Site-Directed Mutagenesis of the Active Site Glutamate in Human Matrilysin: Investigation of Its Role in Catalysis†

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ABSTRACT: Glu-198 of human matrilysin is a conserved residue in the matrix metalloproteinases and is considered to play an important role in catalysis by acting as a general base catalyst toward the zinc-bound water molecule, on the basis of mechanistic proposals for other zinc proteases. In the present study, Glu-198 is mutated into Asp, Cys, Gln, and Ala, and the zinc binding properties, kinetic parameters, and pH dependence of each mutant are determined in order to examine the role of Glu-198 in catalysis. The mutations chosen either modify (C and D) or eliminate (A and Q) the general base properties of residue-198. All the mutants bind 2 mol of zinc per mol of enzyme, indicating that Glu-198 is not crucial to the binding of the catalytic zinc to the enzyme. The value of k_{cat}/K_m for the E198D mutant is only 4-fold lower than that of wild-type enzyme at the pH optimum of 7.5, while that for the E198C mutant is decreased by 160-fold. The E198Q and E198A enzymes containing the mutations that have eliminated the nucleophilic and acid/base properties of the residue are still active, having lower k_{cat}/K_m values of 590- and 1900-fold, respectively. The decrease in activity of all the mutants is essentially due to a decrease in k_{cat}. The k_{cat}/K_m values of the mutants as a function of pH display broad bell-shaped curves that are similar to the wild-type enzyme. The acidic pK_a value is not greatly affected by the change in the chemical properties of residue-198. The similarity in the pH profiles for the mutant and wild-type enzymes indicates that the ionization of Glu-198 is not responsible for the acidic pK_a. Ionization of the zinc-bound water may be responsible for this pK_a since the three His ligands and the scaffolding of the matrilysin catalytic zinc site are different from that observed in carboxypeptidase A and would predict a lower pK_a for the metal-bound water. If the zinc-bound water is the nucleophile in the reaction, the role of Glu-198 in catalysis may be to stabilize the transition state or act as a general acid catalyst after the rate-determining step.

The matrix metalloproteinases (MMPs)[1] are a class of zinc hydrolytic enzymes believed to be important for remodeling the extracellular matrix (ECM) (*1*). MMPs contribute to the catabolism of matrix proteins during pathological processes and normal connective tissue remodeling. Many studies suggest that the elevated levels of MMPs are responsible for rheumatoid arthritis, late-stage tumor progression, and metastasis of tumor cells (*2*−*4*). Recent studies have begun to distinguish the role of each MMP member by the method of immunohistochemical analysis and gene targeting. Wilson et al. (*5*) demonstrated that MMP-7, or matrilysin, is involved in intestinal tumorigenesis using matrilysin-deficient mice. This result suggests that matrilysin, unlike most of the other MMPs, may contribute to early tumor development. This finding extends the general concepts of MMP contributions from early tumor formation to tumor progression and metastasis.

The determination of crystal structures of MMPs complexed with several synthetic inhibitors has accelerated the elucidation of the mechanism of these enzymes by revealing the structural features of their zinc- and calcium-binding sites and the amino acid residues that interact with bound inhibitors (*6*−*12*). Comparison of the structures of matrilysin, thermolysin (TL), and carboxypeptidase A (CPD-A) reveals both similarities and differences in their active sites. A common feature is a catalytic zinc atom that is coordinated by three protein ligands and a nearby ionizable carboxylate group of a Glu residue that is considered to act as a nucleophile or general base (Figure 1). The zinc site is closely similar in regard to the orientation of the metal ligands, the proposed catalytic glutamate residue, and the position at which inhibitors bind (*12, 13*). However, the type of the ligand and the scaffolding of the zinc site is not the same (Figure 1) (*14*). The catalytic zinc of matrilysin is made up of three His residues whereas the zinc atom of thermolysin and CPD-A contains 2 His and 1 Glu. Furthermore, the secondary interactions of the zinc ligands with adjacent side-chain carboxylate groups observed in TL and CPD-A are not observed in matrilysin.

