Zinc Content and Function in Human Fibroblast Collagenase†

Eric B. Springman,‡,§ Hideaki Nagase,|| Henning Birkedal-Hansen,⊥ and Harold E. Van Wart*,▽

Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306, Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66103, Department of Oral Biology and Research Center in Oral Biology, University of Alabama at Birmingham, UAB Station, Birmingham, Alabama 35294, and Institute of Biochemistry and Cell Biology, S3-1, Syntex Discovery Research, Palo Alto, California 94303

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ABSTRACT: The zinc contents of samples of human fibroblast collagenase (HFC) purified by different procedures and of samples purified by the same procedure but prepared for analysis by different dialysis protocols have been determined by atomic absorption spectroscopy. Both the purification method and dialysis conditions affect the zinc stoichiometry. Samples purified with and without the use of a zinc-chelate chromatography step and prepared by dialysis against 1 mM $CaCl_2$ had zinc to enzyme ratios of 1.46 and 1.22, respectively. When the first sample was prepared by dialysis against 0 and 10 mM $CaCl_2$, the values changed to 0.15 and 1.94, respectively. Thus, the zinc content of HFC is critically dependent upon the dialysis conditions used to free the enzyme from adventitious metals. This could account for the disparate reports in the literature that give zinc stoichiometries for members of the matrix metalloproteinase (MMP) family of between 1 and 2. The mechanism of inhibition of the one zinc form of HFC by 1,10-phenanthroline (OP) and 4-(2-pyridylazo)resorcinol has been studied in detail. Inhibition by both chelating agents is time dependent and biphasic. There is an initial, instantaneous inhibition characterized by the involvement of a single inhibitor molecule that corresponds to the formation of a ternary complex between the zinc atom, enzyme, and chelator. This is followed by a second, slower phase involving removal of the zinc atom from the enzyme and its chelation by two molecules of inhibitor. Inhibition of four other human MMPs by OP shows similar characteristics and is thought to occur by the same mechanism.

The matrix metalloproteinases (MMPs)[1] are a family of homologous enyzmes that play a leading role in the catabolism of the macromolecular components of the extracellular matrix in a variety of normal and pathological processes (Van Wart & Mookhtiar, 1990; Birkedal-Hansen et al., 1993). Each family member is capable of hydrolyzing a characteristic set of matrix components, and, collectively, these enzymes are capable of hydrolyzing most of the major extracellular matrix proteins. New human MMPs have continued to be discovered with the family currently consisting of 11 enzymes. However, five of these MMPs have been known the longest and have been the most extensively characterized. Three of these MMPs are produced by fibroblasts, including a collagenase capable of hydrolyzing interstitial collagens (HFC or MMP-1, EC 3.4.24.7), a 72 kDa type IV collagenase or gelatinase A (HFG or MMP-2, EC 3.4.24.24), and a proteinase called stromelysin-1 (HFS or MMP-3, EC 3.4.24.17) that degrades proteoglycan core protein and other matrix components. Two different MMPs are produced by neutrophils, including a distinct collagenase (HNC or MMP-8, EC 3.4.24.34) and a 92 kDa type IV collagenase or gelatinase B (HNG or MMP-9, EC 3.4.24.35).

Ironically, even though the MMPs have been widely recognized to be metalloenzymes and named accordingly, very little attention has been directed to the metallobiochemistry of these enzymes. All of the MMPs contain a catalytic domain that contains the zinc-binding motif HE*xx*H*xx*G*xx*H that is present in astacin (Vallee & Auld, 1992), a crayfish proteinase whose crystal structure reveals this locus to be the site of chelation of an active site metal atom (Bode et al., 1992) confirmed by independent analysis to be zinc (Stocker et al., 1988). Subsequent structural analysis of expressed catalytic domains of HFC (Lovejoy et al., 1994; Borkakoti et al., 1994; Spurlino et al., 1994), HNC (Bode et al., 1994; Stams et al., 1994), and HFS (Gooley et al., 1993) has confirmed that the three His residues of this locus also bind a metal atom in these enzymes. These structures also reveal the presence of a second metal-binding site with a coordination sphere composed of three His and one Asp residues. The location of this site suggests that the bound metal atom stabilizes a loop in these enzymes and serves a structural role.

Although the crystallographic data provide evidence for two metal atoms in these MMPs, direct metal analysis of

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* Author to whom correspondence should be addressed. Tel.: (415) 855-5169. FAX: (415) 354-7554.

‡ Current address: Arris Pharmaceutical Corp., 385 Oyster Point Blvd., South San Francisco, CA 94080. Tel: (415) 737-3240.

§ Florida State University.

|| University of Kansas Medical Center.

⊥ University of Alabama at Birmingham.

▽ Syntex Discovery Research.

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[1] Abbreviations: MMP, matrix metalloproteinase; HFC, human fibroblast collagenase, MMP-1; HFG, human fibroblast gelatinase, MMP-2; HFS, human fibroblast stromelysin-1, MMP-3; HNC, human neutrophil collagenase, MMP-8; HNG, human neutrophil gelatinase, MMP-9; EDTA, disodium ethylenediaminetetraacetic acid; OP, 1,10-phenanthroline; MP, 1,7-phenanthroline; PAR, 4-(2-pyridylazo)resorcinol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Hepps, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonate.

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these enzymes has given confusing results with zinc stoichiometries that vary between 1 and 2 metal atoms per enzyme (Lowry et al., 1992; Salowe et al., 1992; Crabbe et al., 1992; Soler et al., 1994; Willenbrock et al., 1995). The source of the discrepancies in zinc stoichiometries reported for MMPs from different sources and determined by different methods remains unclear. A recent study by Willenbrock and associates (1995) reports that full-length pro-HFS and pro-HFG both contain a single zinc atom, while the corresponding recombinant truncated enzymes lacking the C-terminal domain each contain two zinc atoms. This suggests that the binding constant for one of the zinc atoms is much lower in the full-length enzymes. The inhibition of various MMPs by metal-chelating agents such as 1,10-phenanthroline (OP) has also been widely reported, but no detailed studies have been carried out in which the mechanism of this inhibition has been elucidated. In this study, it is shown that the zinc content of HFC as determined by atomic absorption spectroscopy is dependent upon the purification procedure and method of sample preparation, possibly accounting for the variation in previously reported values for this and other MMPs. The mechanism of inhibition of HFC by 1,10-phenanthroline (OP) and 4-(2-pyridylazo)-resorcinol (PAR) has also been studied in detail.

