Iron-Containing Metallocenes as Active Site Directed Inhibitors of the Proteinase That Cleaves the NH_2-Terminal Propeptides from Type I Procollagen†

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ABSTRACT: Derivatives of ferrocene (dicyclopentadienyliron) (Fc) were examined as active site directed inhibitors of type I procollagen N-proteinase, the enzyme that cleaves the NH_2-terminal propeptides from type I procollagen. The compounds were shown here to be reversible, competitive inhibitors of the enzyme. The effectiveness of the Fc inhibitors varied with modification of the cyclopentadienyl (cp) rings. The monocarboxylic acid (I) and the 1,1'-dicarboxylic acid (II) derivatives of Fc inhibited 50% of the enzymic activity (I_{50}) at concentrations of 1.0 and 0.5 mM, respectively. The K_i values were 0.3 mM for both I and II. Derivatization of the carbonyl α to the cp ring of compound I ($FcCOCH_2CH_2COOH$, III) increased the inhibitory activity (I_{50} = 0.100 mM; K_i = 0.065 mM). Removal of the carbonyl α to the cp ring of III did not improve inhibitory activity: $FcCH_2CH_2COOH$, I_{50} = 2 mM; FcCH=CHCOOH, I_{50} = 1.5 mM. The active inhibitory species apparently contained iron in the 3+ valence state since two ferrocenium derivatives were very effective inhibitors: ferrocenium tetrachloroferrate, IV (I_{50} = 0.030 mM; K_i = 0.004 mM), and carboxyferrocenium hexafluorophosphate, V (I_{50} < 0.1 mM; K_i < 0.05 mM). In addition, reduction of III with ascorbic acid abolished its inhibitory activity. Compounds I and III stabilized the enzyme to heat denaturation in the absence of exogenous calcium; compound IV did not stabilize the enzyme. Further observations indicated that Fc derivatives were specific inhibitors of procollagen N-proteinase. At concentrations that completely inhibited N-proteinase, compounds I, III, and IV showed no significant inhibition of five other proteinases able to cleave NH_2- and COOH-terminal propeptides or propeptide-like fragments from procollagen. The five proteinases tested were procollagen C-proteinase, kallikrein from human plasma, α-chymotrypsin, papain, and thermolysin. In addition, compound I showed no significant inhibition of either bacterial collagenase or vertebrate collagenase.

Type I collagen is assembled in the cell as a precursor called type I procollagen that contains extension propeptides on both the NH_2 and COOH termini of the two α1 chains and the one α2 chain [for reviews, see Fessler and Fessler (1978), Prockop et al. (1979a,b), and Bornstein and Traub (1980)]. The conversion of procollagen to collagen requires cleavage of the propeptides by at least two different endoproteinases (Prockop et al., 1979a,b; Bornstein & Sage, 1980). A procollagen N-proteinase cleaves the NH_2-terminal propeptides (Kohn et al., 1974; Tuderman et al., 1978; Layman, 1981), and a procollagen C-proteinase cleaves the COOH-terminal propeptides from procollagen (Goldberg et al., 1975; Kessler & Goldberg, 1978; Duksin et al., 1978; Leung et al., 1979; Njieha et al., 1982; Hojima et al., 1985b).

The type I procollagen N-proteinase has been extensively purified from chick embryo tendons (Tuderman & Prockop, 1982) and, more recently, from homogenates of whole chick embryos (Tanzawa et al., 1985). The enzyme cleaves type I and type II procollagen but not type III or type IV procollagen. A separate enzyme has been shown to cleave the NH_2-terminal propeptides from type III procollagen (Nusgens et al., 1980; Halila & Peltonen, 1984).

Type I procollagen N-proteinase is a neutral metalloendoproteinase that requires calcium for maximal activity and also possibly a second metal cofactor not yet identified (Tuderman & Prockop, 1982; Tanzawa et al., 1985). The enzyme will cleave only procollagen and pNcollagen[1] that are in a native conformation. Denatured proα1(I) and proα2(I) chains are not cleaved by the enzyme (Tuderman et al., 1978; Tanzawa et al., 1985). Also, mutations that delete amino acids 50 or more residues away from the cleavage site make the protein resistant to N-proteinase (Williams & Prockop, 1983; Sippola et al., 1984).

The N-proteinase is inhibited by metal chelators such as EDTA. Additionally, synthetic peptides with sequences identical with the amino acid sequences found at the Pro-Gln cleavage site of the proα1(I) chain were weak inhibitors of N-proteinase (Morikawa et al., 1980). The enzyme was not inhibited by inhibitors of serine and cysteine proteinases nor by compounds such as ovostatin, α2-macroglobulin, or phosphoramidon or by an inhibitor of several connective tissue

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[1] Abbreviations: pNcollagen, procollagen containing only the NH_2-terminal propeptides; pCcollagen, procollagen containing only the COOH-terminal propeptides; C1, COOH-terminal propeptide from the proα1(I) chain; C2, COOH-terminal propeptide from the proα2(I) chain; Fc, ferrocene [dicyclopentadienyliron(II)]; FMCA, ferrocenemonocarboxylic acid (ferrocenecarboxylic acid); FDCA, 1,1'-ferrocenedicarboxylic acid; Fc^+, ferrocenium [dicyclopentadienyliron(III)]; cp, cyclopentadienyl (η^5-C_5H_5); $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; pNA, *p*-nitroanilide; Brij 35, poly(oxyethylene) 23-lauryl ether; PAB, *p*-aminobenzoic acid; NEM, *N*-ethylmaleimide.

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metalloproteinases (Tuderman & Prockop, 1982; Tanzawa et al., 1985).

Studies with analogues of inhibitory peptides demonstrated that a Phe residue in the P_3[2] position of the proα1(I) chain increased the inhibitory activity and therefore may be important for substrate binding to N-proteinase. The observations that the N-proteinase required a substrate with the correct native conformation and with a hydrophobic residue near the cleavage site suggested that the N-proteinase might be inhibited by metallocenes, aromatic compounds with a cylindrical conformation and a transition metal covalently bound between two cp rings [for review, see Rosenblum (1965)].

