Cleavage of Bovine Collagen I by Neutrophil Collagenase MMP-8

EFFECT OF pH ON THE CATALYTIC PROPERTIES AS COMPARED TO SYNTHETIC SUBSTRATES

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The enzymatic processing of bovine collagen I by neutrophil collagenase (MMP-8) has been monitored at 37 °C, envisaging the occurrence of multiple intermediate steps, following the initial cleavage, which leads to the formation of ¼ and ½ fragments. Further, the first cleavage event has been investigated at 37 °C as a function of pH, and catalytic parameters have been obtained through a global analysis of steady-state kinetic data, such as to get an overall consistent picture of $k_{cat}$, $K_m$, and $K_a$. These data have been compared with those obtained from the catalysis by MMP-8 of two synthetic fluorogenic substrates under the same experimental conditions. The overall behavior can be accounted for by the existence of five protonating groups, which vary to a different extent their $pK_a$ values for the three substrates investigated. The main observation concerns the fact the two of these residues, which play a relevant role in the enzymatic activity of MMP-8, are relatively far from the primary recognition site, and they are coming into action only for large macromolecular substrates, such as bovine collagen I. This finding opens the question of appropriate testing for inhibitors of the enzymatic action of MMP-8, which must take into account, and also of these relevant interactions occurring only with natural substrates.

Matrix metalloproteinases (MMP) are a class of proteolytic enzymes characterized by the presence of a Zn$^{2+}$ atom in the active site, which are able to process enzymatically several components of the extracellular matrix, such as interstitial and basement membranes, collagen, fibronectin, and laminin. Because of their crucial role in the extracellular matrix degradation, they are implicated in the tissue remodeling processes associated to the growth and development and in several pathological conditions, such as rheumatoid arthritis and tumor invasion (1–3). Collagenases, such as fibroblast collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13), are a class of MMPs that specifically cleave several types of native triple-helical collagen (4–6), and in particular collagen I, at a specific peptide bond between Gly775 and the residue in position 776 (which can be either Leu or Ile) (4, 7), leading to the formation of ¼ and ½ fragments. This event is a crucial one because of the fairly slow rate of collagen turnover within the cartilage (8), and it is closely related to the occurrence of important physiological and pathological events (9–11).

Collagen I is one of the major constituents of the extracellular matrix for several tissues, such as skin, tendon, blood vessels, cartilage, bones, and basal laminas (12), and its triple-helical structural arrangement has been the object of several recent studies both on the native molecule (Ref. 13 and references quoted therein) and on model peptides (14, 15). The investigation on the action of MMPs on collagen has been carried out for a long time, and preliminary measurements have been carried out on the activation energy of the catalysis employing porcine collagenases and gelatinases (16). In addition, previous experiments have been reported on the cleavage of native and site-directed mutants of murine collagen by MMP-1, outlining crucial aspects of the recognition mechanism (17). Very recently, proteolysis of single collagen I molecules has been followed by atomic force microscopy (18), confirming that the process follows a simple Michaelis-Menten mechanism and outlining that the results on bulk solution are compatible with those observed on single collagen molecules.

Therefore, the knowledge of the mechanism of collagen cleavage by collagenases indeed is very important for the comprehension of the physiological and pathological processes affecting the extracellular matrix and in perspective for a more effective pharmacological approach to control and eventually inhibit the action “in vivo” of these MMPs. A previous modeling of different cleavage sites of collagen molecules pointed out the possibility that the interaction surface between collagen and collagenases was fairly extended (19). In the present study we have characterized the proton-linked behavior of the proteolytic process leading to the formation of the ¼ and ½ fragments of bovine collagen I by neutrophil collagenase MMP-8 by comparing it to the behavior of the same enzyme with two fluorogenic synthetic substrates to obtain some initial information on the different recognition and cleavage mechanisms operating with small synthetic as well as large macromolecular substrates.

MATERIALS AND METHODS

Recombinant purified neutrophil collagenase MMP-8 was kindly obtained from Dr. G. Murphy (Strangeways Res. Laboratory, Cambridge, United Kingdom). Purity of MMP-8 was measured by SDS-polyacryl-
amide gel electrophoresis according to Laemmli (20). After the gels were run, they were stained using a silver staining kit (Bio-Rad). Zymography was performed as follows: 2 μl of purified MMP-8 was mixed with a 5-fold excess of sample buffer (0.25% Tris, 0.5% SDS, 10% glycerol, and 0.05% bromphenol blue) and run on 12% SDS-polyacrylamide gels containing 0.01 M gelatin or collagen type I, as described previously (21). After electrophoresis, SDS was removed from gels by washing twice for 15 min in 2% Triton X-100. The gels were then incubated at 37 °C for 18 h in the incubation buffer (50 mM Tris/HCl, 0.1 M NaCl, 10 mM CaCl2, 2% Triton X-100), stained with 0.5% Coomassie Blue, and destained in 10% acetic acid and 40% methanol until pale proteinase bands were clearly visible. The proteinase band was further characterized by adding 20 mM EDTA or 0.3 mM 1,10-phenanthroline (MMP inhibitors) or 1 mM phenylmethylsulfonyl fluoride (serine proteinase inhibitor) in the incubation buffer. Protein markers (Sigma) were used as the molecular weight standard.

