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Characterization of Zinc-Binding Sites in Human Stromelysin-1: Stoichiometry of the Catalytic Domain and Identification of a Cysteine Ligand in the Proenzyme

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ABSTRACT: A determination of the zinc stoichiometry of the catalytic domain of the human matrix metalloproteinase stromelysin-1 has been carried out using enzyme purified from recombinant *Escherichia coli* that express C-terminally truncated protein. Atomic absorption spectrometry revealed that both the proenzyme (prostrom^{255}) and the mature active form (strom^{255}) contained nearly 2 mol of Zn/mol of protein. Full-length prostromelysin purified from a mammalian cell culture line also contained zinc in excess of 1 equiv. While zinc in prostrom^{255} could not be removed by dialysis against *o*-phenanthroline, similar treatment of mature strom^{255} resulted in the loss of one-half of the original zinc content. The peptidase activity of the zinc-depleted protein was reduced by >85% but could be restored upon addition of Zn^{2+} or Co^{2+}. Addition of a thiol-containing inhibitor to a CoZn hybrid enzyme resulted in marked spectral changes in both the visible and ultraviolet regions characteristic of sulfur ligation to Co^{2+}. This direct evidence for an integral role in catalysis and inhibitor binding confirms the location of the exchangeable metal at the active site. To examine the environment of zinc in the proenzyme, a fully cobalt-substituted proenzyme was prepared by in vivo metal replacement. The absorbance features of dicobalt prostrom^{255} were consistent with metal coordination by the single cysteine present in the propeptide, although the data do not allow assignment to a particular zinc site. This spectroscopic evidence supports a model proposed for all matrix metalloproteinase proenzymes in which a zinc–cysteine interaction is required for the preservation of latency [Van Wart, H. E., & Birkedal-Hansen, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5578–5582].

Stromelysin-1 is a member of the matrix metalloproteinase (MMP)[1] family of enzymes [reviewed in Woessner (1991)] that are able collectively to degrade all of the components of connective tissue matrices in normal processes such as tissue remodeling as well as in pathological conditions such as arthritis and tumor invasion. Stromelysin, also known as MMP-3, is secreted by synoviocytes and articular chondrocytes in response to the inflammatory mediator interleukin-1 (Murphy et al., 1986; Saus et al., 1988; MacNaul et al., 1991), and its broad substrate range encompasses the major extracellular matrix components of cartilage. A specific cleavage site has recently been identified in human aggrecan digested with stromelysin (Flannery et al., 1992), futher reinforcing the hypothesis that this enzyme is a central agent in the proteolytic destruction of cartilage proteoglycans associated with osteo- and rheumatoid arthritis. Detailed structural information on the enzyme may thus aid the design of potent inhibitors with potential therapeutic value for the treatment of these diseases.

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[1] Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; OP, *o*-phenanthroline; prostrom^{255}, prostromelysin truncated at residue 255; strom^{255}, mature stromelysin truncated at residue 255; HPLC, high-performance liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; ACE, angiotensin converting enzyme.

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Members of the MMP family are characterized by their high primary sequence similarity (~30% identity in the N-terminal catalytic domain), requirement for Ca^{2+}, inhibition of activity by tissue inhibitors of metalloproteinases (TIMP), and secretion as inactive proenzymes which may be activated by proteinases or organomercurials with loss of approximately 80 amino acids from the N-terminus. The presence of zinc has been demonstrated in collagenase (MMP-1) (Seltzer et al., 1977; Swann et al., 1981), and a requirement for this metal as an essential MMP cofactor has been deduced from inhibition by chelating agents such as *o*-phenanthroline (OP). However, a quantitative determination of metal content has not been published for any MMP. Although the identity of the putative metal-coordinating amino acids is unknown, the histidines present in the strictly conserved MMP sequence HExxH are likely ligands based on the crystal structures of bacterial metalloendoproteinases containing the same motif (Vallee & Auld, 1990). The latency of the proenzyme from MMPs has been proposed to arise largely from the interaction of an active site zinc atom with a cysteine residue in the highly conserved sequence PRCGVPDV near the carboxy end of the N-terminal propeptide (Van Wart & Birkedal-Hansen, 1990). Disruption of this bond by one of several pathways is believed to create an open coordination site on the metal which makes it catalytically competent. While mutagenesis experiments have supported this attractive hypothesis (Windsor et al., 1991; Park et al., 1991), direct physical evidence for cysteine ligation of a metal center has been lacking.

