A Rationalization of the Acidic pH Dependence for Stromelysin-1 (Matrix Metalloproteinase-3) Catalysis and Inhibition*

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The pH dependence of matrix metalloproteinase (MMP) catalysis is described by a broad bell-shaped curve, indicating the involvement of two unspecified ionizable groups in proteolysis. Stromelysin-1 has a third pKₐ near 6, resulting in a uniquely sharp acidic catalytic optimum, which has recently been attributed to His₂²₂⁴. This suggests the presence of a critical, but unidentified, S₁' substructure. Integrating biochemical characterizations of inhibitor-enzyme interactions with active site topography from corresponding crystal structures, we isolated contributions to the pH dependence of catalysis and inhibition of active site residues Glu²₀₂ and His²₂₂⁴. The acidic pKₐ 5.6 is attributed to the Glu²₀₂-zinc-H₂O complex, consistent with a role for the invariant active site Glu as a general base in MMP catalysis. The His²₂₂⁴-dependent substructure is identified as a tripeptide (Pro²²¹-Leu²²²-Tyr²²³) that forms the substrate cleft lower wall. Substrate binding induces a β-conformation in this sequence, which extends and anchors the larger β-sheet of the enzyme-substrate complex and appears to be essential for productive substrate binding. Because the PXY tripeptide is strictly conserved among MMPs, this "β-anchor" may represent a common motif required for macromolecular substrate hydrolysis. The striking acidic profile of stromelysin-1 defined by the combined ionization of Glu²₀₂ and His²₂₂⁴ allows the design of highly selective inhibitors.

Matrix metalloproteinases (MMPs)¹ comprise a large family of extracellular calcium- and zinc-dependent, neutral endopeptidases. Substrate specificity among the individual members varies, but, as a group, they are capable of degrading all of the major macromolecules of the extracellular matrix. Consequently, controlled and coordinated MMP activities are essential during physiological events that require tissue remodeling or cellular migration. Proteolytic activity is modulated through a combination of regulated expression, activation, and autodegradation processes, as well as extracellular inhibition by specific natural inhibitors of MMPs, the TIMPs. If any of these interactions becomes even slightly dysregulated, MMP activity can contribute to the pathologies of cancer invasion and metastasis, arthritis, autoimmune disease, tissue ulceration, or cardiovascular disease (1–5). Because modulation of MMP activity could affect the progression of these diseases, MMP inhibitor development has been the target of considerable effort within the pharmaceutical industry (6, 7).

One of the hallmarks of the MMP family is the functionally distinct and structurally independent domain structure of these enzymes. The active site zinc is contained within a catalytic domain, which, when expressed independently, generally exhibits the catalytic activity and substrate specificity of the intact parent molecule (8–10). This truncated catalytic domain has been used successfully for both x-ray crystal and NMR solution studies, yielding structures that agree well with the two full-length MMP structures (reviewed in Refs. 11 and 12). The MMPs share significant primary sequence homology (30–99% identity, 49–99% similarity) (13), and the structures describe a common protein fold (14) consisting of three α-helices packed against a twisted 5-stranded β-sheet. The second helix (αB) and contiguous loop of this domain contain the zinc-binding active site followed by a series of turns that define the S₁' binding site. The substrate cleft is quite shallow, and much of the binding energy and enzyme specificity for substrates and inhibitors seems to derive from interactions within the hydrophobic S₁' pocket (15).

