Roles of the Propeptide and Metal Ions in the Folding and Stability of the Catalytic Domain of Stromelysin (Matrix Metalloproteinase 3)†

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ABSTRACT: Matrix metalloproteinases (MMPs) are an unique class of zinc metalloproteases in that 12 Å from the catalytic zinc site is a second zinc site, the function of which has yet to be determined. In the pro form, the protease is inactive. Here we show that the heat-induced autocatalytic activation of pro to mature MMP3 is bimolecular. Further, the process is modulated by a low-affinity zinc. A mechanism is proposed by which the second zinc site may act as an enzymatic activator for the mature protease. A method for preparing completely metal-free protein is described. Surprisingly, there is a much more dramatic structural change between the apo and holo forms of the mature protein than there is between apo and holo proprotein. Apo mature MMP3 appears to form a native-like stable intermediate structure in which one or more of the tryptophan side chains is more solvent-exposed than in the holo form. Apo MMP3 is remarkably stable to thermal unfolding as monitored by CD; thus the metal ions do not appear to significantly stabilize the secondary structure of the catalytic domain. The apo mature MMP3 intermediate can be unfolded with heat, subsequently refolded, and reactivated by addition of zinc and calcium. Thus for MMP3, unlike subtilisin or α-lytic protease, the propeptide is *not* required for protein folding in a timely fashion and the role of intramolecular chaperone is *not* a universal one for the propeptides of proteases.

MMP3[1] (stromelysin-1) is a member of the matrix metalloproteinase (MMP) family, whose members have been implicated in the breakdown of the extracellular matrix as a result of inflammatory disease (Birkedal-Hansen et al., 1993; Greenwald & Golub, 1994) and in tumor invasion and metastasis (Stetler-Stevenson et al., 1993; Greenwald & Golub, 1994). The MMPs have recently been shown to fall into an evolutionarily related group of proteases for which the term "metzincin" has been coined (Stocker et al., 1995; Bode et al., 1993), but they are distinct from other metzincins in that they bind a second zinc ion distant from the active site (Salowe et al., 1992).

MMPs are synthesized as inactive precursors, which are activated upon removal of the propeptide. One of the roles of the propeptide *in vivo* is that of an intramolecular protease inhibitor. It has previously been shown that the mechanism of inhibition is twofold: via the conserved Cys residue in the C-terminal portion of the peptide, which is a catalytic zinc ligand (Van Wart & Birkedal-Hansen, 1990; Sanchez-Lopez et al., 1988; Park et al., 1991), and through some mechanism involving the N-terminal region of the propeptide (Freimark et al., 1994) and stepwise proteolysis of the propeptide (Nagase et al., 1990; Bergman et al., 1995).

The mature MMPs have one or more structural domains, including a catalytic domain containing the zinc metalloprotease signature sequence HEXXH and a hemopexin-like domain at the C-terminus, and some have one or more cysteine-rich domains [for reviews, see Stocker et al. (1995), Bode et al. (1993), and Birkedal-Hansen et al. (1993)]. Within domains, the amino acid sequences of MMPs are highly conserved. The X-ray crystal and NMR structures of several MMPs truncated at the end of the catalytic domain and bound to active site inhibitors have been solved (Lovejoy et al., 1994; Borkakoti et al., 1994; Gooley et al., 1994; Stams et al., 1994; Spurlino et al., 1994; Reinemer et al., 1994; Bode et al., 1994). An X-ray crystal structure of the pro form of truncated MMP3 has been also solved (Becker et al., 1995; Hardman et al., unpublished experiments).

All MMPs contain a catalytic zinc binding site in which the active site zinc ion is coordinated by three His residues. The fourth ligand is a glutamic acid-bound water or OH^- in the activated mature form. In the pro form, the fourth ligand had earlier been proposed to be the side chain thiolate of a conserved Cys residue at the C-terminal end of the propeptide (Van Wart & Birkedal-Hansen, 1990). This has been confirmed in the X-ray crystal structure of proMMP3, truncated at residue 255 (Becker et al., 1995; Hardman et al., unpublished experiments). Thus, activation involves displacing the Cys residue through what has been termed a

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; APMA, 4-aminophenylmercuric acetate; Tris, tris(hydroxymethyl)aminomethane; OP, 1,10-phenanthroline; PAR, 4-(2-pyridylazo)resorcinol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CD, circular dichroism; Mca peptide, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2; DMSO, dimethyl sulfoxide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MMP3, matrix metalloprotease 3/stromelysin; protMMP3, the pro form of MMP3 truncated at residue 255; tMMP3, the mature form of MMP3 truncated at residue 255 (83−255); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ICP, inductively coupled plasma emission spectroscopy.

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"Cys-switch" mechanism (Van Wart & Birkedal-Hansen, 1990), as well as cleavage of the propeptide. While the *in vivo* mechanism for activation of proMMP3 remains unknown, it has been shown that proMMPs can be activated *in vitro* by several methods, including the use of mercurial compounds, oxidants, extraneous proteases, and heat [summarized in Marcy et al. (1991) and Nagase et al. (1990)].

In addition to the active site zinc, the MMP catalytic domains also contain a second zinc binding site of unknown function. This zinc ion is also coordinated by three His residues, with the fourth ligand provided by an Asp side chain. Contiguous with the second zinc binding loop is a calcium binding site (Borkakoti et al., 1994; Stams et al., 1994). This zinc−calcium loop is also present in the pro form (shown in green in Figure 1) and distinguishes the MMPs from other subclasses of metzincins. There is at least one additional calcium site in MMP1 (Borkakoti et al., 1994) and two additional ones in proMMP3 (Becker et al., 1995; Hardman et al., unpublished experiments). The ligands for the catalytic zinc and the zinc−calcium loop are conserved in the amino acid sequences of all the known MMPs.

The full-length version of proMMP3 contains an 82-residue pro sequence, the catalytic domain (residues 83 to about 247−255), and a hemopexin-like domain (about 250−460) for a total molecular weight of 52 220. The pro sequence does not contain any transition state divalent cation or calcium binding sites (Becker et al., 1995; Hardman et al., unpublished experiments). A method for expressing and purifying a truncated version of proMMP3 (1−255) from *Escherichia coli* has been described (Marcy et al., 1991). Similarly, full-length proMMP3 has been cloned and expressed in *E. coli* (Rosenfeld et al., 1994).

Figure 1 shows two views of the crystal structure of proMMP3, truncated at T255 (Hardman et al., unpublished experiments). The structure of the catalytic domain is superimposable over that of the active mature protein bound to active site inhibitors, within experimental error (Becker et al., 1995; Hardman et al., unpublished experiments). The lower part of the domain consists of a three-helix bundle; the HEXXH zinc binding motif is found in the central helix. A five-strand β sheet partially "wraps" over the helical bundle. The zinc−calcium loop is found between two strands of the β sheet. A second calcium site is found at the other end of the β sheet. The propeptide is a separate 3-helix bundle domain with two extended strands near the N-terminus. The C-terminal region of the propeptide lies in the active site, with the S∂ of Cys 75 in place as the fourth ligand to the active site zinc.