MATERIALS AND METHODS

Materials. Human fibroblast procollagenase (pro-HFC) was purified from the culture medium of human fibroblasts by two different procedures. Method 1 consisted of chromatography first over heparin−Sepharose-4B followed by zinc-saturated iminodiacetate−Sepharose-4B. The collagenase fraction was concentrated on heparin−Sepharose-4B and applied to an Ultrogel AcA-44 column as described previously (Birkedal-Hansen, 1987). Method 2, based on the procedure of Stricklin et al. (1977), consisted of ion-exchange chromatography over CM-cellulose followed by gel filtration through Ultrogel AcA-44. This purification was used in order to avoid the metal-chelate chromatography step with zinc-charged iminodiacetate−Sepharose. Active HFC was prepared by incubating a portion of the pro-HFC prepared by method 2 with 20 μM $HgCl_2$ in 50 mM Hepes, 10 mM $CaCl_2$, 200 mM NaCl, pH 7.5, at 23 °C for 24 h followed by dialysis in a Spectra/Por membrane (molecular weight cutoff, 12 000/14 000) against the same buffer without $HgCl_2$. Pro-HFG and pro-HFS were purified from the culture media of human fibroblasts by the procedures of Seltzer and co-workers (1981) and Ito and Nagase (1988), respectively. Pro-HFS was activated by treatment with 1 mM 4-aminophenylmercuric acetate for 18 h at 37 °C, and the organomercurial activator was removed by gel filtration. HNC and pro-HNG were purified from human buffy coats by the procedure of Mookhtiar et al. (1990). Both pro-HFG and pro-HNG were activated by incubation with 0.1 mM $HgCl_2$ for 2 h at 37 °C, which was followed by removal of the $HgCl_2$ by gel filtration chromatography.

Bovine erythrocyte carbonic anhydrase was purchased from Worthington. Bovine pancreatic carboxypeptidase A, thermolysin (*Bacillus thermoproteolyticus rokko*), imidazole, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Hepes, Hepps, diphenylthiocarbazone (dithizone), and PAR were purchased from Sigma. The samples of carboxypeptidase A and thermolysin were recrystallized before use by dissolution in 10 mM Hepes, pH 7.5, containing 1 M NaCl and dialysis against several changes of 10 mM Hepes. OP was purchased from Lancaster Synthesis, and 1,7-phenanthroline (MP) was purchased from Aldrich. The zinc atomic absorption standard and $CaCl_2$, NaCl, Brij-35, and CCl_4 were purchased from Fisher. Chelex 100 resin was purchased from Bio-Rad. The thio ester substrate Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-OMe was synthesized by Dr. Mohammed Ghafari of the Department of Chemistry at Florida State University by solution phase coupling using t-Boc chemistry and was purified by silica gel chromatography followed by preparative HPLC. All reagents were prepared in metal-free water or buffers.

Metal-Free Conditions. All experiments were carried out under metal-free conditions (Riordan & Vallee, 1988). Demetalated water was prepared by treatment of double distilled, deionized water with Chelex. Concentrated buffer solutions were rendered metal-free by extraction with a solution of dithizone in CCl_4, and subsequent dilutions were prepared using metal-free water. Dialysis membranes were treated with three changes of a 1 mM OP solution followed by four changes of metal-free water at 65 °C and were stored in metal-free water at 4 °C. All pipet tips, storage containers, and cuvettes were cleaned repeatedly with metal-free water and dried before use. Samples of enzymes were generally freed of adventitious metals by dialysis in metal-free Spectra/Por membranes against four changes (12 h per change) of 1 L of metal-free 2 mM Hepes, 1 mM $CaCl_2$, and 250 mM NaCl, pH 7.5. The dialysis buffer was modified to contain 500 mM NaCl and 0.02% Brij-35 for HNC and HNG. In some experiments with HFC, the $CaCl_2$ concentration of the dialysis buffer was varied. All enzyme samples, including the "standards" carbonic anhydrase, carboxypeptidase A, and thermolysin, were examined before and after dialysis by SDS−PAGE according to the method of Laemmli (1970), using the silver-staining method (Hochstrasser et al., 1988) to confirm their purity and ensure that no significant autolysis had occurred.

Zinc Determinations. Zinc was determined by flame atomic absorption spectroscopy with a Perkin-Elmer model 5100 spectrometer using the impact bead configuration, standard flame and detection conditions, and manual sample injection. A standard curve was constructed in the range 0.50−2.5 μM zinc. Enzyme samples were diluted into the linear range of the standard curve using buffer from the final dialysis step. This buffer was also used as the blank. The zinc concentration of each sample was calculated as the mean of three repetitions. Samples of carbonic anhydrase, carboxypeptidase A, or thermolysin prepared by the same method were included with each set of analyses for reference.

Enzyme Concentrations. Enzyme concentrations were determined spectrophotometrically using a Varian model 210 spectrophotometer. The extinction coefficients for the MMPs used in this study were calculated according to the method of Gill and von Hippel (1989) using the reported sequences for HFC and pro-HFC (Whitham et al., 1986), HNC (Devarajan et al., 1990), HFG (Collier et al., 1988), HNG (Wilhelm et al., 1989), and HFS (Wilhelm et al., 1987) and are listed in Table 1. The extinction coefficients used for carbonic anhydrase, carboxypeptidase A, and thermolysin were $\epsilon_{280} = 5.6 \times 10^{-4}$ M^{-1} cm^{-1} (Lindskog, 1960), $\epsilon_{278} = 6.49 \times 10^{-4}$ M^{-1} cm^{-1} (Bargetzi et al., 1963), and $\epsilon_{280} = 6.62 \times 10^{-4}$ M^{-1} cm^{-1} (Ohta et al., 1966), respectively.

