pH- and Temperature-Dependence of Functional Modulation in Metalloproteinases. A Comparison between Neutrophil Collagenase and Gelatinases A and B

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ABSTRACT Metalloproteases are metalloenzymes secreted in the extracellular fluid and involved in inflammatory pathologies or events, such as extracellular degradation. A Zn^{2+} metal, present in the active site, is involved in the catalytic mechanism, and it is generally coordinated with histidyl and/or cysteinyl residues of the protein moiety. In this study we have investigated the effect of both pH (between pH 4.8 and 9.0) and temperature (between 15°C and 37°C) on the enzymatic functional properties of the neutrophil interstitial collagenase (MMP-8), gelatinases A (MMP-2) and B (MMP-9), using the same synthetic substrate, namely MCA-Pro-Leu-Gly≈Leu-DPA-Ala-Arg-NH_2. A global analysis of the observed proton-linked behavior for k_{cat}/K_m, k_{cat}, and K_m indicates that in order to have a fully consistent description of the enzymatic action of these metalloproteases we have to imply at least three protonating groups, with differing features for the three enzymes investigated, which are involved in the modulation of substrate interaction and catalysis by the enzyme. This is the first investigation of this type on recombinant collagenases and gelatinases of human origin. The functional behavior, although qualitatively similar, displays significant differences with respect to what was previously observed for stromelysin and porcine collagenase and gelatinase (Stack, M. S., and R. D. Gray. 1990. *Arch. Biochem. Biophys.* 281:257–263; Harrison, R. K., B. Chang, L. Niedzwiecki, and R. L. Stein. 1992. *Biochemistry.* 31:10757–10762). The functional characterization of these enzymes can have some relevant physiological significance, since it may be related to the marked changes in the environmental pH that collagenase and gelatinases may experience in vivo, moving from the intracellular environment to the extracellular matrix.

INTRODUCTION

Degradation of extracellular proteins by PMN-derived proteinases is a fundamental aspect of normal PMN processes, such as dyapedesis, penetration of tissue barriers, tissue remodeling, and removal of tissue debris and extracellular matrix (Birkedal-Hansen et al., 1993; Blundell, 1994). PMNs contain different proteases and some of these enzymes, namely 92 kDa gelatinase (also called MMP-9) and neutrophil collagenase (also called MMP-8), are zinc- and calcium-dependent endopeptidases (Dioszegi et al., 1995). They are stored in PMN as a proenzymes (Baramova and Foidart, 1995), playing an important role in matrix degradation and in tissue remodeling processes (such as growth and development, ovulation, and wound healing), as well as in some pathological processes, such as tumor invasion, osteoarthritis, periodontitis, and multiple sclerosis (Maeda and Sobel, 1996; Rosenberg et al., 1996; Cossins et al., 1997; Vu et al., 1998). In addition, a 72-kDa gelatinase/type IV collagenase (also called MMP-2) has been identified in several normal and malignant cells (Aimes et al., 1994). Cooperation between interstitial collagenases and gelatinases is thought to be essential during inflammatory and invasive processes, also because MMP-2 seems to have an action intermediate between typical interstitial collagenases and a gelatinase such as MMP-9 (Aimes and Quigley, 1995).

Several papers have dealt with substrate specificities of collagenases and gelatinases (Weingarten et al., 1985; Netzel-Arnett et al., 1993; Niyibizi et al., 1994; Welch et al., 1996) (as well as of other metalloproteinases, such as stromelysin MMP-3), but to date no systematic investigation has been carried out to compare the catalytic properties of human collagenases and gelatinases. Thus, despite meaningful functional differences among them both for synthetic (Nagase and Fields, 1996) and natural substrates (Niyibizi et al., 1994; Tschesche, 1995), the amino acid sequence of the catalytic domain of these metalloproteinases is closely similar (Massova et al., 1997), and the same is true for the main structural aspects (Grams et al., 1995; Morgunova et al., 1999). The only investigation of the pH- and temperature dependence of enzymatic properties was carried out several years ago on porcine collagenases and gelatinases (Stack and Gray, 1989, 1990) and on stromelysin MMP-3 (Izquierdo-Martin and Stein, 1992; Harrison et al., 1992; Stein and Izquierdo-Martin, 1994; Holman et al., 1999). Therefore, a functional comparison between human collagenases and gelatinases may be very important to better characterize the charge distribution of residues involved in substrate recognition and processing of the two classes of enzymes, and to correlate this

Received for publication 16 December 1999 and in final form 15 June 2000.

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Abbreviations used: PMN, polymorphonuclear neutrophils; APMA, *p*-aminophenyl mercuric acetate; CHA, cyclohexylalanine; DPA, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; MCA, (7-methoxycoumarin-4-yl)acetyl; NVA, *nor*-valine; PMSF, phenylmethyl sulfonyl fluoride.

0006-3495/00/10/2138/12 $2.00

information to the different enzymatic action (Massova et al., 1998).

In this work we have investigated the functional behavior of the neutrophil collagenase (MMP-8) and of gelatinase A (also called MMP-2), whose x-ray structure has been solved (Bode et al., 1994; Grams et al., 1995; Morgunova et al., 1999; Dhanaraj et al., 1999). Furthermore, we have also investigated the catalytic properties of gelatinase B (also called MMP-9), whose amino acid sequence is known, but no crystal structure is available up to now, and only molecular modeling has been undertaken (Massova et al., 1997). This study has been focused on the modulation by pH of the catalytic parameters for one substrate (MCA-Pro-Leu-Gly≈Leu-DPA-Ala-Arg-NH2), which was found particularly suited for MMP-2 (Knight et al., 1992; Nagase and Fields, 1996), aiming to begin a clarification of the different mechanism operating in these enzymes as far as substrate recognition and processing are concerned. In addition, the temperature-dependence of catalytic parameters has been measured in order to have some information concerning the energetics associated to the overall enzymatic mechanism.

This aspect of their functional modulation is physiologically relevant because after extrusion from intracellular granules, MMPs indeed may experience large variations in environmental conditions, such as pH and ionic strength. Thus, previous observations on stromelysin MMP-3 (Izquierdo-Martin and Stein, 1992; Harrison et al., 1992; Stein and Izquierdo-Martin, 1994) and porcine synovial collagenases and gelatinases (Stack and Gray, 1989, 1990) showed a complex linkage between catalytic activity and proton uptake and release, allowing the unraveling of the role of specific residues through the use of site-directed mutagenesis (Cha and Auld, 1997).

These MMPs have been cloned from human genes, and they are usually involved in the defense and inflammation mechanism (Hirose et al., 1992; Walakovits et al., 1992), but also in tumor invasion (Heppner et al., 1996; Vu et al., 1998; Fang et al., 2000). Therefore, it seemed relevant to study in more detail the effect of pH on some catalytic parameters of these enzymes, aiming to obtain some differential information. In this way, it may become feasible to initiate the design of more specific drugs, which specifically interact with either one of these enzymes, resulting in a more efficient pharmaceutical effect and avoiding dangerous collateral effects.

MATERIALS AND METHODS

Methods

Recombinant purified matrix metalloproteinases (i.e., MMP-2, MMP-8, and MMP-9) were kindly obtained from Dr. G. Murphy (Strangeways Research Lab., Cambridge, UK). Purity of MMPs was measured by SDS-PAGE according to Laemmli (1970). After gels were run, they were stained using a silver staining kit (Biorad, Hercules, CA). Zymography was performed as follows: 2 μl purified MMPs were mixed with a fivefold excess of sample buffer (0.25 M Tris, 0.8% SDS, 10% glycerol, and 0.05% bromophenol blue) and run on 12% SDS-polyacrylamide gels (SDS-PAGE) containing either 1 mg/ml of gelatine or collagen type I, as previously described (Fisher et al., 1994). 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 buffer pH 7.6, 0.15 M NaCl, 10 mM $CaCl_2$, 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. Proteinase bands were further characterized by adding 20 mM EDTA or 0.3 mM 1,10-phenanthroline (MMP inhibitors), or 1 mM PMSF (serine proteinase inhibitor) in the incubation buffer. Protein markers (Sigma, St. Louis, MO) were used as molecular weight standard.

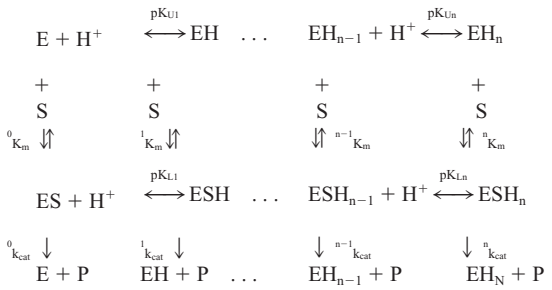
Recombinant purified MMP-8 and MMP-9 proenzymes were activated by incubating 100 μl of a 0.1 μg/ml procollagenase solution with APMA (Sigma) at 37°C for 2 h; this treatment shifts the equilibrium of the conformations toward the open, autocatalytically activated form, involving the cleavage of the region between residues 71 and 81 for MMP-8. MMP-2 was activated as described above at 25°C for 1 h. Because BB-94 (known also as Batimastat, a peptidomimetic MMP inhibitor, kindly provided by British Biotech Pharmaceutical, Cowley, Oxford, UK) fully inhibits stoichiometrically MMPs, we used it to titrate the active amount of enzyme(s).

Enzymatic assay

Enzyme activity was measured using one fluorogenic substrate, namely MCA-Pro-Leu-Gly≈Leu-DPA-Ala-Arg-NH_2 (a gift of Dr. G. Knight, Strangeways). Experiments were carried out at a final 0.01 nM concentration of purified activated MMPs at 37°C in Tris/HCl 50 mM, NaCl 0.1M, $CaCl_2$ 10 mM plus Brij 35 0.05% buffered at different pH values (between 9.0 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 M NaCl, 10 mM $CaCl_2$ and no difference for enzymatic activities between two buffer systems (i.e., MES and Tris/HCl) was observed. The substrate was diluted in DMSO and preliminary experiments demonstrated that the addition of DMSO to the incubation mixture does not affect MMP activity. Assays were carried out by continuously monitoring the increase in fluorescence at 393 nm after excitation at 328 nm, using a Jobin-Yvon spectrofluorimeter (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 $<10\%$ of the substrate was degraded during the assay, and data were normalized and expressed as nanomoles of cleaved substrate/s. Assays were made with substrate concentrations between 5×10^{-7} and 10^{-4} M, i.e., spanning the range of K_m values, and no absorptive quenching effect has been detected.