Given the likely importance of zinc in proenzyme latency and mature enzyme catalysis, we have sought to more rigorously define its presence and function. For this purpose we have used a C-terminally truncated form of the proenzyme containing the putative catalytic domain common to all MMPs (Marcy et al., 1991). This 29K protein (prostrom^{255}) has been shown to activate identically to the wild-type proenzyme, and the corresponding mature 19K protein (strom^{255}) has kinetic constants very similar to those of full-length stromelysin for a peptide substrate and inhibitor. In this paper we describe our initial experiments exploring the role of zinc in (pro)-strom^{255} and our unexpected discovery of a second zinc-binding site.

Materials and Methods

Materials. Reagents were from previously listed sources (Marcy et al., 1991). Inhibitor U19345 [$HSCH_2CH(iBu)$-$COPheNH_2$; Caputo et al., 1987] was prepared according to literature procedures and purified by reverse-phase HPLC. In general, precautions against adventitious metal ion contamination were taken as described (Holmquist, 1988). Buffers and substrate were prepared in water purified to ≥18 MΩ by a Milli-Q reagent water system (Millipore) and further rendered "metal-free" by extraction with 0.02% dithizone (Fluka) in CCl_4 or passage through Chelex-100 resin (Bio-Rad). Spectra/Por 1 dialysis tubing (10-mm flat width, MW cutoff 6000–8000) was treated as recommended (Auld, 1988a).

Enzyme Purification and Assay. Recombinant full-length prostromelysin, purified from a mammalian cell culture line (Koklitis et al., 1991), was a gift of Dr. Michael Lark. It was further purified for metal determinations by analytical size-exclusion HPLC on coupled Biosep SEC-S2000 and SEC-S3000 columns (Phenomenex). The catalytic domain of human stromelysin-1 (prostrom^{255}) containing the N-terminal 255 residues was purified from recombinant *Escherichia coli* as previously described (Marcy et al., 1991). The mature form was prepared by heating the proenzyme at 53 °C for 1 h (Koklitis et al., 1991), followed by gel filtration on a HiLoad Superdex-75 preparative grade column. To prepare zinc-depleted strom^{255}, the protein was dialyzed at 300–400 μM for approximately 20 h at 4 °C versus three changes of 500 volumes of buffer A (50 mM HEPES, 5 mM $CaCl_2$, pH 7.0) containing 2 mM OP, followed by dialysis versus three to four changes of 200 volumes of buffer A alone for an additional 20 h. Strom^{255} activity was measured by cleavage of the peptide [Nle^{11}]substance P (Harrison et al., 1989; Teahan et al., 1989). Assays of metal-depleted enzyme contained buffer A + 0.25 mM peptide and were initiated with 1 μM enzyme. The reactions were terminated after 10 min with 0.2 volume of 4% trifluoroacetic acid. Product was quantitated by HPLC as previously described (Marcy et al., 1991).

Table I: Metal Content of Protein Samples Determined from Atomic Absorption

sample	metal content, mol/mol of protein[a] zinc	cobalt	*n*
prostrom^{255}	1.82 ± 0.06		6
strom^{255}	1.75 ± 0.13		6
OP-treated strom^{255}	0.84 ± 0.07		3
prostromelysin (full length)	1.50 ± 0.04		2
Co-substituted prostrom^{255}, batch 1	1.29	0.75	
Co-substituted prostrom^{255}, batch 2	0.14	1.84	

[a] Values are expressed as the average ± standard deviation for *n* determinations.

