Cooperative Binding of Ca\textsuperscript{2+} to Human Interstitial Collagenase Assessed by Circular Dichroism, Fluorescence, and Catalytic Activity*

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Yan-na Zhang, William L. Dean, and Robert D. Gray§
From the Department of Biochemistry, University of Louisville School of Medicine, Louisville, Kentucky 40292

Dissociation of Ca\textsuperscript{2+} from human interstitial collagenase induced either by chelation with EGTA or by dilution resulted in loss of enzyme activity, a red shifted emission maximum from 334 to 340 nm and quenching of protein fluorescence by 10% at 340 nm. Circular dichroism indicated that secondary structure was unaffected by EGTA. Ca\textsuperscript{2+} binding to the EGTA-treated enzyme as assessed by fluorescence was cooperative (Hill coefficient, 2.9; 50% saturation at 0.4 mM Ca\textsuperscript{2+}). The dependence of catalytic activity on [Ca\textsuperscript{2+}] was also cooperative (Hill coefficient, 1.7–2.0; midpoint [Ca\textsuperscript{2+}], 0.2 mM). The Ca\textsuperscript{2+}-reconstituted protein was indistinguishable from the untreated enzyme by activity and fluorescence measurements. These results demonstrate that removal of Ca\textsuperscript{2+} from full-length collagenase generates a catalytically incompetent, partially unfolded state with native secondary structure but altered tertiary structure characterized by exposure of at least one tryptophyl residue to a more polar environment.

The matrix metalloproteinases mediate remodeling of extracellular matrix in healthy and diseased tissue (1, 2). Sequence analysis of the MMPs,\textsuperscript{1} also referred to as the metzincin proteinase family (3), reveals a characteristic domain structure. The smallest MMP, matrilysin, consists of 19-kDa catalytic domain with an active site zinc ion coordinated within a conserved HEXXHHXXGXXH motif. Proteolytic latency is maintained by an N-terminal propeptide of approximately 10 kDa in which a conserved cysteine is coordinated to the catalytic zinc (4). The larger members of the family, including interstitial collagenases, stromelysins, and gelatinases, possess additional functional and structural domains. In particular, collagenases have a C-terminal domain of about 30 kDa that exhibits sequence homology with hemopexin and vitronectin (5). This so-called pexin domain is essential for the expression of collagenolytic activity; without it, the enzyme retains general peptidase activity but does not hydrolyze native collagen (6). Matrixin and mutant forms of collagenase and stromelysin possessing only a catalytic domain contain a second zinc ion (7–9). This structural zinc is absent from the full-length forms of stromelysin-1 and gelatinase A (10).

Calcium ions are also an essential components of the MMPs (11, 12). Crystallographic analysis of the catalytic domain reveals three bound Ca\textsuperscript{2+} in fibroblast collagenase (13, 14) and two in the neutrophil enzyme (15). Crystallography of full-length porcine MMP-1 also shows three Ca\textsuperscript{2+} in the catalytic portion and two Ca\textsuperscript{2+} in the pexin domain (16). Ca\textsuperscript{2+} undoubtedly binds to similar sites in the pexin domain of human collagenase because the binding sites are conserved. Previous studies suggest that Ca\textsuperscript{2+} stabilizes an active conformation of the MMP that is more stable to denaturants (17) and less susceptible to proteolysis (18). However, the precise stereochemical basis for Ca\textsuperscript{2+} stabilization is unknown because a three-dimensional model of a Ca\textsuperscript{2+}-free MMP is not available for comparison.

To provide additional insight into the relationship between MMP structure and calcium binding, we correlated the effect of Ca\textsuperscript{2+} on catalytic activity and protein structure using intrinsic protein fluorescence and circular dichroism as structural indicators. Ca\textsuperscript{2+} concentration was manipulated either by the chelating agent EGTA or by dilution. EGTA was chosen because its affinity for Ca\textsuperscript{2+} is comparable with that of EDTA,\textsuperscript{2} although its affinity for Zn\textsuperscript{2+} is much lower than that of EDTA (19). The following data document that Ca\textsuperscript{2+} binding to collagenase is cooperative and associated with changes in tertiary but not secondary structure. Furthermore, these changes were independent of the method used to alter calcium concentration.