Recombinant purified MMP-8 proenzyme was activated by incubating 100 μl of a 0.1 μg/ml procollagenase solution with p-aminophenyl mercuric acetate (Sigma) at 37 °C for 2 h; this treatment shifts the equilibrium of the conformation toward the open, autocatalytically activated form involving the cleavage of the region between residues 71 and 81. Because BB-94 (known also as Batimastat, a peptidomimetic MMPs inhibitor, kindly provided by British Biotech Pharmaceutical, Cowley, Oxford, UK) fully inhibits stoichiometrically MMPs, we employed the active amount of enzyme for all experiments.

Bovine collagen I was dissolved in acidified water at room temperature for 2 days. Afterward, the suspension was centrifuged for 1 h at 10,000 × g, and the supernatant containing dissolved collagen was used. The amount of collagen was quantified as described by Bradford (22).

**Fragmentation Experiments—**Activated MMP-8 (0.01 mM final concentration) in 50 mM Tris/HCl, 0.1 mM NaCl, 10 mM CaCl2, and 0.05% bromphenol blue) and run on 12% SDS-polyacrylamide gels as described previously (23), and the gels were stained by using Coomassie Blue (see Fig. 1 described previously (23), and the gels were stained by using Coomassie Blue (see Fig. 1).

Different experiments were performed by using different pH values. Mixtures were kept at 37 °C, and a small amount was harvested at different time intervals and frozen at −80 °C until use. The aliquots were then run on a 12% SDS-polyacrylamide gel electrophoresis, as described previously (21). After electrophoresis, SDS was removed from gels by incubation at 37 °C for 18 h in the incubation buffer (50 mM Tris/HCl, 0.1 M NaCl, 10 mM CaCl2, 2% Triton X-100), stained with 0.5% Coomassie Blue, and destained in 10% acetic acid and 40% methanol until pale proteinase bands were clearly visible. The proteinase band was further characterized by adding 20 mM EDTA or 0.3 mM 1,10-phenanthroline (MMP inhibitors) or 1 mM phenylmethylsulfonyl fluoride (serine proteinase inhibitor) in the incubation buffer. Protein markers (Sigma) were used as the molecular weight standard.

**Circular Dichroism Experiments—**For spectroscopic observations bovine collagen I was entrapped in agarose gel by mixing the solubilized protein to a 0.6% low melting point agarose solution at pH 3.0. The temperature of the agarose solution when collagen was added was 37 °C. After rapid stirring the mixture was poured on a simple gel casting (Mini-protean II, Bio-Rad) and immediately cooled to obtain the gel. Final concentrations were 0.5% for agarose and 1 μM for collagen. The thickness of the homogeneous gel was 1 mm. Shares of suitable size were cut off from the homogeneous gel and kept overnight in buffer solution to reach the desired pH without the formation of aggregates and then used for circular dichroism measurements.

**Circulator Dichroism (CD) spectra in the far UV region (240–190 nm)** were carried out on a Jasco J710 spectropolarimeter. Each spectrum was the average of four measurements. As a starting control, CD measurements were performed on samples of gel-entrapped collagen at pH 3.0 as well as on solubilized collagen at the same pH and concentration conditions. Control measurements have been also performed by using agarose gel. Data obtained were considered as background. The effect of proteolytic fragmentation of bovine collagen I by MMP-8 has been also followed by CD spectra, diffusing MMP-8 (to a final concentration of 0.01 nM) into the slices of agarose gel containing bovine collagen I (see Fig. 1B).