Materials and Methods

Materials. Procollagen C-proteinase from the media of cultured chick tendons (Hojima et al., 1985b) and kallikrein from human plasma (Hojima et al., 1980) were gifts of Dr. Yoshio Hojima, Department of Biochemistry, UMDNJ—Rutgers Medical School. Vertebrate collagenase from rabbit synovial fibroblasts (Brinckerhoff et al., 1979; McMillan et al., 1981; Nagase et al., 1982) and ^{14}C-acetylated collagen (Cawston & Barrett, 1979) were gifts of Dr. Hideaki Nagase, Department of Medicine, UMDNJ—Rutgers Medical School. Bacterial collagenase was a gift from advanced Biofactures Corp., Lynbrook, NY. D-Pro-L-Phe-L-Arg-pNA was purchased from Kabi Diagnostica, Sweden. α-Chymotrypsin, thermolysin, papain, azocasein, methyl α-mannoside, L-ascorbic acid, and pyrrole-2-carboxylic acid were purchased from Sigma Chemical Co., St. Louis, MO. [2,3,4,5-^{3}H]Proline and [2-^{14}C]iodoacetic acid were purchased from Amersham. FMCA, FDCA, and $NaPF_6$ were purchased from Aldrich, Milwaukee, WI. Concanavalin A–Sepharose and heparin–Sepharose were purchased from Pharmacia, Piscataway, NJ. All other chemicals used were of reagent grade purity.

Preparation of Substrates. Radioactively labeled type I procollagen was prepared from the medium of freshly isolated chick embryo fibroblasts incubated with a mixture of ^{14}C-labeled amino acids (Dehm & Prockop, 1972). The ^{14}C-labeled type I procollagen was purified from the medium by chromatography on DEAE-cellulose (Hoffman et al., 1976). Procollagen concentration was measured by circular dichroism spectrophotometry, using a mean residue ellipticity of 6000 deg cm^2 dmol^{-1} at 221 nm (Hayashi et al., 1979).

Type I pNcollagen from the skin of dermatosparactic sheep was a gift from Dr. Peter Bruckner (Miyahara et al., 1983). The purified type I pNcollagen was labeled with [2-^{14}C]-iodoacetic acid, diluted to a final concentration of 0.4 mg/mL and stored as described previously (Tanzawa et al., 1985).

Extraction of Type I Procollagen N-Proteinase. The N-proteinase was extracted from 12 dozen 13-day-old whole chick embryos by using a modification of the procedure of Tanzawa et al. (1985). The crude extract was mixed with 100 mL of concanavalin A–Sepharose equilibrated with 50 mM Tris-HCl buffer, pH 7.4 at 4 °C, containing 0.5 M KCl, 1 mM $CaCl_2$, 0.05% Brij 35, and 0.02% NaN_3 (buffer A). The sample was rotated overnight at 4 °C and used to prepare a column. The column was washed with buffer A until the absorbance of the eluate at 280 nm was less than 0.1. The column buffer was exchanged with 100 mL of buffer A containing 0.5 M methyl α-mannoside, allowed to equilibrate for at least 8 h, and eluted with an additional 400 mL of buffer A containing 0.5 M methyl α-mannoside. The peak of activity was pooled and dialyzed against a 50 mM Tris-HCl buffer, pH 7.4 at 4 °C, containing 0.4 M KCl, 1 mM $CaCl_2$, 0.05% Brij 35, and 0.02% NaN_3 (buffer B). The enzyme sample was then applied to a 10-mL heparin–Sepharose affinity column equilibrated with buffer B. The column was washed with buffer B and eluted with a linear salt gradient of 0.4–1.0 M KCl in the same buffer. The procedure provided enzyme purified over 200-fold, and with an average specific activity of about 225 units/mg[3]. Heparin–Sepharose-purified N-proteinase was used for all inhibition and kinetic studies.

Synthesis of Metallocenes. Ferrocene and ferrocenium derivatives, other than FMCA and FDCA, were synthesized by using procedures described in the literature: 3-ferrocenylpropionic acid, $FcCH_2CH_2COOH$ (Rosenblum, 1963; Schlogel, 1957); 3-ferrocenylpropenoic acid, $FcCH{=}CH_2COOH$ (Osgerby & Pauson, 1958); 3-ferrocenoylpropionic acid, $FcCOCH_2CH_2COOH$ (Rinehart & Curby, 1957); ferrocenium tetrachloroferrate, $Fc^+FeCl_4^-$, and ferrocenium hexafluorophosphate, $Fc^+PF_6^-$ (Neuse, 1985).

3-Propionylferrocenium hexafluorophosphate, $[Fc^+\text{-}CH_2CH_2COOH]PF_6^-$, was prepared with the procedure previously used to synthesize the Fc^+ cation (Neuss, 1985). A solution of 0.2 g (0.8 mmol) of 3-ferrocenylpropionic acid in 2 mL of 98% H_2SO_4 was allowed to stand for 30 min at room temperature. After dilution with ice water to a volume of 20 mL and filtration through Whatman No. 1 filter paper, the solution of 3-propionylferrocenium sulfate was treated with 0.4 g (2.4 mmol) of solid $NaPF_6$. The solution was left stirring for 2 h at 4 °C. The dark blue hexafluorophosphate salt was collected by filtration and dried at room temperature (yield, 0.22 g, 62.5%). A portion of the crude product (96.1 mg) was recrystallized from water/acetone (2:1 v/v). The recrystallized solid was washed with anhydrous diethyl ether and dried at 60 °C for 1 h. Shiny, dark blue plates were obtained (yield, 47.6 mg, 49.5%). Anal. Calcd: C, 38.74%; H, 3.50%. Found: C, 38.40%; H, 3.50%. IR (ν_{max}) 3600–2400 (br, COOH), 3140 (m, C–H stretch), 2980 (m), 2920 (w, CH_2 stretch), 1710 (s, C=O stretch), 1435 (m, CH_2 bend), 1310 (m), 1235 (m), 830 (vs, P–F stretch), 560 cm^{-1} (s, P–F bend).