In CPD-A, Glu-270 is considered to assist the ionization of the metal-bound water based on chemical modification, spectrokinetic, and NMR studies (*14*−*18*). The hypothesis

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[1] Abbreviations: MMP, matrix metalloproteinase; MMP-7, matrilysin; ECM, extracellular matrix; Dns, 5-(dimethylamino)naphthalene-1-sulfonyl; Ampso, 3-[(1,1-dimethyl-2-hydoxyethyl)amino]-2-hydroxypropanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TFA, trifluoroacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, 3-(2,4-dinitrophenyl)-2,3-diaminopropionyl; Cbz, carbobenzoxy; NMR, nuclear magnetic resonance; PAC, perturbed angular correlation of γ-rays.

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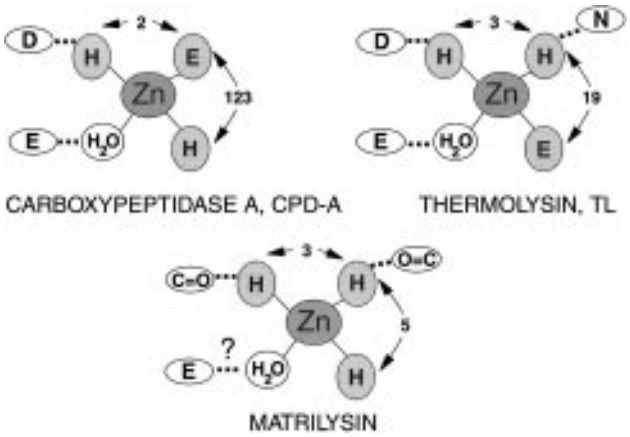

FIGURE 1: The catalytic zinc sites of carboxypeptidase A, thermolysin, and matrilysin.

is also supported by crystallographic studies which show that the interatomic distance from active site zinc to the carboxylate of Glu-270 is about 4.5 Å, a distance consistent with a hydrogen bond between the metal-bound water and Glu-270 (*19, 20*). Calculation of the coordination geometry of the Cd-substituted enzyme from PAC spectra (*21, 22*) and molecular calculations on a model system (*23*) are also in agreement with this assignment. A general base role for Glu-143 in thermolysin catalysis is also proposed on the basis of X-ray crystallographic and computer graphic analyses of thermolysin bound with inhibitors (*24*−*27*) and the proximity of Glu-143 to the zinc-bound water molecule (*28*). This role for the glutamate in catalysis is generally believed to pertain to the MMP family of proteases as well.

The pH dependence of matrilysin catalysis demonstrates that ionization of a group with a pK_a of 4.3 is needed for catalytic activity (*29*). We therefore have focused on Glu-198 as a likely amino acid residue that could be responsible for this pK_a. We developed a recombinant system for the high-level expression and purification of active matrilysin, devoid of its prodomain, that has an advantage for structure−function studies by mutagenesis (*29*). In the present study, the functional role of the active site Glu-198 in catalysis is investigated by comparing the metal binding, kinetic parameters, and pH properties of its mutants with those of the wild-type enzyme. Our results indicate Glu-198 is not essential for catalysis and likely does not act as a general base catalyst. Instead, it may play a role in catalysis through its protonated carboxyl group.

MATERIALS AND METHODS

Materials. A plasmid containing the entire matrilysin cDNA, pUN121, was provided by Dr. R. Breathnach (Strasbourgh, France). *Escherichia coli* BL21(DE3) cells and the pET-11a vector were obtained from Novagen; oligonucleotide primers for mutagenesis were from Promega and Amitof Biotech Inc; restriction and ligation enzymes were from New England Biolabs. The site-directed mutagenesis kit was from Promega. Heparin-agarose was from Sigma; Chelex-100 resin was from Bio-Rad.