MMPs (or matrixins), along with astacins, adamalysins, and serralysins belong to the metzincin superfamily of zinc peptidases (16). This classification is based on two conserved sequence motifs. The first, an extended active site consensus sequence, HEXXHXXXXXX, provides three histidine ligands for the catalytic zinc. The second, an adjacent 1–4 β-tight turn (the "Met-turn"), positions an invariant methionine beneath the zinc to support the active site configuration. This substructure is critically important to the catalytic function of these enzymes and is maintained by an elaborate hydrogen bonding network (17, 18). The metzincins are distantly related to the thermolysin family of metalloproteinases, sharing some structural features and a limited active site motif (HEXXH), and it has been proposed that these enzymes share a similar mechanism of catalysis (19, 11). Both families demonstrate broad bell-shaped curves (pH 4.5–9.5) for the dependence of catalysis on pH, indicating the involvement of two ionizable groups in the proteolytic process (19–21). However, these groups have not been unequivocally identified for either enzyme family, and the specific mechanism of catalysis remains controversial (22, 23). The current availability of a large body of crystal structures of metalloproteinases complexed with substrate analog inhibitors has allowed an extended comparison of structure-function relationships. The correlation of biochemical characteristics with the conserved features of this broader group of enzymes could identify the common mechanistic details of zinc-

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¶ The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; CD, catalytic domain.
catalyzed proteolysis.

Several models have been advanced to describe metalloproteinase catalysis. One of the earliest schemes, proposed for thermolysin (20, 24) and often applied to MMPs (11, 25), describes a mechanism of general base catalysis. In this model, the invariant active site glutamic acid (HExXH, Glu143 in thermolysin, Glu198 in matrilysin, and Glu202 in stromelysin) acts on the scissile peptide bond through a zinc-bound water molecule and is required for catalysis. The water molecule is polarized, but not ionized in the active configuration of the enzyme, resulting in the destabilization of one of the protons between the zinc-bound water and the negatively charged glutamate. The acidic pKₐ of 5 was believed to be too low for the zinc-water complex and was attributed, at least in early models, to the combined system of Glu143 and the zinc-bound water (20).

A recent study of Glu198 mutations in matrilysin suggested that MMP catalysis proceeds through a specific base mechanism (23). The acidic pKₐ of matrilysin was not attributed to the active site glutamic acid because the mutants retained the characteristic bell-shaped curves for the pH dependence of catalysis, describing the same pKₐ values as the wild-type parent. Mutant catalytic efficiency was significantly reduced compared with wild-type matrilysin (Glu→Asp→Cys→Gln→Ala), but it was still detectable, suggesting an auxiliary role for this residue after the initial rate-determining step. In this model, the zinc-hydroxide nucophile attacks the peptide bond directly, and the acidic pKₐ of 4.3 is assigned to the zinc-H₂O complex. It was proposed that Glu198 (proposed pKₐ > 9) is protonated in the enzyme ground state and acts either to stabilize the transition state or as a general acid to protonate the leaving amino group (23). These pKₐ values are quite unusual for the assigned groups, but this was attributed to the exceptionally hydrophobic character of the MMP active site.

The pH activity profile of human stromelysin-1 is somewhat more complicated than that observed for other MMPs due to the presence of a third pKₐ, at about 6 (26, 27). This produces an unusually sharp catalytic optimum at pH 5.9, followed by a broad shoulder of more limited catalysis through pH 9. Catalytic efficiency at neutral pH is about one-third that measured at the pH optimum. This distinctive pH profile has been observed for both the full-length and truncated enzymes, using either natural macromolecular or synthetic peptide substrates (26–29), and appears to be an intrinsic property of the enzyme. The unique pH-dependent activity of stromelysin-1 may provide physiologically important posttranslational regulation because at acidic pH, the latent enzyme undergoes autoactivation more readily (30), binds TIMP-1 less avidly (31), and is more susceptible to autodegradation (8, 26, 27). This phenomenon may play a role in human pathologies as well, because the progression of osteoarthritis (30) and the invasive behavior of some cancer cells (32) are enhanced under localized acidic conditions.