Carboxyferrocenium hexafluorophosphate, $[Fc^+COOH]\text{-}PF_6^-$, was synthesized by using a modification of the methods reported for synthesizing the Fc^+ cation (Neuss, 1985; Nesmeyana et al., 1960). FMCA (1 g, 4.3 mmol) was suspended in 30 mL of anhydrous diethyl ether with vigorous stirring. To the suspension was added 2.3 g (8.5 mmol) of $FeCl_3{\cdot}6H_2O$, turning the solution dark green immediately. The reaction mixture was stirred for at least 12 h at room temperature. The solution was then filtered through a sintered glass funnel, yielding a dark green solid. The solid was suspended in 5 mL of water, and sufficient HCl was added to precipitate unreacted FMCA, followed by filtration to remove the insoluble FMCA. The dark blue filtrate was then treated with 0.73 g (4.3 mmol) of solid $NaPF_6$ and left stirring for 1 h at 4 °C. The dark blue carboxyferrocenium hexafluorophosphate was collected by filtration (yield, 0.15 g, 9.3%). Attempts at recrystallization in H_2O/acetone (2:1 v/v), H_2O, acetone, ethanol, or glacial acetic acid resulted in decomposition of the compound. For elemental analysis, the hexafluorophosphate salt was washed 3 times with 2 mL of boiling diethyl ether to remove any FMCA, or until the ether was colorless. The product, a dark blue powder, was dried in vacuo at room temperature. Anal. Calcd: C, 35.23%; H, 2.69%. Found: C, 34.93%; H, 2.64%. IR (ν_{max}) 3115 (m, C–H

[2] The nomenclature proposed by Schecter and Berger (1967) is used to define the amino acid position (P_1, P_2, P_3, ...) of the substrate, counting to the left from the point of cleavage.

stretch), 2920 (w), 1715 (s, C=O stretch), 1490 (m), 1415 (m), 1300 (m), 830 (vs, P–F stretch), 560 cm^{-1} (s, P–F bend).

Cobaltocenium hexafluorophosphate, $[Co(cp)_2]^+PF_6^-$, carboxycobaltocenium hexafluorophosphate, $[cpCo(C_5H_4COOH)]^+PF_6^-$, and the monoacid chloride [(chlorocarbonyl)cobaltocenium hexafluorophosphate], $[cpCo(C_5H_4COCl)]^+PF_6^-$ were synthesized according to published procedures (Sheats & Rausch, 1970; Sheats & Kirsch, 1973). Diaminocobaltocenium hexafluorophosphate, $[Co(C_5H_4NH_2)_2]^+PF_6^-$, was prepared according to the procedure previously reported (Sheats et al., 1975).

(Aminocarbonyl)cobaltocenium hexafluorophosphate, $[cpCo(C_5H_4CONH_2)]^+PF_6^-$, was prepared by dissolving the monoacid chloride in concentrated NH_4OH. The solid was precipitated by addition of solid $NaPF_6$. It was recrystallized from either acetone/$CHCl_3$ (50:50 v/v) or boiling water: IR (ν_{max}) 3450 (s), 3125 (vs, N–H stretch), 1695 (vs, C=O stretch), 1630 (s, NH_2 bend), 1492 (m), 1428 (m), 1415 (vs, C–N stretch), 1375 (m), 1350 (m), 1195 (w), 1122 (m), 1080 (w), 1050 (w), 1033 (w), 1020 (w), 830 cm^{-1} (vs. P–F stretch).

All compounds synthesized according to literature procedures were of analytical purity. Elemental analyses were performed by Robertson Laboratory, Inc., Florham Park, NJ.

Enzyme Assays. Assays of all enzymes were of initial linear velocity and were carried out in duplicate. Inhibitors were dissolved in a standard assay buffer that consisted of 0.15 M NaCl and 0.02% NaN_3 in 50 mM Tris-HCl, adjusted to pH 7.4 at 35 °C. Metallocene inhibitor solutions were routinely filtered through a 0.22-μm Millex filter (Millipore Corp., Bedford, MA). Appropriate blanks with no enzyme were run in parallel with all inhibition assays.

N-Proteinase activity was measured essentially as described previously (Tanzawa et al., 1985) by using either a rapid assay method or a gel electrophoresis assay. The rapid assay method was carried out by using 20 μL (8 μg) of ^{14}C-labeled pN-collagen as substrate, 10 μL (about 1.2 units)[3] of heparin–Sepharose-purified N-proteinase, and 10 μL of 50 mM $CaCl_2$ in the standard assay buffer. The reaction mixture was adjusted to a final volume of 100 μL with the standard assay buffer. Samples were incubated at 35 °C, and the reaction was stopped by adding 25 μL of 500 mM EDTA in the standard assay buffer. N-Proteinase activity was quantitated by ethanol pptn. as described previously (Tanzawa et al., 1985) and assay of ^{14}C-labeled NH_2-terminal propeptides in the supernatant.

For assay of N-proteinase by the gel electrophoresis method, the reaction mixture contained 5 μL (5000 cpm and 0.5 μg) of type I ^{14}C-labeled procollagen as substrate, 5 μL of 50 mM $CaCl_2$ in the standard assay buffer, and 5 μL (0.6 unit) of heparin–Sepharose-purified N-proteinase. The reaction mixture was adjusted to a final volume of 50 μL with the standard assay buffer. After incubation at 35 °C, the reaction was stopped by adding 12.5 μL of 500 mM EDTA and $^1/_{10}$ volume of 20% $NaDodSO_4$ and by heating the sample at 100 °C for 3 min.

C-Proteinase activity was measured by using the gel electrophoresis method as described by Hojima et al. (1985b) with ^{14}C-labeled procollagen as substrate and 25 μL of heparin–Sepharose-purified C-proteinase [about 0.3 unit as defined by Hojima et al. (1985b)]. After incubation at 35 °C, the reaction was stopped by adding 25 μL of 500 mM EDTA and $^1/_{10}$ volume of 20% $NaDodSO_4$ and by heating the sample at 100 °C for 3 min.