Bacterial Strains and Vectors. The human matrilysin cDNA lacking its prodomain was inserted in the pET-11a expression vector under control of an IPTG-inducible T7 promoter as described (*29*). pALTER-1 was used for the mutagenesis vector. The *E. coli* strains ES1301 *mutS* and JM109 were used for the selection and cloning host, respectively, for mutagenesis. *E. coli* BL21(DE3) was used for the production of the wild-type and mutant enzymes.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out using the Altered Sites II *in vitro* Mutagenesis kit from Promega. The pET-MMP7 constructed for the expression of an active matrilysin was subcloned into pALTER-1 (Amp^s, Tet^r) using *Xba*I and *Hin*dIII and the resulting plasmid pALTER-MMP7 was transformed into *E. coli* JM109. The DNA was then purified and denatured using 2 M NaOH and 2 mM EDTA. The denatured single-stranded template was annealed to mutagenic oligomers, ampicillin repair oligomer, and tetracycline knockout oligomer, and T4 DNA polymerase was used to synthesize the mutant strand of DNA. After ligation, the reaction product was transformed into ES1301 *mutS* cells and the recombinants were screened for their resistance to ampicillin. The selected recombinant DNA was transformed into JM109 cells to stabilize the mutant DNA. The presence of the desired mutation was confirmed by dideoxy sequencing of the area of the mutation. The pALTER-MMP7 containing a desired mutation was directly used for expression of the mutants or subcloned into the pET-11a expression vector.

Expression and Purification of Mutants. The wild-type and mutant matrilysins were overexpressed in *E. coli* BL21-(DE3) and purified as described (*29*). An overnight culture in the amount of 5−10 mL was added to 1 L of LB medium containing 50 μg/mL of ampicillin and grown for 3 h. IPTG (0.1 mM) was added, and the cells were grown another 3−4 h. The cells were harvested and frozen at −20 °C. SDS−PAGE was performed to examine the purity and mobility of the mutant enzymes. Protein concentration during the purification was measured using Bio-Rad assays and bovine serum albumin as the protein standard. Purified enzyme concentration was determined by amino acid analysis or the absorbance at 280 nm using the extinction coefficient of 33 000 M^{-1} cm^{-1}.

Enzyme Assays and Determination of Kinetic Parameters. Enzyme activity was measured with the synthetic fluorescent peptide Dns-PLALWAR as the substrate. Substrate concentrations were determined spectrophotometrically by the absorbance of the dansyl group at 340 nm (ϵ_{340} = 4300 M^{-1} cm^{-1}). Assays (300 μL) were performed over the substrate concentration range (1−5) × 10^{-5} M in 20 mM Hepes buffer at pH 7.5 that contained 10 mM $CaCl_2$ and 0.5 M NaCl. The enzyme concentrations in the assay mixture ranged from 2.4 × 10^{-8} M for the wild-type to 1 × 10^{-6} M for the E198Q and E198A mutants. Each aliquot (40 μL) of the reaction mixture was removed and stopped by addition of TFA to a final concentration of 1%. Initial rates corresponding to less than 10% of the reaction were measured by HPLC (Waters, Nova Pak C_{18} 3 × 150 mm) with a linear acetonitrile gradient (25−55%) in 0.1% TFA at a flow rate of 1 mL/min. Peak areas were determined at 214 nm with a Hewlett Packard 3380 A integrator. The k_{cat} and K_m values were determined from the results at five to six substrate concentrations and nonlinear regression analysis using the ENZFITTER program.

pH Dependence of the Reaction Catalyzed by Matrilysin. The dependence of activity (k_{cat}/K_m) on pH was measured for the mutants over the range of pH 4.25−10 using the following buffers: acetate (4.25−5), Mes (5.5−6.5), Hepes