Data analysis

Values of observed k_{cat}/K_m, k_{cat}, and K_m for MMP-2, MMP-8, and MMP-9 at any given pH and their pH-dependence over the range investigated (i.e., between 4.8 and 9.0) were calculated simultaneously through a global analysis of the whole data set, using two formalisms (i.e., linear Lineweaver-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}, and K_m) must be closely related according to the following general scheme



(Scheme I)

where ES (as well as ESH_x, with $x = 1, 2, \ldots n$) simply refers to the species undergoing the rate-limiting step and ${}^{x}K_m$ (with $x = 0, 1, \ldots n$) refers to all preequilibrium events leading to the species that undertakes the rate-limiting step.

In more detail, the fitting procedure forced the system to be described by n protonation states with n values of k_{cat} and n values of K_m, which must combine then to give n corresponding values of k_{cat}/K_m. Therefore, the fitting of the pH-dependence is internally constrained to obey Scheme I with n values of pK_U and pK_L, which are the same for all three catalytic parameters.

Catalytic parameters were also obtained at different temperatures between 15 and 37°C, and they were analyzed according to the following equation

$$\partial \ln(\text{Par1})/\partial(1/T) = (\text{Par2})/R \qquad (1)$$

where Par1 ($= k_{cat}/K_m$, k_{cat}, or K_m) is a catalytic parameter, and Par2 is E_a (in the case of k_{cat}/K_m and k_{cat}) or ΔH (in the case of K_m).

RESULTS AND DISCUSSION

Fig. 1 shows the pH-dependence of the catalytic parameters (i.e., k_{cat}/K_m, k_{cat}, and K_m) at 37°C for the recombinant wild type forms of human neutrophil collagenase (MMP-8), gelatinase A (MMP-2) and B (MMP-9) with a common substrate, namely MCA-Pro-Leu-Gly≈Leu-DPA-Ala-Arg-NH_2. It is immediately obvious that, unlike what observed for porcine synovial collagenase and gelatinase (Stack and Gray, 1989, 1990), the proton-linked behavior is markedly different among the three metalloproteases investigated, even though at least two protons appear to be involved in the pH-dependent modulation of catalytic parameters for all three MMPs investigated (Fig. 1), as for other MMPs (Stack and Gray, 1990; Harrison et al., 1992; Cha and Auld, 1997; Holman et al., 1999).

Unfortunately, the pH-dependent stability of the three enzymes is quite different, impairing the possibility of extending the investigation over the same pH range, this being particularly evident for pH < 6.0. Thus, while in the case of MMP-8 it has been possible to carry out experiments down to pH 4.8, for gelatinases we were unable to run experiments below pH 6.0 for MMP-9 and below pH 6.4 for MMP-2, limiting to some extent the accuracy of our investigation on these enzymes. Therefore, even though the fitting of data has been obtained only over the pH range of enzyme stability (see below), theoretical curves corresponding to parameters reported in Tables 1–3 are reported for the whole pH range (i.e., 4.5–9.5, see Figs. 1 and 2), but they are dashed outside the limit of the enzyme stability. In addition, at pH > 10 we observe a rapid irreversible decrease of the enzymatic activity in all three metalloproteinases; therefore we decided to ignore this event, which is likely related to some alkaline denaturation of enzymes, and to analyze only data at pH ≤ 9.0.

The most straightforward analysis can be carried out on k_{cat}/K_m, a parameter immediately obtainable from experimental data, being referable to the slope of the steady-state velocity as a function of substrate concentration. The observation of the pH-dependence of k_{cat}/K_m for the three metalloproteinases investigated is reported in Fig. 1 *A*, and it shows that MMP-8 has the fastest overall catalytic rate at all pH values. Furthermore, the effect of pH on this parameter seems rather small both in MMP-2 and MMP-8, at least over the pH 6.0–9.0 range, with a similar proton-linked rate enhancement from pH 9.0 to pH 7.0, while MMP-9 displays over the same range a marked pH-dependence of the same parameter with a bell-shaped behavior (Fig. 1 *A*). A broad bell-shaped pH-dependence of k_{cat}/K_m, as displayed by MMP-2 and MMP-8 (Fig. 1 *A*), is also observed in porcine synovial collagenase and gelatinase (Stack and Gray, 1990) and in matrilysin MMP-7 (Cha and Auld, 1997), whereas a narrow pH-dependent range, such as that shown by MMP-9 (Fig. 1 *A*), has never been observed over the same pH range, stromelysin MMP-3 displaying a similar behavior but at a much lower pH (Harrison et al., 1992; Holman et al., 1999).

The shape of the pH-dependence displayed in Fig. 1 *A* indicates that we have to imply the occurrence of at least two groups whose protonation modulates the proton-linked behavior of k_{cat}/K_m in all three metalloproteinases investigated, thus using $n = 2$ in Scheme I. Solid lines in Fig. 1 *A* correspond to the nonlinear least-squares fitting of data according to the following formalism

$$^{obs}(k_{cat}/K_m) = {}^{0}(k_{cat}/K_m)/P + {}^{1}(k_{cat}/K_m) \cdot K_1[H^+]/P + {}^{2}(k_{cat}/K_m) \cdot K_1K_2[H^+]^2/P \qquad (2a)$$

where ${}^{obs}k_{cat}/K_m$ is the observed parameter at each pH value; ${}^{0}k_{cat}/K_m$, ${}^{1}k_{cat}/K_m$, and ${}^{2}k_{cat}/K_m$ are the parameters in the unprotonated, single-protonated, and double-protonated forms, respectively (see also Scheme I); K_1 and K_2 are the proton-binding association constants for the two protonating groups; and P is the proton binding polynomial.

$$P = 1 + K_1[H^+] + K_1K_2[H^+]^2 \qquad (2b)$$

Indeed, the quality of fitting seems to indicate that $n = 2$ might be appropriate for the description of the system. However, if we limit ourselves to the evaluation of the proton-linked behavior of k_{cat}/K_m we cannot get enough information for the applicability of Scheme I, since both values of k_{cat} and K_m for any protonation level in Scheme I must be such as to give the resulting k_{cat}/K_m. Therefore, the investigation has been extended to the pH-dependence of