Assays Using Protein Substrates. The collagenase activities of HFC (10 nM) and HNC (1 nM) were quantitated by

Table 1: Calculated Extinction Coefficients for Various MMP[a]

MMP	ϵ_{280} ($\times 10^{-4}$), M^{-1} cm^{-1}
pro-HFC	6.83
HFC	6.44
HNC	7.26
HFG	12.3
HNG	10.4
HFS	6.24

[a] Calculated by the method of Gill and von Hippel (1989).

measuring the rate of hydrolysis of soluble [^{3}H]acetylated rat tendon type I collagen in 50 mM Hepes, 1 mM $CaCl_2$, 200 mM NaCl, pH 7.5, at 30 °C as described previously (Mallya et al., 1986). The gelatinase activities of HFG and HNG (both 5 nM) were quantitated by measuring the rate of hydrolysis of [^{3}H]acetylated rat tendon type I gelatin in 50 mM Hepes, 1 mM $CaCl_2$, 200 mM NaCl, pH 7.5, at 37 °C using 15% TCA to precipitate intact gelatin. [^{3}H]gelatin was prepared by boiling [^{3}H]collagen for 5 min immediately prior to initiation of the assay. HFS (2 nM) activity was quantitated by measuring the rate of hydrolysis of [^{3}H]-acetylated casein in 50 mM Hepes, 1 mM $CaCl_2$, 200 mM NaCl, pH 7.5, at 37 °C using 15% TCA to precipitate the intact casein. For the inhibition studies, the enzymes were incubated in assay buffer with variable concentrations of inhibitor for 1 h prior to the start of the assay in a total volume of 950 μL. The assays were initiated by addition of 50 μL of [^{3}H]substrate.

Thio Ester Assay. In order to study the time dependence of inhibition, the rapid assay based on the hydrolysis of Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-OMe described by Weingarten and Feder (1985) was used. Initial rates of hydrolysis were measured in 50 mM Hepes, 1 mM $CaCl_2$, 200 mM NaCl, pH 7.5, containing 200 μM DTNB at 23 °C by continuously monitoring the absorbance at 412 nm using a Varian Model 219 spectrophotometer. In the absence of inhibitor, first-order kinetics were observed for all five enzymes, indicating that $[S_o] \ll K_M$ for all reactions. In some experiments, the enzymes (2 nM HFC, 0.5 nM HNC, 2 nM HFG, 2 nM HNG, or 1 nM HFS) were incubated with inhibitor for variable times in assay buffer in a total volume of 490 μL at 23 °C. The assays were initiated by addition of 10 μL of thio ester substrate to a final concentration of 20 μM. Initial rates were obtained within 1−5 min of initiation of the assay, including 20 s of mixing time. In other studies, HFC was assayed in the presence of inhibitors with no preincubation, and initial rates were determined from the first 30−60 s of the progress curves.

PAR Studies. The chromogenic metal-chelating agent PAR has been used to study the rate of removal of zinc from HFC. The reaction between PAR and $Zn(NO_3)_2$ was studied in detail in order to determine the $\Delta\epsilon_{490}$ value associated with formation of the $(PAR)_2Zn(II)$ complex. The maximum absorbance change associated with formation of this complex occurs at 490 nm and is dependent on the pH and to a lesser degree on the temperature, but is not significantly dependent on [NaCl], [$CaCl_2$], or [PAR] at ratios from 10 to 1000 PAR/Zn. In 50 mM Hepps, 40 mM NaCl, 25 mM $CaCl_2$, pH 8.5, $\Delta\epsilon_{490}$ was found to be 0.078 μM^{-1} cm^{-1}, which is in close agreement with a previously reported value of $\Delta\epsilon_{490}$ = 0.071 μM^{-1} cm^{-1} for $(PAR)_2$ Zn(II) at pH 8.0 (Kaarsholm & Dunn, 1987). HFC (0.8−1.2 μM) was incubated with PAR in 50 mM Hepps, pH 8.5, and the absorbance change at 490 nm was monitored continuously. The rate and extent of this reaction was studied as a function of [PAR], [$CaCl_2$], [NaCl], and temperature.

Table 2: Zinc Stoichiometry of Pro-HFC Compared with Other Zinc Enzymes

enzyme	zinc/enzyme: this work	zinc/enzyme: lit. value	ref
pro-HFC			
method 1, [Ca(II)] = 0 mM	0.15 ± 0.01		
1 mM	1.46 ± 0.08		
10 mM	1.94 ± 0.12		
method 2, [Ca(II)] = 1 mM	1.22 ± 0.04		
HFC (determined by PAR)	1.3		
HFC (catalytic domain)		1	*a*
pro-HFS		1.50 ± 0.04	*b*
		0.90 ± 0.07	*c*
HFS catalytic domain		1.75 ± 0.13	*b*
		2.22 ± 0.11	*d*
truncated pro-HFS		1.82 ± 0.06	*b*
		1.8 ± 0.08	*c*
pro-HFG		1.1 ± 0.18	*c*
truncated pro-HFG		2.6 ± 0.24	*c*
pro-matrilysin		0.97 ± 0.06	*e*
		2.36 ± 0.19	*d*
		2.13 ± 0.39	*d*
matrilysin		2.22 ± 0.11	*d*
carbonic anhydrase	1.10 ± 0.14	1.0	*f*
carboxypeptidase A	1.07 ± 0.10	1.05	*g*
thermolysin	0.88 ± 0.09	1.15 ± 0.17	*h*
angiotensin converting enzyme		1.19	*i*
Clostridium histolyticum collagenase β		1.03	*j*
Clostridium histolyticum collagenase γ		0.81	*j*
Clostridium histolyticum collagenase ζ		0.88	*j*

[a] Lowry et al. (1992). [b] Salowe et al. (1992). [c] Willenbrock et al. (1995). [d] Soler et al. (1994). [e] Crabbe et al. (1992). [f] Lindskog (1960). [g] Vallee and Neurath (1955). [h] Calculated from Latt et al. (1969). [i] Das and Soffer (1975). [j] Bond and Van Wart (1984).