In this study, we have probed the heat-induced autocatalytic processing of the pro form of truncated MMP3 (protMMP3, MW = 28 779) to the mature form (tMMP3, MW = 19 395), as well as the roles of the propeptide and the metal binding sites in the folding and stabilization of the protein. The protein concentration dependence and zinc dependence of autocatalytic activation have been studied. A method for preparing apo pro and apo mature tMMP3 is described, and the difference in structure of those forms from the holo counterparts is examined and discussed. Thermal unfolding studies of both apo- and holoproteins and recovery of active protease from unfolded apoprotein are presented. A preliminary report of this work was disclosed elsewhere (Wetmore & Hardman, 1995).

MATERIALS AND METHODS

Materials. Double distilled water was used throughout. For spectroscopic experiments, proteins were stored in buffers containing HEPES (J. T. Baker Inc., Phillipsburg, NJ), Tris free base (Research Organics, Inc., Cleveland, OH), or MOPS (Sigma Chemical Co., St. Louis, MO), NaCl (EM Science, Gibbstown, NJ), $CaCl_2$ (Fisher Scintific Co., Fairlawn, NJ), and $ZnCl_2$ (Sigma or Alfa Aesar, Ward Hill, MA). Other chemicals used in this study included EDTA and 1,-10-phenanthroline (OP) (Sigma), 4-(2-pyridylazo)resorcinol (PCA) (Acros Organics, Geel, Belgium), perchloric acid (J. T. Baker Inc.), Mca peptide [(7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2] (Bachem Bioscience Inc., King of Prussia, PA), and tricine (Sigma). SDS−PAGE gels and zymogram gels were purchased in precast form from Novex Experimental Technology (San Diego, CA). Chelex 100 resin and low molecular weight protein markers were supplied by Bio-Rad Laboratories (Hercules, CA).

Source of Protein and Purification. The expression plasmid for the truncated form of stromelysin, protMMP3, including residues 1−255, was prepared from the full-length version of proMMP3 (Rosenfeld et al., 1994) as previously described (Marcy et al., 1991). Purification of protMMP3 was carried out according to methods described (Marcy et al., 1991). The preparation of mature tMMP3 was carried out either by using heat activation, as described herein, or by APMA activation (Marcy et al., 1991; Nagase et al., 1990). Protein concentration was carried out using Centriprep 10 concentrators from Amicon (Beverly, MA).

Preparation of Apoprotein. Purified holoprotein was stored at −80 °C in 50 mM HEPES, pH 7.5, 0.1 M NaCl, 10 mM $CaCl_2$, and 0.1 mM $ZnCl_2$. Apo protMMP3 and apo tMMP3 were prepared by adding equal volumes of 4 mM 1,10-phenanthroline (OP), 50 mM HEPES, pH 7.5, and 0.1 M NaCl. Protein was placed on ice for 20 min, followed by addition of $^1/_{17}$ volume of 0.5 M ETDA stock (final concentration of EDTA was 30 mM). Protein was allowed to stand at 4 °C for 1 h to overnight and then concentrated using Amicon Centriprep 10s. When protein reached the desired concentration (typically 0.1 mM or higher), the sample was dialyzed against two 50x volumes of 50 mM HEPES, pH 7.5, 0.1 M NaCl, 2 mM OP, and 3 mM EDTA with Chelex 100 in the dialysate reservoir. Finally, the protein was dialyzed against at least seven 50× volumes of 50 mM HEPES, pH 7.5, and 0.1 M NaCl, again with Chelex 100 in the dialysate reservoir. In handling apoprotein, metal-free procedures were followed throughout (Holmquist, 1988). For spectroscopic experiments, the proteins were dialyzed against a buffer containing 1 mM EDTA in order to eliminate the possibility of metal ion uptake from quartz cuvettes.

The method described here, along with the refolding experiments presented in this paper, have potential importance in developing methods for purifying and storing this class of proteins. Both pro and mature MMPs may be expressed in *E. coli* and refolded and purified from inclusion bodies *in the apo form*. Further, the proteins may be stored indefinitely at 4 °C in their apo forms, preventing autolysis. It is shown here that enzymatic activity is recoverable upon addition of metal ions.

Spectroscopy. Circular dichroism measurements were made on an AVIV Model 62DS (Lakewood, NJ); fluores-

