the recombinant enzyme's activity remained essentially unchanged at 3% of control. Thus, exposing the latent enzyme–inhibitor complex to TFA resulted in partial autoactivation, while the recombinant enzyme appeared to be nearly completely inactivated by exposure to TFA. Although TFA is known to dissociate TIMP-2 from the enzyme, it may be that as the enzyme refolds during the dialysis step, TIMP-2 is able to reassociate with the enzyme and prevent significant autodegradation by enzyme activated by the acid treatment. Clearly, acid treatment does not preserve enzymatic activity in either the enzyme–inhibitor complex or the recombinant enzyme system.

Effects of Repeated Freezing and Thawing on Enzyme Stability. Because repeated cycles freezing and thawing can have a deleterious effect on both enzyme activity and enzyme stability, we examined the effects of multiple cycles of freezing and thawing on autoactivation and autodegradation of the enzyme–inhibitor complex and the recombinant enzyme. Samples of complex and recombinant enzyme were cycled between dry ice (–70 °C) and room temperature (25 °C) for up to 50 cycles. The gelatinolytic activity of each sample was measured using the radiolabeled gelatin assay both before and after pAPMA activation (Figure 6). Neither the enzyme–inhibitor complex nor the recombinant enzyme showed any tendency to undergo autoactivation under these conditions. Both showed less recoverable activity with pAPMA activation, suggesting the possibility of complete inactivation of the enzyme by repeated cycles of freezing and thawing.

DISCUSSION

Extracellular matrix degradation is a carefully controlled process that is regulated at multiple levels from regulation of gene transcription to the inactivation and degradation of the proteolytic enzymes. At the protein level, the rates of enzyme activation, enzyme degradation, and the level of inhibitors determine the amount of active enzyme available for matrix turnover. Some cell lines are known to secrete the latent form of the 72-kDa type IV collagenase as a complex with its specific inhibitor, TIMP-2. This has led to the suggestion that there might exist multiple binding sites for the inhibitor on the proenzyme and that the presence of inhibitor in the latent complex might serve some additional noninhibitory function (Goldberg et al., 1989; Stetler-Stevenson et al., 1989a). Several recent studies have attempted to better characterize the enzyme–inhibitor interaction.

A major approach by several laboratories has been to use proteolytically derived enzyme fragments or recombinant constructs that lacked key domains to define regions of the

