Higher-Order Complex Formation between the 72-Kilodalton Type IV Collagenase and Tissue Inhibitor of Metalloproteinases-2

David E. Kleiner, Jr., Edward J. Unsworth, Henry C. Krutzsch, and William G. Stetler-Stevenson*

Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20892

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ABSTRACT: The collagenases are a class of matrix degradative enzymes whose actions are important in physiological and pathological processes. The human 72-kDa type IV collagenase (matrix metalloproteinase-2) and its proteinase inhibitor, tissue inhibitor of metalloproteinases-2 (TIMP-2), are produced as a proenzyme–inhibitor complex by numerous cell lines. We analyzed the quaternary structure of and enzyme–inhibitor interactions in the native enzyme–inhibitor complex by studying the pattern of complexes demonstrated by molecular weight determination in nondenaturing polyacrylamide gels and evaluating the products formed by reaction of the native complexes with cross-linking agents. Electrophoresis in native polyacrylamide gels demonstrates that approximately 79% of the latent enzyme is present in a 1:1 bimolecular complex with the inhibitor TIMP-2, with 21% present as a complete tetrameric complex of two molecules of collagenase combined with two molecules of TIMP-2. The enzyme complex activated with organomercurials displays a shift to a higher proportion of the bimolecular complex with only 5% present as higher molecular weight complexes. Cross-linking of the latent and active forms of the complex with bis(sulfosuccinimidyl) suberate (BS^3) and bis(sulfosuccinimidyl) tartarate demonstrates both the 1:1 and 2:2 complexes as well as an intermediate form that appears to be a complex composed of two molecules of collagenase and one of TIMP-2. The distribution of cross-linked products is unchanged with the addition of excess TIMP-2 to the reaction mix, implying that the binding sites for TIMP-2 to the initial enzyme–inhibitor complex are all occupied when the stoichiometry is 1 to 1. Cross-linking of the latent complex does not prevent organomercurial-induced activation and loss of an 80 amino acid prosegment. These cross-linked, activated complexes retain the ability to degrade gelatin in radiolabeled gelatin assays with the BS^3-cross-linked, activated complex having a 7-fold higher specific activity than the un-cross-linked control. The BS^3-cross-linked, activated complex is resistant to inhibition by free, exogenous TIMP-2. These results strongly suggest that there are two binding sites for TIMP-2 on collagenase and that binding to one site may decrease the affinity of binding of TIMP-2 to the second site.

Regulation of enzymes involved in the synthesis and degradation of extracellular matrix components has come under intense scientific scrutiny. The control of extracellular matrix degradation is important in numerous physiologic and pathologic processes, including ovulation, implantation of the fertilized ovum, wound repair, bone remodeling, scar formation, and tumor invasion and metastasis (Reddi, 1984; Liotta & Stetler-Stevenson, 1990; Matrisian, 1990; Woessner, 1991). Regulation of this process occurs at many levels with control of transcription, translation, secretion, and activation of the various degradative enzymes. At the protein level, in addition to control of the activation of latent proteinases, there are protein inhibitors of the active enzymes (Stricklin & Welgus, 1983; Stetler-Stevenson et al., 1989a). Numerous enzymes have been implicated as key elements in the role of extracellular matrix degradation, and among these are a class of metalloenzymes known as the collagenases.

The collagenase enzyme family, also known as the matrix metalloproteinases, are a group of proteinases with homologous structural domains and similar modes of activation and activity (Docherty & Murphy, 1990; Stetler-Stevenson, 1990). All exist in both latent and active forms, the latent form losing an approximately 80 amino acid segment from the amino-terminal end of the protein following chemical or enzymatic activation. Within this prosegment is a highly conserved amino acid sequence, P-R-C-G-V/N-P-D, containing an unpaired cysteine residue. These metalloproteinases also share a highly conserved region homologous to the zinc binding domain of thermolysin (Whitham et al., 1986). Previous studies from a number of different laboratories have resulted in a hypothesis for the activation of all metalloproteinases that suggests that the unpaired cysteine residue in the prosegment is coordinated to the zinc atom in the latent enzyme and that disruption of this interaction results in enzyme activation and freeing of a coordination site around the zinc (Stetler-Stevenson et al., 1989b, Nagase et al., 1990; Springman et al., 1990). In vivo mechanisms of activation have been proposed for some of the matrix metalloproteinases, specifically fibroblast interstitial collagenase and stromelysin-1 (He et al., 1989; Nagase et al., 1990), but little is known about the in vivo control of activation for the other members of this class.

In vivo activity of the matrix metalloproteinases also appears to be modulated by the presence of protein inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs).[1] Two different TIMPs have been identified and sequenced; they are known as TIMP-1 (Stricklin & Welgus, 1983) and TIMP-2 (Stetler-Stevenson et al., 1989a; DeClerck et al., 1989) in order of their discovery. They share approximately 40% homology

* Address correspondence to this author at the Laboratory of Pathology, National Cancer Institute, Building 10, Room 2A33, Bethesda, MD 20892.

[1] Abbreviations: TIMP, tissue inhibitor of metalloproteinases; BS^3, bis(sulfosuccinimidyl) suberate; sulfo-DST, bis(sulfosuccinimidyl) tartarate; SDS, sodium dodecyl sulfate; APMA, aminophenylmercuric acetate; EDTA, ethylenediaminetetraacetic acid.