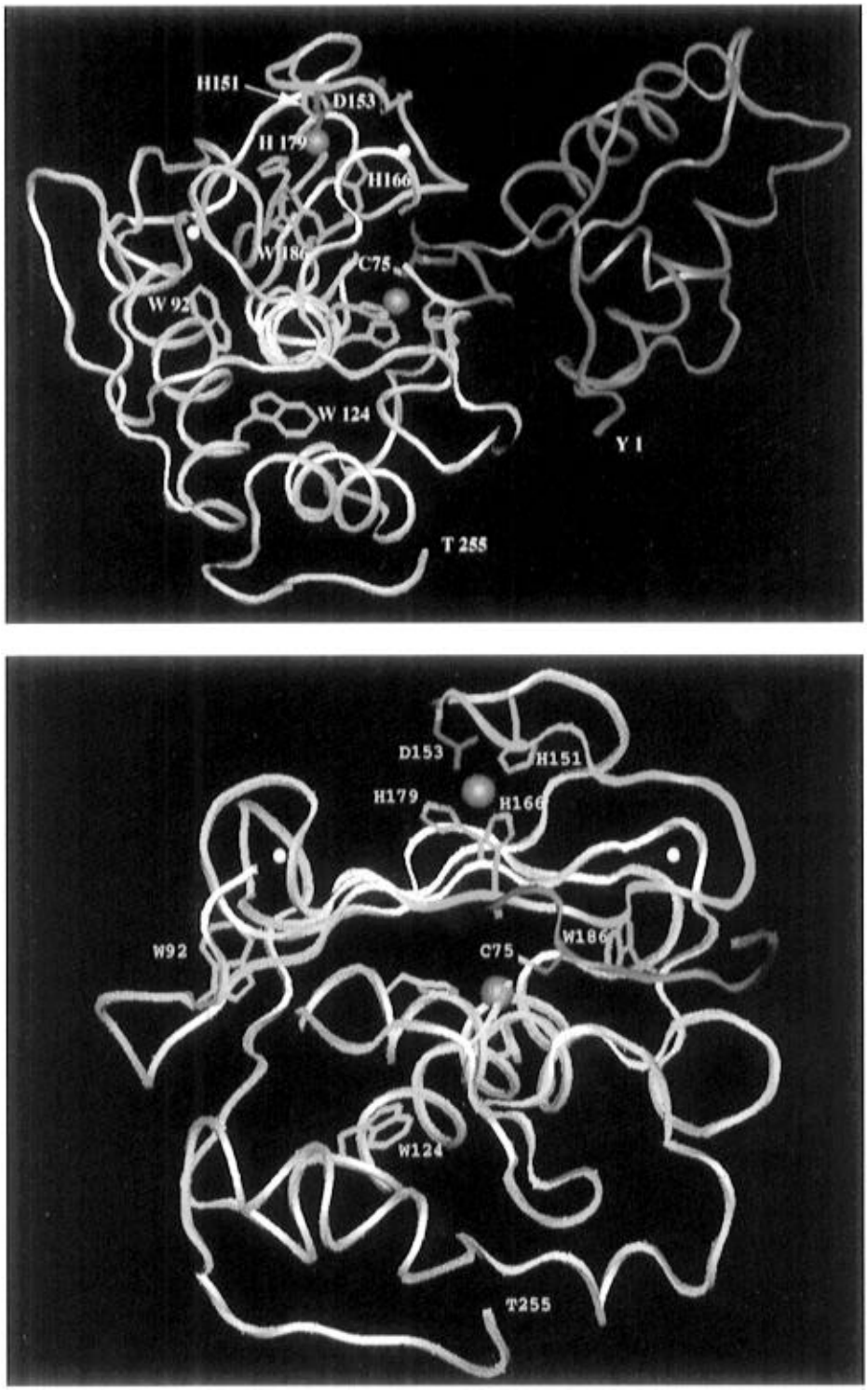

FIGURE 1: α-Carbon backbone representation of the X-ray crystal structure of protMMP3. (A, top) The propeptide is shown in red and the catalytic domain is shown in white. The two zinc ions are red spheres and two of the calcium ions are shown as yellow spheres. The active site zinc ligand Cys 75 is labeled. The active site zinc His ligands are shown but are not labeled. The zinc−calcium loop (H151 through H166) is shown in green. The second zinc site side chain ligands, H151, D153, H166, and H179, are labeled. The Trp side chains are labeled and shown in yellow. The N-terminus, T255, is labeled. (B, bottom) Second view of protMMP3, corresponding to a 90° rotation of view A about a perpendicular axis. Only that part of the propeptide in the active site is shown. This view emphasizes the β structure which forms the base of the second zinc site and the two calcium sites. The color scheme is the same as in view A.

cence data were collected on a SPEX Industries Inc. Model DMIB, equipped with DM3000 software; and UV−vis absorbance measurements were made using a Hewlett-Packard 8450A diode array spectrophotometer.

Enzymatic Assays. The activity of the mature form of tMMP3 was measured using the Mca peptide assay (Knight et al., 1992). The assay involves use of the substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 and follows the increase in emission fluorescence of the 7-methoxycoumarin (Mca) group as cleavage at the Gly−Leu bond eliminates quenching by the 2,4-dinitrophenyl group (Knight et al., 1992). Assays were carried out in a total volume of 0.5 mL in fluorescence cuvettes. The assay

buffer was diluted from a 10× stock of 500 mM tricine, pH 7.5, 100 mM $CaCl_2$, and 0.1 mM $ZnCl_2$. The Mca peptide was freshly diluted in DMSO to 0.3 mM from a 20 mM stock stored at −20 °C. Twenty-five microliters of Mca peptide was added to 425 μL of 1× assay buffer and mixed thoroughly. The reaction was initiated by addition of 50 μL of protein (concentration was typically 0.5 μM). After excitation at 330 nm, emission at 400 nm was monitored over time. A blank where assay buffer was substituted for protein solution was run for every three to five sample assays. Specific activity was calculated as the slope of the linear part of the increase in fluorescence with time divided by protein concentration.

Metal Content Determination: ICP and PAR. ICP metal analysis for calcium and zinc was carried out by the Corporate Center for Analytical Science, DuPont Central Research and Development, Wilmington, DE. Zinc analysis using PAR was carried out on the basis of methods previously described (Hunt et al., 1985). Zinc standards were prepared as stocks and analyzed by ICP to ensure accurate standard curves.

Determination of Extinction Coefficients, ϵ_{280}. The extinction coefficients for protMMP3 and tMMP3 at 280 nm were calculated according to the method of Edelhoch (1967) using estimates for the percentage buried for each chromophore side chain based on the crystal structure. Recently, it has been shown that calculated extinction coefficients based on Edelhoch's method differ from measured values by an average of 3.8% (Pace et al., 1995).

Molecular Modeling. The modeling exercise in which H166 was repostioned was carried out using the program Quanta 4.1.1 (Molecular Simulations Inc.). The starting structure used was the X-ray crystal structure determined in this laboratory (K. Hardman, unpublished experiments). The side chain of H166 was rotated about 180° and positioned such that the Nϵ2 of the His was superimposed over the position that the sulfur atom of C75 would occupy in the proprotein structure. The position of the Nϵ2 of H166 was fixed, and the polypeptide chain from F146 to G171 was reiteratively minimized by CHARMm.

RESULTS AND DISCUSSION

Preparation of the Apoproteins. The development of a method for preparation of apo protMMP3 and apo tMMP3 was crucial to probing the roles of the bound metal ions in the structure and function of the proteins. Several structures of truncated MMPs (Borkakoti et al., 1994; Stams et al., 1994; Reinemer et al., 1994; Lovejoy et al., 1994), including protMMP3 (Becker et al., 1995; Hardman et al., unpublished experiments) (Figure 1), show that at least one well-resolved calcium binding site is contiguous with the loop comprising the second, noncatalytic zinc site. Therefore, it can be reasoned that the binding of zinc and calcium may be cooperative. The method for preparation of apo tMMP3 reported here uses both the zinc chelator 1,10-phenanthroline (OP) and EDTA (see Materials and Methods). The chelators were added to the protein before concentration and then diluted out by dialysis in the presence of an ion chelating resin. The decision to use both OP and EDTA in the preparation of apo protMMP3 was based on the premise that it might be necessary to remove the calcium in order to more easily remove the zinc. Application of the method was successful for both the pro form of tMMP3 and the mature form. The zinc and calcium contents of the treated proteins were checked by ICP analysis (Table 1).

Table 1: Metal Content of Apoprotein

	protein concn (μM)	total zinc ion concn (μM)	total calcium ion concn (μM)
apo protMMP3[a]	112	≤1.5[a,b]	≤2.5[b]
apo tMMP3[a]	153	≤1.5[b]	≤2.5[b]
protMMP3[c,d]	31.0	28.5	nd

[a] Determined by ICP. [b] Values represent the detection limit of the method, which was 0.1 ppm (μg/mL) for both calcium and zinc. [c] Determined by PAR (see Materials and Methods). [d] Starting material for experiment described in Figure 8 in which only the lower affinity zinc was removed.