EXPERIMENTAL PROCEDURES

Materials—A recombinant human fibroblast collagenase expression vector was generously supplied by Dr. G. McGeehan, Glaxo-Wellcome (Research Triangle Park, NC). The recombinant protein, which lacks the propeptide, was expressed in Escherichia coli and purified as described (20). Native procollagenase was purified from culture medium of human umbilical vein endothelial cells (20). The proenzyme was activated with trypsin (20 ng of trypsin/1 µg of procollagenase-1) for 1 h at 37 °C (21); trypsin was subsequently inactivated with soybean trypsin inhibitor. The substrate peptide Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH\textsubscript{2} (DnpS) was synthesized by Stack and Gray (22); Ac-Pro-Leu-Gly-(2-mercapto-4-methyl-pentanoyl)-Leu-Gly-OEt (TPS) was purchased from Bachem (King of Prussia, PA). Type I collagen from calf skin, soybean trypsin inhibitor, 4,4-dithiopyridine, MOPS, and EGTA were from Sigma. CaCl\textsubscript{2} 2H\textsubscript{2}O was from EM Science (Cherry Hill, NJ); it contained 6 ng zinc/g. Brj 35 (prepared and packaged under nitrogen) was from Pierce, and trypsin (sequencing grade) was from Promega (Madison, WI). Zinc reference standard solution was from Fisher.

Methods—MMP activity was measured with DnpS, TPS, or collagen as substrate. Hydrolysis of DnpS by rMMP-1 was assessed by high pressure liquid chromatography as described (22). Hydrolysis of TPS by HUVEC MMP-1 was measured in a continuous spectrophotometric

1 The abbreviations used are: MMP, matrix metalloproteinase; rMMP-1, recombinant human interstitial collagenase; DnpS, Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH\textsubscript{2}; TPS, Ac-Pro-Leu-Gly-(2-mercapto-4-methyl-pentanoyl)-Leu-Gly-OEt; GdnHCl, guanidine hydrochloride; HUVEC, human umbilical vein endothelial cell; MOPS, 3-(N-morpholino)propanesulfonic acid.

2 log K\textsubscript{EDTA} = 10.6 and log K\textsubscript{EGTA} = 11.0 for Ca\textsuperscript{2+}; log K\textsubscript{EDTA} = 16.4 and log K\textsubscript{EGTA} = 12.9 for Zn\textsuperscript{2+} (19).
assay using 4,4′-dithiopyridine to trap the thiol peptide product, HSC(HiBu)-Leu-Gly-OEt (23). Assays were conducted in a 96-well UV-transparent microtiter plate with a Spectramax 250 UV-visible plate reader (Molecular Devices Corp., Sunnyvale, CA) set at 324 nm to record the kinetics of substrate hydrolysis. Collagenolytic activity was assayed with a peptide substrate, the resulting Hill plot also exhibits a maximum near 315 nm, which is strongly red shifted from that expected for tyrosine in aqueous solution (28). A small decrease in emission intensity was observed, suggesting that the Ca$^{2+}$-dependent conformational differences are sensed by tyrosyl as well as tryptophyl residues.

Titration data illustrating the dependence of fluorescence and catalytic activity on Ca$^{2+}$ are shown in the Hill plots of Fig. 3. Ca$^{2+}$ binding as assessed by changes in fluorescence was cooperative (Hill coefficient, 2.9; 50% saturation at 0.43 mM Ca$^{2+}$). When the Ca$^{2+}$ dependence of rMMP-1 activity was assayed with a peptide substrate, the resulting Hill plot also indicated cooperative Ca$^{2+}$ binding (Hill coefficient, 1.7; 50% saturation at 0.23 mM [Ca$^{2+}$]). When the [Ca$^{2+}$] was manipu-
Ca$^{2+}$ Binding to Collagenase