at the amino acid level and are highly internally disulfide-bonded (Williamson et al., 1990). Both inhibitors are smaller than the collagenases, being 28 and 21 kDa, respectively. TIMP-1 and TIMP-2 show inhibitory activity against multiple members of the matrix metalloproteinase family, including interstitial collagenase, stromelysin, and the 72- and 92-kDa type IV collagenases (Docherty & Murphy, 1990). They are also found in association with two latent members of this enzyme group, the 92-kDa and the 72-kDa type IV collagenase, respectively (Stetler-Stevenson et al., 1989a; Goldberg et al., 1989; Wilhelm et al., 1989). In this study, we have focused on the interaction between the 72-kDa type IV collagenase and TIMP-2. This enzyme and inhibitor pair are secreted as a complex from a number of normal and tumor human cell lines, including A2058 melanoma cells, HT1080 fibrosarcoma cells, RPMI-7951 melanoma cells, WI38 fibroblasts, HT-144 melanoma cells, Malme-3M melanoma cells, and A549 lung carcinoma cells (Stetler-Stevenson, unpublished work). The complex formed is tight; denaturation (acid, organic or detergent) is required to separate the inhibitor from the enzyme (Goldberg et al., 1989; Stetler-Stevenson et al., 1989a). Although the presence of the TIMP-2 does not prevent the chemical activation of the enzyme, addition of free TIMP-2 does inhibit the enzymatic activity (Goldberg et al., 1989; Stetler-Stevenson et al., 1989a). These observations beg the question of how TIMP-2 binds to the enzyme and affects activity. Previous experiments have suggested that the enzyme and inhibitor exist in a complex with an overall equimolar ratio (Stetler-Stevenson et al., 1989a; Goldberg et al., 1989); however, these same studies also suggest the possibility of two TIMP-2 binding sites: one, an active-site interaction that inhibits catalytic activity and two, a separate site with a possible role in stabilizing the latent enzyme to autoactivation and with unknown effects on catalytic activity (Howard et al., 1991; Howard & Banda, 1991). Unanswered are questions concerning the interaction of these sites with each other, as well as interactions between enzyme–inhibitor complexes to form discrete aggregates of higher stoichiometry than 1:1. In this study, we examine the native state of latent and chemically activated 72-kDa type IV collagenase–TIMP-2 complex using chemical cross-linking methods and polyacrylamide gel electrophoresis under nondenaturing conditions in order to address these questions as well as to develop methodology that provides a way to further characterize the native enzyme–inhibitor interaction.

Materials and Methods

Materials

Molecular weight standards for native polyacrylamide gel electrophoresis were from Sigma. Molecular weight standards for SDS gel electrophoresis were from BRL. Bis(sulfosuccinimidyl) suberate (BS^3) and bis(sulfosuccinimidyl) tartarate (sulfo-DST) were from Pierce and were used without further purification. SDS–polyacrylamide gels and 10% SDS–gelatin gels were from Novex. The human 72-kDa type IV collagenase–TIMP-2 complex was purified from A2058 melanoma cell conditioned media as previously described (Stetler-Stevenson et al., 1989b). The purified complex was greater than 98% homogeneous with respect to the 72-kDa type IV collagenase and TIMP-2 on reduced SDS–polyacrylamide gels. Gelatin affinity purified, recombinant human 72-kDa type IV collagenase from baculovirus was the gracious gift of Dr. Karl Tryggvason.

Methods

Activation of Type IV Collagenase. Samples of collagenase were activated in the presence of 1 mM aminophenylmercuric acetate (APMA) for 1 h at 37 °C and then prepared for cross-linking experiments by dialysis against cross-linking buffer (50 mM HEPES, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35, pH 7.5) at 4 °C. Collagenase complex activated in this fashion showed essentially complete conversion to the activated 62-kDa form on reduced SDS–polyacrylamide gels. Recombinant collagenase was activated in the presence of 1 mM APMA for 5 min at 37 °C and used immediately.

Enzyme Assay. Enzymatic activity was assayed by measuring degradation of ^{3}H-gelatin in a manner conceptually similar to that of Harris and Krane (1972). Briefly, tritiated type I collagen (NEN) was diluted with cold type I collagen and then denatured at 55 °C for 25 min. Enzyme samples and gelatin (at a final assay concentration of 0.2 mg/mL, 5.8 μCi/mg) were added to buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35, pH 7.5) to a final volume of 220 μL. The reaction was incubated at 37 °C and stopped by the addition of 20 μL of 250 mM EDTA. Proteins were precipitated on ice with the addition of 60 μL of 10% trichloroacetic acid/0.05% tannic acid. The soluble peptide fragments were counted in a liquid scintillation counter.

Experiments evaluating the inhibition of cross-linked complexes by TIMP-2 were performed using an assay enzyme concentration of 0.80 μg/mL protein (9.6 nM for un-cross-linked enzyme–inhibitor complex). Recombinant enzyme tested for inhibition by TIMP-2 was also used at a concentration of 0.73 μg/mL (10 nM) in the assay. Assays were prepared by combining buffer, radiolabeled gelatin, and TIMP-2 (at 0, 5.5, 11, 22, 55, and 110 nM final concentration). Enzyme was added to initiate the reaction, and activity was measured as described above.

Demonstration of the activity in 10% SDS gels containing 1% gelatin (zymography) was carried out as previously described (Brown et al., 1990). Protein concentrations were determined using the Bio-Rad dye binding assay and by measurement of the absorbance at 205 nm (Scopes, 1974).

Purification of TIMP-2. TIMP-2 was separated from the complex by methods similar to those previously described (Stetler-Stevenson et al., 1989a) with the replacement of reverse-phase chromatography by gel filtration. Samples of gelatin affinity purified, latent enzyme–inhibitor complex were diluted with 4 volumes of 0.1% trifluoroacetic acid and 10% acetonitrile and then eluted through a TSK-Gel G3000SW-XL column (Tosohaas) in a DIONEX BIOLC (0.5 mL/min, 0.1% trifluoroacetic acid/10% acetonitrile mobile phase). The enzyme eluted at 15.5 min and TIMP-2 eluted at 20.5 min under these conditions. Fractions containing TIMP-2 were lyophilized and resuspended in 50 mM Tris, pH 7.5.

Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out in a Tris/glycine Laemmli buffer system using prepared single-percentage and gradient gels (Novex). Samples were diluted in SDS-containing sample buffer with 5–10% 2-mercaptoethanol and heated to 95 °C for 5 min prior to being loaded. High molecular weight protein standards (BRL) were used routinely to estimate molecular weights on the basis of the relative mobility. When the gel was to be used for Western blotting, prestained high molecular weight protein standards (BRL) were used. Electrophoresis was carried out at 20-mA constant current until the bromphenol blue dye marker reached the bottom of the gel.

Nondenaturing discontinuous polyacrylamide gels were prepared in a Laemmli buffer system lacking SDS with separating gels containing eight different concentrations of acrylamide (4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, and 10.0%).