Preparation of Dicobalt Prostrom^{255}. In vivo substitution of cobalt for zinc in prostrom^{255} was accomplished using a modification of the procedure described by Speckhard et al. (1977). Recombinant bacteria harboring the truncated stromelysin gene were grown in a zinc-depleted M9 medium supplemented with 0.1% casamino acids and 5 μM cobalt(II) acetate. Media components were treated with dithizone or Chelex as described above to reduce their zinc content. After 12 h of induction with 0.1 mM isopropyl β-D-thiogalactoside, the cells were pelleted and resuspended in 100 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0), 5 mM $CaCl_2$, 0.05% Brij-35, 0.02% sodium azide, 0.1 mM $CoCl_2$, and 1 μg/mL each leupeptin and aprotinin. Cells were then used for prostrom^{255} purification as previously described (Marcy et al., 1991) without additional changes.

Other Methods. Protein concentrations were determined from the absorbance at 280 nm using extinction coefficients calculated from the known amino acid sequences (Whitham et al., 1986) and the data in Mach et al. (1992) and verified by comparison of the spectra of native and denatured proteins as described in Gill and von Hippel (1989): prostrom^{255}, 34380 M^{-1} cm^{-1}; strom^{255}, 28460 M^{-1} cm^{-1}; full-length prostromelysin, 69614 M^{-1} cm^{-1}. Absorption spectra were recorded on 400-μL samples with a 1-cm path length versus a buffer reference in a Cary 3 spectrophotometer. Protein samples for metal determination were diluted with metal-free buffer A to be within the range of 0.1–1 ppm of Zn or 1–5 ppm of Co and analyzed directly by flame atomic absorption spectrometry at Robertson Microlit Laboratories (Madison, NJ). Multielement analysis by inductively coupled plasma emission spectroscopy was performed after acid treatment at the Research Analytical Laboratory of the University of Minnesota.

Results

Zinc Stoichiometry. To verify and quantitate the presence of zinc in stromelysin, analyses were performed by atomic absorption spectrometry on both the proenzyme and mature forms of the C-terminally truncated protein purified from

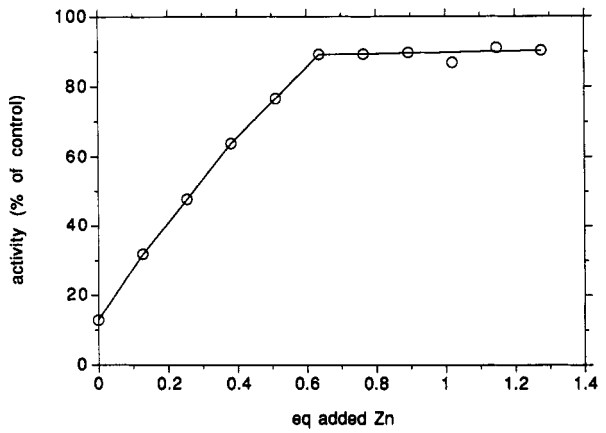

FIGURE 1: Restoration of enzymatic activity to OP-treated strom^{255} (0.8 equiv of Zn) by titration with Zn^{2+}. Peptidase activity was measured as described under Materials and Methods and is expressed as a percentage of untreated control enzyme.

recombinant *E. coli*. As summarized in Table I, the zinc content of both prostrom^{255} and strom^{255} was reproducibly found to be nearly 2 mol/mol of protein. The same result was obtained for prostrom^{255} analyzed by inductively coupled plasma emission spectroscopy which also confirmed the absence of Fe, Mn, Cu, Pb, Ni, Cr, and Cd. The unexpected "excess" zinc bound within this catalytic domain of the full-length protein remained tightly associated during size-exclusion chromatography or dialysis against metal-free buffer. Overnight incubation of prostrom^{255} with 2 equiv of zinc followed by size-exclusion chromatography also had no effect upon metal content. Although it seemed unlikely that a new high-affinity zinc site could be created by truncation of the full-length protein, we also analyzed two full-length 57-kDa prostromelysin samples recovered from mammalian cell cultures and found zinc in excess of 1 equiv to be present in these as well (Table I). We do not currently have an explanation for the slightly lower content of these full-length samples.