RESULTS

Zinc Stoichiometry of Pro-HFC. The zinc content of samples of pro-HFC prepared by two different methods has been determined by atomic absorption spectroscopy. Samples of carbonic anhydrase, carboxypeptidase A, and thermolysin have been subjected to parallel treatment and have been included as standards. The analyses for the latter three well-characterized metalloenzymes gave zinc stoichiometries of 1.10 ± 0.14, 1.07 ± 0.10, and 0.88 ± 0.09 zinc per enzyme, respectively (Table 2). These values agree closely with the respective value of 1.0, 1.05, and 1.15 reported in the literature [Lindskog, 1960; Vallee & Neurath, 1955; calculated from Latt et al. (1969)].

Three sets of samples of pro-HFC purified by method 1 were dialyzed against four changes of 1 L of metal-free 2 mM Hepes, 250 mM NaCl, pH 7.5, at 4 °C containing 0, 1, and 10 mM $CaCl_2$, respectively. The average zinc contents of these three sets of samples give stoichiometries of 0.15 ± 0.01, 1.46 ± 0.08, and 1.94 ± 0.12, respectively. The sample prepared by method 2 and dialyzed against buffer containing 1 mM $CaCl_2$ was found to contain 1.22 ± 0.04 zinc per enzyme. This sample contains less zinc than that prepared by method 1 and dialyzed under the same conditions. Since method 1 involves zinc-chelate chromatography, this step could be responsible for the higher zinc content. These results indicate that pro-HFC has a zinc stoichiometry that depends on the method of purification and on the concentration of calcium ions in the dialysis buffer.

Since all of the dialyses described above were carried out under scrupulously metal-free conditions, it is unlikely that

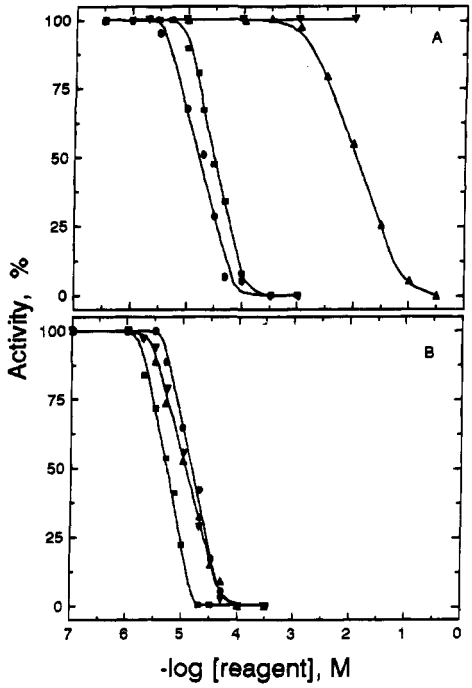

FIGURE 1: (A) Inhibition of the collagenase activity of HFC by (●) OP, (■) PAR, (▲) imidazole, and (▼) MP. (B) Inhibition of the (■) collagenase activity of HNC, the gelatinase activities of (▼) HFG and (▲) HNG, and the (●) caseinase activity of HFS by OP. The enzymes were incubated with the reagents in 50 mM Hepes, 200 mM NaCl, 1 mM $CaCl_2$, pH 7.5, for 1 h at 30 °C, and the assays were initiated by addition of [^{3}H]acetylated substrate.

the loss of zinc during dialysis was due to its replacement by another metal atom. However, in order to investigate this possibility, the metal content of the sample prepared by method 2 was examined by reaction with PAR (see section below on reaction of PAR with HFC for details). On reaction of free zinc with PAR, a 1:2 complex is formed with each PAR molecule contributing three ligands. The formation of this complex results in a large increase in absorbance near 500 nm that allows quantitation of the zinc. PAR also forms chromogenic complexes with other metal atoms such as Co(II), Ni(II), Mn(II), Cu(II), and Fe(II) (Yotsuyanagi et al., 1972; Corsini et al., 1962), and, if these metal atom were present, the zinc contents of the sample measured by atomic absorption and PAR analysis would disagree. After the sample of HFC was boiled to dissociate all bound metal atoms, the zinc content calculated on the assumption that only zinc was present was 1.3 zinc atoms per enzyme. This agrees well with the value of 1.22 obtained from atomic absorption measurements (Table 2). Thus, the lower zinc content of the samples dialyzed against buffers containing the lower $CaCl_2$ concentrations is due to loss of zinc from the enzyme, rather than replacement by another metal atom. All further studies were carried out with the one zinc form of HFC prepared by method 2.

Inhibition of MMPs by Metal-Chelating Agents. A series of inhibition studies has been performed utilizing three reagents which are known to complex with zinc (OP, PAR, and imidazole) and MP, a nonchelating isomer of OP. The MMPs used in these studies were prepared by dialysis against buffer containing 1 mM $CaCl_2$. HFC was preincubated separately with variable concentrations of all four reagents for 1 h. OP, PAR and imidazole all inhibit the collagenolytic activity of HFC in a dose-dependent manner, while MP does not inhibit at all (Figure 1A). The values of pI_{50} determined from these inhibition curves are 4.8 for OP, 4.3 for PAR, and 2.0 for imidazole. The fact that there is inhibition of HFC by OP but not by MP supports the view that inhibition occurs through interaction of the reagent with the intrinsic zinc atom, rather than through nonspecific binding of these hydrophobic compounds at the active site. Similar inhibition studies with OP have been carried out for four other human MMP (Figure 1B). OP inhibits the collagenolytic activity of HNC with $pI_{50} = 5.3$, the gelatinase activities of HFG and HNG with values of 5.0 and 4.9, respectively, and the caseinase activity of HFS with a value of 4.9 (Table 3). Thus, inhibition by OP is a characteristic of the MMP family. The values of these inhibition constants are similar in magnitude to those for carboxypeptidase A (Coombs et al., 1962), thermolysin (Holmquist & Vallee, 1974), and two *Clostridium histolyticum* collagenases (Angleton & Van Wart, 1988) measured at the same pH and temperature (Table 3).