(7−8.5), and Ampso (8.5−10). The buffers for the pH profile were checked for inhibitory or activating effects on the reaction over the concentration range 5−50 mM. The NaCl concentration of 0.5 M ensured essentially constant ionic strength at all pH values. Stopped-flow experiments were employed for the wild-type enzyme as described (*29*). The HPLC method was used for the determination of initial rates of the mutants. The reaction was carried out in 10 mM $CaCl_2$, 0.5 M NaCl, and 50 mM of each buffer at different pH values. Acetonitrile (final concentration of 2.5% (v/v)) was added to the substrate to ensure solubility. The temperature was maintained at 25 ± 0.2 °C. Substrate concentrations, $(1.5-3) \times 10^{-6}$ M ($S << K_m$), and enzyme concentrations, 1.0×10^{-8} to 1.0×10^{-6} M, were used for obtaining k_{cat}/K_m values. Initial rates were measured and k_{cat}/K_m values were calculated directly according to the Michaelis−Menten equation: $v_{init} = k_{cat}[E][S]/([S] + K_m)$ which is equal to $k_{cat}/K_m \times [E][S]$ when $[S] << K_m$.

Analysis of Zinc Content. The zinc content of the mutants and the wild-type matrilysin enzymes was quantified by a model 4100ZL Perkin-Elmer graphite furnace Zeeman atomic absorption spectrophotometer using the absorption peak at 212.9 nm as described before (*29*). After purification of the recombinant protein, samples containing 5−20 μM of protein were dialyzed against 5 mM $CaCl_2$, 5 mM Hepes, pH 7.5 at 4 °C for at least three changes of dialysate. Zinc concentrations were determined from a curve obtained with standard 2.5−20 μg/L zinc samples. The buffer from the last dialysis was used as a blank. Analyses were performed at least in triplicate.

Amino Acid Analysis and N-Terminal Sequencing. Samples for amino acid analysis were hydrolyzed with 6 N HCl (gas phase) at 110 °C under vacuum overnight. The hydrolysate was derivatized using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQTaq) methodology (*30*). *N*-Terminal sequences were determined using a Millgen/Biosearch ProSequencer according to the manufacturer's recommended procedures.

RESULTS

Expression and Purification of the Matrilysin Mutants. The expression vector, pET-MMP7, is subcloned into the mutagenesis vector, pALTER-1, and mutated by the appropriate oligomers for each mutant. The resultant plasmids are identified by restriction enzyme analysis and confirmed by sequencing. The mutant forms of human matrilysin are expressed in *E. coli* using pET-MMP7, as described previously for the wild-type enzyme (*29*). High amounts of all mutant proteins are expressed (around 5−10% of total protein), and the purified proteins are homogeneous as determined by SDS−PAGE. The *N*-terminal sequence for the most active mutant, E198D, is the same as that of the wild-type in this expression system, LFPNS. In contrast, all of the other lower activity mutants give MYSLFPNS, suggesting the more active enzymes autocatalytically remove MYS during the refolding process. The mutant enzymes are generally quite stable on storage at 4 °C, suggesting they have similar three-dimensional folds to the native enzyme.

Zinc Content. The proteins are extensively dialyzed at 10−20 μM concentrations against metal-free 5 mM Hepes and 5 mM $CaCl_2$ at pH 7.5. All samples are diluted in 0.2% HNO_3 solution to a 0.2−1 μM concentration and analyzed for zinc content using a graphite furnace atomic absorption spectrometer. Table 1 shows the content of zinc for each mutant. All the mutants contain two zinc atoms per enzyme molecule which is the same amount of zinc as the wild-type enzyme (*29*). The results indicate that the amino acid replacements for the Glu-198 residue do not drastically alter the catalytic zinc binding site properties.