Recently, it was shown that the additional stromelysin pKₐ represents the ionization of His224 (27). This residue is unique to human stromelysin-1 and resides in the flexible outer loop of the S1′ pocket. In its protonated state, His224 forms a hydrogen bond across the pocket lumen with the backbone carboxyl of Ala217, a residue immediately preceding the Met-turn (33, 34). This interaction appears to be critical to substrate binding because above the pKₐ of His224 (pKₐ 6.2), stromelysin catalytic efficiency decreases dramatically as a result of increasing Kᵢ. The mutation of His224 to glutamine (27) or asparagine (35) releases stromelysin from its acidic optimum and results in mutants that demonstrate the simple bell-shaped curve for pH dependence of catalysis characteristic of other members of the metzincin superfamily. The maximum catalytic efficiency of the H224Q mutant is expressed over a broad pH range (pH 5.8 to 9.5) and was more than twice the value determined for stromelysin at the wild-type acidic optimum. These data indicate that an intact S1′ substructure is essential for enzyme function and that its loss could be responsible for the attenuated activity of the native enzyme at neutral pH. However, the specific substructure has not been identified.

In the studies described here, we use the catalytic domain of human stromelysin-1 (MMP-3 CD) to relate to the pH dependence of inhibition to specific atomic interactions described by x-ray crystal structures of the enzyme-inhibitor complexes. Using a cognate pair of inhibitors bearing either a carboxylic or hydroxamic acid as the zinc-binding functional group, we demonstrate a strong pH dependence of inhibitor potency for human stromelysin-1. Because these inhibitors share the same P1′ group, differences in potency were predicted to be due to interactions with one or more ionizable residues close to the active site zinc. The pH dependence of inhibition was also determined for a hydroxamic acid-containing peptidic inhibitor. This compound has a fairly small P1′ group, which does not displace the His224 hydrogen bond in the S1′ pocket. Comparison of the relationship of Kᵢ to pH for these three compounds allowed us to isolate the specific contributions to inhibitor binding from the ionization of key active site residues. The ionization constants determined from the inhibitor studies could be correlated to these pH values describing the catalytic efficiency of stromelysin-1 over this same pH range, contributing to the characterization of interactions that define the mechanism of catalysis for the broader family of zinc metalloproteinases. This information is useful in the rational design of potent and selective inhibitors for human stromelysin-1.

EXPERIMENTAL PROCEDURES

Materials—Superbroth was purchased from Difgen (Beltsville, MD). Ampicillin and chloramphenicol were from Roche Molecular Biochemicals. The thiopetide substrate Ac-Pro-Leu-Gly-[2-mercapto-4-methylpentanoyl]-Leu-Gly-OEt was from BACHEM Bioscience (King of Prussia, PA). Other chemicals were from Sigma.

Stromelysin-1 Expression and Purification—The construction of the expression vector for human stromelysin-1 catalytic domain has been described (8). Two-liter cultures of the freshly transformed Escherichia coli strain BL21(DE3)pLysS (Novagen, Madison, WI) were grown over-night in shaking flasks at 37 °C in Superbroth supplemented with ampicillin (50 μg/ml) and chloramphenicol (50 μg/ml) and induced with 1 mM isopropyl β-thiogalactopyranoside for 2 h before harvesting by centrifugation. Recombinant enzyme was strongly expressed as insoluble protein under these conditions and represented about 25% of the cellular protein. Active catalytic domain was refolded and purified as described previously (8), except that refolded material was centrifuged, concentrated, dialyzed, and applied directly to a Q-Sepharose column (Amersham Pharmacia Biotech) without ammonium sulfate fractionation.