The present studies, conducted with full-length rMMP-1 that includes both the catalytic and pexin domains, demonstrate that acquisition of catalytic activity by collagenase is coupled to protein conformational changes induced by Ca$^{2+}$ binding. Binding was cooperative as assessed by both structural and activity changes. The observation that a lower Ca$^{2+}$ concentration is required for catalytic activity compared with development of the fluorescence changes suggests that fewer binding sites influence catalysis, whereas the fluorescence changes involve sites other than those necessary for catalytic efficiency. Our studies with the full-length protein extend previous studies conducted with the catalytic domain alone. Lowry et al. (17) showed that both Ca$^{2+}$ and Zn$^{2+}$ stabilized a recombinant collagenase catalytic domain against denaturation by GdnHCl, and Housley et al. (18) reported that Ca$^{2+}$ stabilized the catalytic domain of recombinant stromelysin against heat denaturation. In addition, activation of prostromelysin-1 by an organomercurial in the presence of 0.1 mM Ca$^{2+}$ resulted in autolysis that could be prevented with 5 mM Ca$^{2+}$ (18). These data indicate that Ca$^{2+}$ stabilizes a compact protein structure that is less susceptible to denaturation and proteolysis. Our results show that Ca$^{2+}$ binding to full-length rMMP-1 results in local conformational change(s) that do not detectably alter the secondary structure of the protein but do change tertiary structure as reflected in the environment of tryptophyl and tyrosyl residues. The change in tertiary structure in the absence of secondary structural changes suggests that Ca$^{2+}$ removal may produce a “molten globule-like” structure (20).

The emission maximum of denatured MMP-1 is at 356 nm (20), characteristic of tryptophan in water, whereas the emission maximum of the Ca$^{2+}$-deficient enzyme shifts only to 340 nm. Thus the change in tryptophyl environment brought about by Ca$^{2+}$ release is less drastic than complete unfolding. In our study of GdnHCl-induced denaturation of rMMP-1, we observed a folding intermediate with an emission maximum at 340 nm at 1 M denaturant (20). However, it is unlikely that the Ca$^{2+}$-free form of MMP-1 in the present study is the same as this intermediate because in 1 M GdnHCl, there was an increase, rather than a decrease, in emission intensity.

MMP-1 contains one tryptophyl residue in the propeptide, three in the catalytic domain and four in the pexin domain. Assignment of the fluorescence changes to particular residues is of course impossible from the data at hand. Lovejoy et al. (13) suggested that in 19-kDa collagenase one of the calcium ions and the structural zinc may stabilize a surface loop by fastening it to two $\beta$ strands. Neither the loop nor the two strands contain tyrosyl or tryptophyl side chains that might be directly affected by Ca$^{2+}$. Thus, it is likely that any conformational change induced by Ca$^{2+}$ binding at this site is propagated to other regions of the structure. The two Ca$^{2+}$-free domains of pig collagenase also appear to link structural domains together: $\beta$-sheet-1 to sheet 3 and $\beta$-sheet-4 to sheet 2 (16). Two of the four pexin tryptophyl residues are located within these sheets; Trp$^{349}$ is in sheet 2, and Trp$^{398}$ is in sheet 3. Thus, it is reasonable to assign at least a portion of the Ca$^{2+}$-dependent fluorescence change to them. In addition, both tryptophyls are close in the primary structure to tyrosyl residues [YW$^{249}$A and YW$^{338}$RY (29)]. Variation in the relationship between these groups should influence the efficiency of excitation energy transferred from tyrosyl to tryptophyl residues. However, the quenching of tyrosyl emission observed on Ca$^{2+}$

**TABLE I**

| Addition                  | Activity (pmoles degraded/min) |
|---------------------------|--------------------------------|
| Collagen$^a$  | DnpS$^b$      |
| None                 | 2.7          | 9.7           |
| 7 mM EGTA            | 0            | 0             |
| 7 mM EGTA followed after 10 min by 8 mM CaCl$_2$ | 2.6          | 10.2          |
| 7 mM EGTA followed after 24 h by 8 mM CaCl$_2$ | 2.7          | 11.2          |

$^a$ Activity determined at 25°C for 50 min with 0.7 mg/ml collagen and 0.3 mM rMMP-1.

$^b$ Activity determined at 25°C for 60 min with 20 mM DnpS and 0.2 mM rMMP-1.