The ability to prepare apo MMPs and the results of the refolding experiments (presented in the section The Unfolding of Apo tMMP3 Is Reversible) have potential importance in developing methods for purifying and storing this class of proteins. Mature MMPs can be expressed in *E. coli* and refolded and purified from inclusion bodies *in the apo form*. Further, the proteins can be stored indefinitely at 4 °C in their apo forms, preventing autolysis. It will be shown here that enzymatic activity is recoverable upon addition of metal ions.

The Role of the Metal Ions in the Conformation of the Proteins. There are several potential roles for bound metal ions, including the stabilization of conformation to thermal unfolding. It is well established that the presence of calcium stabilizes catalytically active MMP3 toward autolytic degradation. Calcium modulation of autocatalytic degradation is a general theme for proteases and has been observed in other systems such as thermolysin-like neutral proteases (Drucker & Borchers, 1971; Voordouw & Roche, 1975). Interestingly, it has been shown that calcium is *required for MMP1 activity* (Lowry et al., 1992), and we also have observed calcium-dependent activity for MMP3 (unpublished results). It has been suggested that the role of the second zinc in MMPs also resides in the stabilization of this class of proteins (Borkakoti et al., 1994).

The circular dichroism (CD) spectra of apo protMMP3 and holo protMMP3 are shown in Figure 2A. The spectrum of holo protMMP3 is typical of a protein with a high degree of α-helical secondary structure, showing minima at both 210 and 222 nm. Both forms of the protein show similar intensity of absorbance at 222 nm, although there is a loss of signal in the 210 nm range for apo protMMP3 compared to the holo protein. This suggests that, upon removal of metals (here and throughout the discussion, the use of the term "metals" is meant to include both calcium and zinc, unless otherwise stated), some helix is converted to β structure (which shows a single minimum at 222 nm) or random coil.

The CD spectrum for holo tMMP3 (Figure 2B) shows a loss of signal from 208 nm to 240 compared to the pro form of the protein (Figure 2A). The propeptide has a high degree of helicity (Becker et al., 1995; Hardman et al., unpublished experiments); 32 out of 82 amino acids are found in three helical sections. Thus, the lower negative signal in the mature form can be partly explained by the loss of the propeptide.

The molar ellipticities for both holo protMMP3 and holo tMMP3 (Figure 2) are unusually low in magnitude, for which

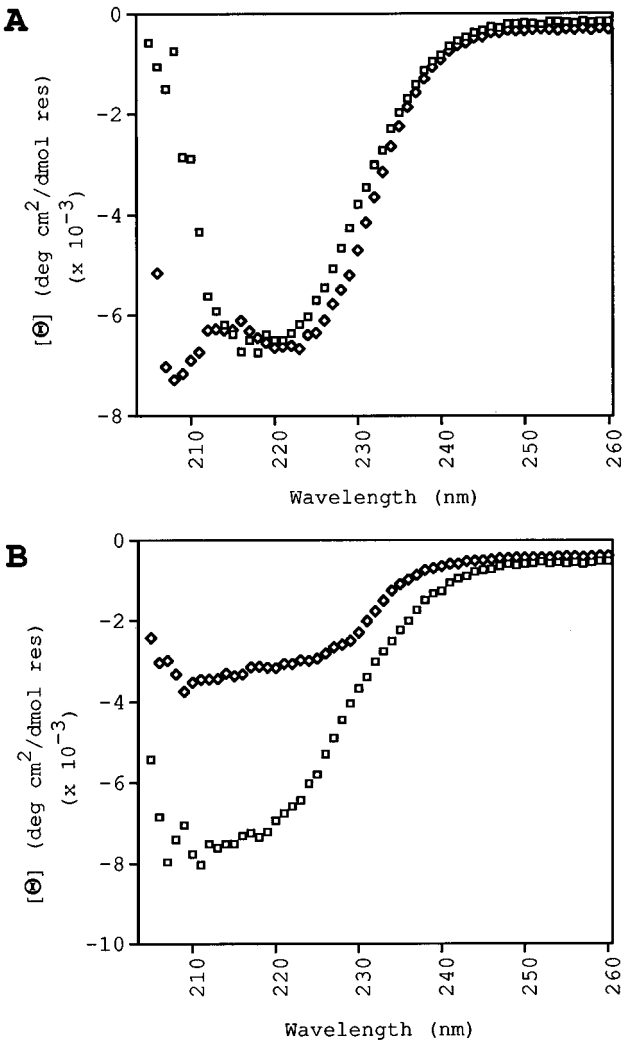

FIGURE 2: Circular dichroism spectra. (A) protMMP3. Open diamonds represent the holo form, and open squares represent the apo form. (B) tMMP3. Open diamonds represent the holo form of tMMP3, and open squares represent the apo form of tMMP3. Buffer conditions were 10 mM MOPS, pH 7.5, and 50 mM NaCl for all samples, with 10 mM $CaCl_2$ and 0.1 mM $ZnCl_2$ in the holo samples or 1 mM EDTA in the apo samples. Apo samples were prepared as described in Materials and Methods prior to dialysis against buffer and EDTA. Scans were taken at 0 °C, and data were averaged over 5−15 s for each point.

we presently have no clear explanation. Removal of metal from mature tMMP3 has a surprising effect on the CD spectra: Figure 2B shows a dramatic *increase* in the magnitude of the negative molar ellipticity. The difference is reproducible between experiments and protein preparations, and protein concentrations have been carefully checked. No evidence of the presence of autolytic fragments was detected in the samples by SDS−PAGE gels, even when overloaded. Other studies have shown that the presence of tryptophan residues in a folded protein can have a significant effect on the far-UV spectra (Vuilleumier et al., 1993; Freskgard et al., 1994; Sherman et al., 1995). If the three Trp residues of tMMP3 are in different chiral environments for the apo and holo forms, this would also account for some of the differences seen in their CD spectra. It is clear that there are significant conformational changes in both pro and mature MMP3 upon removal of metal ions. However, the information provided by the CD spectra is not sufficient to determine the detailed nature of these changes.

The conformational difference between apo and holo mature tMMP3 was further investigated by determining the tryptophan emission spectra (Figure 3). The emission maximum for holo tMMP3 is at 325 nm, whereas the maximum emission for apo tMMP3 is at 340 nm. This shift is suggestive of a change in the environment of the tryptophan side chains, such that one or more of the tryptophans is more solvent-exposed in the apo form than in the holo form (Cantor & Schimmel, 1980). Thus, the fluorescence results provide additional evidence that there is a significant change in the packing of the interior of the protein which affects the environment of the Trp residues in the catalytic domain of tMMP3 upon metal binding.