Samples and standards (lactalbumin, carbonic anhydrase, ovalbumin, bovine serum albumin, and urease) were applied to the top of each gel, and the electrophoresis was carried out at 20 mA/gel constant current until the dye front approached the bottom of the gel. The distance to the dye front and the length of the gel were recorded prior to staining the gels with Coomassie Blue G-250 (Neuhoff et al., 1988). The molecular weights of the unknown bands were calculated from the relative mobilities in the different gel concentrations (Rodbard & Chrambach, 1971).

Laser scanning densitometry was performed on dried gels using an LKB 2202 Ultrascan laser densitometer set for the slowest scan rate (6 mm/min) and an LKB 2226 recording integrator.

Cross-Linking Reactions. Protein samples were prepared for cross-linking by dialysis against cross-linking buffer (as above). Stock solutions of the cross-linking reagents (BS^3 and sulfo-DST) were prepared fresh in cross-linking buffer immediately prior to use. In a typical reaction, proteins, buffer, and cross-linking reagent were combined in a polypropylene tube, and the reaction was allowed to proceed for 15 min at room temperature. The reaction was stopped and excess cross-linking reagent consumed by the addition of a 0.1 volume of 50 mM HEPES/50 mM 2-aminoethanol, pH 7.5. The reaction products were analyzed by SDS–polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol.

Amino Acid Analysis. Cross-linked enzyme was prepared by the procedure given above using an enzyme complex concentration of 5 μM (based on a calculated molecular mass of 93 kDa for the latent complex and 83 kDa for the active complex) and a BS^3 concentration of 1 mM. Samples of cross-linked complex were isolated for amino acid analysis by SDS electrophoresis in 8% gels followed by electroelution of Coomassie R-250 stained bands in a Bio-Rad Model 422 electroelutor using a dialysis membrane trap with a 12 000-dalton cutoff. The electroelution buffer was 50 mM ammonium bicarbonate/0.1% SDS, and the electrophoresis was carried out at 10 mA per tube constant current for 5.5 h. SDS was removed from the sample (Henderson, 1979), and samples of 1–1.5 μg were subjected to amino acid analysis as previously described (Stetler-Stevenson et al., 1989a).

Western Blot Analysis. Western blot analysis was carried out as previously described (Brown et al., 1990) using rabbit polyclonal antibodies raised against TIMP-2 and two synthetic peptides, A1–17 and A472–490 (amino acids 1–17 and 472–490 of latent type IV collagenase) (Wacher et al., 1990).

Results

Native Molecular Weight Determination. The native molecular weights of free TIMP-2, latent collagenase–inhibitor complex, and APMA-activated collagenase–inhibitor complex were evaluated on native polyacrylamide gels at eight different concentrations of acrylamide. The latent type IV collagenase–TIMP-2 complex migrated as three distinct bands at high gel concentrations (Figure 1, lane 1); at lower gel concentrations, the two faster migrating bands merged. Activated type IV collagenase complex migrated as three distinct bands at all tested gel concentrations (Figure 1, lane 2). TIMP-2 migrated as two bands with a connecting smear (Figure 1, lane 3). The identity of these assignments was confirmed by Western blots of 7% native polyacrylamide gels using antibody against TIMP-2, A1–17, and A472–490. Anti-TIMP-2 reacted with all of the Coomassie-stained bands, while anti-A472–490 reacted only with the bands in the active and latent collagenase lanes (data not shown). Anti-A1–17 reacted only with bands in the latent collagenase lane, consistent with the loss of this peptide epitope upon activation with APMA (Stetler-Stevenson et al., 1989b).

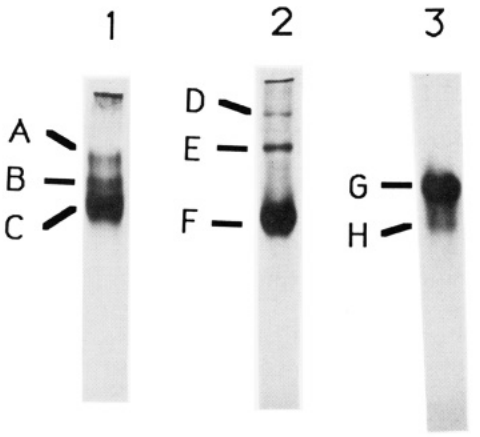

FIGURE 1: Migration of enzyme–inhibitor complex and free inhibitor in an 8% native polyacrylamide gel. Lane 1, latent collagenase–TIMP-2 complex showing three bands (A–C); lane 2, APMA-activated collagenase–TIMP-2 complex showing three bands (D–F); lane 3, TIMP-2 alone showing two bands (G and H).

Table I: Calculated Molecular Masses from Native Polyacrylamide Gel Electrophoresis and Relative Percentages of Each Complex

	protein bands (see Figure 1)	mol mass (kDa)[a]	integrated area (%)
latent complex	A	193 (35)	21
	B	82 (14)	79[b]
	C	103 (18)	
active complex	D	420 (80)	1
	E	183 (35)	4
	F	82 (14)	95
TIMP-2	G	30 (5)	*b*
	H	29 (5)	

[a] Values of standard error are given in parentheses. [b] The scanning densitometer was unable to separate bands B and C and also bands G and H.

The logarithm of the relative mobilities of each of the standards and samples was plotted against gel concentration to obtain the retention coefficient (slope of this line) for each band. The logarithm of the retention coefficients of the standards was then plotted against the logarithm of their molecular weights to obtain a standard curve (Ferguson plot) for the unknown samples. Molecular masses calculated from this standard curve are shown in Table I for each band of the three samples. Although separate molecular mass determinations were carried out for bands B and C and for bands G and H, these pairs of bands probably represent molecular species with the same molecular mass and different charges.

Laser scanning densitometry was used to estimate the relative abundance of each of the higher molecular mass complexes. The percentages of total integrated intensity for each lane (as measured by scanning of the 8% native gel) are indicated in Table I. Despite a slow scan rate, the laser scanner was not able to separate bands B and C (latent collagenase complex) and bands G and H (TIMP-2). In the latent complex, at a protein concentration of 1 mg/mL, about one-fifth of the sample migrated at a molecular mass consistent with a heterotetramer, while the active complex, at the same protein concentration, showed only 5% higher molecular mass complexes (bands D and E).