**Enzymatic Activity—**Enzyme activity was measured employing two fluorogenic substrates, namely MCA-Pro-cyclohexylamine-Gly-γ-norvaline-His-Ala-DPA-NH₂ (i.e. MCA-1) and MCA-Pro-Leu-Gly-γ-Leu-DPA-Ala-Arg-NH₂ (i.e. MCA-2); both substrates were a gift of Dr. G. Knight, Strangeways Res. Laboratory, Cambridge, U.K. Experiments were carried out at a final 0.01 mM concentration of purified activated MMP-8 (0.1 mM Tris/HCl, 0.1 mM NaCl, 10 mM CaCl₂ and 0.05% Brij 35) buffered at different pH values (between 10.5 and 4.8). The pH value was measured before and after the experiment, and only measurements in which no pH change was observed have been taken into account. Experiments at pH 4.8, 5.7, and 6.2 have been also performed in 5 mM MES, 0.1 mM NaCl, 10 mM CaCl₂, and no difference for enzymatic activities between two buffer systems (i.e. MES and Tris/HCl) was observed. The substrate was diluted in Me₂SO, and preliminary experiments at all pH values investigated demonstrate that the addition of Me₂SO to the incubation mixture does not affect MMP-8 activity. In the case of synthetic substrates, MCA-1 and MCA-2 assays were carried out by continuously monitoring the increase in fluorescence at 395 nm after excitation at 328 nm, using a Jobin-Yvon spectrophotometer (model JY-3), and the amount of substrate catalyzed was calibrated at every pH, letting the catalysis reaction go to completion and measuring the amplitude of the signal; only experiments for which a linear dependence on substrate concentration has been observed have been used for the analysis. The measurement of the initial velocity has been referring to a time period over which less than 10% of the substrate was degraded during the assay, and data were normalized and expressed as nanomoles of cleaved substrate/s.

**Data Analysis—**Values of observed k_{cat, K_m}, k_{cat, K_m}, and K_m for MMP-8 at any given pH and the pH dependence over the range investigated (i.e. between 4.5 and 10.0) were calculated simultaneously through a global analysis of the whole data set, employing two formalisms (i.e. linear Lineaweaver-Burk and sigmoidal Michaelis) for determining the observed parameter at a given pH value. Fitting of catalytic parameters was constrained to an internally full consistent picture, such that at any protonation level values of all three parameters (i.e. k_{cat, K_m}, k_{cat, K_m}, and K_m) must be closely related. Thus, the fitting procedure forced the system to be described by n protonation states with n values of k_{cat, K_m} and n values of K_m, which must combine then to give n corresponding values of k_{cat, K_m}. The fitting of the pH dependence is internally constrained to obey a scheme with n values of pK_{i} and pK_{i}, which are the same for all three catalytic parameters. Furthermore, because pK_{i} values are expected to be independent on substrate, in the fitting procedure we have forced these values to be the same for all three substrates (i.e. MCA-1, MCA-2, and bovine collagen I). A general description of this system may be referred to in Scheme 1, where ES (as well as ESH₂ with x = 1, 2, ..., n) simply refers to the species undergoing the rate-limiting step and K_m (with x = 0, 1, ..., n) refers to all preequilibrium events leading to the species that undertake the rate-limiting step.

A comprehensive comparative global analysis has been carried out on the catalytic parameters for synthetic substrates and bovine collagen I altogether according to Scheme 1, employing different numbers of protonating groups, to obtain the whole picture of the proton-linked modulation of the MMP-8 enzymatic activity.

Such an approach has indicated that five is the minimum number of MMP-8 residues potentially involved in the functional interaction with natural as well as synthetic substrates to account for the observed pH dependence of the enzymatic properties of MMP-8; in the following discussion, we will refer to these five residues as residue i corresponding to that displaying a pK_{i} < 1. Therefore, the application of Scheme 1 with five protonating groups leads to fairly complex equations for the analysis of catalytic parameters, such as:

$$k_{rel} = \frac{k_{cat}}{k_{cat}} = \frac{k_{cat}}{k_{cat}}$$

(Eq. 1)

where k can be either k_{cat, K_m} or k_{cat, K_m} (i = 0...n) are the protonating equilibrium constants (with K_{m} = 1, K_{i} = K_{i} in the case of k_{cat, K_m} and k_{cat, K_m}).
RESULTS AND DISCUSSION