Enzyme Assays—Because there are no mechanistic differences between thioester and amide hydrolysis for human stromelysin-1 (29), enzyme activity could be determined from the initial rates for the hydrolysis of a thiopetide substrate coupled to the reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (8). The change of absorbance at 405 nm was monitored continuously at room temperature using a SPEC-TRAMax 340 microplate reader (Molecular Devices, Sunnyvale, CA). A 50 μM substrate, 1 mM CaCl₂, 100 mM N-(morpholino)ethanesulfonic acid, 10 mM Tris·HCl buffer (pH 8.0) was chosen to provide constant ionic strength over the buffering range of the studies (36). Enzyme concentration was varied from 5 to 21 nM to yield a uniform rate of reaction at each pH. For each determination, inhibitors were included in the assay at approximate concentration, resulting in a final reaction mixture concentration of 2% dimethyl sulfoxide. At basic pH the substrate can undergo hydroxide-catalyzed hydrolysis, and at acidic pH, the 2-nitro-5-thiobenzoate product of 5,5′-dithio-bis(2-nitrobenzoic acid) becomes protonated (pKₐ 4.5) and undetectable, limiting the practical range of the study to pH 5–8. The spontaneous background rate of reaction at each pH condition...
Km was subtracted from the enzyme-catalyzed rate for all reaction velocity determinations. 

Steady State Kinetic Determinations—The inhibitors being studied bind in the enzyme active site (34). Using experimentally derived IC50 values, inhibitor binding affinity, $K_i$, could be calculated from the relationships $K_i = IC_{50}/[1 + [S]/K_m]$ or $K_i = (IC_{50} - [E]/2)/([S]/K_m)$, where the $K_i$ value was comparable to the enzyme concentration (37, 38). Estimations of $K_m$ and $k_{cat}$ at each pH were obtained from plots of reaction velocity versus substrate concentration assuming a Michaelis-Menten mechanism and using substrate concentrations ranging from 0 to 2 mM. The least squares fit of the data to a hyperbola was calculated using the equation $v = (K_m[S]/([1 + [S]/K_m]))$ (see Refs. 28 and 39). The thiopeptolide substrate has no ionizable groups between pH 5 and 9, so the pH dependence of $k_{cat}/K_m$ represents the ionization of the free enzyme (39). The data for pH dependence were fit to the equation for the ionizing system described in Fig. 1 using the general relationship for a pH-dependent variable $L_1$ (Equation 1) (see Refs. 28 and 39).

$$L_1 = \frac{[H^+]^{K_{L1}}[H^+]^{K_{L1}}}{{[H^+]^{K_{L1}}[H^+]^{K_{L1}}}}$$

The pH dependence of $k_{cat}/K_m$, illustrated in Fig. 1 describes a mechanism in which two catalytic routes lead to product, and $L_{E3}$ and $L_R$ equal zero. Where an inhibitor binds to only one form of the enzyme, the relationship of $L_{E1}$ to pH can be used to determine the $pK_a$ values of inhibition. For Compound I, $L_{E3}$, $L_{E1}$ and $L_R$ are equal to zero. For Compound II and U24522, $L_{E3}$, $L_{E1}$, and $L_R$ are equal to zero. This study was designed to investigate the acidic pH dependence of stromelysin-1 catalysis and inhibition. Because the practical basic pH limit of the assay system was near pH 8, the value for $pK_{a3}$ was determined from the pH dependence of $k_{cat}/K_m$ and set at 8.5 in the inhibitor relationships. No further work was done to characterize that parameter. Theoretical curves were fit to the data and solved for $pK_a$ and $pK_{aa}$ using the KaleidaGraph software (Synergy Software, Reading, PA).

Inhibitors—Compound I and Compound II were synthesized as part of the medicinal chemistry program supporting the development of selective MMP-3 inhibitors (40, 41). U24522 was synthesized at Parke-Davis as a reference standard representing a broad-spectrum, substrate-based MMP inhibitor (42). The inhibitor-enzyme complexes have been described (33, 34).