Cross-Linking of the Latent Enzyme–Inhibitor Complex. In order to further characterize the interaction of the collagenase and TIMP-2, the enzyme–inhibitor complex was cross-linked using two homobifunctional cross-linking agents

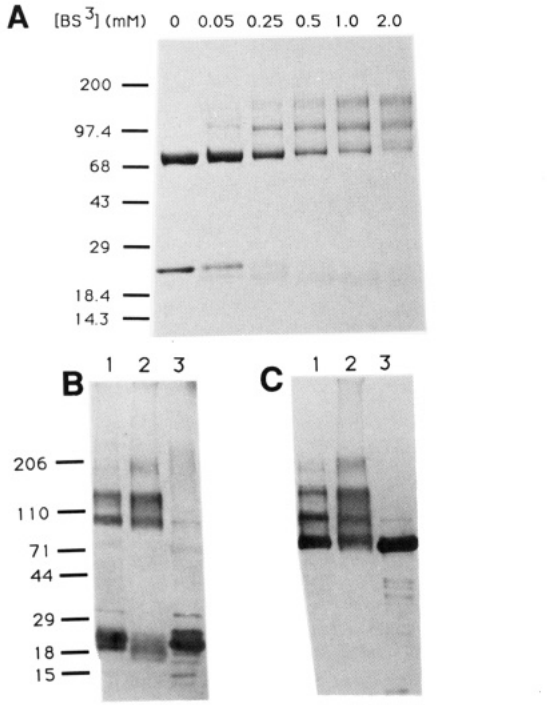

FIGURE 2: Analysis of cross-linking of latent complex by BS^3 on a 4–20% gradient gel. (A) Cross-linking of 5 μM complex by progressively increasing concentrations of BS^3. Positions of molecular mass standards (in kilodaltons) are given along the side. When no BS^3 is added (left lane), the complex dissociates into collagenase (72 kDa) and TIMP-2 (21 kDa). (B and C) Western blots of cross-linked and un-cross-linked latent complex using anti-TIMP-2 (B) and anti-A1–17 (C). Lane 1, latent complex cross-linked with 5.0 mM sulfo-DST; lane 2, latent complex cross-linked with 2.0 mM BS^3; lane 3, un-cross-linked latent complex. Approximate molecular masses of prestained molecular mass standards are given in kilodaltons.

that form amide bonds with primary amino groups. These agents were bis(sulfosuccinimidyl) suberate (BS^3), a non-cleavable cross-linker with an 11.4-Å spacer, and bis(sulfosuccinimidyl) tartarate (sulfo-DST), a cleavable cross-linker with a 6.4-Å spacer. Reactions of cross-linkers with the complex were evaluated using varying concentrations, temperatures, and incubation times. Incubation times longer than those used for routine experiments (15 min) did not alter the product distribution as detected on Coomassie-stained gels. Increasing the reaction temperature to 37 °C or lowering it to 4 °C changed the reaction rate without significantly altering the product distribution (data not shown). Conditions used for routine cross-linking at room temperature used concentrations of 1–2 mM BS^3 or 5 mM sulfo-DST with a 15-min incubation time. This represents a 200–1000-fold molar excess of cross-linking reagent over enzyme complex. Figure 2A shows the results of a cross-linking experiment in which the latent complex was exposed to varying concentrations of BS^3 and then analyzed by reduced SDS electrophoresis on a 4–20% gradient gel. Three new bands appear as the concentration of BS^3 is increased: two prominent bands at approximately 100 and 140 kDa and one very faint band at 200 kDa. The TIMP-2 band gradually disappears, and the latent collagenase band diminishes and shifts to a slightly higher molecular mass (75–80 kDa). We estimated the relative amounts of the products cross-linked by 2.0 mM BS^3 (sixth lane, Figure 2A) using scanning densitometry and automated integration. This method gives the following relative peak areas: TIMP-2, 3.8%; 75 kDa, 22.1%; 100 kDa, 33.3%; 130 kDa, 34.7%; 200 kDa, 6.1%. In contrast to cross-linking by BS^3, sulfo-DST cross-linking yields only 5–10% intermolecularly cross-linked complexes (mainly the 100-kDa complex), with the balance of the products being intramolecularly cross-linked collagenase and TIMP-2.

Table II: Leucine:Isoleucine Ratios for Isolated, Cross-Linked Complexes and Corresponding Predicted Collagenase:TIMP-2 Ratios

app mol mass (kDa)	Leu:Ile ratio	predicted collagenase:TIMP-2 ratio
75	1.43	>3.5
100	1.10	1.0
140	1.34	2.1

Molecular weight determination in SDS gels depends on the assumption that all of the unknowns and standards have the same charge-to-mass ratio and the same three-dimensional shape. Since intra- and intermolecular cross-links prevent complete unwinding of the peptide backbone by SDS even in reduced SDS gels, and because each cross-linking reaction adds to the molecular weight of the protein, the molecular weight assignments of the cross-linked proteins may not be representative of their true molecular weight. Preliminary assignments of the identities of the three new bands following treatment of type IV collagenase and TIMP-2 complex with cross-linking agents were made as follows (predicted molecular masses in parentheses). The 100-kDa band is thought to be a 1:1 complex of collagenase to TIMP-2 (93 kDa), a complex identified previously on the basis of amino acid composition analysis of native enzyme–inhibitor complex (Stetler-Stevenson et al., 1989a). The 200-kDa band represents a 2:2 complex of collagenase to TIMP-2 (186 kDa) as seen in native polyacrylamide gels. The band seen at 140 kDa represents some intermediate complex such as a 2:1 collagenase–TIMP-2 complex (165 kDa), a 1:2 collagenase–TIMP-2 complex (114 kDa), or a type IV collagenase dimer (144 kDa).