Fig. 1A shows the electrophoretic pattern of the bovine collagen I fragmentation by human neutrophil collagenase MMP-8 at pH 7.0 and 37 °C. It has been reported by many other authors that proteolysis of collagen is occurring through a very specific recognition mechanism, which displays different determinant factors for the various types of collagen (19). In the case of collagen I this process brings about the cleavage of a specific peptide bond between Gly775 and the residue in position 776 (which can be either Leu or Ile) (4, 7), leading to the formation of 1/4 and 3/4 fragments. This event is also observed in Fig. 1A, and the two populations are indicated on the side portion of the electrophoretic pattern. Obviously, the amount of the two populations varies as a function of the incubation time of MMP-8 with bovine collagen I, and the temporal evolution is reported in Fig. 1B for the processing of 1 μM bovine collagen I with 0.01 nM MMP-8 with both fragments showing a bell-shaped time behavior of their concentration. Such a feature suggests the occurrence of a serial proteolytic pathway, which implies the further fragmentation of both the 3/4 and the 1/4 fractions. Furthermore, Fig. 1B shows that the temporal evolution of the two fragments is not perfectly overlapping, indicating the occurrence of an intermediate fragmentation state. Therefore, we have tried to model this process with a minimum reaction scheme (Scheme II), where A is the whole collagen, B is the 3/4 fragment, D is the 1/4 fragment, C is the intermediate fragmentation state (e.g. 1/2 fragment, as in our modeling of Scheme I), and E is the smallest fragmentation state (i.e. 1/n of D, in our case n = 2). The representation appears adequate to describe the process, at least to a first approximation, even though other schemes might likely turn out to be equally good.

![Electrophoretic pattern of collagen I fragmentation](image)

Fig. 1. A, electrophoretic pattern of the bovine collagen I (at a concentration of 1 μM) fragmentation at 37 °C by MMP-8 (final concentration 0.01 nM) at different time intervals (as indicated). The main 3/4 and 1/4 fragments are marked on the side portion of the figure. B, concentration of 1/4 and 3/4 fragments of bovine collagen I as a function of time after addition of MMP-8 (0.01 nM final concentration). Experimental values have been obtained by measuring the optical density of bands reported in A with a gel scanner. The continuous lines have been obtained by simply applying the catalytic parameters reported in Table II to obtain k1 in Scheme II and thus the production of species B and D after the first step (see Scheme II). The values of k1 (0.95±0.02 h⁻¹), k2 (0.04±0.01 h⁻¹), and k3 (0.12±0.03 h⁻¹) have been obtained by least-squares fitting of experimental data according to a Runge-Kutta algorithm applied to Scheme II. C, circular dichroism spectra of agarose gel-entrapped bovine collagen I as a function of time (as indicated) after exposure to proteolytic fragmentation by MMP-8. For further details, see text.
Fig. 1C reports the circular dichroism spectrum of the gel-entrapped bovine collagen I in the UV region between 190 and 240 nm during the proteolytic processing by MMP-8 under the same experimental conditions employed for the experiment reported in Fig. 1B. Gel entrapping avoids formation of collagen aggregates and fibrils at a physiological pH range. The CD spectra so obtained are quite in agreement with those obtained for collagen-related peptides (24, 25) showing the characteristic collagen triple-helix maximum in the 223–225 nm range. This result allows us to have a “quasinative” model for evaluating structural rearrangements during proteolytic processes on collagen. It appears evident that over the first 20 h, during which the 3/4 and the 1/4 fragments are building up (see Fig. 1B), very minor changes can be detected for the dichroic spectrum, suggesting that the unwinding of the molecule is very limited, if any. On the other hand, during the following 30 h a large variation of the spectrum is observed (see Fig. 1C), in particular a great decrease of the peak intensity at 223–225 nm, which corresponds to a large fraction of the further fragmentation of the collagen molecule (see Fig. 1B).

As an internal control, acid solution of bovine collagen I and/or gel-entrapped collagen samples at the pH and temperature condition of the proteolytic assays were monitored for a time longer than 50 h, showing no significant changes in CD spectra (data not shown). Therefore, such observations suggest that the 3/4 and the 1/4 fragments retain most of the secondary structural arrangement of the native collagen, whereas further fragmentation leads to a significant unwinding of the molecule.

In view of these observations, which indicate that upon the first cleavage step no major structural change is occurring in the collagen fragments, the investigation has been primarily focused on the first step of the proteolytic processing of bovine collagen I by MMP-8, comparing it to the behavior observed with two synthetic substrates whose cleavage site closely resembles that of the natural substrate. In this way, we can observe the role of possible additional regions of the macromolecular natural substrate on the enzymatic action of the neutrophil collagenase.