Of the three Trp residues shown in Figure 1, W124 and W186 are fully buried, while W92 is about 20% solvent-exposed. Proton exchange NMR data also indicate that the side chain amide protons of W124 and W186 are very tightly bound, but the side chain amide proton of W92 exchanges readily (Sharon Archer, personal communication). The latter residue is also found at the farthest distance from either of the two zinc ions (14.7 Å from the catalytic zinc and 13.1 Å from the second zinc). W186 is nearly equidistant from both zincs: 11.6 Å from the active site zinc and 12.2 Å from the second zinc. W124 is 9.7 Å from the active site zinc. We conclude that the red shift in the Trp emission spectra for the apoprotein is more likely due to an increase in the solvent exposure of W124 and/or W186 than of W92.

The unfolding transition of apo mature tMMP3 upon heating is cooperative (Figure 4A). Thus, while the conformation of apo mature tMMP3 is different from that of the holo form, the apo form of the protein is a thermodynamically stable intermediate. The midpoint of the unfolding transition occurs somewhere between 70 and 75 °C and reaches a plateau at 80 °C. These data show that the conformation of tMMP3 is remarkably stable to thermal unfolding even in the absence of metals. There was a slight loss of protein material upon unfolding of apo tMMP3, but otherwise the second melt overlays the first, indicating that unfolding is reversible. The reversibility of unfolding has been observed after heating apo tMMP3 to as high as 96 °C, although loss of material is slightly greater (results not shown).

In the case of holo tMMP3, unfolding of the protein coincided with precipitation, which occurred at 80 °C (Figure 4B). The increase in (negative) signal as the temperature is increased beyond 80 °C is consistent with a scattering effect of the precipitate. An SDS−PAGE gel analysis of the precipitate revealed the appearance of some autolysis products, approximately 20% of the total material (results not shown). It is reasonable to suggest that some unfolding event precedes the precipitation of holo tMMP3, and the precipitation itself may be a result of the presence of metals in the solution interacting in a nonspecific way with the unfolded intermediate. Thus, the loss of signal at Θ_{222} is due to some combination of unfolding (loss of secondary structure), autolysis, and precipitation. The unfolding of holo protMMP3 (Figure 4C) will be discussed in the section The Heat Activation of protMMP3 Can Be Followed by CD.

The temperature at which the thermal unfolding (accompanied by precipitation with associated autolysis) of holo mature tMMP3 occurs, 65°−80 °C, is the same temperature range over which apo tMMP3 unfolds reversibly. Since we cannot determine the endpoint of the holo tMMP3 unfolding

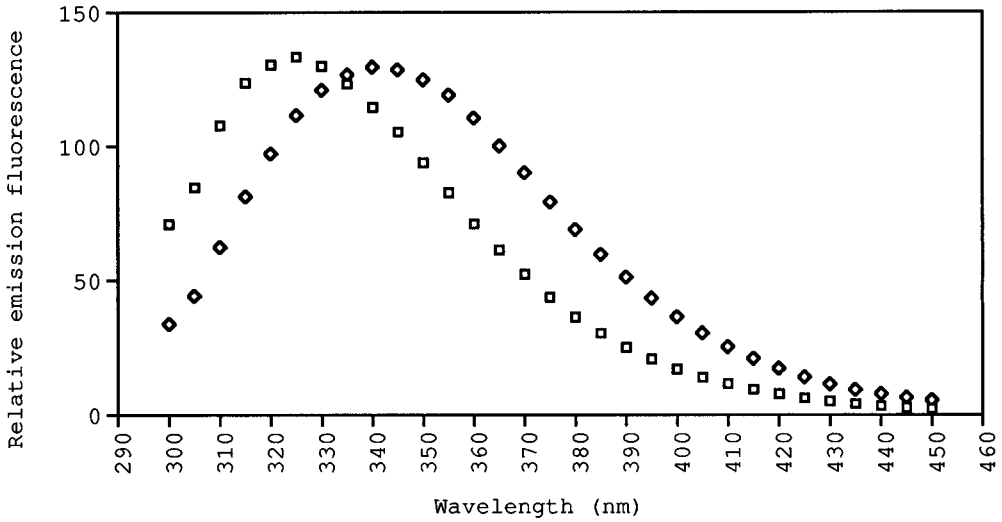

FIGURE 3: Tryptophan emission spectra for tMMP3. Open squares represent holo tMMP3, and open diamonds represent apo tMMP3. Excitation was at 295 nm. Data were averaged over 5 s for each point. Buffer conditions were 10 mM MOPS, pH 7.5, and 50 mM NaCl for both samples, with 10 mM $CaCl_2$ and 0.1 mM $ZnCl_2$ in the holo sample or 1 mM EDTA in the apo sample. Data have been normalized for protein concentration.

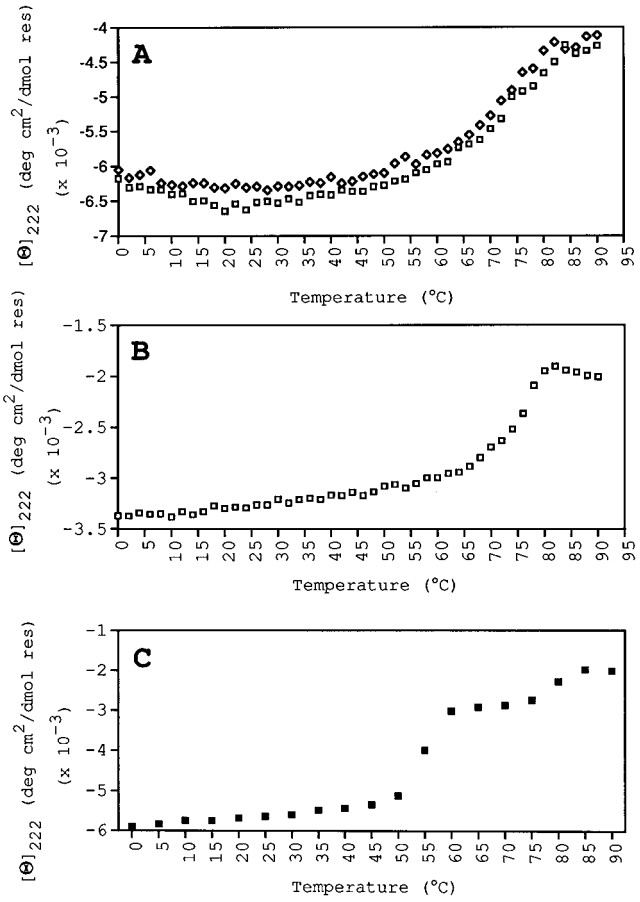

FIGURE 4: Thermal unfolding of tMMP3 followed by change in ellipticity at 222 nm. (A) apo tMMP3. Open squares represent the initial thermal scan. Open diamonds represent a second scan of the same sample, after being cooled to 0 °C. Buffer conditions were 10 mM MOPS, pH 7.5, 50 mM NaCl, and 1 mM EDTA. (B) Holo tMMP3 and (C) holo protMMP3. Buffer conditions were 10 mM MOPS, pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$, and 0.1 mM $ZnCl_2$. In all cases, samples were allowed to equilibrate at each temperature for 3 min, and data were averaged over 30 s for each point.