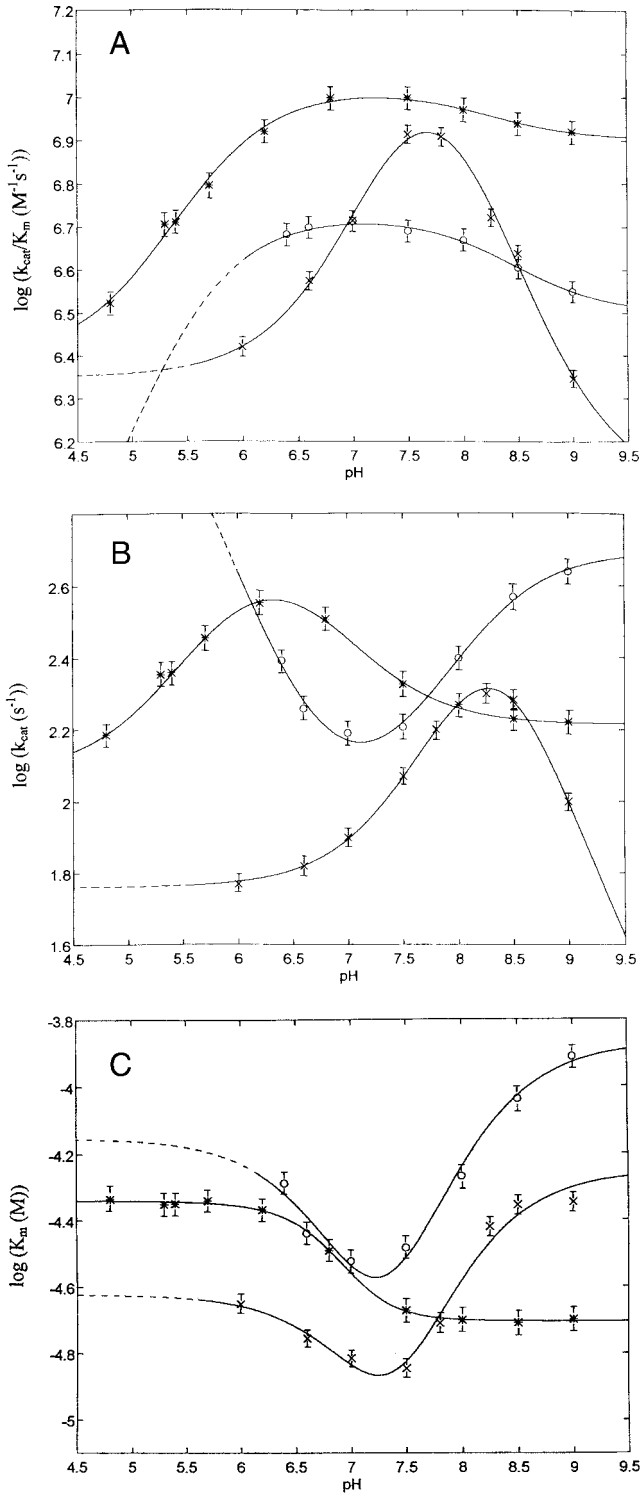


FIGURE 1 pH-dependence of k_{cat}/K_m (*A*), k_{cat} (*B*), and K_m (*C*) for neutrophil collagenase MMP-8 (∗), gelatinase A MMP-2 (○), and gelatinase B MMP-9 (×) at 37°C. Theoretical fitted curves are reported as solid lines within the range of pH values where experimental data have been obtained. However, they are reported as dashed lines over the range of pH values where the lack of experimental data renders theoretical curves less reliable. (*A*) Theoretical lines have been obtained by nonlinear least-squares fitting of experimental data using Eq. 2a. The following parameters have been obtained for MMP-2 (○): ${}^0k_{cat}/K_m = 3.17\ (\pm 0.42)$ E6 $M^{-1}\,s^{-1}$, ${}^1k_{cat}/K_m = 5.31\ (\pm 0.63)$ E6 $M^{-1}\,s^{-1}$, ${}^2k_{cat}/K_m = 5.00\ (\pm 1.21)$ E5 $M^{-1}\,s^{-1}$, $K_1 = 2.16\ (\pm 0.95)$ E8 M^{-1}, $K_2 = 3.07\ (\pm 2.79)$ E5 M^{-1}; for MMP-9 (×): ${}^0k_{cat}/K_m = 1.22\ (\pm 0.15)$ E6 $M^{-1}\,s^{-1}$, ${}^1k_{cat}/K_m = 1.93\ (\pm 0.23)$ E7 $M^{-1}\,s^{-1}$, ${}^2k_{cat}/K_m = 2.25\ (\pm 0.31)$ E6 $M^{-1}\,s^{-1}$, $K_1 = 6.07\ (\pm 2.42)$ E7 M^{-1}, $K_2 = 4.27\ (\pm 1.48)$ E7 M^{-1}; for MMP-8 (∗): ${}^0k_{cat}/K_m = 8.01\ (\pm 0.72)$ E6 $M^{-1}\,s^{-1}$, ${}^1k_{cat}/K_m = 1.04\ (\pm 0.09)$ E7 $M^{-1}\,s^{-1}$, ${}^2k_{cat}/K_m = 2.51\ (\pm 0.21)$ E6 $M^{-1}\,s^{-1}$, $K_1 = 1.37\ (\pm 0.55)$ E8 M^{-1}, $K_2 = 5.00\ (\pm 1.98)$ E5 M^{-1}. (*B*) Theoretical lines have been obtained by nonlinear least-squares fitting of experimental data using Eq. 2c. The following parameters have been obtained for MMP-2 (○): ${}^0k_{cat} = 4.96\ (\pm 0.31)$ E2 s^{-1}, ${}^1k_{cat} = 8.69\ (\pm 0.59)$ E1 s^{-1}, ${}^2k_{cat} = 2.77\ (\pm 0.22)$ E3 s^{-1}, $K_1 = 1.55\ (\pm 0.48)$ E8 M^{-1}, $K_2 = 1.49\ (\pm 1.31)$ E5 M^{-1}; for MMP-9 (×): ${}^0k_{cat} = 1.02\ (\pm 0.12)$ E1 s^{-1}, ${}^1k_{cat} = 6.30\ (\pm 0.53)$ E2 s^{-1}, ${}^2k_{cat} = 5.77\ (\pm 0.49)$ E1 s^{-1}, $K_1 = 1.71\ (\pm 0.68)$ E8 M^{-1}, $K_2 = 2.63\ (\pm 0.97)$ E8 M^{-1}; for MMP-8 (∗): ${}^0k_{cat} = 1.64\ (\pm 0.12)$ E2 s^{-1}, ${}^1k_{cat} = 5.26\ (\pm 0.43)$ E2 s^{-1}, ${}^2k_{cat} = 1.20\ (\pm 0.11)$ E2 s^{-1}, $K_1 = 5.25\ (\pm 2.03)$ E6 M^{-1}, $K_2 = 7.04\ (\pm 3.77)$ E5 M^{-1}. (*C*) Theoretical lines have been obtained by nonlinear least-squares fitting of experimental data using Eq. 2d. The following parameters have been obtained for MMP-2 (○): ${}^0K_m = 1.34\ (\pm 0.22)$ E-4 M, $K_{U1} = 3.71\ (\pm 2.51)$ E4 M^{-1}, $K_{U2} = 9.77\ (\pm 5.26)$ E9 M^{-1}, $K_{L1} = 1.35\ (\pm 0.82)$ E8 M^{-1}, $K_{L2} = 5.13\ (\pm 3.83)$ E6 M^{-1}; for MMP-9 (×): ${}^0K_m = 5.55\ (\pm 0.62)$ E-5 M, $K_{U1} = 4.17\ (\pm 2.35)$ E4 M^{-1}, $K_{U2} = 1.32\ (\pm 0.97)$ E10 M^{-1}, $K_{L1} = 1.10\ (\pm 3.76)$ E8 M^{-1}, $K_{L2} = 1.17\ (\pm 0.56)$ E7 M^{-1}; for MMP-8 (∗): ${}^0K_m = 1.98\ (\pm 0.17)$ E-5 M, $K_{U1} = 3.52\ (\pm 1.66)$ E6 M^{-1}, $K_{U2} = 3.43\ (\pm 1.39)$ E7 M^{-1}, $K_{L1} = 3.11\ (\pm 1.47)$ E6 M^{-1}, $K_{L2} = 1.68\ (\pm 0.73)$ E7 M^{-1}, applying Eq. 2c. For further details, see text.

k_{cat} (Fig. 1 *B*), another parameter that can be directly obtained from experimental data by the extrapolation of the steady-state velocity to very large substrate concentrations, and also in this case we require at least two protonating groups that modulate the proton-linked behavior of k_{cat} according to the following formalism

$$ {}^{obs}k_{cat} = {}^0k_{cat}/P + {}^1k_{cat} \cdot K_1[H^+]/P + {}^2k_{cat} \cdot K_1K_2[H^+]^2/P \quad (2c)$$

where ${}^{obs}k_{cat}$ is the observed parameter at each pH value, ${}^0k_{cat}$, ${}^1k_{cat}$, and ${}^2k_{cat}$ are the parameter in the unprotonated, single-protonated, and double-protonated forms, respectively (see also Scheme I); K_1, K_2, and P have the same meaning as applied above. The quality of fitting obtained for the pH-dependence of k_{cat} (*solid lines* in Fig. 1 *B*) indeed seems to suggest that also in this case two protons may be sufficient for a satisfactory description of the system. Like k_{cat}/K_m, the pH-dependence of k_{cat} shows a significant difference among the three enzymes (see Fig. 1 *B*), which display three distinct pH ranges over which the proton-linked modulation is operative, namely between pH 9.0 and 7.0 for MMP-9, between pH 8.0 and 5.0 for MMP-8, and between pH 9.0 and 6.0 for MMP-2 (Fig. 1 *B*). These data clearly indicate that the three MMPs differ for pK_a values of the groups involved in the modulation of the rate-limiting step of the enzymatic action. However, it is very important to outline that pK_a values, which have been obtained from the pH-dependence of k_{cat}/K_m (Fig. 1 *A*) and of k_{cat} (Fig. 1 *B*) and are reported in Table 1, are clearly different each other, at least in the case of MMP-8 and MMP-9, suggesting