Fig. 2 displays the pH-dependent behavior of k_{cat}/K_m (A), k_{cat} (B), and K_m (C) for MMP-8 with two synthetic substrates, namely MCA-1 and MCA-2 (see “Materials and Methods”). The global analysis of catalytic parameters for the proteolytic processing of these two synthetic substrates indicates that a satisfactory description of enzymatic properties requires the involvement of at least three protonating groups to account for the proton-linked functional modulation. However, it must be outlined that the different amino acid sequence of the two substrates seems to have some influence on the absolute values of the catalytic parameters as well as on the pK_a shifts observed upon substrate binding, thus resulting in a different pH-dependent profile for the catalysis of the two substrates (see Fig. 2). Thus, whereas values of pK_a for the free enzyme (i.e. pK_{U} values) are independent on the substrate, as expected (see Table I), in the substrate-bound enzyme (i.e. pK_{L} values) these values strongly depend on the substrate. Such an observation is very important, because it may represent relevant information for the final target of mapping the functional role played by different residues involved in substrate binding and cleavage.

This appears unequivocal in the case of the natural substrate bovine collagen I (see Fig. 3 and Table I), where a marked displacement of the optimal overall activity (i.e. k_{cat}/K_m) toward a much more alkaline pH with respect to synthetic substrates is observed (see Fig. 3A), suggesting that in this case an important functional role could be played by residues characterized by fairly high pK_a values. A global analysis of the catalytic behavior of MMP-8 suggests that at least five residues must be involved to account for the overall functional modulation with the two synthetic substrates and the bovine collagen.

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*G. F. Fasciglione, S. Marini, S. D’Alessio, V. Politi, and M. Coletta, submitted for publication.*
of the three synthetic and natural substrates, but there is some overlapping as well as some specific behavior for one substrate and not for the other one. The situation is rendered more cumbersome by the fact that not all residues modulating one parameter (e.g. \( K_m \) through a \( pK_a \) shift upon substrate binding) are affecting another one (e.g. the catalytic rate constant \( k_{cat} \)) and vice versa.

In particular, in the case of MCA-1 three residues appear to be involved in the modulation of \( K_m \) (see Table I), displaying a \( pK_a \) shift (i.e. \( \Delta pK_a = +0.21 \), \( \Delta pK_a = -0.05 \), and \( \Delta pK_a = -0.30 \)); thus, a bell-shaped pH dependence of \( K_m \) is observed (see Fig. 2C), because \( \Delta pK_a = 3 \) brings about an increase of substrate binding affinity, whereas further \( pK_a \) shifts (i.e. \( \Delta pK_a = 4 \) and \( \Delta pK_a = 5 \)) induce a decrease of the substrate association constant. On the other hand, \( k_{cat} \) is mostly affected by \( pK_1 \) (6.71 ± 0.11) and \( pK_3 \) (5.85 ± 0.09) and only to a very marginal extent by \( pK_4 \) (7.36 ± 0.09) (see Table I). Therefore, if we take a general overview of the modulation mechanism for the enzymatic catalysis of MCA-1 by MMP-8 we can say that the interaction of MCA-1 (i) does not affect the protonation of the two residues with higher \( pK_a \) values (i.e. \( pK_1 \) and \( pK_2 \)), (ii) raises the value of \( pK_3 \), bringing about proton uptake by this residue, but this has a very modest effect on the rate-limiting step kinetic constant, (iii) decreases significantly the value of \( pK_5 \), and to a lesser extent that of \( pK_4 \), with varying effects on \( k_{cat} \) (see Fig. 2B). In fact, protonation of residue with \( pK_1 \) greatly increases the catalytic rate-limiting step, whereas the opposite occurs upon protonation of the group characterized by \( pK_3 \). As a whole, the overall catalytic rate constant \( k_{cat}/K_m \) for MCA-1 shows an enhancement upon the protonation of residue 3, mostly because of the increased substrate affinity, which is further accentuated by the protonation of residue 4, mostly because of the raised \( k_{cat} \) (see Table II). The decrease of both \( K_m \) and \( k_{cat} \) upon the protonation of residue 5 leads to a marked decrease of the \( k_{cat}/K_m \) (see Fig. 2A).