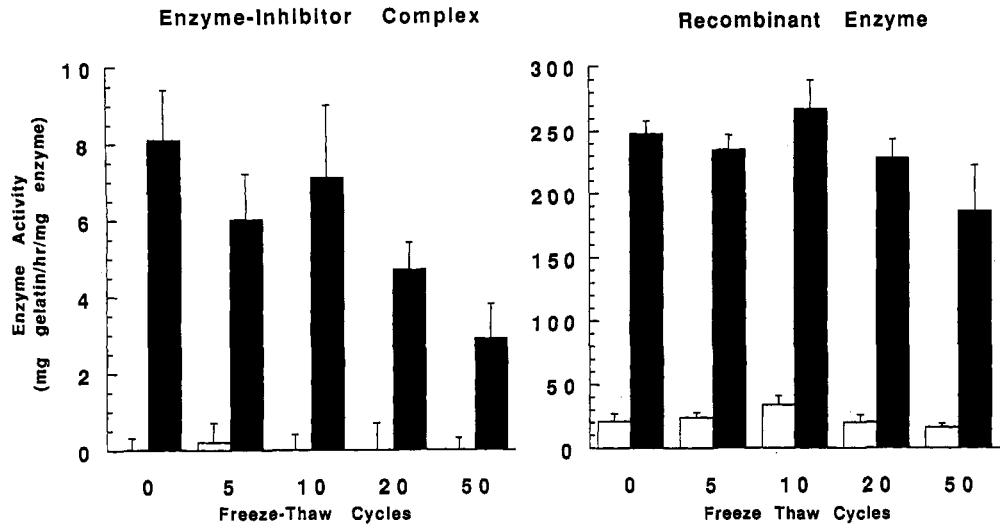

FIGURE 6: Enzyme activity following repeated freezing and thawing. Gelatinolytic activity of the latent enzyme–inhibitor complex and the latent recombinant enzyme was measured before and after pAPMA activation as noted under Methods. Open boxes indicate activity of latent preparations, and the filled boxes are activity following organomercurial activation. Note the different scales used for gelatinolytic activity of the enzyme–inhibitor complex as opposed to the recombinant enzyme.

enzyme involved in binding the inhibitor. Howard and Banda (1991) studied the binding of TIMP-2 to autoproteolytically derived fragments of the enzyme that had been recovered from acid separation of the complex and proposed the existence of a binding site within the C-terminal domain. Because the enzyme underwent some degree of autoactivation during the course of the separation, they proposed that binding of TIMP-2 to this site stabilized the latent enzyme against autoactivation. They also analyzed the binding of radiolabeled TIMP-2 to the protein fragments and showed that binding occurs both to fragments containing the active site/metal-binding domain and to the C-terminal (hemopexin-like) domain. The binding of TIMP-2 to the metal-binding domain fragment was prevented by 1,10-phenanthroline. The K_d measured for the binding of TIMP-2 to the C-terminal and active-site domain fragments was 0.42 and 0.72 nM, respectively, and so TIMP-2 interacts with each of these sites with approximately the same binding affinity.

Evidence for a binding site within the C-terminal domain is also supported by recent work on recombinant deletion mutants of the latent 72-kDa type IV collagenase (Fridman et al., 1992; Murphy et al., 1992). These papers describe the design and characterization of deletion mutants in which portions of the C-terminal domain were eliminated. These truncated enzymes were able to undergo pAPMA-induced activation associated with a decrease in mass consistent with the loss of the 80 amino acid propeptide. The mutant enzymes cleaved gelatin in both zymograms and soluble radiolabeled assays with levels of activity approximately the same as the full length recombinant enzyme. Disruption of the C-terminal domain prevented TIMP-2 binding to the latent enzyme forms. In addition, active forms of the deletion mutants were more resistant to the inhibitory effects of both TIMP-1 (Murphy et al., 1992) and TIMP-2. These results support the hypothesis that there is a site in the C-terminal domain for TIMP-2 which is necessary for binding of TIMP-2 to the enzyme and for the maximum inhibitory effect.

Previously, we have approached the analysis of the enzyme–inhibitor interaction using cross-linking reagents (Kleiner et al., 1992). Cross-linking of latent enzyme to inhibitor failed to prevent pAPMA induced activation or cleavage of the 80 amino acid amino-terminal domain. Cross-linked complex was fully capable of digesting gelatin in soluble gelatinolytic assays and in gelatin impregnated SDS gels (zymograms). After pAPMA activation, the cross-linked complex showed a specific activity for gelatin degradation that was significantly higher than uncrosslinked pAPMA activated control enzyme–inhibitor complex and was resistant to inhibition by exogenous free TIMP-2. This apparent augmentation of specific activity was ascribed to restriction of a portion of the TIMP-2 to a noninhibitory site; under these conditions activation of cross-linked latent complex resulted in a specific activity that was 30–40% of control recombinant enzyme. This is an increase in specific activity when compared with non-cross-linked activated complex which retains a specific activity that is only 5% of control recombinant enzyme in the absence of TIMP-2. When the order of cross-linking and activation was reversed, and the activated enzyme–inhibitor complex was cross-linked, there was no difference in the specific activity or TIMP-2 inhibitory profiles between the cross-linked complex and uncross-linked control. Furthermore, the cross-linked complex of activated enzyme and inhibitor showed no digestion of gelatin in the zymogram assay at the relative mobility expected for a 1:1 cross-linked complex. This is consistent with the observation that, upon activation, the active site of the enzyme is free to interact with TIMP-2. This inhibitory interaction takes place rapidly (within the time scale of the pAMPA activation) and results in enzyme which is significantly (~95%) inhibited relative to activated recombinant enzyme assayed in the absence of TIMP-2. From these studies we proposed a model for enzyme–inhibitor interaction in which there are at least two sites of interaction for the inhibitor on the enzyme. On the latent enzyme only one site is exposed and cross-linking the inhibitor to this site does not interfere with activation or enzymatic activity.