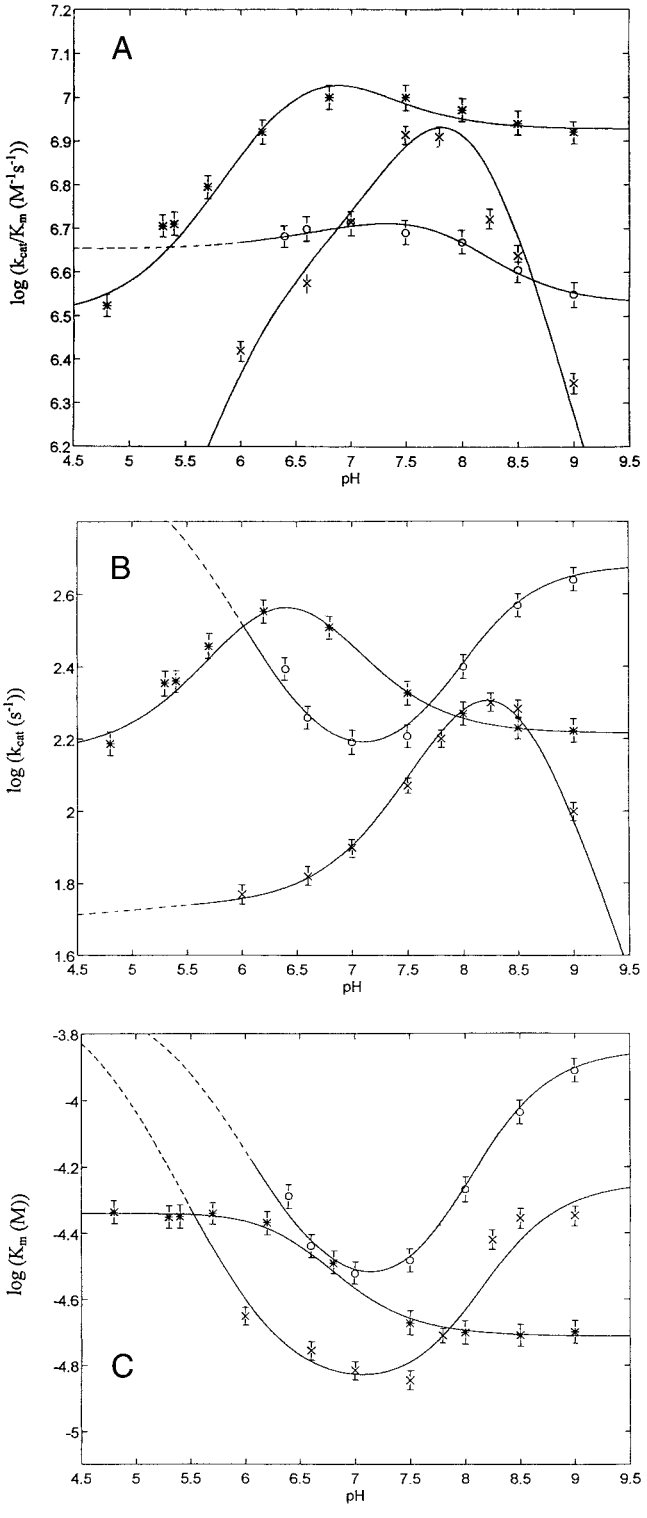


FIGURE 2 pH-dependence of k_{cat}/K_m (*A*), k_{cat} (*B*), and K_m (*C*) for neutrophil collagenase MMP-8 (∗), gelatinase A MMP-2 (○), and gelatinase B MMP-9 (×) at 37°C. Theoretical fitted curves are reported as solid lines within the range of pH values where experimental data have been obtained. However, they are reported as dashed lines over the range of pH values where the lack of experimental data renders theoretical curves less reliable. Theoretical lines have been obtained by nonlinear least-squares global fitting of experimental data using Eqs. 3a–d. The following parameters have been obtained for MMP-2 (○): ${}^0k_{cat}/K_m$ = 3.36 (±0.57) E6 $M^{-1}s^{-1}$, ${}^1k_{cat}/K_m$ = 5.74 (±0.63) E6 $M^{-1}s^{-1}$, ${}^2k_{cat}/K_m$ = 5.16 (±0.71) E6 $M^{-1}s^{-1}$, ${}^3k_{cat}/K_m$ = 4.50 (±0.67) E6 $M^{-1}s^{-1}$ (*A*), ${}^0k_{cat}$ = 4.81 (±0.31) E2 s^{-1}, ${}^1k_{cat}$ = 3.49 (±0.19) E2 s^{-1}, ${}^2k_{cat}$ = 1.14 (±0.12) E2 s^{-1}, ${}^3k_{cat}$ = 9.53 (±0.87) E2 s^{-1} (*B*), ${}^0K_m$ = 1.43 (±0.26) E-4 M (*C*), K_{U1} = 9.82 (±1.17) E7 M^{-1}, K_{U2} = 4.89 (±1.04) E7 M^{-1}, K_{U3} = 3.33 (±0.89) E6 M^{-1}, K_{L1} = 2.29 (±0.78) E8 M^{-1}, K_{L2} = 1.35 (±0.81) E8 M^{-1}, K_{L3} = 3.52 (±1.13) E5 M^{-1}. The following parameters have been obtained for MMP-9 (×): ${}^0k_{cat}/K_m$ = 1.75 (±0.17) E4 $M^{-1}s^{-1}$, ${}^1k_{cat}/K_m$ = 1.84 (±0.21) E7 $M^{-1}s^{-1}$, ${}^2k_{cat}/K_m$ = 4.42 (±0.37) E6 $M^{-1}s^{-1}$, ${}^3k_{cat}/K_m$ = 2.41 (±0.22) E5 $M^{-1}s^{-1}$ (*A*), ${}^0k_{cat}$ = 1.15 (±0.41) s^{-1}, ${}^1k_{cat}$ = 4.70 (±0.49) E2 s^{-1}, ${}^2k_{cat}$ = 5.53 (±0.52) E1 s^{-1}, ${}^3k_{cat}$ = 5.04 (±0.44) E1 s^{-1} (*B*), ${}^0K_m$ = 5.71 (±0.42) E-5 M (*C*), K_{U1} = 1.12 (±0.79) E8 M^{-1}, K_{U2} = 7.48 (±1.18) E7 M^{-1}, K_{U3} = 1.13 (±0.93) E6 M^{-1}, K_{L1} = 2.52 (±1.04) E8 M^{-1}, K_{L2} = 1.47 (±1.12) E8 M^{-1}, K_{L3} = 6.57 (±1.21) E4 M^{-1}. The following parameters have been obtained for MMP-8 (∗): ${}^0k_{cat}/K_m$ = 8.45 (±0.75) E6 $M^{-1}s^{-1}$, ${}^1k_{cat}/K_m$ = 1.22 (±0.11) E7 $M^{-1}s^{-1}$, ${}^2k_{cat}/K_m$ = 1.12 (±0.13) E7 $M^{-1}s^{-1}$, ${}^3k_{cat}/K_m$ = 3.12 (±0.27) E6 $M^{-1}s^{-1}$ (*A*), ${}^0k_{cat}$ = 1.64 (±0.14) E2 s^{-1}, ${}^1k_{cat}$ = 3.42 (±0.34) E2 s^{-1}, ${}^2k_{cat}$ = 5.22 (±0.49) E2 s^{-1}, ${}^3k_{cat}$ = 1.45 (±0.09) E2 s^{-1} (*B*), ${}^0K_m$ = 1.94 (±0.22) E-5 M (*C*), K_{U1} = 1.41 (±1.17) E7 M^{-1}, K_{U2} = 5.62 (±1.09) E6 M^{-1}, K_{U3} = 1.12 (±1.32) E6 M^{-1}, K_{L1} = 9.78 (±1.16) E6 M^{-1}, K_{L2} = 3.43 (±0.98) E6 M^{-1}, K_{L3} = 1.15 (±1.36) E6 M^{-1}. For further details, see text.

TABLE 1 pK_a values for the pH-dependence of catalytic parameters in MMP-2, MMP-8, and MMP-9, at 37°C

	MMP-2	MMP-8	MMP-9
k_{cat}/K_m			
pK_{a1}	8.33 ± 0.16	8.14 ± 0.15	7.78 ± 0.15
pK_{a2}	5.49 ± 0.28	5.70 ± 0.14	7.63 ± 0.13
$k_{cat}t$			
pK_{a1}	8.19 ± 0.12	6.72 ± 0.14	8.23 ± 0.17
pK_{a2}	5.17 ± 0.28	5.85 ± 0.19	8.42 ± 0.14
K_m			
pK_{U1}	4.57 ± 0.25	6.55 ± 0.16	4.62 ± 0.17
pK_{U2}	9.99 ± 0.20	7.53 ± 0.15	10.12 ± 0.17
pK_{L1}	8.13 ± 0.23	6.49 ± 0.17	8.04 ± 0.13
pK_{L2}	6.71 ± 0.22	7.22 ± 0.16	7.07 ± 0.15

that the applicability of Scheme I with two protonating groups (i.e., $n = 2$) might be insufficient for an overall description of the system.

In order to have more insight into the actual mechanism of proton modulation for catalytic parameters in the three MMPs, the analysis has been then further extended to the pH-dependence of K_m (Fig. 1 *C*), even though in this case the interpretation of the proton-linked behavior is less straightforward, since this parameter is determined indirectly from experimental data. The pH-dependence of K_m has been carried out using the following formalism

$$ {}^{obs}K_m = \frac{{}^0K_m \cdot (1 + K_{U1}[H^+] + K_{U1}K_{U2}[H^+]^2)}{(1 + K_{L1}[H^+] + K_{L1}K_{L2}[H^+]^2)} \quad (2d) $$

where ${}^{obs}K_m$ is the observed parameter at each pH value and ${}^0K_m$ is the Michaelis-Menten equilibrium constant in the unprotonated form; K_U and K_L are the proton binding association constants to the free enzyme and to the enzyme:

substrate complex, respectively. In the case of K_m, it is interesting to observe that while both gelatinases display a bell-shaped pH-dependence characterized by a peak value of substrate affinity over the pH 7.0–7.5 range, collagenase MMP-8 shows a single pH-dependent transition (Fig. 1 *C*). However, pK_a values obtained by the analysis of the pH-dependence of K_m (Table 1) appear even more unconciliable with those resulting from the analysis of the other two parameters. Furthermore, values of K_m at any protonation level are incompatible with k_{cat} to give a correct value of k_{cat}/K_m, which is instead demanded by the applicability of Scheme I.

Therefore, an analysis of data for k_{cat}/K_m, k_{cat}, and K_m using two protonating groups, although able to satisfactorily describe the pH-dependence of the separate three catalytic parameters (*solid curves* in Fig. 1, *A–C*) for all three metalloproteinases investigated, is absolutely unsatisfactory when we try to insert these values in an overall consistent picture of the system. It is worth noticing that this deeper information can be obtained only by analyzing the three catalytic parameters altogether.

We have therefore carried out a global fitting analysis of data for each one of the three metalloproteinases investigated, constraining the outcomes to give a single set of pK_{Ur} and pK_{Lr} values, as well as of ${}^{r}k_{cat}/K_m$, ${}^{r}k_{cat}$, and ${}^{r}K_m$, ($r = 0, 1, \ldots, n$, see Scheme I), so as to satisfactorily describe the pH-dependence of all three catalytic parameters simultaneously for a given MMP. Solid lines in Fig. 2, *A–C* display the result of such an approach with three protonating groups (i.e., $n = 3$, see Scheme I) according to the following formalism

$$ {}^{obs}(k_{cat}/K_m) = {}^{0}(k_{cat}/K_m)/P + {}^{1}(k_{cat}/K_m)\cdot K_1[H^+]/P + {}^{2}(k_{cat}/K_m)\cdot K_1K_2[H^+]^2/P + {}^{3}(k_{cat}/K_m)\cdot K_1K_2K_3[H^+]^3/P \quad (3a) $$

$$ {}^{obs}k_{cat} = {}^{0}k_{cat}/P_2 + {}^{1}k_{cat}\cdot K_{L1}[H^+]/P + {}^{2}k_{cat}\cdot K_{L1}K_{L2}[H^+]^2/P + {}^{3}k_{cat}\cdot K_{L1}K_{L2}K_{L3}[H^+]^3/P \quad (3b) $$

$$ {}^{obs}K_m = \frac{{}^{0}K_m\cdot(1 + K_{U1}[H^+] + K_{U1}K_{U2}[H^+]^2 + K_{U1}K_{U2}K_{U3}[H^+]^3)}{(1 + K_{L1}[H^+] + K_{L1}K_{L2}[H^+]^2 + K_{L1}K_{L2}K_{L3}[H^+]^3} \quad (3c) $$

where ^{obs}par (par = k_{cat}/K_m or k_{cat} or K_m) is the observed parameter at each pH value, ^{0}par, ^{1}par, ^{2}par, and ^{3}par is the parameter in the unprotonated, single-protonated, double-protonated, and triple-protonated forms, respectively (see also Scheme I); and K_U and K_L are the proton binding association constants to the free enzyme and to the enzyme: substrate complex, respectively. We have also imposed that pK_a values regulating k_{cat} must correspond to pK_L values (which was impossible in the case of $n = 2$), and that pK_a values influencing k_{cat}/K_m can only be either pK_U or pK_L (and in Eq. 3a are simply reported as pK_r); P is the proton-binding polynomial

$$ P = 1 + K_1[H^+] + K_1K_2[H^+]^2 + K_1K_2K_3[H^+]^3 \quad (3d) $$

where above considerations apply (i.e., where K_rs are K_Ls for k_{cat} and they can be either K_Us or K_Ls for k_{cat}/K_m). Solid curves in Fig. 2, *A–C* correspond to the global fitting analysis according to the above-mentioned constraints, where a better description of the pH-dependence of k_{cat}/K_m has been obtained using pK_Us obtained from the proton-linked behavior of K_m (Table 2).