Table 1: Zinc Content of Matrilysin Mutants

protein	zinc mol/mol of protein[a]	*n*
wild-type	1.91 ± 0.08	4
E198D	2.10 ± 0.11	3
E198C	2.01 ± 0.13	5
E198Q	2.01 ± 0.45	3
E198A	2.19 ± 0.02	4

[a] Values are expressed as the average ± standard deviation for *n* determinations.

Table 2: Kinetic Parameters of the Wild-Type and Mutant Matrilysins[a]

enzyme	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (s^{-1} M^{-1})	fold decrease	specific activity (%)
wild-type	2.7	2.4×10^{-5}	1.2×10^{5}	1	100
E198D	0.61	2.1×10^{-5}	2.9×10^{4}	4.1	24
E198C	0.014	1.8×10^{-5}	7.7×10^{2}	160	0.63
E198Q	0.0036	1.8×10^{-5}	2.0×10^{2}	590	0.17
E198A	0.00051	7.9×10^{-6}	6.5×10^{1}	1900	0.054

[a] Substrate used is DnsPLALWAR; assay conditions are 20 mM Hepes, pH 7.5, 10 mM $CaCl_2$, and 0.5 M NaCl at 25 °C.

Kinetic Characterization of Glu-198 Mutants. Kinetic parameters of the wild-type and Glu-198 mutants are determined using Dns-PLALWAR as a substrate at pH 7.5 (Table 2). The effect of the mutations on activity is generally due to a decrease in the value of k_{cat}. The k_{cat} for the E198D mutant decreases 4.1-fold compared to the wild-type activity, while the k_{cat} values for other mutants are lower, ranging from 200-fold (E198C) to 5300-fold (E198A). The K_m values of the Glu-198 mutants are quite similar to that of the wild-type enzyme. No K_m value is greater than the native enzyme. The k_{cat}/K_m values for the mutants range from a 4- to a 1900-fold reduction in activity.

pH Dependence of Glu-198 Mutants. In order to examine whether changes in the chemical properties of amino acid residue 198 produce alterations in the pH dependence of peptide hydrolysis, the steady-state kinetic parameters for the wild-type and Glu-198 mutants are measured at a substrate concentration about 10- to 20-fold below its K_m value at pH 7.5. No marked decrease in the K_m value occurs at pH values of 5 or 9 for the E198D, E198Q, or wild-type enzyme (data not shown). The pH dependence of the k_{cat}/K_m value for the wild-type enzyme is maximal in the pH region 5 to 9 and decreases on either side resulting in a broad bell-shaped curve characterized by an acidic pK_a of 4.3 and a basic one of 9.6. Similar behavior is exhibited by the E198D, E198C, E198Q, and E198A mutants, although the k_{cat}/K_m values decrease at acidic pH values slightly faster than does the wild-type (Figure 2). The pK_a values for the mutants are given in Table 3. The mutation of Glu-198 to Asp, Cys, Gln, and Ala apparently has little effect on the acidic and alkaline pK_a values compared to that of the wild-type. The most distinct change is observed in the acidic side of E198D mutant. The observed pK_a value of 5.6 is 1.3 unit higher than that of the wild-type which may be surprising

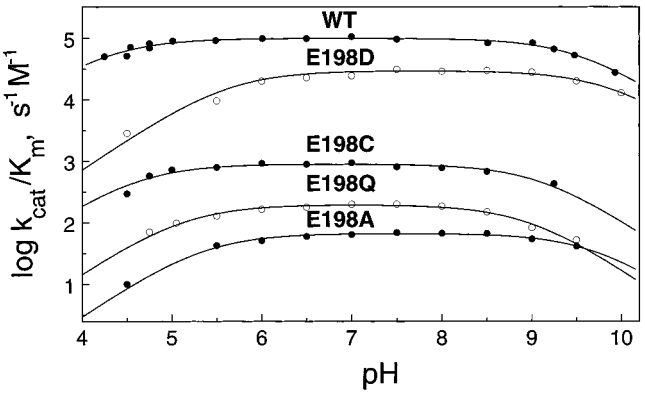

FIGURE 2: The pH dependence of the k_{cat}/K_m values for the wild-type (wt), E198D, E198C, E198Q, and E198A matrilysin at 25 °C. Buffer conditions are given in Materials and Methods section.