Depletion and Replacement of Catalytic Zinc. Preliminary experiments with metal chelators established that *o*-phenanthroline (OP) was a more potent inhibitor of strom^{255} than 8-hydroxyquinoline, 2,2′-bipyridyl, or pyridinedicarboxylate. The mode of action of OP was determined from the dependence of inhibition upon OP concentration according to the equation $\log (V_c/V_i - 1) = -\log K_I + n \log [I]$ (Auld, 1988b). A slope value of approximately 3 was obtained from this plot (data not shown), indicating that inhibition was due to removal of metal from the enzyme rather than the formation of a complex in situ. Consistent with these kinetic results, dialysis of mature strom^{255} against OP was successful in removing zinc from the enzyme; however, in repeated experiments, the treated protein retained one-half of its original zinc content (Table I). When 5 equiv of Zn was added back to the zinc-depleted protein followed by dialysis against metal-free buffer, the original metal content was restored, indicating the process to be reversible. In contrast to the behavior of the mature enzyme, the zinc content of prostrom^{255} was unchanged after dialysis against OP. It thus appears that a portion of the zinc in the proenzyme becomes accessible to chelators upon loss of the N-terminal propeptide, while the rest of the metal remains inaccessible.

The peptidase activity of strom^{255} toward the substrate [Nle^{11}]substance P was reduced by >85% by the metal depletion procedure but could be regenerated to 90% of the original level by addition of Zn^{2+} (Figure 1). Although a high enzyme concentration (1 μM) was used in activity assays of zinc-depleted protein to minimize the effect of adventitious metal ion contamination, the difficulty in completely removing trace amounts of zinc, particularly from the substrate, may account for the 13% residual activity observed in this experiment. Consistent with this hypothesis, the activity of the zinc-depleted protein did not increase linearly with enzyme concentration; the actual remaining activity of this preparation was estimated to be 6%. The titration in Figure 1 demonstrates that the catalytic activity restored was proportional to the amount of Zn^{2+} (~77% activity restored by the addition of ~0.65 equiv of Zn). These results are consistent with the involvement of the zinc in the chelator-accessible site in the catalytic mechanism.

Table II: Metal Competition for Zinc-Depleted Enzyme[a]

entry	first metal	second metal	rel activity
1			23
2	Zn		100
3	Zn	Co	99
4	Zn	Cd	98
5	Co		79
6	Co	Zn	96
7	Co	Cd	36
8	Cd		8
9	Cd	Zn	54

[a] 10 μM zinc-depleted strom^{255} was preincubated for 15 min with 1 equiv of metal followed by the addition of either no metal or 1 equiv of a second metal for another 15 min. Activity was determined as described under Materials and Methods after diluting the protein 10-fold into buffer containing substrate.

To further characterize this accessible site, several divalent first-row transition metal ions were surveyed at 1 mM for their ability to substitute for zinc. Both Mn^{2+} and Ni^{2+} were inactive as replacements, while Fe^{2+} restored partial activity (~30%). Only Co^{2+} generated activity (80%) comparable to Zn^{2+}. Cu^{2+} and Cd^{2+} decreased the apparent residual activity of the metal-depleted enzyme to 7% and 3%, respectively. Since Cu^{2+} is also an inhibitor of the native enzyme at concentrations <1 mM (unpublished results), it is difficult to reach any conclusion about its specific interactions with the zinc-depleted protein. The apparent inhibition of zinc-depleted enzyme by Cd^{2+}, however, could be observed at concentrations as low as 1 μM (stoichiometric with the protein), a level which did not inhibit native enzyme at all.

An explanation for this phenomenon is that Cd^{2+} competes effectively with adventitious zinc in the assay for the vacant metal-binding site on the protein. Table II summarizes data that demonstrated directly the competition among metals. After preincubation of metal-depleted enzyme with 1 equiv of Zn^{2+}, neither Co^{2+} nor Cd^{2+} altered the activity of the Zn^{2+}-reconstituted enzyme (entries 2–4). The more weakly bound Co^{2+}, however, was substantially displaced by the subsequent addition of either Zn^{2+}, which increased activity from 79% to 96% (entries 5 and 6), or Cd^{2+}, which decreased activity from 79% to 36% (entries 5 and 7). Zn^{2+} was also able to displace Cd^{2+} from enzyme reconstituted with that metal (entries 8 and 9).[2] These results indicate that several alternative metals with potentially useful spectroscopic properties can be exchanged for zinc in the catalytic center of strom^{255}.