The overall behavior observed in all three MMPs investigated is qualitatively similar to that reported for stromelysin MMP-3 (Harrison et al., 1992) and it has been sketched in Fig. 3, where two groups (i.e., *A* and *B*) are shown to affect the protonation properties of the Zn^{2+}-bound H_2O, even though they cannot be definitely identified. Furthermore, although we have no direct proof that the observed pH dependence of the catalytic parameters is not affected by variations of the rate-limiting step (thus rendering the measured pK_a as apparent values), we can consider this possibility as a remote one in view of previous investigations on different systems (Stack and Gray, 1990; Harrison et al., 1992) and of the fact that at pH 7.0 and 9.0 the temperature-dependence shows linear Arrhenius plots (see below). As a matter of fact, in other serine proteases the change in the rate-limiting step has been shown to occur at extreme pH values (i.e., $\leq$4.5, see Antonini and Ascenzi, 1981). Furthermore, the full applicability of Scheme I for three protonating groups represents a strong indication that these pK_a values are real ones, even though residues to which they refer may indeed come into play at different steps along the catalytic pathway. Therefore, even though we are aware that pK_a values reported in Table 2 may not all be intrinsic parameters, we deem it important to analyze them with a reasonable confidence that in the range between pH 5 and 9 they have a physical meaning.

TABLE 2 pK_a values from global analysis of the pH dependence of catalytic parameters in MMP-2, MMP-8, and MMP-9 at 37°C

	pK_U	pK_L
MMP-2		
pK_1	7.99 ± 0.09	8.36 ± 0.12
pK_2	7.69 ± 0.08	8.13 ± 0.11
pK_3	6.52 ± 0.08	5.54 ± 0.09
MMP-9		
pK_1	8.05 ± 0.08	8.40 ± 0.09
pK_2	7.87 ± 0.07	8.18 ± 0.08
pK_3	6.04 ± 0.09	4.82 ± 0.08
MMP-8		
pK_1	7.15 ± 0.08	6.99 ± 0.09
pK_2	6.75 ± 0.07	6.53 ± 0.08
pK_3	6.05 ± 0.09	6.05 ± 0.07

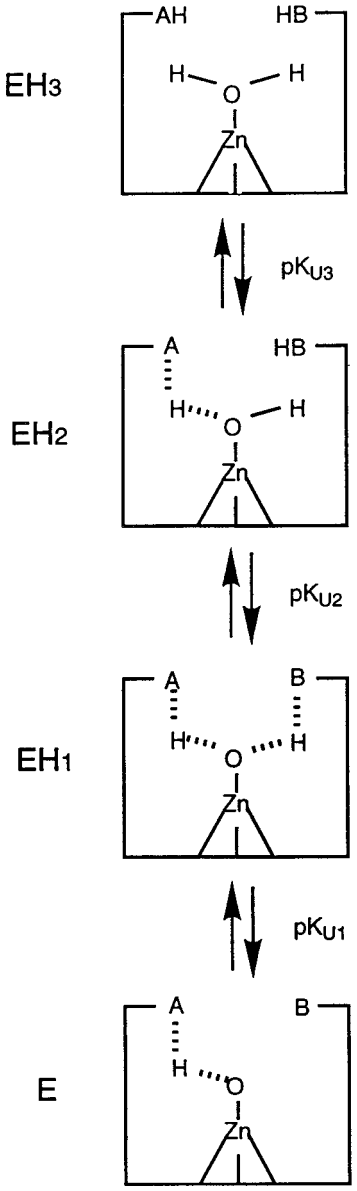


FIGURE 3 Sketched representation of the protonation events connected to the modulation of catalysis by MMPs in the free enzyme (modified from Harrison et al., 1992).

From pK_a values reported in Table 2 a clearcut difference between MMP-8 and the two gelatinases emerges. Thus, both gelatinases display two catalytically relevant groups, with pK_a values ranging in the free enzyme between 7.6 and 8.1, which undergo a pK_a shift of $\approx+0.4$ pH units upon substrate binding, and a third group with a $pK_a = 6.52 \pm 0.08$ in MMP-2 and 6.04 ± 0.09 in MMP-9, which both undergo a pK_a shift of ≈-1.1 pH unit upon substrate binding (see Table 2). Therefore, in gelatinases, substrate interaction with enzyme brings about proton uptake from two groups with higher pK_a values, which can be possibly referred as to a Zn^{2+}-bound H_2O (Vallee and Auld, 1990) and a histidyl residue (B group), and proton release by a third more acid residue (A group).

Somewhat different is the proton modulation mechanism operative in the collagenase MMP-8, because their pK_a values (namely $pK_a = 7.15 \pm 0.08$ and 6.75 ± 0.07 in the free enzyme) are essentially one pH unit lower than in both MMP-2 and MMP-9. Furthermore, unlike the two gelatinases, substrate binding in MMP-8 brings about a pK_a shift for these two groups of ≈-0.2 pH units, which is in the opposite direction as compared to the other two enzymes. In addition, there is a more acid residue with a $pK_a = 6.05 \pm 0.09$ (thus closely similar in the free enzyme to the two gelatinases, mostly to MMP-9, see Table 2), which is unaffected by substrate binding, though influencing the catalytic mechanism. This behavior, which accounts for the lack of a bell-shaped pH-dependence displayed by K_m in MMP-8 (Fig. 2 *C*), is clearly indicating that the environment in the close proximity of the active site is drastically different in MMP-8 as compared to that of gelatinases. Therefore, a much lower pK_a value of groups involved in the proton-linked modulation results in MMP-8, also suggesting that the mode of substrate binding should markedly differ, bringing about only the proton release from the two groups.

A closer look at steady-state kinetic parameters describing the enzymatic mechanism of the three MMPs investigated (Table 3) clearly shows that in both gelatinases the optimization of the catalysis is accomplished through a delicate balancing between the two groups protonating with higher pK_a values. Thus, in MMP-9 the first protonation event (leading to species 2; Fig. 3) brings about a marked enhancement of k_{cat} (by about two orders of magnitude) and some increase for the substrate affinity, while in MMP-2 the same phenomenon, though inducing a similar increase for substrate binding, shows a slight decrease for k_{cat} (Table 3). However, the occurrence of the second protonation (i.e., the formation of species 3; Fig. 3) leads in both cases to a further increase of substrate affinity accompanied a substantial decrease of k_{cat} (Table 3). The overall effect is a marked increase of k_{cat}/K_m in MMP-9 (mostly related to the enhancement of the rate-limiting step efficiency). This is immediately counterbalanced by the decrease of k_{cat} upon the second protonation, while in MMP-2 the opposite proton-linked effect on k_{cat} and on K_m (Table 3) leads to a very weak pH-dependence of k_{cat}/K_m (Fig. 2 *A*). Therefore, in both gelatinases the overall catalytic rate is maximized after the first protonation in species 2 (Fig. 3), which brings about a dramatic increase for substrate affinity (see Table 3). The formation of species 3 (with the protonation of group B) induces in both enzymes a further increase of substrate affinity (counterbalanced by a decrease of k_{cat}) with only a slight decrease of k_{cat}/K_m (being somewhat more marked in MMP-9 than in MMP-2). This feature allows both molecules to extend the efficiency of the overall enzymatic activity over a larger pH range in a modulated fashion (Fig. 2 *A*). However, the protonation of the A group, leading to

TABLE 3 Catalytic parameters for different protonation states in MMP-2, MMP-9, and MMP-8. Numbering of species corresponds to that used in Fig. 3

	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (M)
MMP-2			
Species 1	$3.36 (\pm 0.57) \times 10^6$	$4.81 (\pm 0.31) \times 10^2$	$1.43 (\pm 0.26) \times 10^{-4}$
Species 2	$5.74 (\pm 0.63) \times 10^6$	$3.49 (\pm 0.19) \times 10^2$	$6.10 (\pm 0.48) \times 10^{-5}$
Species 3	$5.16 (\pm 0.71) \times 10^6$	$1.14 (\pm 0.12) \times 10^2$	$2.21 (\pm 0.17) \times 10^{-5}$
Species 4	$4.50 (\pm 0.67) \times 10^6$	$9.53 (\pm 0.87) \times 10^2$	$2.11 (\pm 0.19) \times 10^{-4}$
MMP-9			
Species 1	$1.75 (\pm 0.17) \times 10^4$	$1.15 (\pm 0.41) \times 10^0$	$5.71 (\pm 0.42) \times 10^{-5}$
Species 2	$1.84 (\pm 0.21) \times 10^7$	$4.70 (\pm 0.49) \times 10^2$	$2.55 (\pm 0.18) \times 10^{-5}$
Species 3	$4.42 (\pm 0.37) \times 10^6$	$5.53 (\pm 0.52) \times 10^1$	$1.25 (\pm 0.13) \times 10^{-5}$
Species 4	$2.41 (\pm 0.22) \times 10^5$	$5.04 (\pm 0.44) \times 10^1$	$2.07 (\pm 0.18) \times 10^{-4}$
MMP-8			
Species 1	$8.45 (\pm 0.75) \times 10^6$	$1.64 (\pm 0.14) \times 10^2$	$1.94 (\pm 0.22) \times 10^{-5}$
Species 2	$1.22 (\pm 0.11) \times 10^7$	$3.42 (\pm 0.34) \times 10^2$	$2.80 (\pm 0.24) \times 10^{-5}$
Species 3	$1.12 (\pm 0.13) \times 10^7$	$5.22 (\pm 0.49) \times 10^2$	$4.65 (\pm 0.43) \times 10^{-5}$
Species 4	$3.12 (\pm 0.27) \times 10^6$	$1.45 (\pm 0.09) \times 10^2$	$4.65 (\pm 0.51) \times 10^{-5}$

the formation of species 4 (Fig. 4), brings about a dramatic decrease of the catalytic activity due to the simultaneous decrease of k_{cat} and an increase of K_m (Table 3), thus decreasing the affinity for substrate.