To sort out these possibilities and confirm the stoichiometric assignments, the cross-linked complexes were analyzed by Western blot (Figure 2B,C) and by amino acid analysis following isolation of the 75-, 100-, 140-, and 200-kDa bands by electroelution. The Western blot with anti-TIMP-2 demonstrates the presence of TIMP-2 in the 100-, 140-, and 200-kDa bands as well as bands at the level of un-cross-linked TIMP-2. The blot using anti-A1–17 as the primary antibody shows the presence of the latent collagenase in the bands at 75, 100, 140, and 200 kDa.

The leucine:isoleucine ratio was used to evaluate the ratio of collagenase to TIMP-2 in the amino acid analysis (Stetler-Stevenson et al., 1989a). TIMP-2 contains a high number of these hydrophobic residues in the opposite ratio to the latent type IV collagenase. The large difference in the ratio from TIMP-2 (0.39) to the 1:1 collagenase–TIMP-2 complex (1.10) to the latent collagenase (1.56) as well as the relative stability of leucine and isoleucine to the conditions of amino acid analysis makes this ratio a useful indication of the stoichiometry of an enzyme–inhibitor complex. The leucine:isoleucine ratio for the isolated bands and the corresponding predictions of the TIMP-2:collagenase ratio are summarized in Table II. The amount of protein collected for the 200-kDa band was too small to give a reliable amino acid analysis. This analysis confirms the preliminary assignments of the 75- and 100-kDa bands and suggests a complex composed of a 2:1 ratio of collagenase to TIMP-2 for the 140-kDa band.

Cross-Linking of the Complex in the Presence of Added TIMP-2. The activated complex is inhibited by the addition of exogenous TIMP-2, suggesting the possibility that TIMP-2 interacts with the enzyme at more than one site (Stetler-Stevenson et al., 1989a; Goldberg et al., 1989). Cross-linking the complex in the presence of added TIMP-2 might demon-

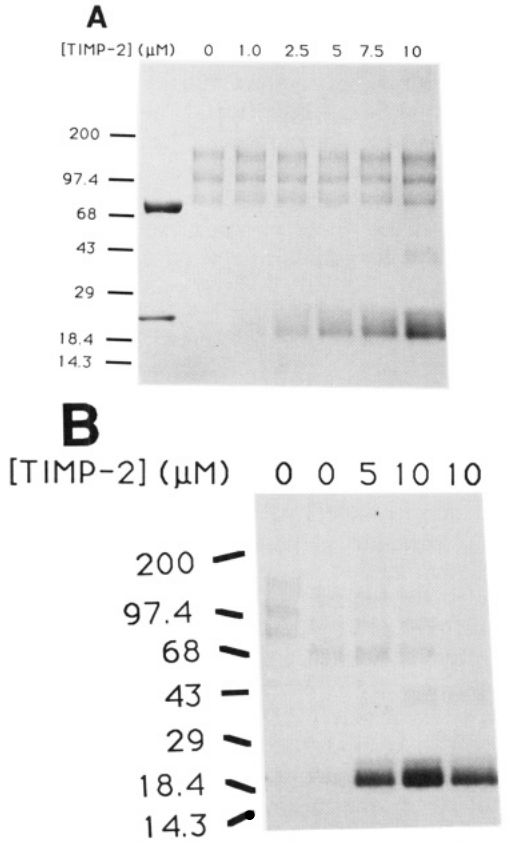

FIGURE 3: Cross-linking of latent and APMA-activated complex in the presence of exogenous TIMP-2. (A) Latent complex. The BS^3 concentration was 2 mM and the latent complex concentration was 5 μM. The first lane shows un-cross-linked complex for comparison. (B) APMA-activated complex. Concentrations of BS^3 and enzyme complex as in (A). The first lane shows cross-linking of latent complex, and the last lane shows cross-linking of 10 μM TIMP-2 alone for comparison.

strate multiple binding sites by a change in the pattern of cross-linked complexes. Thus, samples of latent and APMA-activated complex (5 and 3 μM, respectively) were cross-linked with BS^3 (2 mM) with and without added TIMP-2 (1–10 μM). The complex was incubated for 15 min at room temperature with the additional TIMP-2 prior to addition of the cross-linking agent. The results of these experiments are shown in Figure 3A (latent) and Figure 3B (activated). Note that when activated complex is cross-linked, the same pattern of higher molecular weight bands is seen, though each band is shifted to a lower molecular weight, reflecting the loss of the 80 amino acid prosegment during activation. At the higher concentrations of added TIMP-2, a new band develops at 43 kDa, consistent with the formation of a TIMP-2 dimer. Although the intensity of staining of the bands varies somewhat from lane to lane, the relative ratio between the bands of any given lane appears to remain unchanged. The visual impression is confirmed by laser scanning densitometry. Thus, this method fails to demonstrate the presence of a second binding site for the TIMP-2 on active or latent type IV collagenase.

Activation of the Collagenase Complex following Cross-Linking. Latent complex was activated with APMA for 1 h at 37 °C following cross-linking with either BS^3 or sulfo-DST. A control sample was treated in the same fashion except for the addition of the cross-linker. The results are shown in Figure 4. In each case, following activation, there is a reduction in the molecular weight of each band consistent with loss of the prosegment, indicating the presence of autoproteolytic activity. When equivalent samples of activated

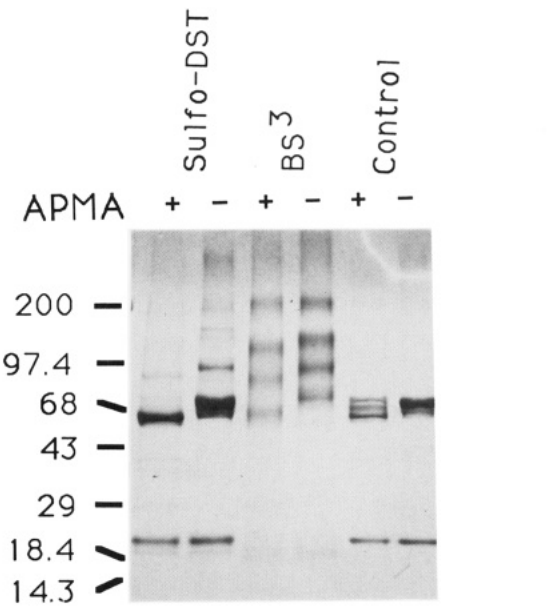

FIGURE 4: APMA activation of previously cross-linked complexes. Samples were incubated for 1 h at 37 °C with 1 mM APMA prior to being loaded. All samples were treated in an identical fashion except for the addition of the cross-linking agents.