Spectral Properties of a CoZn Hybrid Strom^{255}. The addition of Co^{2+} to zinc-depleted strom^{255} generated a broad, weak absorption envelope between 450 and 640 nm ($\epsilon \approx 100$

[2] Entry 4 of Table II suggests that the addition of Zn^{2+} to Cd^{2+}-reconstituted enzyme in entry 9 should have fully reactivated the protein. A closer examination revealed that Cd^{2+} displacement required a significantly longer reaction time (>1 h) than allowed in this experiment (unpublished results); this phenomenon was not investigated further.

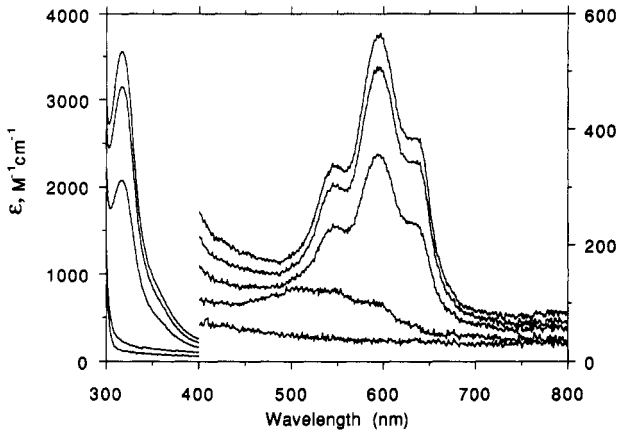

FIGURE 2: Electronic spectra of cobalt-substituted strom^{255} and its complex with thiol-containing inhibitor U19345. The cuvette contained 73 μM zinc-depleted strom^{255} (0.8 equiv of Zn) in 50 mM HEPES and 5 mM $CaCl_2$, pH 7.0 (bottommost spectrum). The remaining spectra, from bottom to top, represent the subsequent additions of 1.4 equiv of Co^{2+} and 0.5, 1.0, and 1.5 equiv of inhibitor, respectively. A control experiment demonstrated that no absorption was formed between Co^{2+} and the inhibitor at the same concentrations in the absence of enzyme.

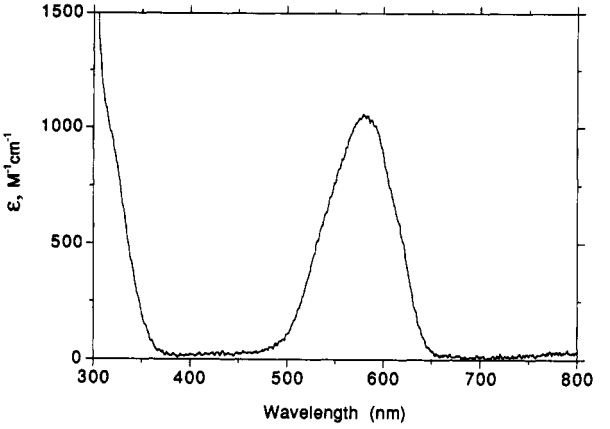

FIGURE 3: Electronic spectrum of dicobalt prostrom^{255}. The protein (50 μM; batch 2 in Table I) was scanned in 50 mM HEPES and 5 mM $CaCl_2$, pH 7.0. The corresponding region of the dizinc proenzyme had no absorption above 305 nm.