Table 3: Comparison of Values of n and pI_{50} for the Inhibition of Five Human MMPs by OP with Values Reported for Other Zinc Enzymes

enzyme	n	pI_{50}	ref
HFC	1.8	4.8	this work
HNC	1.8	5.3	this work
HFG	1.5	5.0	this work
HNG	1.6	4.9	this work
HFS	1.9	4.8	this work
carboxypeptidase A	2.3	4.2	*a*
thermolysin	1.8	4.4	*b*
Clostridium histolyticum collagenase γ	1.8	4.0	*c*
Clostridium histolyticum collagenase ζ	1.8	4.4	*c*

[a] Coombs et al. (1962). [b] Holmquist and Vallee (1974). [c] Angleton and Van Wart (1988).

Mechanism of MMP Inhibition by OP. From a replot of the inhibition data shown in Figure 1, it is possible to calculate the apparent number of inhibitor molecules (n) required for inhibition (Table 3). A derivation from the equilibrium equation for an enzyme−inhibitor interaction by the method of Johnson et al. (1942) yields the following relationship

$$\log[(v_o/v_I) - 1] = n \log [I] - \log K_{app} \qquad (1)$$

where v_o is the uninhibited rate and v_I is the rate at [I]. Thus, a plot of $\log[(v_o/v_I) - 1]$ vs log [I] allows a determination of n from the slope, pI_{50} from the x-intercept, and pK_{app} from the y-intercept. Accordingly, analysis of the data in Figure 1A indicates that the values of n for inhibition of HFC with OP, PAR, and imidazole are 1.8, 2.0, and 1.2, respectively. The values of n for the inhibition of the other four MMPs by OP are 1.8 for HNC, 1.5 for HFG, 1.6 for HNG, and 1.9 for HFS. Values of n of more than 1 indicate that the mode of inhibition by this agent is the removal of a catalytically important metal ion (Coombs et al., 1962). This mode of inhibition by OP can occur through two distinct pathways. Metal ions can be removed from the enzyme via complexation of free metal that is transiently dissociated by OP or via the transient formation of a ternary complex between enzyme, zinc, and OP followed by loss of zinc from the enzyme (Felber et al., 1962; Kidani & Hirose, 1977).

Time-Dependence of Inhibition of MMP by OP. The time dependence of inhibition by a chelating agent can help to distinguish between different possible mechanisms of

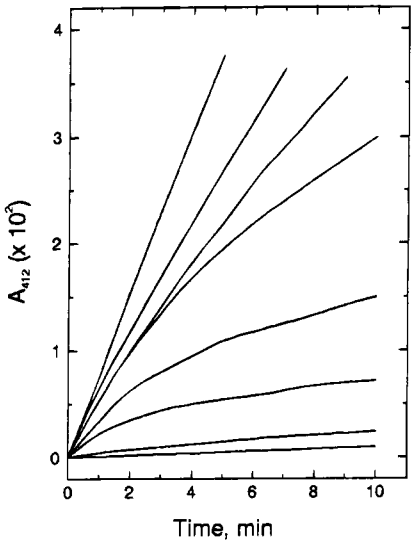

FIGURE 2: Progress curves for the hydrolysis of 20 μM thiopeptide by HFC in the presence of (from top to bottom) 0, 50, 100, 150, 400, 600, and 1000 μM OP. The bottom curve is the blank rate in the absence of OP and HFC. The assays were carried out in 50 mM Hepes, 200 mM NaCl, 1 mM $CaCl_2$, pH 7.5, at 25 °C containing 200 μM DTNB.

inhibition. Such studies are best carried out with a rapid, continuously recording assay. Thus, the effect of OP on the hydrolysis of the thio ester Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-OMe by HFC has been investigated. Progress curves (A_{412} vs time) for the reaction carried out in the presence of variable concentrations of OP, MP, and imidazole have been obtained. In these experiments, there was no preincubation of HFC with the inhibitor. No inhibition of HFC by MP was observed after any time of incubation. Progress curves in the presense of imidazole were monophasic (data not shown). For example, 3 mM imidazole reduced the activity of HFC to 74% within 20 s, and no further decrease was observed after 2 h. In contrast, the progress curves in the presence of OP are biphasic with an initial phase with higher rate of substrate hydrolysis that equilibrates to a second phase with a lower rate over a time period that varies with the concentration of OP (Figure 2).

To study the approach of this inhibition to equilibrium, initial rates measured over the first 30−60 s of the progress curves have been acquired as a function of the incubation time of HFC with OP at 23 °C. These measurements clearly show that the inhibition is time dependent with an equilibration time that decreases as the concentration of OP in the assay is increased, but typically approaches a constant level within 1−2 h (Figure 3A). A similar time dependence was observed for the inhibition of HNC, HFG, HNG, and HFS by OP (Figure 3B). In order to characterize the two phases of inhibition further, the values of n and pI_{50} describing the inhibition of HFC by OP and PAR were measured for both (Table 4). When assays were carried out in the presence of variable concentrations of OP or PAR and initial rates were determined with no preincubation of enzyme and inhibitor, the values of n were 1.1 and 1.0 for OP and PAR, respectively, indicating inhibition by a single inhibitor molecule. After 2 h of preincubation with OP or PAR, however, these values increased to 1.9 and 1.8, respectively, in agreement with the values from the collagenase assays