RESULTS

pH Dependence of Catalysis for the Human Stromelysin-1 Catalytic Domain—A plot of the relationship of $k_{cat}/K_m$ to pH for human stromelysin-1 catalysis yields a complex curve with a sharp acidic optimum at pH 5.9, followed by a broad shoulder of significantly reduced catalytic activity (Fig. 2). The observed catalytic efficiency at pH 7.0 is one-third that measured at pH 6.0. This lowered catalytic efficiency is dominated by increasing $K_m$, whereas $k_{cat}$ remains essentially constant over this pH range. The $pK_a$ values derived from the pH dependence of stromelysin catalysis describe the ionization of three groups involved in the catalytic process (28). This relationship indicates that there are two pathways to product for stromelysin catalysis (Fig. 1). Although the substrate can bind productively to two forms of the enzyme, $E_H$ and $E_L$, the catalytic efficiency associated with the $E_H$ complex is significantly attenuated compared with $E_H$.S. For example, we find that $(k_{cat}/K_m)_{E_H}$ is equal to 102,890 M$^{-1}$ s$^{-1}$, whereas $(k_{cat}/K_m)_{E_L}$ is 5393 M$^{-1}$ s$^{-1}$.

pH-dependent Inhibitor Interactions—Three compounds were selected to study the pH dependence of inhibitor potency for stromelysin-1 (Fig. 3). The cognate pair, Compound I and Compound II, was chosen to isolate titratable interactions with active site residues. Crystal structures of inhibitor-MMP-3 CD complexes indicate that these two compounds share a common binding mode within the S1’ pocket, and differences in inhibitor potencies should be attributable to specific interactions between the inhibitor zinc-binding groups and residues in the immediate vicinity of the catalytic zinc. We estimate that in aqueous solution, the $pK_a$ of the carboxylic acid of Compound I is between 4 and 5, whereas the $pK_a$ of the hydroxamic acid of Compound II is about 9. Thus, over the pH range of these studies, Compound I is predominantly ionized, and Compound II is mostly neutral.

We were specifically interested in characterizing the pH profile of Glu202 in the stromelysin-1 active site. Typically, the $pK_a$ of a side-chain carboxyl group in a protein is between 4.0 and 4.8, but this value depends on the hydrophobic character of the local environment and has been reported as high as 7.3 for some proteins (43). The ability of Glu202 to act as a general base in stromelysin-catalyzed hydrolysis depends on this residue being ionized in the catalytically active form of the enzyme. To act as a general acid or to stabilize the transition state as proposed for matrilysin (23), it should be protonated throughout this pH range.

Titratable interactions specific to the S1’ pocket were additionally probed with U24522. This peptidic inhibitor and Compound II both have a hydroxamate as their zinc-chelating group but differ significantly in their interactions with the S1’ subsite. U24522 has a relatively small P1’ group, and the His254 hydrogen bond across the pocket is intact in the enzyme-inhibitor complex (33). Much of the inhibitor potency of this compound results from main chain contacts in the substrate cleft, and U24522 was predicted to mimic substrate interactions with the enzyme.

In contrast, diphenylpiperidine inhibitors, the class of compounds represented by Compound I and Compound II, bind...
deeply in the S1’ pocket and derive considerable binding energy from aromatic interactions with His224 (35). The binding of these inhibitors replaces the normal His224 hydrogen bond with an indirect bidentate hydrogen bond to the Ala117 and Thr215 backbone carbonyls through a tightly bound water molecule (34). Inhibitor-MMP-3 CD complexes were crystallized between pH 5.5 and 6.5, where sufficient protonated His224 is available to support the formation of this stabilizing (direct or indirect) hydrogen bond. In fact, we have been unable to identify a crystallographic condition for S1’-binding inhibitors that does not result in either the direct or indirect His224 hydrogen bond, supporting the previously reported biochemical importance of this substructure (27). If this S1’ substructure is required for inhibitor binding as well as catalysis, the potencies of all three compounds will decrease as pH increases above the pK_{a} of His224.

**Configuration of the Active Site in Inhibitor-Enzyme Crystal Structures**—Carboxylate and hydroxamate inhibitors form complexes that require different coordination states of the active site zinc (II) ion (Fig. 4). In all cases, the zinc-bound water has been displaced by the inhibitor, with different functionalities within the zinc-chelating groups assuming the position of the catalytic water in the proposed reaction intermediate.