In this work, we have tried to carefully evaluate and clarify the role of TIMP-2 as it pertains to enzymatic autoactivation and autodegradation. We have found that reconstitution of the 72-kDa type IV collagenase from trifluoroacetic acid leads to the recovery of a slight amount of gelatinolytic activity and multiple proteolytic (or acid-hydrolyzed) fragments. As previously reported (Howard et al., 1991a), additional gelatinolytic activity was not recoverable after organomercurial treatment. When latent enzyme–inhibitor complex and latent recombinant enzyme were exposed to trifluoroacetic acid and then dialyzed back into neutral buffer, the gelatinolytic activity

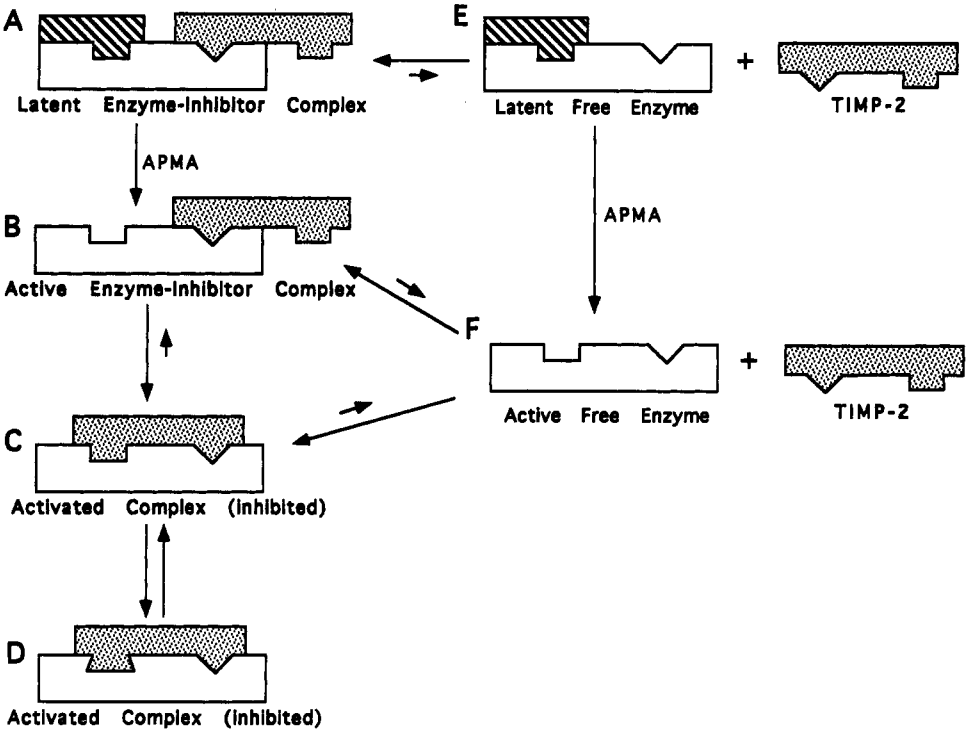

FIGURE 7: Scheme showing activation of type IV collagenase in the presence of TIMP-2. The letters A–F correspond to different enzyme or enzyme–inhibitor forms. The proteins and binding sites are represented symbolically. TIMP-2 is shown as the stippled box with two potential binding domains corresponding to sites on the active enzyme (open box). The amino-terminal domain of the type IV collagenase which is lost following organomercurial-induced activation is shown as the diagonally hatched box.