Collagenolytic activity of vertebrate collagenase was measured by using a modification of the method of Nagase et al. (1983). For assay by gel electrophoresis, 25 μL (75 μg) of reconstituted ^{14}C-acetylated collagen fibrils (Cawston & Barrett, 1979) was used as substrate in a total volume of 250 μL containing 100 μL of vertebrate collagenase [about 0.1 unit as defined by Nagase et al. (1983)], 50 μL of 50 mM $CaCl_2$ in the standard assay buffer, and 75 μL of standard assay buffer. After incubation at 37 °C, the reaction was stopped by adding 25 μL of 500 mM EDTA and 100 μL of 10% $NaDodSO_4$ and by heating at 100 °C for 3 min. Collagenolytic activity was also assayed by using a rapid assay method in a volume of 300 μL containing 50 μL of ^{14}C-acetylated collagen (150 μg), 30 μL of 50 mM $CaCl_2$ in the standard assay buffer, 100 μL (about 0.1 unit) of vertebrate collagenase, and 120 μL of the standard assay buffer. After incubation at 37 °C the reaction was stopped by centrifugation of the fibrils at 13000*g*. The cleavage products in the supernatant were quantitated by counting 200 μL of the supernatant in a liquid scintillation counter. The collagenolytic activity of bacterial collagenase was measured with the rapid assay method for vertebrate collagenase described above.

Kallikrein activity was measured by using both a spectrophotometric assay with D-Pro-L-Phe-L-Arg-pNA as the substrate and gel electrophoresis assay with ^{14}C-labeled procollagen as substrate. For the spectroscopic assay (Hojima et al., 1985a), 25 μL of a 25 μg/mL solution of kallikren in was preincubated with 25 μL of the standard assay buffer for 10 min at room temperature. The assay mixture contained 20 μL of preincubated kallikrein (0.25 μg), 0.1 mM D-Pro-L-Phe-L-Arg-pNa, and the standard assay buffer in a final volume of 1000 μL. Activity was assayed by the increase in absorbance at 405 nm. The reaction for the gel electrophoresis assay was carried out in a volume of 50 μL containing 5 μL of ^{14}C-labeled procollagen (25000 cpm and 1.5 μg), 10 μL of preincubated kallikrein (0.12 μg), and 35 μL of the standard assay buffer. After incubation at 35 °C, the reaction was stopped by adding 5 μL of a 1 mg/mL solution of soybean trypsin inhibitor and $^1/_{10}$ volume of 20% $NaDodSO_4$ and by heating at 100 °C for 3 min.

Thermolysin activity was assayed against azocasein as substrate at room temperature as described previously (Nagase et al., 1982, 1983), but the procedure was modified by using the standard assay buffer. The activity of thermolysin against ^{14}C-labeled procollagen was measured by gel electrophoresis of the reaction products as described above. The reaction was carried out in a volume of 50 μL containing 5 μL of ^{14}C-labeled procollagen (25000 cpm and 1.5 μg), 10 μL of 50 mM $CaCl_2$ in the standard assay buffer, 5 μL of thermolysin (1 ng) in the standard assay buffer containing 10 mM $CaCl_2$, and 30 μL of standard assay buffer. The reaction mixture was incubated at 35 °C and stopped by adding 5 μL of 0.5 M EDTA and $^1/_{10}$ volume of 20% $NaDodSO_4$ and by heating at 100 °C for 3 min.

α-Chymotrypsin activity was assayed by using azocasein as substrate under the same conditions as the thermolysis assay described previously (Nagase et al., 1982, 1983) with 50 μL of α-chymotrypsin (1.25 μg) and the standard assay buffer. After incubation at 35 °C, the reaction was stopped with 2.5 mL of 3% trichloroacetic acid and filtered. Activity was quantitated by measuring the absorbance of the filtrate at 366 nm. The gel electrophoresis assay for α-chymotrypsin using ^{14}C-labeled procollagen as substrate was the same as that

[3] One unit of N-proteinase activity is defined as 1 μg of pNcollagen cleaved/h at 35 °C and is equivalent to 0.5 unit as defined by Tanzawa et al. (1985). One absorbance unit at 280 nm is assumed to be 1 mg of protein.

Table I: Effect of Ferrocene Derivatives on Activity of Proteinases Able To Cleave NH_2- and COOH-Terminal Propeptides and Propeptide-like Fragments from Procollagen

	% act.[a]					
	FMCA		$FcCOCH_2CH_2COOH$		$Fc^+FeCl_4^-$	
enzyme	1000 μM	5000 μM	100 μM	500 μM	50 μM	250 μM
N-proteinase	50	0	50	0	25	0
C-proteinase	93	85	75	*b*	91	98
kallikrein	91	90	117	94	116	83
α-chymotrypsin	88	91	101	95	98	90
papain	100	96	96	98	102	99
thermolysin	91	80	93	91	114	97

[a] Enzymic activity was determined by the gel electrophoresis assays using ^{14}C-labeled procollagen as substrate as described under Materials and Methods. [b] Compound could not be assayed at a concentration of 500 μM because it caused precipitation of the procollagen substrate and cleavage products in the presence of the $NaDodSO_4$ buffer used for gel electrophoresis.

described above for thermolysin using 0.1 μg of α-chymotrypsin, but without $CaCl_2$ in the incubation mixture. After incubation at 35 °C, the reaction was stopped by adding 5 μL of 50 mM PSMF in isopropyl alcohol and $^1/_{10}$ volume of 20% $NaDodSO_4$ and by heating at 100 °C for 3 min. Papain activity was measured by a gel electrophoresis assay using ^{14}C-labeled procollagen as substrate. The buffer for this assay was 50 mM NaH_2PO_4 (pH 6.2 at 35 °C) containing 0.15 M NaCl, 5 mM EDTA, and 5 mM cysteine. The reaction was carried out in a final volume of 50 μL containing 5 μL of ^{14}C-labeled procollagen (25 000 cpm and 1.5 μg), 5 μL of papain (0.2 μg) in the assay buffer, and 40 μL of the assay buffer. After incubation at 35 °C, the reaction was stopped by adding 5 μL of 0.2 M iodoacetamide in absolute ethanol and $^1/_{10}$ volume of 20% $NaDodSO_4$ and by heating at 100 °C for 3 min.