Table 3: pK_a Values of the Wild-Type and Mutant Matrilysins[a]

protein	acidic pK_a	alkaline pK_a
wild-type	4.3	9.6
E198D	5.6	9.9
E198C	4.6	9.1
E198Q	5.1	9.0
E198A	5.4	9.7

[a] The pK_a values are calculated by the equation $k_{cat}/K_{m(observed)} = k_{cat}/K_{m(pH\ independent)}/(1 + [H^+]/K_{a1} + K_{a2}/[H^+])$. The error in the p$K_a$ values is approximately ± 0.1 units for all enzymes.

since an Asp and Glu terminal carboxylate are expected to have similar pK_a values.

DISCUSSION

Glu-198 mutants are made by site-directed mutagenesis, and their zinc binding, kinetic parameters, and pH-activity dependence properties are characterized to examine the catalytic importance of the carboxylate of Glu-198 in matrilysin catalysis. The mutants are purified as described previously (*29*). All purified mutants are stable at 4 °C, are homogeneous as judged by SDS−PAGE, and contain two zinc atoms per protein molecule (Table 1) suggesting they have three-dimensional folding similar to the wild-type.

In the matrilysin−hydroxamate complex structure, the carboxylate group of Glu-219 (equivalent to Glu-198 in our numbering) clearly forms a hydrogen bond with the oxygen (2.6 Å) and nitrogen (3.0 Å) of the hydroxamate group of the inhibitor (*12*). The interaction of Glu-219 with atoms that mimic the main-chain nitrogen atoms of the substrate has been interpreted as evidence for a role of Glu-219 in general base catalysis comparable to that for Glu-270 and Glu-143 in CPD-A and TL, respectively. Some information is available on the functional role of the conserved glutamate in MMPs. The identical glutamate in human gelatinase A and fibroblast collagenase has been mutated and characterized by the activation of proenzyme, TIMP-1 binding, and catalysis (*31, 32*). The difficulty in activating the proforms of the mutants made it difficult to characterize them. Nevertheless, the highly decreased activity of the mutants suggested the importance of the glutamate in catalysis. The low but detectable activities of Ala and Gln mutants on a gelatin zymogram assay were explained by another residue in the enzyme that takes the place of the glutamate as a general base in catalysis (*31*). However, the available structural data on MMPs show that no alternative residues are observed within the vicinity of the substrate binding site capable of acting as a nucleophile.

The results presented in our study indicate Glu-198 plays a role but is not essential for catalysis, as its replacement by Asp, Cys, Gln, and Ala still retains catalytic activity. The order of the catalytic activity is Glu (wild-type) > Asp >> Cys > Gln > Ala. The decrease of activity is mainly due to a decrease in k_{cat} of the enzyme (Table 2). The differences of the activity of each mutant could come from the different effects of the substituted side chains in the active site environment. Asp is chosen because it has a pK_a similar to that of Glu but is shorter by one carbon atom, thus potentially reducing its efficiency of abstracting a proton from the zinc-bound water molecule. Cys has a much different type of potential nucleophile (SH vs COOH) that has a much higher pK_a than Glu. Gln is the same size as Glu and can form hydrogen bonds but, unlike Glu, is unable to act as a general base catalyst. Ala has no acid/base, hydrogen-bonding, or hydrophobic properties. The modification of the acid/base properties or length of side chain leads to decreased, but a relatively small effect on catalysis. The value of k_{cat}/K_m of E198D mutant is only 4-fold lower than that of wild-type. The detectable activities (>0.05% of k_{cat}/K_m for the wild-type) of E198Q and E198A indicate that the nucleophilic and acid/base properties of the carboxyl group are not obligatory for catalysis. The minor effect on the activity of the E198D mutation also suggests that the direct nucleophilic attack of Glu-198 on the substrate carbonyl to form a covalent anhydride intermediate is not likely due to the shortened distance results from the Asp replacement.