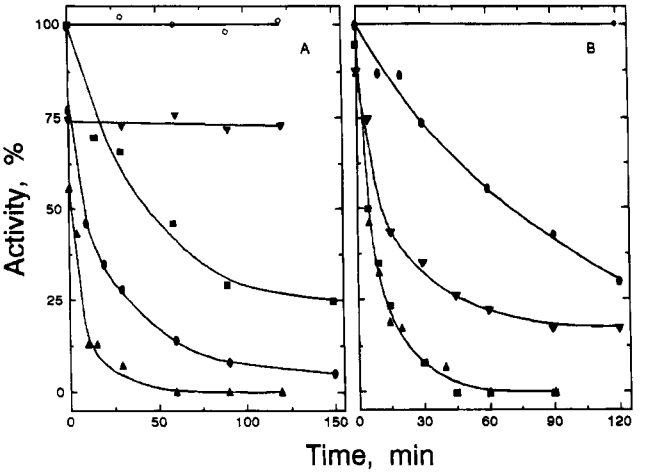

FIGURE 3: Time dependence of inhibition of MMP by OP. (A) Inhibition of HFC by (■) 30, (●) 50, and (▲) 100 μM OP, (▼) 3 mM imidazole, and (○) 10 mM MP. (B) Inhibition of (●) HNG by 50 μM OP, (▼) HFS by 30 μM OP, (▲) HNC by 20 μM OP, and (■) HFG by 100 μM OP. There was no inhibition of any of these MMP by 10 mM MP after 2 h (○). Incubations and assays were carried out in 50 mM Hepes, 200 mM NaCl, 1 mM $CaCl_2$, pH 7.5, at 23 °C. Thiopeptide (20 μM) and DTNB (200 μM) were added at the indicated times to initiate the assays.

Table 4: Time Dependence of pI_{50} and n for the Inhibition of HFC by OP and PAR

	no preincubation		2 h preincubation	
inhibitor	pI_{50}	n	pI_{50}	n
OP	3.8	1.1	5.7	1.9
PAR	3.7	1.0	5.3	1.8

(Table 3). This indicates that the inhibition observed at equilibrium is the result of a process involving two molecules of inhibitor per enzyme. Correspondingly, the values of pI_{50} increased as a result of the 2 h preincubation from 3.8 to 5.7 for OP and from 3.7 to 5.3 for PAR. Thus, the inhibition of HFC by OP and PAR is a two-step process with an initial phase characterized by involvement of a single molecule of chelator followed by a second, more potent phase involving chelation of zinc by two molecules of inhibitor.

Reaction of PAR with HFC. The reaction of 100 μM PAR with 1.2 μM $Zn(NO_3)_2$ is complete within the manual mixing time. However, the reaction of PAR with 1.2 μM HFC in 50 mM Hepps, 40 mM NaCl, 25 μM $CaCl_2$, pH 8.5, at 23 °C is time dependent, biphasic, and similar in appearance to the behavior observed for the inhibition of HFC by OP (Figure 4). The final change in absorbance at the completion of the reaction indicates that 1.1 atoms of zinc have been removed from each molecule of HFC by the PAR. As already described above, this value increases slightly to 1.3 zinc atoms per enzyme when the sample is boiled to ensure that all of the metal has dissociated. First-order plots of data from these progress curves show two distinct phases and allow the determination of the apparent rate constants (k_{obs}) for each phase. A plot of the apparent first-order rate constants, k_{obs}, for the fast and slow phases of the reaction of HFC with PAR as a function of [PAR] reveals that the rate of the fast step increases linearly with [PAR] while the slow step saturates at [PAR] > 300 μM (Figure 5A). Denoting the enzyme with its active site zinc atom as [(HFC)-Zn(II)] and the form from which the zinc has been removed as (HFC), this behavior is consistent with the following

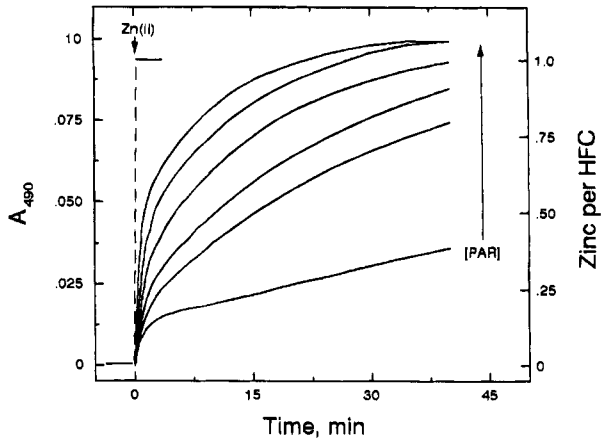

FIGURE 4: Reaction of 1.2 μM $Zn(NO_3)_2$ with 100 μM PAR, and 1.2 μM HFC with (from bottom to top) 30, 60, 100, 200, 300, and 600 μM PAR. All reactions were carried out in 50 mM Hepps, 40 mM NaCl, 25 μM $CaCl_2$, pH 8.5, at 25 °C.

mechanism under pseudo-first-order conditions ([PAR] ≫ [(HFC)Zn(II)])

$$[(HFC)Zn(II)] \underset{k_{-1}}{\overset{k_1[PAR]}{\rightleftharpoons}} [(HFC)Zn(II)]PAR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}$$

$$(HFC) + Zn(II)PAR \underset{k_{-3}}{\overset{k_3[PAR]}{\rightleftharpoons}} (HFC) + Zn(II)PAR_2 \quad (2)$$

where the first step is fast, the second is slow, and the third is very fast. Since step 3 follows a much slower step, it is kinetically invisible. In this mechanism,

$$k_{obs}^{fast} = k_{-1} + k_1[PAR] \quad (3)$$

where the y-intercept of the fast phase in Figure 5A gives $k_{-1} = 0.056$ min^{-1} and the slope gives $k_1 = 0.62$ mM^{-1} min^{-1}. The dissociation constant for the formation of the [(HFC)Zn(II)]PAR complex, K_{PAR}, equals k_{-1}/k_1 and has a value of 90 μM. This corresponds to a pK_{PAR} value of 4.0, which is in good agreement with the pI_{50} value of 3.7 observed above for the instantaneous inhibition of HFC by PAR (no preincubation of the enzyme with inhibitor). The relationship between k_{obs}^{slow} and k_2, k_{-2}, and K_{PAR} is shown below.