M^{-1} cm^{-1}) in the visible region of the electronic spectrum (Figure 2). Significant perturbations to these d → d transitions occurred upon titration with the thiol-containing inhibitor U19345. The addition of more than 1 equiv of the compound was required for the nearly full development of the spectrum (~90%) because of the elevated K_i for the CoZn hybrid enzyme relative to the dizinc enzyme (6 vs 1 μM, respectively). As seen in Figure 2, the entire absorption envelope was greatly intensified and red-shifted by approximately 50 nm. Distinct bands became observable with the maximum central absorption at 595 nm ($\epsilon \approx 560$ M^{-1} cm^{-1}) and side bands at 547 and 634 nm. In addition, an intense new feature appeared in the ultraviolet region at 316 nm ($\epsilon \approx 3600$ M^{-1} cm^{-1}) which can be assigned to a sulfur → cobalt charge-transfer transition from comparison with established models of cobalt–sulfur complex ions and cobalt-substituted metalloproteins containing cysteine ligands (Sugiura, 1978; Vallee & Galdes, 1984). Since strom^{255} contains no cysteinyl residues, the coordinating sulfur must be supplied by the bound inhibitor. This direct evidence for the integral role of the exchangeable metal in inhibitor binding, coupled with its demonstrated essential role in catalysis, confirms that the location of the exchangeable metal is in the enzyme active site.

Spectrum of Fully Cobalt-Substituted Prostrom^{255}. Since treatment of prostrom^{255} with OP had no effect upon the protein's zinc content, we exploited the interchangeability of Co^{2+} and Zn^{2+} by employing an in vivo substitution methodology (Speckhard et al., 1977) to incorporate the spectroscopic reporter into the proenzyme. *E. coli* containing the expression plasmid for the truncated prostromelysin gene were grown and induced in a minimal medium depleted of zinc and supplemented with cobalt. Metal analysis of the purified prostrom^{255} revealed varying levels of success using the in vivo replacement strategy (Table I), although in both batches the combined content of zinc and cobalt was 2 mol/mol of protein, indicating specific substitution into the zinc sites. In the best preparation nearly complete substitution occurred, and the high cobalt content was apparent from the purple color of the concentrated protein. As seen in Figure 3, dicobalt-substituted prostrom^{255} has an intense visible absorbance with a maximum at 580 nm ($\epsilon \approx 1000$ M^{-1} cm^{-1}). Notable as well is the major band in the near-ultraviolet which overlaps the aromatic amino acid absorption and appears as a shoulder at 320 nm ($\epsilon \approx 1000$ M^{-1} cm^{-1}) that extends out to 360 nm. These features are qualitatively similar to the spectrum of cobalt-substituted mature enzyme complexed with the thiol inhibitor (Figure 2). In the proenzyme, however, the S → Co charge transfer can only arise from an endogenous ligand, the single cysteine present in the propeptide of prostrom^{255}.

Discussion

The expression and purification of a stable C-terminally truncated catalytic domain of human fibroblast stromelysin have made detailed structural characterization possible that would be difficult with the relatively unstable and heterogeneous full-length protein. We have begun with the elucidation of the roles of zinc in proenzyme latency and in mature enzyme catalysis and inhibition. Earlier observations of inhibition by metal chelators and the restoration of activity by zinc indicated that stromelysin was, like the more extensively studied MMP collagenase, a metalloenzyme (Okada et al., 1986). There has yet to be, however, a published determination of metal stoichiometry for any member of the growing list of matrix metalloproteinases. The discovery of a second intrinsic zinc-binding site in stromelysin was unexpected, and it remains to be determined whether other members of the MMP family will also contain more than one zinc atom. The noncompetitive inhibition of human neutrophil collagenase by certain heavy metals led to the proposal of an extrinsic metal-binding site in that enzyme (Mallya & Van Wart, 1989), but further evidence for its existence has not been reported. The occurrence of more than one transition metal binding site in a peptidase is rare in the literature, although not unprecedented. Aminopeptidases from bacterial (Prescott et al., 1985) and mammalian (Burley et al., 1991) sources containing two zinc sites have been identified. Neither protein is likely to be a useful model, however, as the structural and functional interactions between metal sites in these enzymes have no parallels in stromelysin. A recent reexamination of angiotensin converting enzyme (ACE) revealed that the somatic isozyme has a stoichiometry of 2 mol of zinc/mol of protein (Ehlers & Riordan, 1991). In this case a second site was not completely unanticipated since the primary structure has, in contrast to stromelysin, tandem homologous domains containing the same metal binding site motif.