Also in the case of MCA-2 there are three protonating groups that appear to be involved in the modulation of \( K_m \) (i.e. \( \Delta pK_a = +0.05 \), \( \Delta pK_a = -0.16 \), and \( \Delta pK_a = -0.24 \), see Table I), two of which (namely \( pK_3 \) and \( pK_4 \)) are the same involved in the case of MCA-1, and they show a \( pK_a \) decrease on MCA-2 binding, bringing about a decrease of the substrate affinity (corresponding to an increase of \( K_m \), see Fig. 2C and Table I). A third residue, which displays a higher \( pK_a \) value (i.e. \( pK_1 \)), appears specific to the interaction of MCA-2 with MMP-8, and its \( pK_a \) increase upon substrate binding is responsible for the enhancement of substrate affinity (corresponding to a decrease of \( K_m \) ) as the pH lowered from 10 to 8 (see Fig. 2C). Furthermore, whereas the protonation of the residue characterized by \( pK_1 \) does not influence the rate-limiting step, the protonation of the residues characterized by \( pK_3 \) and \( pK_4 \) affects \( k_{cat} \) as well (see Table II), bringing about in both cases a significant rate enhancement (see Fig. 2B). In addition, a third residue, whose protonation is not influenced by the substrate interaction (i.e. \( pK_5 \)), also affects \( k_{cat} \), inducing a marked reduction of this rate constant (see Table II). The overall catalytic behavior for the enzymatic processing of MCA-2 shows a slight increase
of $k_{\text{cat}}/K_m$ after the protonation of residue 1, which is completely because of the decrease of $K_m$; after the protonation of residue 3 we have a marked rise of $k_{\text{cat}}/K_m$, totally referable to an increase of $K_m$, whereas the protonation of residue 4 keeps $k_{\text{cat}}/K_m$ essentially unchanged because of a balancing between an increase of $K_m$ and a decrease of substrate affinity (corresponding to an increase of $K_m$, see Fig. 2C). Protonation of residue 5 leads to a decrease of $k_{\text{cat}}/K_m$, which mirrors the behavior of $k_{\text{cat}}$, $K_m$ being unchanged because $\Delta pK_5 = 0$.

As a whole, the catalytic behavior of these two synthetic substrates displays some similairities, but also differences, likely related to the variation of amino acid sequence of the peptide. In particular, for both substrates (i) residue 2 does not appear to play any role, (ii) after protonation of residue 3 the affinity is closely similar, (iii) the highest $k_{\text{cat}}$, which is observed after the protonation of residue 4, has a closely similar value. On the other hand, several differences can be outlined between the two synthetic substrates, so that although binding of MCA-1 brings about a raising of $pK_3$, in the case of MCA-2 we observe a decrease of $pK_3$ (see Table I), indicating that the interaction mode with this residue is different for the two substrates; MCA-1 brings about a proton uptake, whereas MCA-2 leads to a proton release. Furthermore, although only the interaction of MCA-1 with MMP-8 induces a specific variation on the protonation properties of residue 5, in the case of MCA-2 we observe a $pK_5$ shift of residue 1, which is unchanged upon interaction of MMP-8 with MCA-1.

A different situation can be observed for bovine collagen I, in this case at least four residues are modulating $K_m$ (see Table I), three of which are overlapping with those involved in the functional modulation of synthetic substrates (being characterized by $\Delta pK_1 = -3.66$, $\Delta pK_2 = -2.05$, and $\Delta pK_4 = -2.15$), whereas the other one is specific of the interaction of MMP-8 with bovine collagen I (i.e. $\Delta pK_2 = -2.79$). In addition, the interaction of bovine collagen I with MMP-8 is characterized by a sequence of protonating groups, which alternatively increase (e.g. $\Delta pK_1$ and $\Delta pK_4$) and decrease (e.g. $\Delta pK_2$ and $\Delta pK_3$) the substrate binding affinity; the resulting $pH$ dependence is then more complex than for the synthetic substrates (see Fig. 3C). However, only three of these residues (namely residue 2, residue 3, and residue 4) seem to be relevant for the modulation of $k_{\text{cat}}$: the first two bring about a rate enhancement and the third leads to a dramatic reduction of the kinetic constant for the rate-limiting step (see Table II). The overall catalytic behavior displays a steep raising of $k_{\text{cat}}/K_m$ at very alkaline $pH$, which is wholly attributable to a marked increase of collagen binding affinity related to the proton uptake of residue 1. Proton release of residue 2 upon collagen binding brings about a drastic decrease of substrate binding affinity, which induces a pronounced reduction of $k_{\text{cat}}/K_m$ (only partially compensated by the increase of $k_{\text{cat}}$ upon protonation of this residue in the substrate-bound form, see Table II). A drastic increase in the catalytic efficiency occurs upon protonation of residue 3 in the collagen-bound form, whose proton release upon substrate binding is, however, accompanied by a marked decrease of the affinity for collagen, bringing about a modest but meaningful reduction of the overall enzymatic activity $k_{\text{cat}}/K_m$ (see Fig. 3A). Proton uptake of residue 4 drastically reduces $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ in spite of a moderate increase of collagen binding affinity (as indicated by the decrease of $K_m$, see Fig. 3C).