of the recombinant enzyme was almost completely abolished while the enzyme–inhibitor complex did retain some activity and underwent significant autoactivation, despite the presence of TIMP-2. It seems reasonable therefore to conclude that acid treatment of the enzyme results in denaturation and irreversible inactivation of the majority of the enzyme sample. This in turn suggests that conclusions based on such preparations should be questioned, since the majority of the sample would not represent correctly folded enzyme. This would be true even if the fragments were further purified by gelatin-affinity chromatography, since, by analogy to work done on fibronectin (Ingham et al., 1989; Skorstengaard et al., 1986), the gelatin-binding domain is probably heavily internally disulfide bonded and would be expected to retain its tertiary structure despite harsh conditions (Isaacs et al., 1989).

The stability experiments using latent and pAPMA-activated enzyme–inhibitor complex and recombinant enzyme reveal several findings. First, in agreement with the work of Okada et al. (1990), we find that the latent recombinant enzyme shows no autoactivation for the first 6 h. With prolonged (24 h) incubation at 37 °C there is partial (20%) autoactivation (Figure 2). The latent complex shows no autoactivation at all during this time period (Figure 1). Autodegradation is seen best in the experiments with pAPMA-activated enzyme (Figure 2). The recombinant enzyme begins to show low molecular weight cleavage products almost immediately, while the activated complex shows no apparent autoproteolytic activity during the 24-h incubation. This is unexpected because there is only a 20-fold difference in specific activity at the start of the experiment. If autodegradation were solely dependent on overall enzyme activity, one would predict the appearance of cleavage products from the enzyme–inhibitor complex beginning about 2 h into the experiment. Since no degradation products are observed even after 24 h at 37 °C, we must consider other explanations. Possibilities include protection of susceptible cleavage sites by TIMP-2, subtle alteration of the enzyme's substrate specificity by the presence of TIMP-2, or stabilization of the tertiary structure of the enzyme by TIMP-2 so that the cleavage sites remain hidden. Alternatively, the recombinant enzyme may be inherently less stable that the native enzyme and so might be more susceptible to autodegradation. Despite the fact that no autodegradation was observed with activated enzyme–inhibitor complex, the enzymatic activity as measured by solution methods declined over the 24-h period. The gelatinolytic activity measured by zymography remained unchanged, suggesting that the enzyme remained structurally intact or at most only underwent reversible changes. Formation of protein aggregates does not seem to play a role in the observed loss of activity based on evaluation of the complex by native polyacrylamide gel electrophoresis or cross-linking experiments. We would propose that the loss of activity observed upon incubation of the activated enzyme–inhibitor complex at 37 °C is due to the formation of a more stable interaction. This would account for the reversibility of the inhibition by zymography, since that procedure separates the enzyme from the inhibitor. It would also suggest that the observation of similar half-times for loss of activity in both the enzyme–inhibitor complex and recombinant enzyme is coincidental rather than due to a similar mechanism. Despite the actual mechanism involved, the observation of activity decline has particular relevance for experimental protocols which make use of prolonged enzyme incubations following activation. In this study we tried to use the shortest gelatinolytic assay times consistent with a reasonable amount of experimental error.

On the basis of these results and on previously published work by our laboratory and others, we represent a model for the interaction of TIMP-2 and type IV collagenase before and after organomercurial-induced activation (Figure 7). The scheme shows two binding sites on both TIMP-2 and type IV collagenase. On the enzyme, there is a C-terminal domain