The high activity for the Asp mutant of the corresponding glutamate is also observed in other MMPs, such as gelatinase A and collagenase (*31, 32*). Replacement of Glu with Asp gives a 3- to 5-fold reduction in specific activity toward McaPLGLDpaAR substrate. The high and detectable activity of Glu mutants observed in MMPs is in contrast to mutations of the corresponding glutamate of the thermolysin-like neutral endopeptidase 24.11 (*33*), the neutral protease of *Bacillus stearothermophilus* MK232 (*34*), the neutral protease of *B. subtilis* (*35*), and mouse aminopeptidase A (*36*) (Table 4). No residual activity is detected toward synthetic peptides, casein, or gelatin even when the Glu residue is replaced with an Asp. Similar results are found in the case of the serine protease from *B. amiloliquefaciens*, in which mutations of the Ser and His located in the catalytic triad decrease k_{cat} by a factor of 2×10^6 (*37*). The marked contrast in the present result on matrilysin and those of the thermolysin-like enzymes implies that the conserved glutamate in matrilysin and other MMPs may play a different functional role in their catalysis from that proposed for the thermolysin-like enzymes. The lower catalytic efficiency of the E198A and E198Q mutants compared to the E198D and E198C enzymes suggests that acid/base properties of residue-198 are still important in catalysis.

In order to further probe the role of Glu-198, the pH dependence of the steady-state kinetic parameters was examined. The pH dependence of k_{cat}/K_m for the wild-type enzyme indicates the catalytically competent form of the enzyme requires ionization of a group having a pK_a of 4.3 (*29*). Glu-198 is a reasonable candidate based on the active site structure (Figure 1), the pK_a value, and previous mechanistic studies on CPD-A (*14*−*23*). If the γ-carboxylate of Glu-198 is responsible for the acidic pK_a of matrilysin,

Table 4: Site-Directed Mutagenesis of the Conserved Active Site Glutamate in Metalloproteases

	mutant form	substrate	activity	ref
neutral endopeptidase 24.11	Asp, Val	[tyrosyl-3,5-^{3}H]Leu-enkephalin	ND[a]	33
B. stearothermophilus neutral protease	Asp, Gln	casein	ND	34
B. subtilis neutral protease	Ala, Ser, Trp, Arg, Leu, Val, Cys	Cbz-Ala-Leu-Ala	ND	35
mouse aminopeptidase A	Asp, Ala	α-L-glutamyl-β-naphthylamide (GluNA)	ND	36
human fibroblast collagenase (MMP-1)	Asp	casein	35.2%	32
		McaPLGLDpaAR	39.1%	
	Gln	casein	ND	
		McaPLGLDpaAR	ND	
human gelatinase A (MMP-9)	Asp	McaPLGLDpaAR	10%	31
	Gln, Ala	McaPLGLDpaAR	ND	
		gelatin	0.01%	

[a] ND = not detectable.

we can expect an alteration of the pK_a when the chemical property of the side chain is altered. However, the pH profiles for k_{cat}/K_m for the mutant and wild-type enzymes are very similar and thus independent of the acid/base properties of the residue (Figure 2; Table 3). The largest change is observed for E198D where the pK_a value increases by 1.3 pH unit. This is quite striking since the pK_a value for the carboxylate group of an Asp is usually similar to that for a Glu residue. The Cys mutant is expected to increase the pK_a close to 8.0, but the observed pK_a value is increased only by 0.3. The pK_a of 4.6 is similar to that of the mutation of Tyr-219 to Phe which is not thought to be responsible for the pK_a (Cha and Auld, unpublished result). The Gln and Ala mutants, lacking any possible acid/base properties, also slightly increase the pK_a values by 0.8 and 1.1, respectively. Thus, although the pK_a of 4.3 for the pH−activity profile for the wild-type enzyme is reasonable for a carboxylate group such as Glu-198, the results of the pH dependence of k_{cat}/K_m for the Glu-198 mutants argue against it being responsible for the acidic pK_a of matrilysin.