Gel Electrophoresis. For all gel electrophoresis assays, $^1/_5$ volume of 5× gel electrophoresis sample buffer containing 10% $NaDodSO_4$, 50% glycerol, and 0.005% bromphenol blue in 1.25 M Tris-HCl (pH 6.8 at room temperature) was added, and the samples were heated for 3 min at 100 °C. All samples were reduced by adding mercaptoethanol to a concentration of 2%, followed by heating at 100 °C for 3 min. Samples were dialyzed against 1× gel electrophoresis sample buffer and applied to the gel. Electrophoresis was carried out with $NaDodSO_4$–polyacrylamide slabs of 1.5 mm thickness and stacking gels of 4.5% polyacrylamide. The separating gels were 6% polyacrylamide in assays for N-proteinase, thermolysin, α-chymotrypsin, and papain activities and 9% polyacrylamide in assays for C-proteinase and kallikrein activities. Fluorograms were prepared to visualize cleavage products (Laskey & Mills, 1975). The separating gels for the collgenase assays were 7.5% polyacrylamide, and cleavage products were determined by using Coomassie Brilliant Blue (Berger et al., 1985).

Analysis of Kinetic Data. All kinetic constants for N-proteinase were determined from data obtained only by using the rapid assay method. The type of inhibition and kinetic consts. were detd. either by plotting $1/v$ vs. inhibitor concentration at different substrate concentrations (Dixon, 1953) or by plotting $1/v$ vs. $1/s$ at different inhibitor concentrations (Lineweaver & Burk, 1934). Values of I_{50} were used only in screening the inhibitors (Naqui, 1983).

For assays in which the ^{14}C-labeled reaction products were separated on electrophoretic gels, the dried gels were analyzed with the Beta Scanning System (Automated Microbiology Systems, Inc., San Diego, CA).

Results

Inhibition of N-Proteinase by FMCA. In initial experiments, FMCA was found to be an inhibitor of N-proteinase.

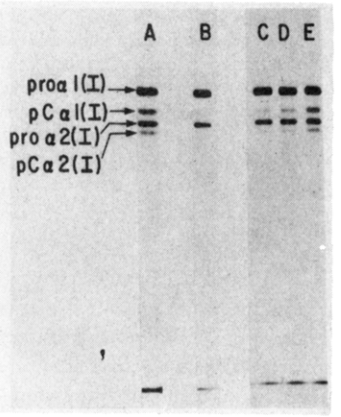

FIGURE 1: Inhibition of N-proteinase by FMCA. Gel electrophoresis assay using ^{14}C-labeled procollagen as substrate: (Lane A) uninhibitied reaction mixture; (lane B) reaction mixture inhibited with 100 mM EDTA; (lane C) reaction mixture inhibited with 5 mM FMCA; (lane D) reaction mixture inhibited with 3 mM FMCA; (lane E) reaction mixture inhibited with 1 mM FMCA.

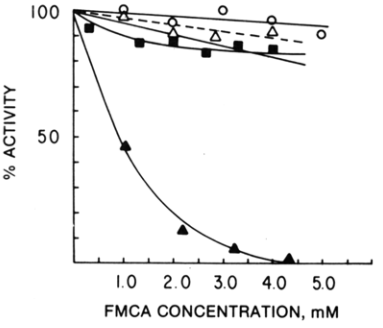

FIGURE 2: Effect of FMCA on procollagen-processing enzymes and collagen-degrading enzymes: cleavage of ^{14}C-labeled pNcollagen by N-proteinase (▲—▲) and of ^{14}C-labeled procollagen by C-proteinase (○—○); cleavage of ^{14}C-acetylated collagen by bacterial collagenase (■—■) and by vertebrate collagenase (△---△). Assays were carried out as described under Materials and Methods.

As shown in Figure 1, cleavage of both the proα1(I) and the proα2(I) chains was inhibited. The I_{50} was 1 mM, and complete inhibition was obtained with a concentration of about 5 mM. No difference in the degree of inhibition was observed when pNcollagen was used as the substrate (Figure 2). FMCA in the same concentrations did not inhibit cleavage of the COOH-terminal propeptides from the procollagen substrate by procollagen C-proteinase (Figure 2, Table I). Also, FMCA did not inhibit cleavage of type I collagen by bacterial collagenase or by vertebrate collagenase (Figure 2). Similarly, FMCA did not inhibit the cleavage of NH_2- and COOH-terminal propeptide-like fragments from either the proα1(I) or proα2(I) chains of procollagen by kallikrein,[4]

Table II: Inhibition of N-Proteinase by Fc and Fc^+ Derivatives

compd	I_{50} (mM)[a]	K_i (mM)[b]
(A) Fc Derivatives		
FMCA	1.0	0.30
FDCA	0.5	0.20
$FcCOCH_2CH_2COOH$	0.10	0.065
$FcCH_2CH_2COOH$	2.0	nd[c]
FcCH=CHCOOH	1.5	nd
(B) Fc^+ Derivatives		
$Fc^+FeCl_4^-$	0.030	0.004
$Fc^+PF_6^-$	0.035	nd
$[Fc^+COOH]PF_6^-$	(0.10)[d]	(0.050)[d]
$[Fc^+CH_2CH_2COOH]PF_6^-$	2.0	nd

[a] Determined by gel electrophoresis and rapid assay. [b] Determined by rapid assay. [c] nd, not determined. [d] Values in parentheses could not be measured accurately because of the instability of the oxidized state. As determined by the absorbance at 629 nm, the oxidized $[Fc^+COOH]PF_6^-$ appeared to reach an equilibrium with the reduced form, FMCA, with a ratio of the oxidized to reduced species of about 1:3. This conclusion was supported by the observation that, after the equilibrium was reached, addition of ascorbic acid, to reduce the Fc^+COOH cation, abolished the absorbance at 629 nm. The inhibition was not caused by the release of Fe^{3+} since the addition of ascorbic acid to $FeCl_3$ under the assay conditions increased, rather than decreased, the absorbance at 629 nm. Thus, the actual inhibitor constants are probably 4-fold lower than the values indicated in parentheses.

Table III: Inhibitors of N-Proteinase That Are in Part Structurally Related to Ferrocene Derivatives[a]

inhibitor	concn (mM)	inhibition (%)[b]
ascorbic acid	5	10
$FeCl_3$	0.1	50
pyrrole-2-carboxylic acid	1	50
furan-2-carboxylic acid	5	25
$[Co(cp)_2]^+PF_6^-$	5	30
$[cpCo(C_5H_4COOH)]^+PF_6^-$	5	45

[a] No inhibition was seen with 5 mM concentrations of $NaPF_6$, L-phenylalanine, benzoic acid, phenol, or imidazole. [b] N-Proteinase activity determined by rapid assay as described under Materials and Methods.