Collagenase MMP-8 is operating in a slightly different way, and the proton-linked regulation is occurring at significantly lower pH values. Thus, the overall activity is only moderately affected by the first protonation event (going from species 1 to species 2, see Fig. 3), because an increase of k_{cat} is partially compensated by an increase of K_m, which decreases the substrate affinity, unlike gelatinases (see Table 3). Protonation of the B group, leading to species 3, leaves the overall catalytic activity through an almost perfect balancing between an increase of k_{cat} and an increase of K_m (Table 3) unaffected. Therefore, differently from gelatinases in this case, the protonation of the B residue enhances the rate-limiting step and decreases the substrate affinity, though contributing as well to keep the overall enzymatic activity high over a wider pH range. Such a behavior confirms the idea that substrate interaction with these two groups must be very different in MMP-8 with respect to gelatinases, because its binding brings about a proton release in MMP-8 and a proton uptake in MMP-2 and MMP-9. As in gelatinases, the protonation of the more acid A group (Fig. 3) leads to a drastic reduction of the overall catalytic activity but, unlike MMP-2 and MMP-9, this takes place only through a decrease of the rate-limiting step process (Table 3), suggesting again that substrate interaction with this residue is different in MMP-8 with respect to both gelatinases.

Deeper information on the different energetic contributions to the catalytic mechanism may come from the temperature-dependence of the different parameters in the three MMPs investigated. Therefore, we have carried out the temperature-dependence between 15°C and 37°C of the substrate enzymatic processing by the three enzymes, an approach that should allow obtaining a further level of knowledge of the system. At the two temperatures we have calculated the activation parameters contributing directly to the overall phenomenon (for k_{cat}/K_m, see Figs. 4 *A* and 5 *A*) as well as those for the rate-limiting step (for k_{cat}, see Figs. 4 *B* and 5 *B*), and indirectly also those referring to the formation of the complex ES undergoing the catalytic rate-limiting step (i.e., K_m; see Figs. 4 *C* and 5 *C*). The parameters are reported in Table 4 and allow the definition of similarity and variations among the three enzymes investigated.

At pH 7.0 (Fig. 4) the two gelatinases MMP-2 and MMP-9 display essentially the same overall activity (somewhat smaller than for the neutrophil collagenase MMP-8, see Fig. 2 *A*), but the above-mentioned analysis permits us to put in evidence that this close similarity is the result of differing contributions from various processes affecting the whole phenomenon. Thus, even though both in MMP-2 and MMP-9 the formation of the rate-limiting complex ES (corresponding to the intermediate whose enzymatic processing determines the rate-limiting step of the whole catalysis) is for this substrate endothermic and characterized by a an entropy gain, the relative values of ΔH and ΔS render the free energy for the ES formation less negative in MMP-2 than in MMP-9 (Table 4). This difference is compensated by a faster rate-limiting step for MCA-2 enzymatic processing in MMP-2 than in MMP-9, mostly through a less negative activation entropy of the process. Therefore, these two gelatinases clearly display a similar enzymatic mechanism, where the formation of the ES complex is driven by an entropy gain (differing between the two enzymes), while the catalytic step is characterized in both enzymes by a negative activation entropy, though to a different extent (Table 4).

Completely different from what is observed in gelatinases appears to be the energetic contribution of various steps to the enzymatic activity of the neutrophil collagenase MMP-8. In this case, the formation of the rate-limiting

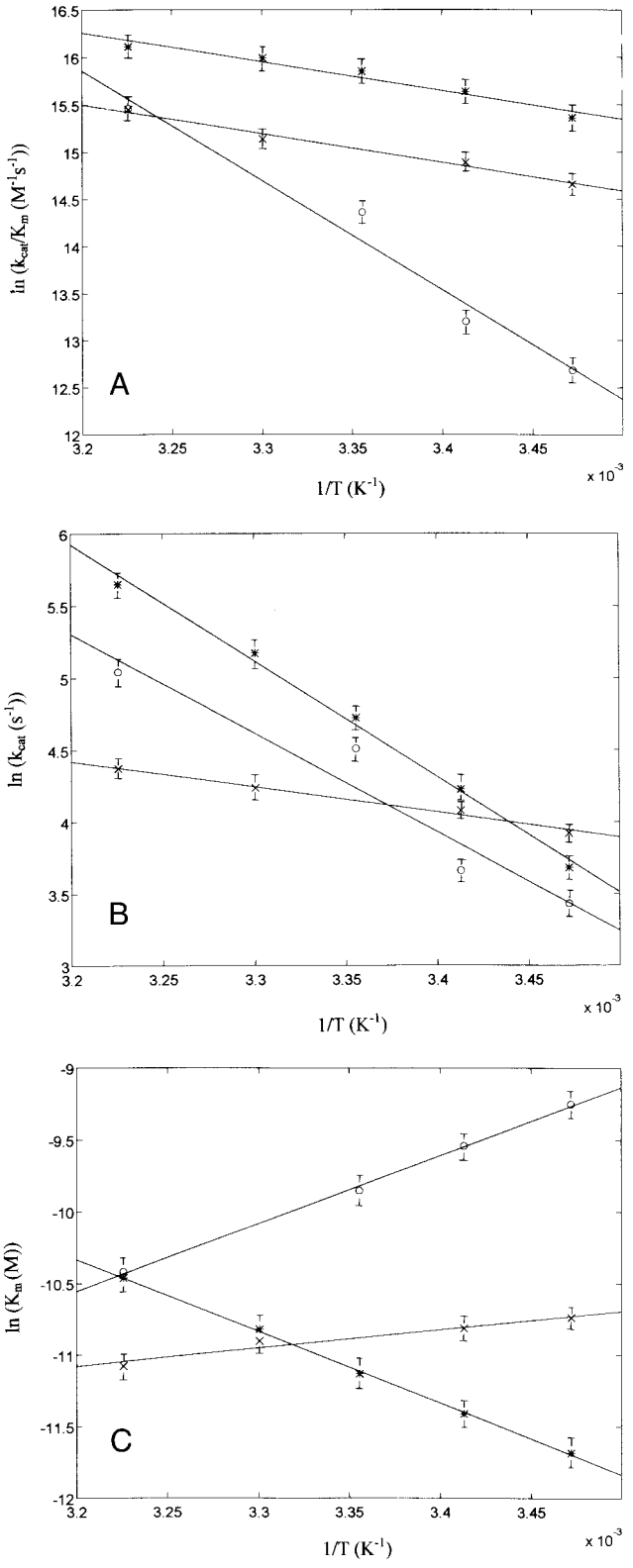


FIGURE 4 Temperature-dependence of k_{cat}/K_m (*A*), k_{cat} (*B*), and K_m (*C*) at pH 7.0 for neutrophil collagenase MMP-8 (∗), gelatinase A MMP-2 (○), and gelatinase B MMP-9 (×). Solid lines have been obtained by nonlinear least-squares fitting of experimental data, applying Eq. 1. Data are reported in Table 4. For further details, see text.

TABLE 4 Thermodynamic and activation parameters for the enzymatic activity of MMP-2, MMP-8, and MMP-9 at 37°C at pH 7.0 and pH 9.0

		MMP-2	MMP-8	MMP-9
k_{cat}/K_m				
$\Delta G^{\ddagger}$ (kJ/mol)	pH 7.0	+37.3 ± 1.9	+35.5 ± 1.8	+37.3 ± 1.7
	pH 9.0	+39.3 ± 1.9	+36.0 ± 1.8	+38.2 ± 1.9
$\Delta H^{\ddagger}$ (kJ/mol)	pH 7.0	+93.6 ± 4.5	+22.5 ± 3.6	+22.6 ± 3.9
	pH 9.0	+3.7 ± 1.2	+36.6 ± 4.0	+40.0 ± 4.2
$T\Delta S^{\ddagger}$ (kJ/mol)	pH 7.0	+56.3 ± 5.1	−13.0 ± 4.8	−14.7 ± 4.6
	pH 9.0	−34.5 ± 4.2	+0.6 ± 2.0	+1.6 ± 2.1
k_{cat}				
$\Delta G^{\ddagger}$ (kJ/mol)	pH 7.0	+64.0 ± 4.2	+62.4 ± 3.9	+65.8 ± 4.2
	pH 9.0	+61.4 ± 3.8	+63.7 ± 4.0	+65.0 ± 4.1
$\Delta H^{\ddagger}$ (kJ/mol)	pH 7.0	+54.3 ± 3.2	+64.0 ± 3.7	+12.0 ± 2.9
	pH 9.0	+147.8 ± 9.7	+18.5 ± 2.7	+86.9 ± 8.2
$T\Delta S^{\ddagger}$ (kJ/mol)	pH 7.0	−9.7 ± 3.2	+1.5 ± 2.6	−53.8 ± 5.9
	pH 9.0	+86.4 ± 7.2	−45.2 ± 4.8	+21.9 ± 3.2
K_m				
ΔG^{*} (kJ/mol)	pH 7.0	−26.8 ± 3.6	−27.0 ± 3.8	−28.5 ± 3.8
	pH 9.0	−23.2 ± 3.4	−27.8 ± 3.7	−25.7 ± 3.4
ΔH^{*} (kJ/mol)	pH 7.0	+39.3 ± 5.2	−41.5 ± 5.3	+10.6 ± 4.2
	pH 9.0	−144.1 ± 12.3	+18.0 ± 4.5	−46.0 ± 5.8
$T\Delta S^{*}$ (kJ/mol)	pH 7.0	+66.1 ± 8.4	−14.5 ± 4.9	+39.1 ± 6.7
	pH 9.0	−120.9 ± 13.1	+45.8 ± 5.6	−20.3 ± 5.2

*Parameters are expressed as association equilibrium constants.

complex ES is strongly exothermic for this substrate and characterized by a significant entropy loss. Therefore, MMP-8 behaves in an opposite fashion as compared to the two gelatinases, even though the overall free energy turns out to be closely similar to that displayed by MMP-2 (see Fig. 2 *C* and Table 4). The faster overall enzymatic activity shown by MMP-8 with this substrate at pH 7.0 (Fig. 2 *A*) is completely referable to a faster rate-limiting step of this enzyme with respect to the two gelatinases, wholly attributable to the slightly positive activation entropy of the process (as compared to the negative value observed in both gelatinases; see Table 4). Such an outcome is in keeping with the evidence of a different mode for substrate binding between collagenase and gelatinase, as mentioned above, and the different entropic contribution suggests that the structural organization of H_2O could be playing a role in characterizing this difference.