It appears clear as there are different types of groups regulating the enzymatic behavior of MMP-8, and their role has a more or less general relevance. As a matter of fact, residues 3 and 4 seem the most central ones, because their $pK$ values are always affected by substrate binding, suggesting their involvement in the interaction, and their protonation always influences the rate-limiting step $k_{\text{cat}}$, indicating that they are also involved in the formation of the catalytic intermediate(s) (see Tables I and II). Such a behavior indeed suggests that their location is very close to the active site and/or the primary specificity site(s), and their involvement is occurring for all types of substrates, even though the extent of the effect indeed may depend on the amino acid sequence of the substrate. On the other hand, residue 5, whose protonation in the substrate-bound species is important for the modulation of $k_{\text{cat}}$ in all substrates investigated (see Table II), is usually not involved in the substrate interaction, because its $pK_5$ does not vary (except for the binding of MCA-1) upon the formation of the ES complex (see Table I). Residue 1 appears involved only in the recognition process (in the case of MCA-2 and bovine collagen I), whereas residue 2 comes into play only for the modulation of the enzymatic activity on bovine collagen I (see Tables I and II). This behavior suggests that residue 5 is crucial for the enzymatic activity, such that its protonation essentially abolishes the function of the enzyme, but it does not modulate the activity. Residue 1 appears to lie not in the immediate proximity of the active site, but its modulatory role appears very much

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**Table II**

|                  | $K_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ |
|------------------|----------------|-------|----------------------|
|                  | $s^{-1}$       |      | $M$                  | $s^{-1}$       |
| **MCA-1**        |               |       |                      |                |
| Unprotonated     | $2.46 \pm 0.19 \times 10^2$ | $4.57 \pm 0.56 \times 10^{-5}$ | $5.38 \pm 0.67 \times 10^6$ |
| 1-protonated     | $2.46 \pm 0.19 \times 10^2$ | $4.57 \pm 0.56 \times 10^{-5}$ | $5.38 \pm 0.67 \times 10^6$ |
| 2-protonated     | $2.46 \pm 0.19 \times 10^2$ | $4.57 \pm 0.56 \times 10^{-5}$ | $5.38 \pm 0.67 \times 10^6$ |
| 3-protonated     | $2.50 \pm 0.22 \times 10^{-2}$ | $2.79 \pm 0.35 \times 10^{-5}$ | $8.96 \pm 0.74 \times 10^6$ |
| 4-protonated     | $5.17 \pm 0.35 \times 10^{-2}$ | $3.15 \pm 0.29 \times 10^{-5}$ | $1.64 \pm 0.26 \times 10^7$ |
| 5-protonated     | $1.80 \pm 0.19 \times 10^{-2}$ | $6.22 \pm 0.43 \times 10^{-5}$ | $2.58 \pm 0.35 \times 10^7$ |
| **MCA-2**        |               |       |                      |                |
| Unprotonated     | $1.64 \pm 0.14 \times 10^2$ | $2.08 \pm 0.25 \times 10^{-5}$ | $7.88 \pm 0.67 \times 10^6$ |
| 1-protonated     | $1.64 \pm 0.14 \times 10^2$ | $2.08 \pm 0.25 \times 10^{-5}$ | $7.89 \pm 0.75 \times 10^6$ |
| 2-protonated     | $1.64 \pm 0.14 \times 10^2$ | $2.08 \pm 0.25 \times 10^{-5}$ | $7.89 \pm 0.75 \times 10^6$ |
| 3-protonated     | $2.24 \pm 0.33 \times 10^{-2}$ | $2.65 \pm 0.29 \times 10^{-5}$ | $1.22 \pm 0.14 \times 10^7$ |
| 4-protonated     | $5.62 \pm 0.51 \times 10^{-2}$ | $4.60 \pm 0.36 \times 10^{-5}$ | $1.22 \pm 0.12 \times 10^7$ |
| 5-protonated     | $1.48 \pm 0.11 \times 10^{-2}$ | $4.60 \pm 0.36 \times 10^{-5}$ | $3.22 \pm 0.29 \times 10^6$ |
| **Bovine Collagen I** |           |       |                      |                |
| Unprotonated     | $4.70 \pm 0.45 \times 10^{-1}$ | $9.58 \pm 0.68 \times 10^{-4}$ | $4.91 \pm 0.27 \times 10^9$ |
| 1-protonated     | $4.70 \pm 0.45 \times 10^{-1}$ | $9.58 \pm 0.68 \times 10^{-4}$ | $4.92 \pm 0.33 \times 10^9$ |
| 2-protonated     | $1.50 \pm 0.12 \times 10^{-1}$ | $1.28 \pm 0.13 \times 10^{-4}$ | $1.17 \pm 0.14 \times 10^9$ |
| 3-protonated     | $1.12 \pm 0.13 \times 10^{-3}$ | $1.44 \pm 0.13 \times 10^{-2}$ | $7.81 \pm 0.54 \times 10^9$ |
| 4-protonated     | $1.09 \pm 0.11 \times 10^{-2}$ | $1.02 \pm 0.09 \times 10^{-4}$ | $9.50 \pm 0.69 \times 10^9$ |
| 5-protonated     | $1.09 \pm 0.11 \times 10^{-2}$ | $1.02 \pm 0.09 \times 10^{-4}$ | $9.50 \pm 0.69 \times 10^9$ |
related to the ionic properties of the substrate, because its influence on MCA-2 binding might be connected to the presence of an Arg residue. In the case of residue 2, its behavior suggests that the location is fairly separate from the catalytic site and/or the primary specificity site(s), probably lying outside the inter-action surface between MMP-8 and small synthetic substrates.