Only one of the two zinc atoms present in mature strom^{255} could be removed by dialysis against OP, a common procedure employed for the preparation of apoenzymes (Wagner, 1988). The correlation between peptidase activity and zinc occupancy

of this exchangeable site (Figure 1) suggests that the removable zinc is at the enzyme's active site. Spectroscopic evidence from the cobalt–zinc hybrid enzyme confirms this assignment. The addition of cobalt to zinc-depleted strom^{255} generated a catalytically competent enzyme with a broad, weak absorption in the visible portion of the electronic spectrum (Figure 2). These absorption features are comparable to those of other cobalt-substituted zinc metalloproteinases that have been characterized crystallographically and are indicative of irregular tetrahedral geometry (Vallee & Galdes, 1984). The sensitivity of these d → d transitions to changes in metal coordination and/or geometry has been exploited to demonstrate the interactions of the metal in carboxypeptidase A, thermolysin, and ACE with inhibitors containing coordinating thiol or carboxyl functionalities (Holmquist & Vallee, 1979; Bicknell et al., 1987). The CoZn hybrid of strom^{255} is a superb example of this phenomenon. As seen in Figure 2, the addition of the thiol-containing inhibitor U19345 to strom^{255} containing Co^{2+} in the exchangeable site shifts the visible absorption to higher wavelengths and markedly intensifies its molar absorptivity. In addition, the appearance of the intense new ultraviolet feature at 316 nm characteristic of S → Co charge transfer provides direct evidence that the inhibitor supplies a ligand to the metal. While the strom^{255} spectrum qualitatively resembles spectra obtained for cobalt-substituted carboxypeptidase A, thermolysin, and ACE complexed with thiol-containing inhibitors, the intensity of the bands is matched only by the ACE complex with the drug captopril (D-2-methyl-3-mercaptopropanoyl-L-proline) (Bicknell et al., 1987). The distinct positions and intensities of the visible and charge-transfer maxima in all four zinc metalloenzymes emphasize the differences in their active site coordination geometries.

In contrast to the mature enzyme, both zinc sites in the proenzyme are inaccessible to chelators. In order to introduce a spectroscopic probe into prostrom^{255}, we relied upon the general tolerance of zinc enzymes toward cobalt as a functional surrogate (Vallee & Galdes, 1984). The in vivo substitution strategy successfully employed for a constitutive bacterial RNA polymerase (Speckhard et al., 1977) was also successful for our induced enzyme (Table I). Purification without reexchange for adventitious zinc was aided by the sequestration of the metal within the folded proenzyme. The spectrum of dicobalt prostrom^{255} shown in Figure 3 has intense absorption bands in the visible centered on 580 nm and in the ultraviolet manifested as a shoulder at 320 nm on the aromatic amino acid envelope. These spectral features bear the signature of sulfur coordination as discussed above for the mature enzyme complexed with a thiol-containing inhibitor.

The only endogenous residue in prostrom^{255} able to supply a thiol ligand is cysteine-75 located in the propeptide sequence PRCGVPDV that is highly conserved in all MMPs sequenced to date (Woessner, 1991). The coordination of this cysteine to zinc has been proposed to be a crucial structural feature of proenzyme latency (Van Wart & Birkedal-Hansen, 1990). The spectroscopic observation of a protein sulfur ligand to cobalt in prostrom^{255} provides direct confirmation of cysteine coordination in a MMP. There are few examples outside of the MMP family of a zinc site containing only one cysteine. Sorbitol dehydrogenase from human liver (Maret, 1989) and β-lactamase II from *Bacillus cereus* (Sutton et al., 1987) have a single cysteine ligand, but in both cases the zinc is present in an open active site and is available for coordination by substrate molecules during turnover. The cysteine-coordinated zinc in prostromelysin, however, shows no evidence of accessibility. Its apparent role as a labile structural site is distinct from the more classical examples of zinc structural sites, e.g., alcohol dehydrogenase, aspartate transcarbamoylase, and zinc fingers, all of which contain at least two cysteine ligands (Vallee & Auld, 1990).