site available on the latent enzyme (Fridman et al., 1992; Howard & Banda, 1991; Murphy et al., 1992), represented by the triangular indentation, and an inhibitory site available only on the active enzyme (Kleiner et al., 1992), represented by the rectangular indentation. TIMP-2 is shown as having two distinct binding sites corresponding to the sites on type IV collagenase. This seems reasonable based on cross-linking experiments and on evidence that latent type IV collagenase–TIMP-2 complex can act as an inhibitor (Kolkenbrock et al., 1991). Enzyme–inhibitor complexes are in equilibrium with free enzyme and TIMP-2 (Figure 7, transitions A to E, B to F, and C to F), and as the relative size of the reaction arrows indicates, complex formation is favored over dissociation. This equilibrium has been directly shown by exchange experiments using radiolabeled TIMP-2 (Howard et al., 1991b), and equilibrium constants have been determined both kinetically (Murphy et al., 1992) and by radiolabeled TIMP-2 binding experiments (Howard & Banda, 1991). Organomercurial activation (transitions A to B and E to F) results in loss of the 80 amino acid propeptide via an autoproteolytic cleavage (Stetler-Stevenson et al., 1989b). Cross-linking of TIMP-2 to the C-terminal domain site on the latent enzyme does not interfere with this reaction (Kleiner et al., 1992). After activation, the enzyme–inhibitor complex is now in equilibrium between inhibited and noninhibited forms of the complex and with free enzyme and inhibitor (transitions B to C, B to F, and C to F). The equilibrium between noninhibited forms of the complex (B) and inhibited forms (C) favors inhibition. This is shown by comparison of cross-linked-then-activated complex to activated-then-cross-linked complexes on gelatin zymograms (Kleiner et al., 1992). The relative contribution to proteolytic activity of the free enzyme (F) versus the noninhibited complex (B) is not known. Following activation, the free enzyme undergoes autoproteolytic cleavage to active and inactive fragments associated with a decline in specific activity. This pathway is not observed in the presence of a stoichiometric equivalent of TIMP-2. Activation of the enzyme–inhibitor complex is also associated with a slow first order loss of activity, without evidence of autoproteolytic activity or aggregate formation. A possible explanation for this result is slow formation of a more stable, inhibited form of the complex (transition C to D). We have chosen to show TIMP-2 interacting with both sites simultaneously, which has not been definitively shown by experimental evidence. However, truncated forms of the enzyme which lack the C-terminal site are not inhibited as well as the full-length enzyme (Fridman et al., 1992; Murphy et al., 1992), suggesting that binding to the inhibitory site is adversely affected by absence of the C-terminal site. For simplicity, we have also shown only one molecule of TIMP-2 interacting with the two binding sites on the activated enzyme although it is also possible that two molecules of TIMP-2 could bind simultaneously. However, cross-linking studies using both the latent and activated enzyme have not demonstrated binding of a second molecule of TIMP-2 to the enzyme. We feel that this model is consistent with the experimental data in this work and with previously published data, and it forms a basis for further experiments evaluating the interaction between the 72-kDa type IV collagenase and TIMP-2.

From the data presented, we conclude that TIMP-2 does not have a significant role in preventing the autoactivation of the latent 72-kDa type IV collagenase. Instead, the data suggest that TIMP-2 may have a role in modulating the autoproteolytic degradation of the enzyme. The physiological relevance of the inhibition of this autodegradative activity has not yet been determined; however, one might hypothesize a dual mode of action for the enzyme depending on the presence or absence of TIMP-2. When TIMP-2 is not present, as may be the case for synovial cells (Okada et al., 1990), or is present in less than stoichiometric amounts (Ward et al., 1991), secretion and activation of limited amounts of enzyme might result in a burst of high catalytic activity that is subject to rapid autodegradation of the enzyme. If some autoproteolysis results in active enzyme fragments which lack the C-terminal domain, these would be expected to be resistant to inhibition by TIMP-2 (Murphy et al., 1992). However, when secreted as an enzyme–inhibitor complex, as is observed in cultures of human melanoma and bovine endothelial cells, activation of the proenzyme–inhibitor complex would result in a low level of matrix degrading activity that is resistant to rapid autodegradation.

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