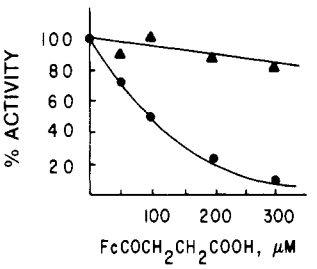

FIGURE 3: Effect of oxidation state of $FcCOCH_2CH_2COOH$ on inhibitory activity against N-proteinase. Nitrogen was bubbled through reaction buffers to remove dissolved oxygen. The N-proteinase and pNcollagen samples were degassed under vacuum, and assay tubes were sealed under a nitrogen atmosphere. Reaction mixtures contained no ascorbic acid (●) and 1 mM ascorbic acid (▲). Rapid assays for N-proteinase activity were carried out as described under Materials and Methods.

α-chymotrypsin, papain, or thermolysin (Table I). Finally, FMCA inhibited less than 10% of the cleavage of azocasein by α-chymotrypsin or thermolysin and of the cleavage of D-Pro-L-Phe-L-Arg-pNa by kallikrein (not shown). In addition to establishing the specificity of FMCA as an inhibitor of N-proteinase, the results suggested that the inhibition of N-proteinase was not explained by binding of FMCA to the cleavage site of procollagen.

Inhibition of N-Proteinase by Fc Derivatives. In further experiments, other Fc derivatives were examined as inhibitors of N-proteinase (Table IIA). The dicarboxylic acid derivative, FDCA, inhibited N-proteinase with an I_{50} that was half the I_{50} of FMCA. Derivatization of the carbonyl α to the cp ring to generate $FcCOCH_2CH_2COOH$ lowered the I_{50} 10-fold relative to FMCA. However, omission of the carbonyl α to the cp ring of $FcCOCH_2CH_2COOH$ to give $FcCH_2CH_2COOH$ increased the I_{50} to a value twice that of FMCA. Similarly, addition of an acrylic acid side chain to Fc to give FcCH=CHCOOH resulted in a 1.5-fold increase of the I_{50} relative to FMCA. Thus, the results demonstrated that selective modification of the side chain increased the inhibitory activity of FMCA.

Since ferrocenes are known to undergo rapid oxidation–reduction reactions (Rosenblum, 1965), the inhibition of N-proteinase by ferrocenes may be explained by the presence of a small amount of ferrocene derivative in the oxidized state. Ascorbic acid has been shown to reduce ferrocenium salts rapidly back to ferrocene (Rosenblum, 1965). Here we found that ascorbic acid did not significantly affect N-proteinase activity (Table III). However, addition of ascorbic acid under anaerobic conditions to an incubation mixture containing $FcCOCH_2CH_2COOH$ abolished the inhibitory activity of this derivative (Figure 3). Thus, the results suggested that the active inhibitory species is the oxidized metallocene.

Inhibition of N-Proteinase by Fc^+ Derivatives. Metallocenes containing iron in the 3+ valence state were next examined. $Fc^+FeCl_4^-$ was a strong inhibitor of N-proteinase with an I_{50} of 30 μM (Table IIB). The inhibition by $Fc^+FeCl_4^-$ was not caused by the counterion $FeCl_4^-$, since the same I_{50} was obtained with $Fc^+PF_6^-$ and the PF_6^- anion was itself not inhibitory (Table III). Fe^{3+} in the form of $FeCl_3$ was inhibitory, but the I_{50} was 3-fold greater than the I_{50} of $Fc^+FeCl_4^-$ (Table III). Also, spectral analysis of $Fc^+FeCl_4^-$ by the absorption peak of Fc^+ at 619 nm and the absorbance of $FeCl_3$ below 440 nm indicated that the molar ratio of $FeCl_4^-$ to Fc^+ in the assay conditions was only about 1:3. Finally, although Fc^+ can decompose and thereby release Fe^{3+} (Goldberg & Martin, 1964; Neuse, 1985), assay of Fc^+ at its absorbance peak indicated that only about 30% of the compound was degraded under the standard assay conditions for N-proteinase. Therefore, the results further indicated that the Fc^+ cation is the inhibitory species.

Examination of additional Fc^+ derivatives showed that the derivative with a carbonyl α to the cp ring, $[Fc^+COOH]PF_6^-$, was a strong inhibitor of N-proteinase (Table IIB), but that $[Fc^+CH_2CH_2COOH]PF_6^-$, without a carbonyl α to the cp ring, was a weak inhibitor of N-proteinase (Table IIB).

As shown in Table I, $Fc^+FeCl_4^-$ was similar to FMCA in that it was apparently specific for the N-proteinase. It did not significantly inhibit either C-proteinase, kallikrein, α-chymotrypsin, or thermolysin at concentrations needed for 50% and 100% inhibition of N-proteinase. $FcCOCH_2CH_2COOH$ was also apparently specific in that it did not inhibit kallikrein, α-chymotrypsin, papain, and thermolysin at concentrations needed for 50% and 100% inhibition of N-proteinase (Table I). $FcCOCH_2CH_2COOH$, however, produced partial inhibition of the C-proteinase at a concentration of 100 μM; it could not be assayed at higher concentration because it caused precipitation of the substrate and cleavage products when $NaDodSO_4$ was added to the reaction mixture.

Other Aromatic Inhibitors of N-Proteinase. The possibility that inhibition of N-proteinase was caused by the aromatic nature of ferrocenes was also examined. Phenol, benzoic acid,

[4] The cleavage of C1 and C2 propeptide-like fragments from procollagen by kallikrein was first observed in our laboratory by Dr. Yoshio Hojima (personal communication).

and phenylalanine did not affect enzymic activity (Table III). Pyrrole-2-carboxylic acid inhibited N-proteinase with an I_{50} of 1 mM. Furan-2-carboxylic acid was a weaker inhibitor, and imidazole was not inhibitory at concentrations less than 5 mM.