The identification of the five residues is a very complex task, but some considerations can be made to identify potential candidates. In the case of residues 3 and 4, their pK_a values suggest that they could be histidyl residues, possibly His162 (involved in the interaction with synthetic inhibitors, see Ref. 27), but other candidate(s) might be one (or more) of the histidyl residue(s) coordinating the catalytic Zn^{2+} atom. Thus, although other groups might be as well responsible for this proton-linked modulation, the protonation state of N_o or N_p of the imidazole could be important in modulating the reactivity of the Zn^{2+} atom. In any case, these two residues, whose pK_a are affected by both small synthetic and large macromolecular substrates (see Tables I and II), appear to undergo a much larger environmental change upon interaction with collagen than with synthetic substrates (as indicated by the much larger ΔpK_a shifts, see Table I). It clearly suggests that the interaction of MMP-8 with bovine collagen I brings about a very large and widespread induced fit conformational change of the enzyme, which drastically differs from that occurring upon binding small synthetic substrates. Therefore, even groups, which are not in the immediate proximity of the catalytic site, may undergo relevant variations of their inter-action network, and thus of their protonation properties. This seems to be the case for residues 1 and 2, even though we have to distinguish their substrate-linked effect. In fact, residue 1 displays only an effect on K_m (and thus indirectly on k_cat/K_m, but not on k_cat, see Table I), which indicates that this additional residue is likely coming into play only as a main element of the interacting surface of MMP-8, being observable only for the interaction with MCA-2 and with bovine collagen I. This residue takes up protons upon substrate binding, being always protonated in the substrate-bound form and likely representing a crucial anchoring charged residue for the substrate molecule. On the other hand, residue 2 shows an effect on both k_cat and K_m but only for bovine collagen I, suggesting that this is a residue that plays a role also in the correct orientation of the substrate for its enzymatic processing. This might mean that either (i) it is a residue far enough from the active site but that exerts a sufficient constraint on the substrate (when it interacts) to become crucial in facilitating the correct orientation for the enzymatic cleavage or (ii) it is a residue that is not influenced by the small conformational change induced by synthetic substrate(s). This being the case, the behavior observed indicates that only the gross structural change following the interaction with bovine collagen I alters its pK_a, pushing it to play a role in modulating the catalytic rate-limiting step. In either case, collagen binding induces a proton release by this substrate (when it interacts) to become crucial in facilitating the catalytic Zn^{2+} atom and whose protonation would lead to the cleavage of the Zn^{2+}-imidazol bond and to the complete unactivation of the enzyme. This being true, it might be possible to envisage the possibility that the most acid portion of the pH dependence of catalytic activity in MMP-8 is characterized by a variation of the coordination state for the catalytic Zn^{2+} atom.

In conclusion, it appears clear as MMP-8 displays a wide range of interactions with substrates, this being especially true for large macromolecular substrates, which involve both residues in the close proximity to the active site, but also residues quite far apart from the catalytic cleft. This behavior, which is not unexpected and was somehow already predicted on the basis of structural modeling (19), has been characterized here for the first step of the enzymatic processing of bovine collagen I (leading to the fragmentation into 1⁄4 and 3⁄4 species, see Ref. 4), which is closely similar to the natural substrate of MMP-8. It envisages a very complex interplay among different portions of the enzyme molecule in the substrate recognition and/or processing, as also suggested by the processing of collagen by MMP-1 (17). Therefore, such an investigation indeed may represent a first basis for the comprehension of molecules, which efficiently interfere at different levels with the cleavage of physiological substrates by MMP-8 as well as by other collagenses.

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Cleavage of Bovine Collagen I by Neutrophil Collagenase MMP-8: EFFECT OF pH ON THE CATALYTIC PROPERTIES AS COMPARED TO SYNTHETIC SUBSTRATES

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