$$k_{obs}^{slow} = k_{-2} + k_2\frac{[PAR]}{[PAR] + K_{PAR}} \quad (4)$$

Since the y-intercept of the slow phase in Figure 5A is very close to zero, $k_{-2} \approx 0$, this indicates that, under the conditions of this assay, the removal of zinc from HFC by PAR is essentially irreversible. Consequently, all of the ternary complex will eventually be converted into (HFC) and Zn(II)$(PAR)_2$ as equilibrium is approached.

The effect of varying the [$CaCl_2$], [NaCl], and temperature on these two phases has been investigated. For the reaction with 0.8 μM HFC with 200 μM PAR in 50 mM Hepps, 40 mM NaCl, pH 8.5, at 23 °C containing variable concentrations of $CaCl_2$, the fast phase of these reactions is unaffected while the rate of the slow phase is markedly lowered at higher Ca(II) concentrations (Figure 5B). In the presence of 3 mM Ca(II), the rate of the slow phase approaches zero and the amplitude of the observed absorbance change is 50% of that of reactions carried out at low [Ca(II)]. PAR does not complex Ca(II) directly, nor does it have any effect on

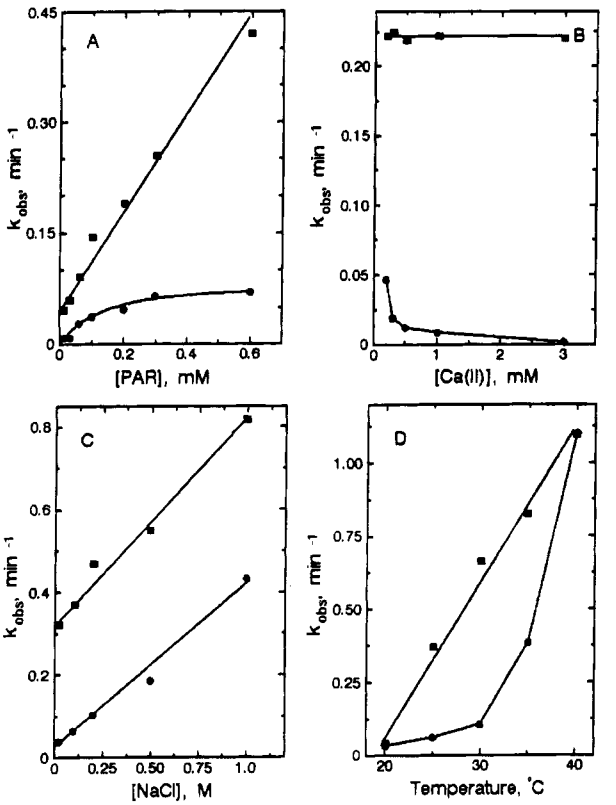

FIGURE 5: Variation of the apparent first-order rate constants, k_{obs}, for the (■) fast and (●) slow phases of the reaction of HFC with (A) 10, 30, 60, 100, 200, 300, and 600 μM PAR in 50 mM Hepps, 40 mM NaCl, 25 μM $CaCl_2$, pH 8.5, at 25 °C, (B) 200 μM PAR in 50 mM Hepps, 40 mM NaCl, pH 8.5, at 25 °C in the presence of 0.2, 0.3, 0.5, 1 and 3 mM $CaCl_2$, (C) 600 μM PAR in 50 mM Hepps, 25 μM $CaCl_2$, pH 8.5, at 25 °C in the presence of 0.025, 0.10, 0.20, 0.50, and 1.0 M NaCl, and (D) 600 μM PAR in 50 mM Hepps, 40 mM NaCl, 25 μM $CaCl_2$, pH 8.5, at 20, 25, 30, 35, and 40 °C.

the formation of its complex with zinc (Kaarsholm & Dunn, 1987). Thus, these effects of Ca(II) are due to its interaction with HFC. This suggests that the effect of high [Ca(II)] is to stabilize the ternary complex and prevent the dissociation of [(HFC)Zn(II)]PAR to (HFC) and Zn(II)PAR.

For the reaction of 1.2 μM HFC with 600 μM PAR in 50 mM Hepps, 25 μM $CaCl_2$, pH 8.5, at 23 °C containing variable concentrations of NaCl, the rates of both phases increase linearly with increasing [NaCl] (Figure 5C). This suggests that NaCl both facilitates formation of the [(HFC)-Zn(II)]PAR ternary complex as well as its conversion to (HFC) and $(PAR)_2$Zn(II). This is consistent with a loosening of the tertiary structure of HFC at higher [NaCl] that makes it easier for the first PAR molecule to bind and also easier for the zinc ion to dissociate from HFC and is likely a general ionic strength effect rather than a specific interaction between NaCl and HFC. The effect of increasing the temperature from 20 to 40 °C on the reaction of 0.9 μM HFC with 600 μM PAR in 50 μM Hepps, 40 μM NaCl, 25 μM $CaCl_2$, pH 8.5, increases the rates of both the fast and slow phases of the reaction (Figure 5D). The rate of the fast phase increases linearly with temperature, whereas the rate of the slow phase increases parabolically. The reactions become markedly less biphasic at higher temperatures due to the apparent convergence of the rate constants for the fast and slow phases. The effect of increased temperature is to increase the rate of formation of the [(HFC)Zn(II)]PAR ternary complex and its

dissociation to (HFC) and Zn(II)PAR, presumably due to disruption of the tertiary structure of HFC.