reaction (due to precipitation and autolysis), it is not possible to determine the midpoint. It is expected to be slightly higher than the midpoint for the unfolding reaction for the apo form (Figure 4A,B). Therefore, it would not be reasonable to suggest that the metals make no contribution to protein stability. However, the data do suggest that some unfolding event that leads to autolysis in holo tMMP3 coincides with the equilibrium unfolding of apo tMMP3. These results can be contrasted to those of similar studies carried out on thermolysin, where the contribution of the binding of calcium to thermal stability is well established (Drucker & Borchers, 1971; Voordouw & Roche, 1975). CD studies on holo thermolysin showed that a thermal unfolding transition occurred between 80 and 90 °C, but protein treated with EDTA showed an unfolding transition between 40 and 55 °C (Dahlquist et al., 1976). In the case of MMP3, the apo form of tMMP3 is remarkably stable to thermal unfolding. The binding of metals may stabilize later events on the unfolding pathway which are not accessible by following Θ_{222}. Additionally, the binding of metals may modulate the kinetics of unfolding.

The Unfolding of Apo tMMP3 Is Reversible. The results from Figure 4 show not only that the propeptide is *not* required for tMMP3 folding but also that the metals are not required. In order to test this further, a sample of apo tMMP3 which had been unfolded at 90 °C and refolded by cooling was assayed for activity in the presence of both calcium and zinc. Figure 5 shows a zymogram in which samples from before and after thermal unfolding show activity toward the substrate gelatin. Additionally, the sample which had been thermally unfolded was assayed using the Mca peptide substrate (see Materials and Methods). The results, shown in Table 2, indicate that the activity of the unfolded protein is recoverable by cooling and the subsequent addition of metals, although conditions for maximal recovery have not yet been optimized, and some loss of material is observed under these conditions.

The Role of the Propeptide in the Conformation and Stability of the Catalytic Domain. As discussed earlier, apo mature tMMP3 can be thermally unfolded, refolded, and reactivated by addition of metals. Thus, the propeptide *is not required* for the proper folding of the catalytic domain of stromelysin. Studies on other protease systems have shown that the propeptides of the subtilisins (Zhu et al., 1989;

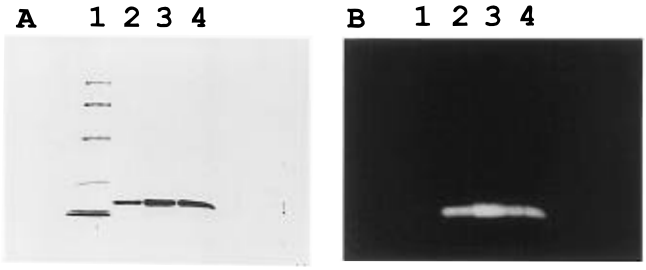

FIGURE 5: Zymogram showing recovery of enzymatic activity of apo tMMP3 after thermal unfolding. (A) 10% SDS−PAGE gel and (B) 10% SDS−PAGE gel with added gelatin. Conditions for zymogram development were as recommended by manufacturer, except that 0.1 mM $ZnCl_2$ was added to the developing buffer. In both gels, lane 1 shows molecular weight markers (97.4, 66, 45, 31, 21.5, 14.5 kDa); lane 2, control (holo tMMP3); lane 3, apo tMMP3 before thermal unfolding; and lane 4, apo tMMP3 after thermal unfolding.

Table 2: Recovery of Activity of Apo tMMP3 after Thermal Unfolding As Determined by Mca Peptide Assay

	specific activity (ΔI_{400}) (s^{-1} mol^{-1}) ($\times 10^7$)
tMMP3 control[a]	4.83 ± 1.29
refolded tMMP3	1.24 ± 0.63

[a] Protein was expressed in the soluble fraction of *E. coli* cultures as the pro truncated form and activated to the mature truncated form using APMA.

Ikemura et al., 1987) and α-lytic protease (Silen & Agard, 1989; Silen et al., 1989) *are* required for efficient folding and act as intramolecular chaperones (Ohta et al., 1991). A recent study on the hydrolase cathepsin D shows that its propeptide, like that of MMP3, is not required for folding (Fortenberry & Chirgwin, 1995). Thus, the role of intramolecular chaperone is *not* a universal one for the propeptides of proteases.

One of the implications of the refolding and regeneration of activity of apo mature tMMP3 is that the catalytic domains of MMPs may be expressed in high yields in *E. coli* and purified from inclusion bodies in the apo form, eliminating concerns for autoproteolysis during refolding and purification steps. It has been shown by others that the catalytic domain of MMP3 can be refolded from *E. coli* inclusion bodies in the holo form (Ye et al., 1992). We have refolded tMMP3 from inclusion bodies in the apo form (results not shown).

From residue 90 on, the α-carbon backbones of both mature tMMP3 and protMMP3 are virtually superimposable. The crystal structures of protMMP3 (in which part of the propeptide occupies the active site) compared to various inhibitor-bound mature tMMP3 structures show that the presence of the propeptide does not alter the structure of the catalytic domain when the metal sites are occupied (Becker et al., 1995; Hardman et al., unpublished experiments). However, it is possible that the presence of the propeptide modulates the structure of the catalytic domain *in the apo form* of the enzyme such that the ellipticity of the protein is significantly affected by its absence (Figure 2). Stabilization of the apo form of protMMP3 by the propeptide would explain why the differences in molar ellipticities between the apo and holo forms are much more dramatic in the mature forms of the protein than in the pro forms.

The Heat Activation of protMMP3 Can Be Followed by CD. Figure 4C shows the changes in molar ellipticity of

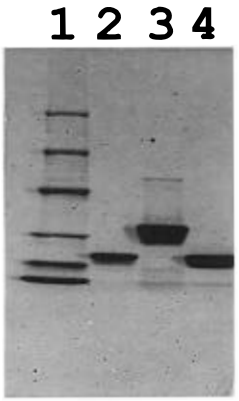

FIGURE 6: 8−16% gradient SDS−PAGE gel showing typical pro to mature tMMP3 heat activation experiment. Lanes: 1, molecular weight markers (97.4, 66, 45, 31, 21.5, 14.5 kDa); 2, APMA-activated tMMP3 (MW = 19 395); 3, protMMP3 before heat activation (MW = 28 779); 4, heat-activated tMMP3.