cross-linked and non-cross-linked complex were tested for their ability to solubilize gelatin in the radiolabeled gelatin digestion assay, the measured activities were 14 (non-cross-linked enzyme), 96 (BS^3-cross-linked), and 29 (sulfo-DST-cross-linked) mg of gelatin h^{-1} (mg of enzyme)$^{-1}$. For comparison, APMA-activated human recombinant 72-kDa (type IV collagenase had a specific activity of 186 mg of gelatin h^{-1} (mg of enzyme)$^{-1}$. Measured rates of control samples not treated with APMA were less than 1% of these values for each experiment. Thus, prior cross-linking of the complex inhibits neither the APMA-induced activation of the enzyme complex nor the proteolytic cleavage of a complex substrate into soluble fragments. BS^3-cross-linked complex had half the gelatinolytic activity of an equivalent sample of pure recombinant human enzyme, and both of these were much more gelatinolytically active than the un-cross-linked, activated complex.

Inhibition of Activated and Cross-Linked Complexes by TIMP-2. Activated and cross-linked complexes were assayed for inhibition by TIMP-2 and compared to the inhibition of similar concentrations of activated, un-cross-linked complex and activated recombinant 72-kDa type IV collagenase. The TIMP-2 concentrations ranged from half the molar concentration of enzyme complex to a 10-fold excess. The results are shown in Figure 5A. The un-cross-linked complex, sulfo-DST-cross-linked complex, and recombinant enzyme all show 90% or greater inhibition with the addition of 1 equiv of TIMP-2 although the recombinant enzyme had an initial activity much greater than that of the enzyme complex. In contrast to these three experiments, complex cross-linked by BS^3 is resistant to inhibition by TIMP-2; 90% inhibition is not achieved until the concentration of added TIMP-2 is 10 times greater than the concentration of complex. Since most of the complex exposed to BS^3 is present as cross-linked complexes of enzyme and inhibitor (Figure 4), this suggests that the binding of TIMP-2 to latent enzyme is at a site which may interfere with the binding of TIMP-2 to an inhibitory site. It is apparent that reaction with a cross-linking agent alone does not produce this effect since sulfo-DST-cross-linked complex does not show this pattern of inhibition.

In order to further characterize the binding of TIMP-2 to the enzyme, we repeated the inhibition experiments using

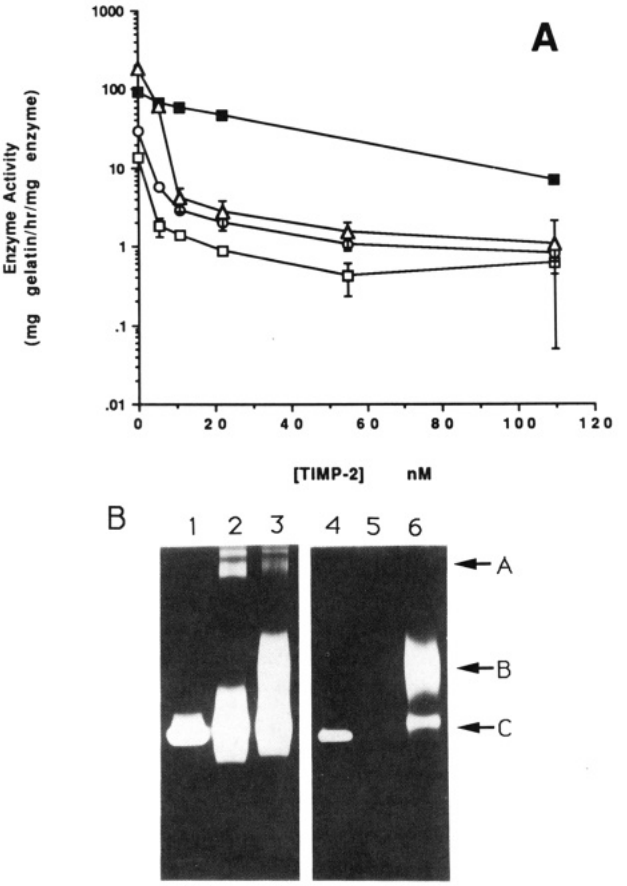

FIGURE 5: Inhibition of cross-linked and activated complexes by TIMP-2. (A) The assay concentration of un-cross-linked complex was 9.6 nM, and recombinant human 72-kDa type IV collagenase concentration was 10 nM. The protein concentration of cross-linked complexes was adjusted to equal the protein concentration of the un-cross-linked complex. Gelatinolytic activity (log scale) is plotted as a function of TIMP-2 concentration: recombinant human type IV collagenase (△); un-cross-linked enzyme–inhibitor complex (□); BS^3-cross-linked complex (■); sulfo-DST-cross-linked complex (○). (B) Gelatin zymogram comparing the order of activation and BS^3 cross-linking with and without TIMP-2 inhibition. Each lane was loaded with 5 ng of protein. Lanes 1 and 4, activated, un-cross-linked complex; lanes 2 and 5, activated and then BS^3-cross-linked complex; lanes 3 and 6, BS^3-cross-linked and then activated complex. The two halves of the gel were treated identically except that lanes 4–6 were incubated in buffer containing 1 μg/mL TIMP-2. Side legends: (A) higher-order complexes; (B) 1:1 cross-linked enzyme–inhibitor complex; (C) activated enzyme, not cross-linked to TIMP-2.