Raising the pH to 9.0 (Fig. 5) brings about a significant reduction for the overall enzymatic activity for all three enzymes (Fig. 2 *A*), although the pH increase is accompanied by a decrease of the rate-limiting step k_{cat} for MMP-8 and MMP-9, and by a rate enhancement in the case of MMP-2 (Fig. 2 *B*). However, for MMP-2 this behavior is regulated by the activation entropy, which becomes positive upon pH raising, whereas in the case of MMP-9 the reduction of k_{cat} at more alkaline pH is totally due to an enhancement of the enthalpic barrier, which overwhelms the positive effect of the activation entropy observed in this gelatinase as well. Completely different is the proton-linked behavior of the collagenase MMP-8, in which the pH in-

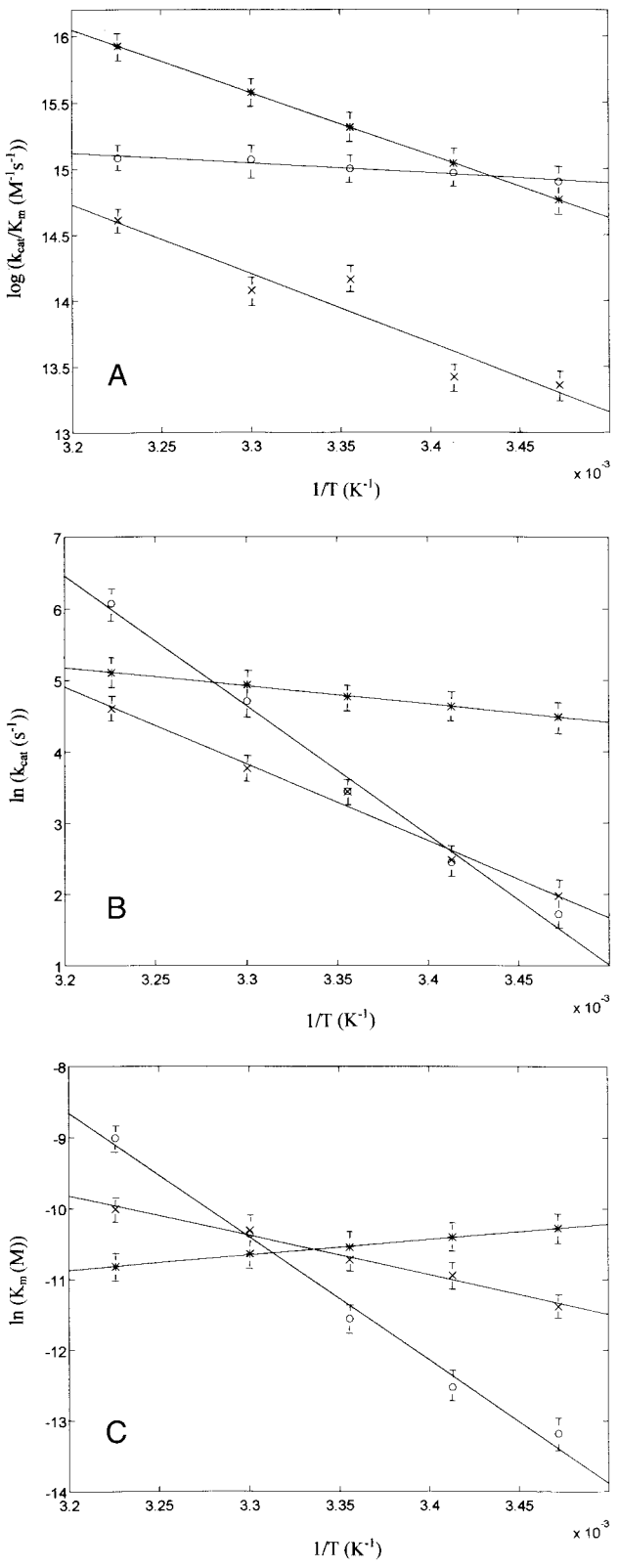


FIGURE 5 Temperature-dependence of k_{cat}/K_m (*A*), k_{cat} (*B*), and K_m (*C*) at pH 9.0 for neutrophil collagenase MMP-8 (∗), gelatinase A MMP-2 (○), and gelatinase B MMP-9 (×). Solid lines have been obtained by nonlinear least-squares fitting of experimental data, applying Eq. 1. Data are reported in Table 4. For further details, see text.

crease leads to a strongly negative activation entropy, and thus to a decrease of k_{cat}.

Closely similar for the two gelatinases is the pH effect on K_m, being mainly represented in both cases by a marked decrease of the stabilization energy for the ES complex as the pH is raised (Fig. 2 *C*). It underlies a common behavior for this parameter, which can be referred for both MMP-2 and MMP-9 to a progressive loss of the entropy upon complex formation as the pH is increased (going from positive values at neutral pH to very negative values of ΔS at alkaline pH; see Table 4). However, in the collagenase MMP-8 only a very limited variation for K_m is observed over the same pH range, this being related to an enthalpy/entropy compensation (Table 4 and Fig. 2 *C*).

A structural interpretation of the different catalytic behavior displayed by these three enzymes is not straightforward, although the x-ray structure has been solved for the catalytic domains of MMP-8 (Bode et al., 1994; Grams et al., 1995) and, to a lower resolution, of MMP-2 (Dhanaraj et al., 1999) as well as of the whole pro-MMP-2 (Morgunova et al., 1999), allowing to formulate some working hypothesis for the groups involved in the proton-linked functional modulation. Thus, it would be plausible to identify Glu-198 as the A residue with the lowest pK_a value in three MMPs ($pK_U = 6.54 \pm 0.08$ for MMP-2, 6.04 ± 0.09 for MMP-9, and 6.05 ± 0.09 for MMP-8; see Table 2), even though it has been reported that in matrilysin MMP-7 its substitution by uncharged residues, such as Gln and Ala, does not alter significantly the pK_a of the acidic transition (Cha and Auld, 1997). Obviously, the influence of Glu-198 might be different between MMP-7 (where site-directed mutants have been tested) and the enzymes investigated in our study, so that the evidence reported for MMP-7 might be not relevant for MMP-8, MMP-2, and MMP-9. As a matter of fact, in stromelysin MMP-3 a very recent investigation of the pH-dependence of catalytic parameters indicates that a low pK_a (≅5.6; see Johnson et al., 2000) may be attributable to the Glu-Zn^{2+}-H_2O complex. However, in our case the lowest pK_U values may also find an additional explanation, and residue A could instead be proposed to be one of the histidyl residues coordinating the catalytic Zn^{2+}, and in fact the acidic pK_a value(s) ($= 6.05 \pm 0.07$ in MMP-8, 5.54 ± 0.09 in MMP-2, 4.82 ± 0.08 in MMP-9 for the substrate-bound forms; see Table 2) might well be compatible with the protonation of the N_ϵ from a imidazole coordinated to a metal (thus displaying a lower pK_a value). In the case of residue B, because the observed pK_a values may certainly be referred to an uncoordinated histidyl residue ($pK_{U1} = 7.69 \pm 0.08$ for MMP-2, 7.87 ± 0.07 for MMP-9, and 6.75 ± 0.07 for MMP-8, see Table 2), a potential candidate indeed is His-162, which is conserved in most MMPs (including MMP-2, MMP-9, and MMP-8; see Massova et al., 1998), and which has been shown to interact with an inhibitor of MMP-8 (Grams et al., 1995). The Zn^{2+}-bound H_2O might be referred to the group with the highest pK_a value

($pK_U = 7.99 \pm 0.09$ for MMP-2, 8.05 ± 0.08 for MMP-9, and 7.15 ± 0.08 for MMP-8; see Vallee and Auld, 1990; Mock and Stanford, 1996), even though in the case of matrilysin MMP-7 and in MMP-3 it has instead been proposed to be responsible for the very low pK_a observed (Cha and Auld, 1997; Johnson et al., 2000), in view of the possible interaction with the protonated Glu-198 (see above). More complex is the task of understanding why 1) pK_a values of Zn^{2+}-bound H_2O and of His-162 are much lower in MMP-8 than in both MMP-2 and MMP-9; and 2) collagenase and gelatinases display a different mode of substrate binding, as indicated by the differing behavior of K_m. Thus, the structure of the active site appears similar in MMP-8 and in MMP-2 (Grams et al.,. 1995; Morgunova et al., 1999), suggesting that the structural determinants should be the same for collagenases and gelatinases, even though the resolution level may not be high enough to detect small but relevant variations between the two enzymes. Obviously, an important difference between MMP-8 and the two gelatinases is the presence, in the latter ones, of the long fibronectin II-like sequence close to the active site, which is inserted between the fifth β strand and helix 2 of the catalytic domain (Morgunova et al., 1999). This structural difference indeed might be responsible for a different charge distribution in the close proximity of the catalytic site, and thus for the marked variation of pK_a values observed between collagenases (i.e., MMP-8) and gelatinases (i.e., MMP-2 and MMP-9; see Table 2). This possibility is further strengthened by the recent proposal that in MMP-3 a histidyl residue, which participates in shaping the β-anchor interacting with a tripeptide, might display a very low pK_a value ($\cong$6.0; see Johnson et al., 2000). However, we cannot also exclude that in different enzymes differing residues are responsible for the various proton-linked transitions, and that residues present in all enzymes play different enzyme-specific roles. In this respect, an additional important difference between the two gelatinases and the collagenase concerns the residue in position 151 (according to the numbering of Grams et al., 1995), which is Ser in MMP-8 and Tyr in both MMP-2 and MMP-9. Because this residue is proposed to be involved in the binding of the *N*-terminus of the substrate (or inhibitor; Grams et al., 1995), the presence of Tyr in gelatinases has been considered important to force the substrate to bind the active site of MMP-2 and MMP-9 as an extended β-strand (Massova et al., 1997). Therefore, the phenolic ring of Tyr could be responsible for a different arrangement of the substrate inside the recognition site, and thus for the substrate-linked effect on pK_a values between collagenase and gelatinases (see Table 2). However, a final statement on this aspect must await higher resolution on the x-ray structure of gelatinases.