Since five-membered aromatic ring containing compounds appeared to be weak inhibitors of N-proteinase (Table III), metallocenes containing Co^{3+} were tested for inhibition of N-proteinase. $[Co(cp)_2]^+PF_6^-$ and $[cpCo(C_5H_4COOH)]^+PF_6^-$ were weak inhibitors of N-proteinase and produced a 30% and 45% inhibition of activity, respectively, at a concentration of 5 mM (Table III). Similarly, $[cpCo(C_5H_4CONH_2)]^+PF_6^-$ and $[Co(C_5H_4NH_2)_2]^+PF_6^-$ produced only a 30% inhibition of N-proteinase activity at concentrations of 5 mM (not shown).

Mode of Fc Inhibition of N-Proteinase. Since Fc and Fc^+ derivatives contain a metal atom, the observed inhibition might have been caused by displacement of a metal cofactor needed for enzymic activity (Tuderman et al., 1982; Tanzawa et al., 1983). No change in the degree of inhibition by FMCA was noted in experiments in which 0, 5, and 10 mM (final concentration) calcium was added to incubation mixutres. This suggested that inhibition was not caused by a chelation or displacement effect, whereby an essential metal cofactor was removed from the enzyme by the inhibitor. Additional evidence regarding this point was that the metalloenzymes C-proteinase, bacterial collagenase, vertebrate collagenase, and thermolysin were not inhibited by FMCA (Figures 1 and 2; Table I).

Experiments in which the N-proteinase was preincubated with FMCA, $FcCOCH_2CH_2COOH$, or $Fc^+FeCl_4^-$ for 30 min at 35 °C before the reaction was started by addition of substrate showed the same degree of inhibition as those in which the enzyme was not preincubated with the inhibitors. This suggested that formation and dissociation of the enzyme–inhibitor complex was a rapid equilibrium process. The type of inhibition by FMCA, $FcCOCH_2CH_2COOH$, and $Fc^+FeCl_4^-$ was shown to be competitive by the methods of Dixon (1953) and Lineweaver and Burk (1934), with K_i values of 0.3 mM, 0.065 mM, and 0.004 mM, respectively (Table II). The reversibility of the inhibitors was also demonstrated by dialysis experiments. Enzyme activity, which had been completely inhibited with 5 mM FMCA, was restored to 80–110% of the uninhibited control by dialysis against the standard assay buffer at either 4 or 35 °C.

Finally, it is known that inhibitors are capable of stabilizing enzymes to heat denaturation. Previous observations indicated that calcium increased the thermal stability of N-proteinase (Tanzawa et al., 1985). Here, two Fc derivatives, FMCA and $FcCOCH_2CH_2COOH$, were shown to stabilize the enzyme in the absence of calcium (Figure 4). For reasons that were not apparent, $Fc^+FeCl_4^-$ did not stabilize the enzyme to heat denaturation.

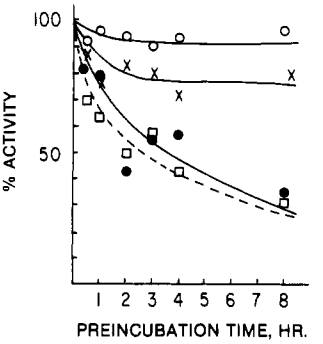

FIGURE 4: Protection of N-proteinase against heat denaturation by iron-containing metallocenes in the absence of calcium. Heparin–Sepharose-purified N-proteinase was dialyzed extensively against the standard assay buffer to remove calcium. Samples containing enzyme, Fc derivative, and assay buffer (total volume, 70 μL) were incubated at 35 °C for the times indicated. At the appropriate time intervals, 10 μL of 50 mM $CaCl_2$ in the standard assay buffer and 20 μL of ^{14}C-labeled pNcollagen were added and were incubated at 35 °C by the rapid assay method as described under Materials and Methods. Incubation mixtures contained no Fc derivative (●—●), 0.5 mM FMCA (○—○), 50 μM $FcCOCH_2CH_2COOH$ (×—×), and 25 μM $Fc^+FeCl_4^-$ (□---□).

Discussion

The chemistry and biochemistry of metallocenes have been studied extensively in the past [for reviews, see Rosenblum (1965) and Dombrowski et al. (1986)]. They are hydrophobic molecules, but when derivatized with polar substituents, they are sparingly soluble in water. Also, the metallocene "sandwich" structure is a stable one in that the metal ion can only be dislodged by completely destroying the molecule with harsh conditions such as strong alkali or by heating to more than 185 °C (Langheim & Wenzel, 1972; Langheim et al., 1975; Schneider & Wenzel, 1982).

Metallocene derivatives were used previously to design enzyme inhibitors by modifying the side chains to resemble substrates of the enzymes. For example, ferrocene derivatives of the penicillin and cephalosporin pharmacophores were synthesized as inhibitors of β-lactamase (Edwards et al., 1975, 1976, 1977, 1979a,b). Also, ferrocenylcholine derivatives have been shown to inhibit butyrylcholinesterase and ferrocenyl carbamate derivatives have been shown to inhibit acetylcholinesterase with K_i values of 10^{-4}–10^{-7} M (Kim, 1978; Hetnarski et al., 1980). Similarly, β-ferrocenylalanine, which structurally resembles phenylalanine, was synthesized as an inhibitor of phenylalanine hydroxylase and of L-amino acid decarboxylase (Hanzlik et al., 1979). However, the K_i values observed with this inhibitor were relatively high (about 1–7 mM).

We initially tested the metallocenes as inhibitors of N-proteinase with the hypothesis that their aromatic nature and cylindrical conformation might approximate the native conformation of the site in procollagen that is cleaved by the N-proteinase. The results indicated that the ferrocene nucleus itself was an inhibitor of N-proteinase. The inhibitory activity, however, depended on both the metal ion in the metallocene and on its valence state. More specifically, ferrocenes containing Fe^{2+} were not inhibitors of the N-proteinase, whereas their oxidized form, the ferrocenium cations containing Fe^{3+}, were strong inhibitors of the enzyme. Additionally, the results presented in this paper suggested that a carbonyl α to the cyclopentadienyl rings increased the inhibitory effectiveness on N-proteinase.