samples of complex in which the order of activation and cross-linking were reversed. This would allow TIMP-2 to bind to an inhibitory site revealed by activation and loss of the amino-terminal profragment. The specific activities of activated and then cross-linked complex were 12 (no cross-linking agent), 9.7 (BS^3-cross-linked), and 10.1 (sulfo-DST-cross-linked) mg of gelatin h^{-1} (mg of enzyme)$^{-1}$. All three samples show similar inhibition profiles for TIMP-2 (data not shown), and these were essentially the same as the inhibition curve for un-cross-linked complex in Figure 5A. Figure 5B shows how the order of activation and cross-linking affects gelatinolytic activity on gelatin zymograms. In the absence of TIMP-2 inhibition, the major difference caused by changing the order of activation and cross-linking was that when the complex is activated first, there is no zone of clearing where one expects the 1:1 complex. Yet when latent complex is cross-linked first, this zone of clearing is as prominent as the zone due to un-cross-linked enzyme. When the zymogram is incubated in the presence of 1 μg/mL TIMP-2, the amount of proteolytic activity associated with all enzyme and enzyme–inhibitor species is reduced. However, when cross-linking precedes activation, there appears to be less inhibition of gelatinolytic activity associated with the 1:1 complex. The order of activation and cross-linking has a marked effect on both the BS^3- and sulfo-DST-cross-linked preparations. Cross-linking following activation produces a pool of complexes where the TIMP-2 is principally bound to an inhibitory site. Activation of previously cross-linked complexes would leave the TIMP-2 bound to the noninhibitory site available on the latent enzyme.

Discussion

The 72-kDa type IV collagenase and TIMP-2 are secreted as a complex from a number of different normal and tumor cell lines. The stoichiometry of the secreted complex is 1:1 based on amino acid composition analysis of purified complex (Stetler-Stevenson et al., 1989a). At neutral pH in a nondenaturing buffer system, the equilibrium between complex and free enzyme and TIMP-2 strongly favors complex formation. In the native polyacrylamide gels used for molecular weight determination, no bands in the latent or activated complex lanes comigrated with the bands in the TIMP-2 lane. Thus, the level of free TIMP-2 in these solutions is undetectable by this method. Enzyme activation can take place despite the presence of TIMP-2 on the complex although the addition of exogenous TIMP-2 will completely inhibit activation (Goldberg et al., 1989). These facts raise questions about the physiologic role of the TIMP-2 in the complex. We have studied the enzyme–inhibitor complex in native polyacrylamide gels to look for higher-order complexes and with cross-linking agents as a structural probe and as a step toward identifying binding sites of TIMP-2 on the 72-kDa proenzyme.

Evidence from the native molecular weight studies and also from the cross-linking experiments demonstrates the ability of the latent and activated complexes to form dimeric complexes (heterotetramers of two type IV collagenase molecules and two TIMP-2 molecules) with molecular masses of 160–180 kDa. Furthermore, activation of the non-cross-linked, native proenzyme–inhibitor complex was found to have a significant effect on dimeric complex formation. Prior to activation, approximately 20% of the enzyme–inhibitor complexes were found as dimeric complexes. However, following organomercurial activation, this fraction dropped to around 5%. These results suggest that the dimeric complex formation is mediated or stabilized through interactions involving the 80 amino acid prosegment that is lost upon activation. The physiological significance of the observation is not clear, but the formation of multimeric complexes in enzymes is not unknown and can have significant effects on catalytic activity. It is also possible that such complex formation might be mediated by two distinct binding sites on the enzyme and the inhibitor, such that each TIMP-2 molecule binds to different sites on the two enzyme molecules.

This possibility is also suggested by the detection of a complex generated by cross-linking that has an apparent stoichiometry of 2:1 collagenase:TIMP-2. Such a complex might arise as a result of a direct collagenase–collagenase interaction and a collagenase–TIMP-2 interaction or as two collagenase–TIMP-2 interactions. This last possibility is strongly suggested by amino acid composition analysis of the 140-kDa complex and implies the presence of two collagenase binding sites on TIMP-2. However, the apparent 2:1 stoichiometry might also be due to an adventitious comigration in the gel of collagenase dimers and 1:2 collagenase–TIMP-2 complexes. In either case, the results of the Western blot (Figure 2B,C) indicate the presence of both TIMP-2 and collagenase in the 140-kDa band and so support the obser-

vation of a higher-order complex.

The observations noted above and previous studies on the interaction of TIMP-2 and collagenase have suggested the possibility of two sites of interaction between TIMP-2 and type IV collagenase (Stetler-Stevenson, 1990; Stetler-Stevenson et al., 1989a; Goldberg et al., 1989; Howard et al., 1991). We attempted to detect this interaction using the two cross-linking agents. If additional molecules of TIMP-2 bound to the enzyme, one might expect to see a shift in the distribution of cross-linked complexes to higher molecular weights. Nonetheless, despite the addition of a 2-fold excess of TIMP-2 to both the active and latent complexes (sufficient excess to abolish enzymatic activity), we could demonstrate no change in the pattern of products generated by cross-linking. If the hypothesis of two distinct binding sites is correct, then either the binding was too transient for the cross-linking reaction to be effective or there were no primary amines within the maximum cross-linking distance (11.4 Å). Alternatively, binding of the first molecule of TIMP-2 to collagenase might sterically hinder binding of a second TIMP-2 molecule.

The site of TIMP-2 binding to the latent type IV collagenase enzyme does not appear to directly involve or sterically block the active site or substrate binding site. This is shown by the experiments evaluating the ability of cross-linked complex to be activated by APMA and carry out proteolytic cleavage of gelatin, a relatively large substrate. TIMP-2 is also apparently not cross-linked to the prosegment since activation of cross-linked complex still results in a reduction of molecular weight and loss of an amino-terminal epitope similar to that seen in non-cross-linked enzyme (Stetler-Stevenson et al., 1989b). The apparent activity of the cross-linked complex is actually increased relative to that of non-cross-linked complex, approaching the level of activity seen with recombinant enzyme (where no TIMP-2 is present in the assay). This may be a reflection both of the inability of cross-linked TIMP-2 to dissociate and interact with the active site of the enzyme and of the possibility that TIMP-2 may be cross-linked to the enzyme in such a way so as to interfere with the binding of a second inhibitor molecule at the active site. It is also possible to demonstrate gelatinolytic activity of the cross-linked complexes in gelatin gels (Figure 5B), indicating that not all the activity can be coming from the 75-kDa band (collagenase exposed to cross-linker but not cross-linked to TIMP-2).