In conclusion, this analysis of the proton-linked and temperature-dependent modulation of the enzymatic action of MMP-2, MMP-9, and MMP-8 allows unraveling of some major differences between collagenases and gelatinases, but also underlines some variations and similarities in the fine regulation of their function. Although it is not possible yet to describe to a great molecular detail the functional differences detected, it is important to begin a structural-functional analysis to distinguish the mode of action of these enzymes in order to trigger the design of enzyme-specific inhibitors.

The authors acknowledge Elisabetta Bennici for her expert support and Sara Sherwood for editing the manuscript.

This work was supported by the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST COFIN 9803184222).

REFERENCES

Aimes, R. T., D. L. French, and J. P. Quigley. 1994. Cloning of a 72 kDa matrix metalloproteinase (gelatinase) from chicken embryo fibroblasts using gene family PCR: expression of the gelatinase increases upon malignant transformation. *Biochem. J.* 300:729–736.

Aimes, R. T., and J. P. Quigley. 1995. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J. Biol. Chem.* 270:5872–5876.

Antonini, E., and P. Ascenzi. 1981. The mechanism of trypsin catalysis at low pH. Proposal for a structural model. *J. Biol. Chem.* 256: 12449–12455.

Baramova, E., and J. M. Foidart. 1995. Matrix metalloproteinase family. *Cell Biol. Int.* 19:239–242.

Birkedal-Hansen, H., W. G. I. Moore, M. K. Bodden, L. J. Windsor, B. Birkedal-Hansen, A. DeCarlo, and J. A. Engler. 1993. Matrix metalloproteinases: a review. *Crit. Rev. Oral Biol. Med.* 4:197–250.

Blundell, T. L. 1994. Metalloproteinase superfamilies and drug design. *Nat. Struct. Biol.* 1:73–75.

Bode, W., P. Reinemer, R. Huber, T. Kleine, S. Schnierer, and H. Tschesche. 1994. The x-ray crystal structure of the catalytic domain of human neutrophil collagenase inhibited by a substrate analogue reveals the essentials for catalysis and specificity. *EMBO J.* 13:1263–1269.

Cha, J., and D. S. Auld. 1997. Site-directed mutagenesis of the active site glutamate in human matrilysin: investigation of its role in catalysis. *Biochemistry.* 36:16019–16024.

Cossins, J. A., J. M. Clements, J. Ford, K. M. Miller, R. Pigott, W. Vos, P. van der Valk, and C. J. A. de Groot. 1997. Enhanced expression of MMP-7 and MMP-9 in demyelinating multiple sclerosis lesions. *Acta Neuropathol.* 94:590–598.

Dhanaraj, V., M. G. Williams, Q.-Z. Ye, F. Molina, L. L. Johnson, D. F. Ortwine, A. Pavlovsky, J. R. Rubin, R. W. Skeean, A. D. White, C. Humblet, D. J. Hupe, and T. L. Blundell. 1999. Crystal structure of gelatinase A catalytic domain. *Croat. Chem. Acta.* 72:575.

Dioszegi, M., P. Cannon, and H. E. van Wart. 1995. Vertebrate collagenases. *Methods Enzymol.* 248:413–431.

Fang, J., Y. Shing, D. Wiederschain, L. Yan, C. Butterfield, G. Jackson, J. Harper, G. Tamvakopoulos, and M. A. Moses. 2000. Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. *Proc. Natl. Acad. Sci. U.S.A.* 97:3884–3889.

Fisher, C., S. Gilbertson-Beadling, A. Powers, G. Petzold, and R. Poorman. 1994. Interstitial collagenase is required for angiogenesis in vitro. *Dev. Biol.* 162:499–510.

Grams, F., P. Reinemer, J. C. Powers, T. Kleine, M. Pieper, H. Tschesche, R. Huber, and W. Bode. 1995. X-ray structures of human neutrophil collagenase complexed with peptide hydroxamate and peptide thiol inhibitors. Implications for substrate binding and rational drug design. *Eur. J. Biochem.* 228:830–841.

Harrison, R. K., B. Chang, L. Niedzwiecki, and R. L. Stein. 1992. Mechanistic studies on the human matrix metalloproteinase stromelysin. *Biochemistry.* 31:10757–10762.

Heppner, K. J., L. M. Matrisian, R. A. Jensen, and W. H. Rodgers. 1996. Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced response. *Am. J. Pathol.* 149: 273–282.

Hirose, T., R. A. Reife, G. N. Smith, R. M. Stevens, C. L. Mainardi, and K. A. Hasty. 1992. Characterization of type-V collagenase (gelatinase) in synovial fluid of patients with inflammatory arthritis. *J. Rheumatol.* 19:593–599.

Holman, C. M., C.-C. Kan, M. R. Gehring, and H. E. van Wart. 1999. Role of His-224 in the anomalous pH dependence of human stromelysin-1. *Biochemistry.* 38:677–681.

Izquierdo-Martin, M., and R. L. Stein. 1992. Transition-state structural features for the association of metalloproteases with phosphorous-containing inhibitors. *J. Am. Chem. Soc.* 114:1527–1528.

Johnson, L. J., A. G. Pavlovsky, A. R. Johnson, J. A. Janowicz, C.-F. Man, D. F. Ortwine, C. F. Purchase II, A. D. White, and D. J. Hupe. 2000. A rationalization of the acidic pH dependence for stromelysin-1 (matrix metalloproteinase-3) catalysis and inhibition. *J. Biol. Chem.* 275: 11026–11033.

Knight, C. G., F. Willenbrock, and G. Murphy. 1992. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett.* 296:263–266.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–683.

Maeda, A., and R. A. Sobel. 1996. Matrix metalloproteinases in the normal human central nervous system, microglial nodules, and multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.* 55:300–309.

Massova, I., F. Fridman, and S. Mobashery. 1997. Structural insights into the catalytic domains of human matrix metalloprotease-2 and human matrix metalloprotease-9: implications for substrate specificities. *J. Mol. Model.* 3:17–30.

Massova, I., L. P. Kotra, R. Fridman, and S. Mobashery. 1998. Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J.* 12:1075–1095.

Mock, W. L., and D. J. Stanford. 1996. Arazoformyl dipeptide substrates for thermolysin. Confirmation of a reverse protonation catalytic mechanism. *Biochemistry.* 35:7369–7377.

Morgunova, E., A. Tuuttila, U. Bergmann, M. Isupov, Y. Lindqvist, G. Schneider, and K. Tryggvason. 1999. Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed. *Science.* 284: 1667–1670.

Nagase, H., and G. B. Fields. 1996. Human matrix metalloproteinase specificity studies using collagen sequence-based synthetic peptides. *Biopolymers.* 40:399–416.

Netzel-Arnett, S., Q.-X. Sang, W. G. I. Moore, M. Navre, B. Birkedal-Hansen, and H. E. van Wart. 1993. Comparative sequence specificities of human 72- and 92-kDa gelatinases (type IV collagenases) and PUMP (matrilysin). *Biochemistry.* 32:6427–6432.

Niyibizi, C., R. Chan, J.-J. Wu, and D. Eyre. 1994. A 92 kDa gelatinase (MMP-9) cleavage site in native type V collagen. *Biochem. Biophys. Res. Commun.* 202:328–333.

Rosenberg, G. A., J. E. Dencoff, N. Correa, M. Reiners, and C. C. Ford. 1996. Effect of steroids on CSF matrix metalloproteinases in multiple sclerosis: relation to blood-brain barrier injury. *Neurology.* 46: 1626–1632.

Stack, M. S., and R. D. Gray. 1989. Comparison of vertebrate collagenase and gelatinase using a new fluorogenic substrate peptide. *J. Biol. Chem.* 264:4277–4281.

Stack, M. S., and R. D. Gray. 1990. The effect of pH, temperature, and D_2O on the activity of porcine synovial collagenase and gelatinase. *Arch. Biochem. Biophys.* 281:257–263.

Stein, R. L., and M. Izquierdo-Martin. 1994. Thioester hydrolysis by matrix metalloproteinases. *Arch. Biochem. Biophys.* 308:274–277.

Tschesche, H. 1995. Human neutrophil collagenase. *Methods Enzymol.* 248:431–449.

Vallee, B. L., and D. S. Auld. 1990. Active-site zinc ligands and activated H_2O of zinc enzymes. *Proc. Natl. Acad. Sci. U.S.A.* 87:220–224.

Vu, T. H., J. M. Shipley, G. Bergers, J. E. Berger, J. A. Helms, D. Hanahan, S. D. Shapiro, R. M. Senior, and Z. Werb. 1998. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell.* 93:411–422.

Walakovits, L. A., V. L. Moore, N. Bhardwaj, G. S. Gallick, and M. W. Lark. 1992. Detection of stromelysin and collagenase in synovial fluid from patients with rheumatoid arthritis and posttraumatic knee injury. *Arthritis Rheum.* 35:35–42.

Weingarten, H., R. Martin, and J. Feder. 1985. Synthetic substrates of vertebrate collagenase. *Biochemistry.* 24:6730–6734.

Welch, A. R., C. M. Holman, M. Huber, M. C. Brenner, M. F. Browner, and H. E. van Wart. 1996. Understanding the P1′ specificity of the matrix metalloproteinases: effect of S1′ pocket mutations in matrilysin and stromelysin-1. *Biochemistry.* 35:10103–10109.