FIG. 3. Dependence of fluorescence intensity and activity of MMP-1 on [Ca$^{2+}$] as analyzed by Hill plots. rMMP-1 was diluted to 0.2 $\mu$M in 150 mM Tris-HCl, 5 mM CaCl$_2$, 200 mM NaCl, 50 $\mu$M ZnSO$_4$, 0.05% Brij 35, at pH 7.6. EGTA (6 mM) was added, and fluorescence intensity at 348 nm (excitation at 280 nm) was monitored as a function of added [Ca$^{2+}$]. Fractional saturation (r) with Ca$^{2+}$ was calculated from the equation, $r = (F_{\text{max}} - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}})$, where $F_{\text{max}}$ and $F_{\text{min}}$ are the fluorescence intensities at the highest, lowest, and any given Ca$^{2+}$ concentration, respectively. Hydrolysis of DnpS (20 $\mu$M) by rMMP-1 (0.2 $\mu$M) was determined under conditions described in Ref. 22. HUVEC MMP-1 was assayed in the absence of EGTA in a continuous spectrophotometric assay using TPS (23). A stock enzyme solution containing 5 mM CaCl$_2$ was diluted into 50 mM MOPS, 200 mM NaCl, 0.05% Brij 35, 500 $\mu$M 4,4-dithiopyridine, at pH 7.0, to a final [Ca$^{2+}$] of 0.125 mM. Individual assays were then conducted with increasing amounts of CaCl$_2$. Reactions were initiated by adding substrate to a final concentration of 100 $\mu$M. Maximal activity was achieved at 1 mM Ca$^{2+}$ for both rMMP-1 and HUVEC MMP-1. For the activity measurements, $r = v/v_{\text{max}}$, where $v_{\text{max}}$ is the activity at 1 mM Ca$^{2+}$ and $v$ is the activity at any individual [Ca$^{2+}$]. Hill coefficients and [Ca$^{2+}$] for half saturation were estimated by linear regression to be 2.9 and 0.43 mM for the fluorescence data (A), 1.7 and 0.2 mM for the rMMP-1 (B), and 2.0 and 0.2 mM for HUVEC MMP-1 (C).

Related by dilution rather than EGTA, cooperative Ca$^{2+}$ binding to native HUVEC MMP-1 was also observed (Hill coefficient, 2.0; 50% saturation at 0.20 mM Ca$^{2+}$). Because similar results were obtained whether [Ca$^{2+}$] was altered by EGTA or dilution, we conclude that the effects of the chelating agent result from Ca$^{2+}$ dissociation from the enzyme rather than removal of catalytic Zn$^{2+}$.

To determine if the effects of Ca$^{2+}$ on activity and fluorescence were reversible, we compared the catalytic activity of the recombinant enzyme with DnpS and collagen prior to Ca$^{2+}$ removal, after Ca$^{2+}$ removal with EGTA, and after reconstitution with Ca$^{2+}$. Previous reports (11, 12) indicate that prolonged incubation of collagenase with EDTA resulted in removal of zinc and irreversible denaturation of the protein. The data in Table I show that activity against both DnpS and collagen was completely lost on treatment with EGTA. Loss of activity and fluorescence changes were reversed by adding Ca$^{2+}$, even 24 h after removal of the metal ion.

**DISCUSSION**

The present studies, conducted with full-length rMMP-1 that includes both the catalytic and pexin domains, demonstrate that acquisition of catalytic activity by collagenase is coupled to protein conformational changes induced by Ca$^{2+}$ binding. Binding was cooperative as assessed by both structural and activity changes. The observation that a lower Ca$^{2+}$ concentration is required for catalytic activity compared with development of the fluorescence changes suggests that fewer binding sites influence catalysis, whereas the fluorescence changes involve sites other than those necessary for catalytic efficiency. Our studies with the full-length protein extend previous studies conducted with the catalytic domain alone. Lowry et al. (17)
dissociation was accompanied by decreased, rather than increased, tryptophyl emission, as would be expected if altered transfer efficiency were the sole basis for the altered fluorescence.

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Ca\(^{2+}\) Binding to Collagenase