There is a striking difference in the pattern of TIMP-2 inhibition seen with the BS^3-cross-linked complex as compared to inhibition of un-cross-linked complex, sulfo-DST-cross-linked complex, and recombinant human type IV collagenase. While the latter three all showed significant inhibition by addition of 1 equiv of TIMP-2, the BS^3-cross-linked complex retained 60% of its initial activity. This level of activity approximately correlates with the fraction of complexes with intermolecular cross-links present in this preparation. The resistance to the inhibitory effects of TIMP-2 is not due to the mere reaction of cross-linker with free amino groups, since the sulfo-DST-cross-linked complex does not show the same pattern of inhibition. Since cross-linking with BS^3 results in the efficient production of cross-linked complexes, it is reasonable to conclude that cross-linking of TIMP-2 to the latent enzyme interferes with the binding of a second molecule of TIMP-2 at the inhibitory site after the cross-linked complex is activated. Interference of binding does not appear to be absolute since the addition of a 10-fold excess of TIMP-2 to the BS^3-cross-linked, activated complex results in the loss of greater than 90% of the initial activity.

Recently published work (Howard et al., 1991; Howard & Banda, 1991) on binding of TIMP-2 to fragments of 72-kDa type IV collagenase generated by autoproteolytic activity suggested the existence of a second binding site for TIMP-2 on the enzyme. Our data confirm and add to the information in these studies by providing evidence for a distinct site occupied by TIMP-2 on the latent enzyme and by showing that binding to this site interferes with binding of TIMP-2 to the inhibitory site. Furthermore, when complex was activated and then cross-linked with BS^3, the 1:1 complex showed no gelatinolytic activity on a zymogram, showing that, once activated, TIMP-2 can bind to the active site to inhibit gelatinolytic activity.

On the basis of these data, several models of enzyme–inhibitor interaction can be proposed. First, in the latent enzyme, TIMP-2 occupies a site with little effect on enzyme activity. Dissociation of TIMP-2 from this site is not required for organomercurial activation or for gelatinolytic activity. Upon activation, a second binding site is revealed which may be occupied by a second molecule of TIMP-2. Alternatively, when TIMP-2 binds to the active form of the enzyme, a single molecule of TIMP-2 may be able to occupy both sites. Murphy and co-workers (Murphy et al., 1991) have shown that an amino-terminal truncated form of TIMP-1 has the inhibitory activity. We have proposed that an amino-terminal domain of TIMP-2 has the same inhibitory function and that a carboxyl-terminal domain of TIMP-2 may contain the binding domain for the site available on the latent enzyme (Stetler-Stevenson et al., 1990). This model is further supported by evidence that the latent complex of 72-kDa type IV collagenase and TIMP-2 can inhibit activated 92-kDa type IV collagenase (Kolkenbrock et al., 1991), implying that in the latent complex the inhibitory domain of TIMP-2 is free to interact with other matrix metalloproteinases. Such interactions between enzyme–inhibitor complexes and free enzyme or other complexes may provide an explanation for the existence of higher-order complexes.

These experiments provide evidence for more complicated quaternary structure to the type IV collagenase–TIMP-2 complex than has been shown before, specificially that the enzyme and inhibitor can form dimeric complexes. The cross-linking experiments confirm this observation and demonstrate the presence of a 2:1 intermediate complex of collagenase and TIMP-2. Furthermore, cross-linking of TIMP-2 to the latent enzyme fails to prevent activation or catalytic activity, confirming the existence of a binding site distinct from the inhibitory site. Binding of TIMP-2 to the noninhibitory site interferes with binding of free TIMP-2 to the second site by a mechanism which is still unclear. The cross-linking agents provide a useful tool for exploring the physical interaction between the enzyme and the inhibitor. We are currently engaged in trying to isolate specific peptide sequences on the two proteins that are involved in cross-linking. These experiments will provide further characterization and localization of the binding interaction between TIMP-2 and type IV collagenase.

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Anionic Phospholipids Are Essential for α-Helix Formation of the Signal Peptide of prePhoE upon Interaction with Phospholipid Vesicles

Rob C. A. Keller,*,‡ J. Antoinette Killian,‡ and Ben de Kruijff‡,§

Centre for Biomembranes and Lipid Enzymology, Department of Biochemistry of Membranes, and Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

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ABSTRACT: The conformational consequences of the interaction of the PhoE signal peptide with bilayers of different types of phospholipids was investigated using circular dichroism. It was found that interaction of the signal peptide with anionic phospholipid vesicles of dioleoylphosphatidylglycerol and dioleoylphosphatidylserine results in induction of high amounts of α-helical structure of 70% and 57%, respectively. Upon addition of the signal peptide to cardiolipin vesicles, less but still significant α-helical structure was induced (29%). In contrast, no α-helix formation was observed upon the interaction of the signal peptide with zwitterionic dioleoylphosphatidylcholine vesicles. In bilayers of dioleoylphosphatidylcholine with dioleoylphosphatidylglycerol, it was shown that in the presence of 100 mM NaCl a minimum amount of 50% of negatively charged lipid was required for induction of the maximal percentage of α-helix, whereas in the absence of salt a minimum amount of 35% of negatively charged lipid was necessary. Induction of α-helix structure appeared to be correlated with functionality, since, in a less functional analogue of the PhoE signal peptide, the PhoE-[Asp$^{-19,20}$] signal peptide, less α-helix was induced than in the wild-type PhoE signal peptide. It is proposed that the interaction with anionic phospholipids is essential for a functional conformation of the PhoE signal sequence during protein translocation.

Proteins synthesized in the cytoplasm of *Escherichia coli* cells but destined for the periplasmic space or outer membrane have to cross the plasma membrane. In this translocation process, proteinaceous components (Oliver & Beckwith, 1981; Collier et al., 1988; Bieker et al., 1990) as well as phospholipids (De Vrije et al., 1988; Lill et al., 1990; De Vrije et al., 1990; Kusters et al., 1991) are involved. Proteins which are exported from the cytoplasm of *E. coli* are synthesized as precursor

* To whom correspondence should be addressed.
‡ Centre for Biomembranes and Lipid Enzymology.
§ Institute of Molecular Biology and Medical Biotechnology.

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