The ferrocenium derivates showed competitive kinetics with respect to the procollagen substrate. The binding of the compounds to the enzyme to form the enzyme–inhibitor complex was a rapid equilibrium process, and most importantly, the inhibition observed was reversible upon dialysis. Also, the inhibitors appeared to be specific inhibitors of N-proteinase in that they did not inhibit seven enzymes able to cleave both collagenous and noncollagenous substrates.

Metallocenium cations are known to interact with biological molecules by forming charge-transfer complexes with conjugated ring systems, such as the purine and pyrimidine bases of DNA and RNA and the aromatic side chains of amino acids (Kornicker & Vallee, 1969). Increasing the size of the conjugated system of the cyclopentadienyl ring by addition of an acyl or aryl group shifts the reduction potential of the compound to a more positive value, making it a better electron

acceptor. Conversely, substitution of the cyclopentadienyl rings with electron-donating groups, such as an alkyl group, shifts the reduction potential to less positive values.

The interaction of metallocenium derivatives with the side chains of amino acids in N-proteinase may explain the inhibition observed here. Both the Fc^+ and Fc^+COOH cations, which were very effective inhibitors of N-proteinase, are good electron acceptors, and the $Fc^+CH_2CH_2COOH$ cation, which was a poor inhibitor of N-proteinase, is a weaker electron acceptor. Therefore, the Fc^+ and Fc^+COOH cations may be binding to an area of high electron density in the substrate-binding or active site of the N-proteinase, possibly by forming a charge-transfer complex. The analogous cobaltocenium derivatives have molecular dimensions and physical properties similar to those of Fc^+ cations but have low reduction potentials and thus have difficulty in forming charge-transfer complexes with aromatic side chains of amino acids (Kornicker & Vallee, 1969). As demonstrated here, they were not inhibitors of N-proteinase.

The most effective ferrocene derivatives tested here inhibited procollagen N-proteinase with a K_i of 4 μM. By appropriate modification, it may be possible to design even more effective inhibitors of the enzyme that may be useful in vivo for controlling the excessive deposition of collagen seen in scarring and other fibrotic conditions.

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Registry No. FMCA, 1217-42-7; FDCA, 1293-87-4; $FcCOCH_2CH_2COOH$, 1291-72-1; $FcCH_2CH_2COOH$, 12261-46-0; FcCH=CHCOOH, 12154-65-3; $Fc^+FeCl_4^-$, 1287-09-8; $[Fc^+COOH]PF_6^-$, 102922-79-2; $Fc^+PF_6^-$, 11077-24-0; $[Fc^+CH_2CH_2COOH]PF_6^-$, 102922-81-6; $[Co(CP)_2]^+PF_6^-$, 12427-42-8; $[CpCo(C_5H_4COOH)]^+PF_6^-$, 41576-48-1; $FeCl_3$, 7705-08-0; $[CpCo(C_5H_4CONH_2)]^+PF_6^-$, 60293-76-7; $CpCo(C_5H_4COCl)$, 12427-45-1; furan-2-carboxylic acid, 88-14-2; pyrrole-2-carboxylic acid, 634-97-9; procollagen *N*-proteinase, 68651-94-5.

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Amino Acid Sequence of a Basic *Agkistrodon halys blomhoffii* Phospholipase A2. Possible Role of NH_2-Terminal Lysines in Action on Phospholipids of *Escherichia coli*†

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ABSTRACT: A basic (p*I* = 10.2) phospholipase A2 of the venom of the snake *Agkistrodon halys blomhoffii* is one of a few phospholipases A2 capable of hydrolyzing the phospholipids of *Escherichia coli* killed by a bactericidal protein purified from human or rabbit neutrophil granules. We have shown that modification of as many as 4 mol of lysine per mole of the phospholipase A2, either by carbamylation or by reductive methylation [Forst, S., Weiss, J., & Elsbach, P. (1982) *J. Biol. Chem. 257*, 14055–14057], had no effect on catalytic activity toward extracted *E. coli* phospholipids or the phospholipids of autoclaved *E. coli*. In contrast, modification of 1 mol of lysine per mole of enzyme substantially reduced activity toward the phospholipids of *E. coli* killed by the neutrophil protein. To explore further the role of lysines in the function of this phospholipase A2, we determined the amino acid sequence of the enzyme and the incorporation of [^{14}C]cyanate into individual lysines when, on average, 1 lysine per molecule of enzyme had been carbamylated. After incorporation of approximately 1 mol of [^{14}C]cyanate per mole of protein, the phospholipase A2 was reduced, alkylated, and exhaustively carbamylated with unlabeled cyanate. The amino acid sequence was determined of the NH_2-terminal 33 amino acids of the holoprotein and of peptides isolated after digestion with trypsin and *Staphylococcus aureus* V-8 protease. The protein contains 122 amino acid residues, 17 of which are lysines. The NH_2-terminal region is unique among more than 30 phospholipases A2 previously sequenced because of its high content of basic residues (His-1, Arg-6, and Lys-7, -10, -11, and -15). The four NH_2-terminal lysines accounted for approximately 50% (about equally distributed) of the total incorporated [^{14}C]cyanate. On the basis of these findings and evidence that the NH_2-terminal region of phospholipases A2 is a functionally important α-helix, we raise the possibility that the cluster of basic residues in the outwardly directed face of this helix is involved in the action of the basic *A. h. blomhoffii* phospholipase A2 on *E. coli* killed by the bactericidal protein of neutrophils.

Phospholipases A2 represent a class of lipolytic enzymes with many common structural and functional features. Their highly conserved nature has been revealed by amino acid sequence analysis of >30 secretory phospholipases A2 from multiple animal sources (Dufton et al., 1983) and by X-ray crystallographic studies (Dijkstra et al., 1983; Brunie et al., 1985). The invariant regions are known to contain the sites concerned with catalysis, Ca^{2+} binding, and substrate recognition (Verheij et al., 1981). Although phospholipases A2 are very similar in many respects, in their action on particular substrates they exhibit major functional differences (Verheij et al., 1980; Hanahan et al., 1980; Zwaal et al., 1975; Dufton & Hider, 1983; Weiss et al., 1979) that presumably are determined by variable structural properties. How such structural differences are related to the ability of a given phospholipase A2 to act in a specific biological event has not been carefully examined.

Our work concerns the determinants of phospholipase A2 action on the envelope phospholipids of *Escherichia coli* (Weiss

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