The hydroxamic acid group of U24522 (or Compound II) provides the fourth and fifth ligands to a trigonal bipyramidal zinc complex (Fig. 4A) that mimics the reaction intermediate formed as the zinc-bound water attacks the scissile peptide bond (Fig. 4B). The inhibitor carbonyl corresponds to the substrate α-carbonyl group, and the hydroxyl group replaces the catalytic water. The nitrogen of the hydroxamate provides some of the interactions predicted for the scissile amide group, but the transposition of carbon and nitrogen compared with a peptide bond precludes truly analogous interactions. The zinc configuration places the carbonyl oxygen atom in the trigonal plane and the hydroxyl oxygen at one apex near Glu202. Crystal structures of hydroxamic acid inhibitors complexed with MMP-3 CD (34) indicate that the inhibitor hydroxyl group forms a hydrogen bond with one of the Glu202 ε-oxygens. However, the substrate-bound enzyme complex probably includes a bidentate coordination between the catalytic water and both oxygens of Glu202 (Fig. 4B). The active site carboxylate could thus act as a general base by enhancing the zinc-catalyzed polarization of the catalytic water through the negatively charged Oε-1, while positioning the emerging hydroxide for a nucleophilic attack on the α-carbon through hydrogen bonding to Oε-2. An additional hydrogen bond between the “catalytic” proton and the adjacent scissile amino group in the proposed intermediate allows the simultaneous catalytic transfer of a proton to the leaving amino group and formation of the hydroxyl-α-carbon bond. Because the neutral hydroxamic acid inhibitors require that the active site glutamate be negatively charged for effective interaction, the binding efficiency for Compound II and U24522 will decrease with pH below the pK_{a} of Glu202.

The carboxylate of Compound I provides the fourth ligand in a tetracoordinated zinc complex that corresponds to the proposed transition state adopted by the enzyme-substrate complex as the scissile peptide bond is cleaved. Fig. 4C shows the active site configuration from the Compound I-MMP-3 CD crystal structure, and Fig. 4D represents a model of the corresponding transition state. The extra carbon bond between the carboxylate and the amide group of the inhibitor mimics the increasing distance between the peptide leaving groups as the tetrahedral intermediate collapses. The crystal structure indicates that a single proton must be shared between Oε-1 of Glu202 and the inhibitor carboxylate to avoid the repulsion resulting from two negatively charged groups in such close proximity. In the proposed tetrahedral intermediate, this proton would be shuttled from the newly formed carboxylic acid to the α-amino leaving group. By analogy to the inhibitor complex, this transfer is facilitated by a transient association with Glu202 and the resulting salt bridge between Glu202 and the newly generated protonated primary amine could provide additional stabilization for the transition state. Glu202 must be protonated to allow Compound I to bind because the principal active site interaction is mediated through a negatively charged inhibitor carboxylate. Consequently, Compound I is expected to lose potency above the pK_{a} of Glu202.

**pH Dependence of Inhibitor Potency**—Fig. 5 illustrates the pH dependence for K_{i} of these three inhibitors for stromelysin-1. The number of protons transferred as pH increases can be determined from the slopes of the Dixon-Webb plots of these relationships. The expected pK_{a} values of the Compound I carboxylate (pK_{a} < 5) and the Compound II and U24522 hydroxamic acids (pK_{a} > 8) are outside of the pH range of these studies and are not considered further. The two acidic pK_{a} values defined by the pH dependence of the K_{i} values for the inhibitors (Table I) are consistent with the mechanism described in Fig. 1 if pK_{a,1} = 5.6 is attributed to Glu202 and pK_{a,2} = 6.3 to His224.

Compound II and U24522 show first order dependence of inhibitor potency on pH with pK_{a} values of 5.6 and 6.2. The K_{i} values increase above and below these pK_{a} values and remain constant in between. The pH dependence of K_{i} in the acidic region is consistent with the involvement of two ionizable groups in inhibitor binding, with the tightest inhibitor-enzyme complexes formed with EH2, where Glu202 is ionized and His224 is protonated.