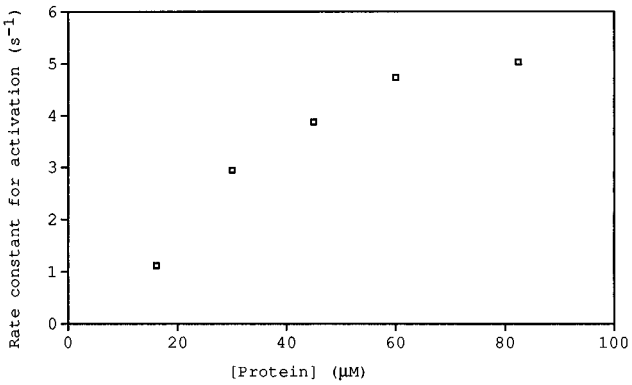

FIGURE 7: Protein concentration dependence of pro to mature heat activation of tMMP3. Activation was followed by change in Θ_{222} with time at 52 °C, and the rate constant was determined as the slope of the linear part of the curve. Buffer conditions were 10 mM MOPS, pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$, and 2 equiv of added $ZnCl_2$ per protein molecule.

protMMP3 upon heating. A highly cooperative transition between 50 and 60 °C was found to reflect the heat-induced autocatalytic activation of protMMP3 to tMMP3. The second transition at 80 °C results from unfolding and precipitation of the activated protein in the same manner as is observed in Figure 4B. Thus, the autocatalytic activation of protMMP3 can be followed by the change in ellipticity at 222 nm. Further experiments showed that the optimum temperature for efficient activation without further degradation products being generated was 52 °C. The SDS−PAGE gel in Figure 6 shows the results of a typical heat-induced autocatalytic activation experiment.

The Autocatalytic Activation Is Bimolecular. The application of CD experiments to study autocatalytic activation of protMMP3 allowed the study of the effect of protein concentration on the kinetics of autocatalytic activation (Figure 7). The ellipticity at 222 nm of the protein was monitored as a function of protein concentration, while the temperature was maintained at 52 °C. The rate constant for activation is dependent on protein concentration. Noting that the propeptide is further degraded upon release (Figure 6, lane 4 shows no accumulation of propeptide), the shape of the curve is consistent with the propeptide products competing with intact proenzyme as substrate for activated tMMP3. These results clearly show that a bimolecular event is involved in the activation reaction, although the data do not exclude the

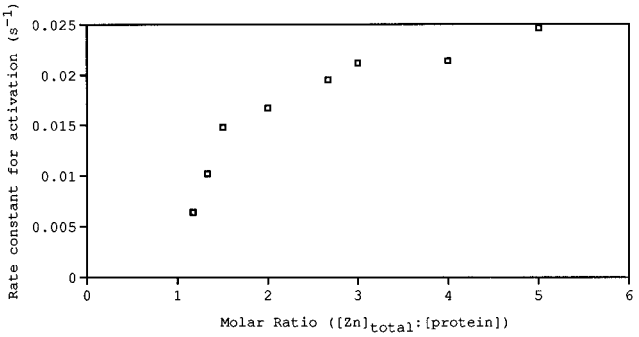

FIGURE 8: Zinc concentration dependence of pro to mature heat activation of tMMP3. Activation was followed by change in Θ_{222} with time at 52 °C, and the rate constant was determined as the slope of the linear part of the curve. Buffer conditions were 10 mM MOPS, pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$, and varying amounts of zinc. Initial zinc concentration was determined by ICP analysis (Table 1). Molar ratio is ratio of zinc molecules per protein molecule. Protein concentration was 30 μM.

possibility that, in addition to this bimolecular reaction, there is a unimolecular initial or concomitant reaction. Furthermore, the rate-limiting step for activation is not an unfolding step, since a rate-limiting unfolding step would not show protein concentration dependence.

Zinc Dependence of Autocataytic Activation. Figure 8 shows the effect of added zinc on autocatalytic activation. Before the titration experiment was started, protMMP3 was dialyzed against a buffer which contains 10 mM $CaCl_2$ but no zinc. The dialyzed protein was analyzed for zinc content using the optically active chelator PAR (see Materials and Methods) and was found to contain one molecule of zinc per protein molecule (Table 1). The titration experiment shows that the rate constant for activation is clearly zinc concentration dependent. Further, the first inflection point of the curve lies between 1 and 2 molar equiv of zinc, indicating that a second, less tightly bound, zinc modulates the autocatalytic process.

Previous investigators report that at least one zinc ion is very tightly bound in the MMP catalytic domains; however, conflicting interpretations appear with respect to the relative affinity of the second zinc ion (Willenbrock et al., 1995; Soler et al., 1994; Salowe et al., 1992; Crabbe et al., 1992). The disagreements in reported zinc content of MMPs may reflect complex ligand binding patterns in this class of proteins. Comparison between studies is not possible in a rigorous way, as buffer conditions (such as whether or not calcium was present) and protein concentrations are different or not reported.

Our results (Figure 8 and Table 1) indicate that one zinc site of protMMP3 is not tightly bound and that this zinc site modulates the activation process. If the catalytic zinc were the titrating site, then the mechanism would be straightforward. If the titrating zinc is at the second zinc site, at least two possibilities exist: it might affect the enzymatic activity of the enzyme through long-range packing effects on the stucture around the active site, or zinc binding to the second zinc site could modulate the conformation of the proprotein so as to make it a better substrate. In the following section we outline a third possibility.

A Suggested Role for the Second Zinc. The role of the second zinc has been presumed to reside in the stabilization of the MMPs (Borkakoti et al., 1994). However, the metzincin proteases astacin and adamalysin do not have the second zinc site even though they are found in environments much more harsh than that of the MMPs (the crayfish digestive tract and snake venom, respectively). Furthermore, our results do not support the role of stabilization. The thermal unfolding of apo tMMP3 shown in Figure 4A suggests the T_m for the apo form is very close to that of the holo form, although the ΔH, $T\Delta S$, ΔG, or ΔC_p of thermal unfolding may be different. Therefore, we suggest that the conservation of this zinc site in this class of proteases represents a more specific structural function than that of stabilization.

We offer the following speculation on the role of the second zinc site. It is assumed from three-dimensional structural information that the contiguous loop which forms the second zinc and first calcium binding sites (H151 through H166, Figure 1) becomes very mobile if the second zinc site is not occupied. (It has been observed that this loop region is subject to proteolysis during protein purification, with the primary clip site being between H166 and A167; Bud Hillman, personal communication.) The loop may become fully solvent-exposed (thereby exposing W186) and then fold over toward the active site pocket, such that H166 (or, less likely, H151) *replaces the water ligand on the active site zinc*. With the second zinc site unoccupied, calcium site 1 would likely be destabilized and lose affinity for its ion. These shifts in local conformation would have the effects of inhibiting the enzyme, where the His would be fulfilling much the same role as the Cys residue of the inhibitory propeptide. This analogous mechanism might be referred to as a "His switch". This mechanism would explain (a) the calcium requirement for enzymatic activity, (b) the zinc-dependent activity reported here (Figure 8) and by others (Salowe et al., 1992), and (c) the inhibition of activity by the addition of EDTA, which is not expected to remove tightly bound zinc at neutral pH. Thus, one of the roles of the second zinc would be to control, or "turn on", enzymatic activity of the mature enzyme. This mechanism could well involve cooperativity with calcium binding at site 1, i.e., the binding of calcium might help pull the loop away from the active site and help preorganize the active site pocket (particularly S1′) for substrate binding.