DISCUSSION

Although it has been widely recognized for some time that the MMP are zinc enzymes, the current view of their metal content remains somewhat confused. There have been conflicting reports of zinc to enzyme stoichiometries in human MMPs (Table 2). The results presented here on the zinc content of full-length pro-HFC indicate that the stoichiometry is dependent upon the way in which the sample is prepared. This includes both the method of purification as well as the dialysis protocol used to free the enzyme of loosely bound, adventitious metal ions. There was a higher quantity of zinc in pro-HFC which had been applied to a zinc-charged iminodiacetate resin (method 1) than in samples prepared by a protocol (method 2) in which this step was not used. With respect to the sample preparation protocol, the concentration of calcium ions in the dialysis buffer is an important parameter. The structures of the catalytic domains of HFC (Lovejoy et al., 1994; Borkakoti et al., 1994; Spurlino et al., 1994), HFS (Gooley et al., 1993), and HNC (Bode et al., 1994; Stams et al., 1994) all show that there is at least one calcium ion that stabilizes the tertiary structure of these enzymes. Dialysis in the absence of calcium leads to a very low, substoichiometric zinc content and concomitant loss of catalytic activity, probably due to denaturation of the enzyme. When the dialysis is performed in the presence of 1 and 10 mM calcium, the zinc stoichiometry is increased to 1.46 and 1.94, respectively. These results suggest that the widely different zinc stoichiometries reported for the MMP discussed above could be attributable to their method of preparation.

The inhibition of individual MMP by metal-chelating agents has been reported by many workers. The inhibition of metalloenzymes by metal-chelating agents can occur by three possible mechanisms: scavenging by the chelator of free metal spontaneously released from the enzyme, formation of a ternary complex between the chelator and the enzyme-bound metal, or removal of the metal from the enzyme by the chelating agent after the formation of a ternary intermediate (Baker, 1988; Auld, 1988). These three mechanisms exhibit characteristics that can be used to distinguish them from each other. Inhibition solely due to the formation of a ternary complex is usually reversible, essentially instantaneous, and requires only one molecule of inhibitor ($n = 1$). The two mechanisms which result in loss of metal by the enzyme characteristically exhibit irreversibility, time-dependence, and binding of the metal by more than one inhibitor molecule ($n > 1$). If the removal occurs via the formation of a ternary complex, then the inhibition is predicted to be biphasic. Inhibition of the one-zinc form of HFC and of samples of HNC, HFG, HNG, and HFS prepared in an identical manner shows that the inhibition by OP is time-dependent and, on completion, requires more than one molecule of OP. Consequently, the inhibition of these enzymes by OP must result in removal of the catalytic zinc ion from these enzymes. The inhibition of HFC by OP is biphasic with an initial, instantaneous phase characterized by involvement of a single chelator molecule, followed by a second phase involving chelation of zinc by two molecules of inhibitor. Thus, the mechanism of inhibition of these MMPs by OP is through the initial formation of a ternary complex with OP followed by the slower removal of the zinc.

This view of the inhibition of these MMPs by chelating agents is supported by the studies with the chromogenic metal-chelating agent PAR. HFC is also inhibited by PAR in the same biphasic manner as OP. Since the complexation of metals by PAR can be monitored spectrophotometrically, the time course of the interaction of PAR with the zinc atom in HFC has been observed directly. These experiments confirm that PAR interacts with the zinc atom of HFC in a two-step process. The fast phase of the PAR−HFC reaction corresponds to the binding of one molecule of PAR to [(HFC)Zn(II)] to form the ternary complex [(HFC)Zn(II)]-PAR. The second phase corresponds to the slow release of the Zn(II)PAR complex from the enzyme followed by the rapid (essentially instantaneous) reaction with a second molecule of PAR to form the $Zn(II)(PAR)_2$ complex and (HFC). The observation of this two-step interaction has afforded the unexpected opportunity to study the influence of several environmental parameters on the zinc removal process. Calcium ions stabilize the terniary complex that is formed with PAR, apparently by strengthening the affinity of the enzyme for zinc and decreasing the off-rate of zinc−PAR complex from the active site. Increasing either the [NaCl] or temperature has the opposite effect and destabilizes the [(HFC)Zn(II)]PAR ternary complex.

The studies described herein support the view that the MMPs contain a catalytically essential zinc atom located at the active site. In HFC, this zinc ion is ligated by the three strictly conserved histidine residues, His_{218}, His_{222}, and His_{228} (Lovejoy et al., 1994; Borkakoti et al., 1994; Spurlino et al., 1994). This is the same zinc ion that is believed to ligate the sulfhydryl group of the cysteine switch residue located in the propeptide of pro-HFC (Springman et al., 1990) and other MMP zymogens (Van Wart & Birkedal-Hansen, 1990) as a mechanism for maintaining latency. The crystal structure of HFC reveals a second metal atom, presumed to be zinc, that is coordinated by four other conserved residues (His_{168}, Asp_{170}, His_{183}, and His_{196}) located in a surface loop. This zinc ion is believed to serve a structural role by stabilizing the conformation of this loop. Interestingly, this loop is also stabilized by a calcium ion that is octahedrally coordinated by the carboxylate or carbonyl groups of six other strictly conserved residues. Since the conformation of this loop is stabilized by both the structural zinc ion and calcium ion, it is plausible that the binding constants for these two metal atoms are dependent on the presence of each other. Specifically, the affinity of the structural zinc ion for the enzyme may be greater at higher calcium concentrations, thus explaining the dependence of the zinc content of pro-HFC samples on the calcium ion concentration in the dialysis buffer. The fact that the one-zinc form of HFC studied here has full activity raises the interesting question of how essential the structural zinc atom is for activity. Once again, this could depend on the calcium ion concentration, since high concentrations could stabilize this loop effectively even in the absence of a structural zinc ion, accounting for the full activity of the one-zinc form of HFC.

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