An alternative role for Glu-198 is its involvement in catalysis as a general acid catalyst or to stabilize the transition state. The high activities of the wild-type and E198D enzymes compared to those of the other mutants suggest that Glu or Asp at this position is a critical requirement for high catalytic activity. The environment of the Glu-198 in matrilysin is quite hydrophobic since it is surrounded by the side chains of the highly conserved Ala and Phe residues that border the third and fourth His ligands to the "structural" zinc site. The effect of this hydrophobic environment might be to shift the pK_a of the Glu-198 to abnormally high values. It would remain in a protonated state in the ground state of the enzyme. It therefore could serve to stabilize a tetrahedral intermediate in the transition state through its protonated carboxyl group. The removal of this group would be expected to decrease activity but probably to a lesser extent than the loss of a crucial nucleophile.

The reduction of the catalytic activities (10^2 to 10^3-fold) observed in E198C, E198Q, and E198A by eliminating the carboxyl group compared to the wild-type is of the same order as that obtained when the His-231 residue proposed to stabilize the transition state of thermolysin is mutated to Ala or Phe (*38*). In addition, the mutation of Arg-127 of rat CPD-A to Lys, Met, or Ala reduces the k_{cat} value for the hydrolysis of Cbz-Gly-Gly-Phe by 50-, 1100-, and 1700-fold, respectively (*39*). The K_m values are increased only about 10-fold for all three mutants. Moreover, there is a direct correlation in the increase in K_i values for the potent phosphonate analogs of tripeptides (*40*) and the decrease in k_{cat}/K_m values for the corresponding peptides (*41*). The X-ray crystal structures of complexes of bovine CPD-A with phosphonate tripeptide inhibitors (*42*, *43*) reveal that the guanidinium group of Arg-127 interacts by a possible hydrogen bond with the phosphinyl oxygen (2.8−3.0 Å) that is coordinated to the zinc. These results have been interpreted as indicating that Arg-127 is important to catalysis by stabilization of the transition state. Therefore, Glu-198 in matrilysin may play a role for stabilizaton of its transition state through its protonated carboxyl group. Alternatively, it may act as a general acid catalyst after the rate-determining step by protonating the leaving amine product. In this case, the loss of this interaction might reduce activity by changing the rate-determining step. If Glu-198 is not the general base catalyst, the pK_a of 4.3 for matrilysin will likely be that for the ionization of the metal-bound water.

In summary, matrilysin-catalyzed hydrolysis of peptides likely occurs by the direct nucleophilic attack of the ionized zinc hydroxide on the carbonyl group of the substrate to form a tetrahedral intermediate. Glu-198 assists the zinc in stabilizing the transition state or acts as a general acid by donating a proton to the leaving amine as the metal-bound tetrahedral intermediate collapses to products in the next step. The proposed pK_a for the zinc-bound water in matrilysin is very low. However, the catalytic zinc of matrilysin is composed of three His residues, none of which are hydrogen bonded to an adjacent Asp residue as in CPD-A and thermolysin (Figure 1). The absence of a Glu carboxylate ligand for the catalytic zinc and indirect hydrogen bonds with Asp would increase the charge on the zinc in matrilysin as compared to CPD-A and thermolysin. This, in turn, would be expected to reduce the pK_a for the zinc-bound water. Further pre-steady-state kinetic and spectroscopic studies of native and cobalt-substituted matrilysin will help to further ascertain the steps in the reaction in which the metal-bound water and Glu-198 are involved in catalysis.

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