A modeling exercise was undertaken in order to assess whether the His-switch mechanism is possible. The second zinc ligand H166 was modeled as if the side chain Cα and Cβ had rotated about the two peptide bonds, bringing it close to the catalytic zinc (see Materials and Methods for details of the modeling exercise). The results are shown in Figure 9. It is possible to place the Nϵ of H166 within 2.1 Å of the zinc atom, such that the Nϵ occupies essentially the same position as the S atom of Cys 75 in the pro structure, which is 2.3 Å from the zinc. The minimized model shows a change in the Cα−Cα distance of H166 before and after the exercise of 0.85 Å, while the Cβ−Cβ distance changes by 1.80 Å. Standard peptide geometry is conserved. The hydrogen bond network of the β sheet structure which includes H166 is *completely conserved*, with the following minimal changes to the length of the hydrogen bonds: H179 N−H166 O, 2.67−3.10 Å; H179 O−H166 N, 3.00−3.18 Å; D181 N−L164 O, 3.17−2.93 Å.

It is stressed that the modeling exercise was intended to determine the *minimum* structural changes necessary to fit a zinc 2 ligand into position to be a fourth ligand to the

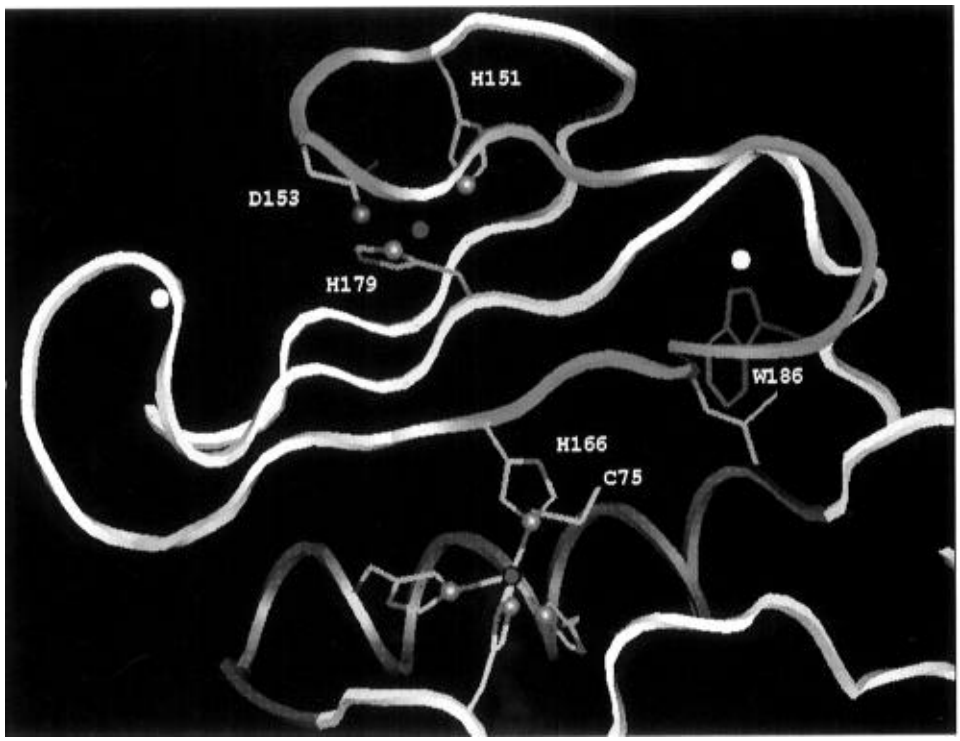

FIGURE 9: Model of minimal changes required to place the side chain of H166 into the fourth ligand position of the active site zinc. The zinc ions are in red and the calcium ions are shown in yellow. The peptide backbone of the loop from H151 through H166 is shown in green. The β structure forming the base of the second zinc site is shown in yellow, and the central α-helix which contains two of the His ligands for the catalytic zinc site is shown in red.

catalytic zinc. It is not unreasonable to expect more extensive changes to the structure upon demetalization of the H151−H166 loop, including but not necessarily limited to folding of the solvated loop into the active site in a similar way to which the propeptide occupies the active site cleft in the proprotein structure (in both cases the direction of the strand is reversed from that of peptide-based inhibitors), complete loss of integrity of the calcium 1 site, and L164 moving such that the Leu side chain fits into the S1′ site. With regard to the last possibility, a transition state analogue MMP inhibitor described recently (Stams et al., 1994) has a Leu side chain in the P1′ position. While there may be more than one conformational state of the loop under these conditions, the internal conformation and packing of the molecule need not change significantly. This would be consistent with our results showing that the apo mature protein, which is missing both zincs, is similar to the holoprotein in its stability to thermal unfolding.

Finally, we recall that the H166−H151 loop is conserved in the MMPs and that the presence of this loop distinguishes the MMPs from the related metzincins astacin and adamalysin (Bode et al., 1993). Although both of these enzymes have similar β structural motifs, the crayfish digestive enzyme astacin has a short loop in place of the MMP zinc−calcium loop, and the snake toxin adamalysin has a helix in place of the loop. Thus, the proposed function of the zinc−calcium loop in MMPs as a switch for modulating the activity of the mature enzyme, sensitive to local zinc concentrations in the extracellular matrix, would provide a good evolutionary pressure for conservation of the structure in this class of proteases. Future efforts will focus on testing the His-switch mechanism experimentally using techniques such as X-ray crystallography and NMR spectroscopy.

CONCLUSIONS

To summarize, we have undertaken an examination of the role of the propeptide in folding and stability of tMMP3 and of factors which modulate the autocatalytic activation of this enzyme. The propeptide is *not* required for folding of the catalytic domain. The propeptide *is* required for maintaining a latent form of the protein, as previously demonstrated (Freimark et al., 1994). Autocatalytic activation of prot-MMP3 to tMMP3 is protein concentration dependent; therefore, autocatalytic activation is a bimolecular process. The rate-limiting step for activation is not an unfolding step. Activation is zinc concentration dependent, implying that a low binding affinity zinc modulates either the activation process specifically or the activity of tMMP3 in general. We have speculated that the second zinc site may be part of a His-switch activation mechanism in the mature form of the enzyme. Upon dissociation of the zinc from the second site, one of the freed His ligands can replace bound water at the catalytic zinc site, inhibiting catalytic activity.

The binding of metal ions (zinc and/or calcium) provides minimal stabilization of the catalytic domain against thermal unfolding, as monitored by changes in circular dichroism. Apo tMMP3 appears to have a native-like intermediate structure in which one or more Trp side chains is more solvent-exposed than in the holo form. We have speculated that the Trp(s) involved is (are) W124 and/or W186. This intermediate apo structure can be thermally unfolded and refolded, after which enzymatic activity is recovered upon addition of metals.

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