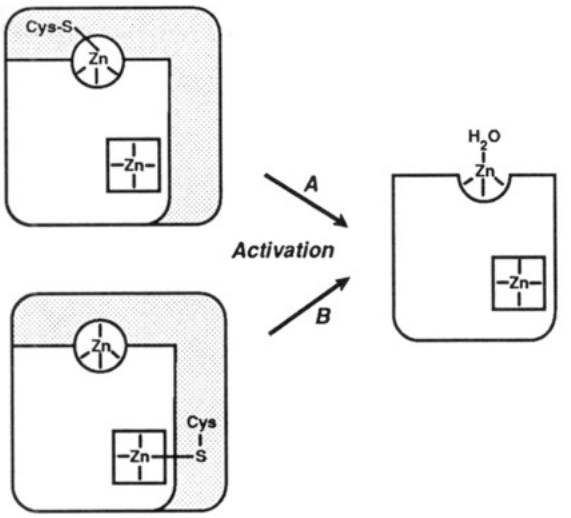

FIGURE 4: Structural models for prostromelysin. The shaded region represents the N-terminal propeptide. In path A, a catalytic site is created when the Cys-75 ligand is displaced by a water molecule during activation; the other zinc site plays no role in the process. In path B, Cys-75 coordinates the zinc that is noncatalytic and nonexchangeable in the mature enzyme; the catalytic site zinc is blocked by some steric and/or conformational constraint until activation releases the propeptide. No coordination number or geometry of the metal is implied.

Our experiments do not allow us to conclude which of the two metal sites in prostromelysin interacts with the sulfhydryl side chain of the propeptide cysteine. The original "cysteine switch" model posits that the thiol(ate) ligand is replaced by a water molecule upon activation to create the active site. In light of our new information, the simplest application of the model to stromelysin would have the cysteine coordinating the zinc atom that becomes catalytic and exchangeable upon activation. However, it is at least a formal possibility that the "switch" site corresponds to the nonexchangeable zinc site of the mature enzyme; in this case the propeptide interferes in some way with the access of substrates or chelators to the zinc in the catalytic site. These alternative models are depicted in Figure 4. Further experimentation is required to distinguish between these possibilities and identify the individual role(s) of each zinc site.

Other than the cysteine-75 in the proenzyme of stromelysin, no other zinc ligands have been definitively identified in MMPs. Limited amino acid sequence similarity to bacterial proteinases such as thermolysin has directed attention to the histidines present in the metal-binding motif HExxH (located at residues 201–205 in prostromelysin), but there is no conserved glutamate 20 residues C-terminal to this sequence analogous to the third zinc ligand of that enzyme. Amino acid sequence alignment of the putative catalytic domains of MMPs has identified other candidates for ligands to the two zinc atoms (Murphy et al., 1991). Among the eight histidines present in mature strom^{255}, six are strictly conserved in the family at positions 151, 166, 179, 201, 205, and 211. Conserved residues with carboxylate side chains include apartates 107, 114, 141, 153, 158, 177, 181, 183, 237, and 238 and glutamates 184 and 202. Furthermore, it has been noted previously (Stöcker et al., 1990) that the conserved MMP sequence HExxHxxGxxH present at residues 201–211 in stromelysin has striking parallels with a phylogenetically

diverse group of proteins resembling the crayfish metalloproteinase astacin (Dumermuth et al., 1991) as well as bacterial metalloproteinases from *Erwinia* and *Serratia* genera (Delepelaire & Wandersman, 1989; Braunagel & Benedik, 1990) and snake venom metalloproteinases (Takeya et al., 1990; Sanchez et al., 1992). The importance of the glutamate and the first and third histidines of this sequence in transin (rat stromelysin) was demonstrated by the loss of activity toward casein upon mutation of any one of these residues (Sanchez-Lopez et al., 1988). Thus it seems possible that the zinc ligands in the catalytic site of stromelysin include histidines 201, 205, and 211, while the noncatalytic site may utilize a constellation of other conserved histidine and/or aspartate residues. Testing these hypotheses is the subject of our continuing research in this area.

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