The pH dependence of K_{i} for Compound I also indicates the involvement of two ionizable groups in inhibitor binding. K_{i} remains constant below pK_{a,1} increases with a slope of 1 between pK_{a,1} and pK_{a,2}, and shows second order dependence on pH above pK_{a,2}. This relationship indicates that Compound I...
**DISCUSSION**

Using the results from the inhibitor studies described here, it is possible to assign a $pK_{a,I}$ of 5.6 to the Glu$^{202}$-H$_2$O-zinc complex because all three inhibitors displace the zinc-bound water during binding to the catalytically active MMP-3 CD. This identification of $pK_{a,I}$ is consistent with our parallel characterization of the pH dependence of $k_{cat}/K_m$. These data indicate that the active site glutamic acid of stromelysin is ionized in the active EH$_2$ and EH forms of the enzyme (Figs. 1 and 4), as required for a role as the general base in zinc-catalyzed hydrolysis. This proposed mechanism has a significant implication for the design of synthetic inhibitors of metalloproteinases. It is apparent from the pH dependence of inhibitor potency for human stromelysin-1. The model for the equations used to fit these relationships is consistent with the proposed mechanism of inhibitor binding.

**Inhibitor Analog**

**Reaction Intermediate**

**General Base Catalysis**

**Cleavage of Scissile Bond**

**Compound I**

**Compound II and U24522**

**Table I** summarizes the $pK_a$ values that define the pH dependence of both inhibition and catalysis for human stromelysin-1. The model for the equations used to fit these relationships is consistent with the proposed mechanism of inhibitor binding.

**Active site coordination of Compound I and Compound II with stromelysin catalytic domain and proposed reaction intermediates. A, trigonal bipyramidal coordination of the stromelysin-1 active site zinc complexed with hydroxamate inhibitors Compound II and U24522. B, proposed reaction intermediate corresponding to hydroxamate inhibitor analog representing the attack of the polarized catalytic water on the scissile peptide bond. C, tetrahedral coordination of zinc complexed with Compound I. D, proposed transition state corresponding to the carboxylate inhibitor analog representing the cleavage of the scissile peptide bond.**

**General Base**

**Attack of Scissile Bond**

**Catalytic Water**

**EH2**

**EH1 through the ionization of Glu202 results in the formation of EH2. Overall, there is a net transfer of two protons from EH$_2$I complex to form EH + I through the ionization of Glu$_{202}^{}$ (p$K_{a,I}$) and the deprotonation of His$_{224}^{224}$ (p$K_{a,2}$).

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**FIG. 4.** Active site coordination of Compound I and Compound II with stromelysin catalytic domain and proposed reaction intermediates. A, trigonal bipyramidal coordination of the stromelysin-1 active site zinc complexed with hydroxamate inhibitors Compound II and U24522. B, proposed reaction intermediate corresponding to hydroxamate inhibitor analog representing the attack of the polarized catalytic water on the scissile peptide bond. C, tetrahedral coordination of zinc complexed with Compound I. D, proposed transition state corresponding to the carboxylate inhibitor analog representing the cleavage of the scissile peptide bond. E, proposed model of the zinc-bound catalytic water polarized by negatively charged active site glutamic acid (Glu$^{202}$ in stromelysin-1).

can only form an effective complex with EH$_{II}$, where both Glu$_{202}^{}$ and His$_{224}^{224}$ are protonated. Below $pK_{a,I}$, Glu$_{202}^{}$ is neutral, whereas the inhibitor is ionized, and $K_r$ remains at its limiting value. From $pK_{a,I}$ to $pK_{a,2}$, the ionization of Glu$_{202}^{}$ results in the formation of EH$_2$. Overall, there is a net transfer of two protons from EH$_2$I complex to form EH + I through the ionization of Glu$_{202}^{}$ ($pK_{a,I}$) and the deprotonation of His$_{224}^{224}$ ($pK_{a,2}$).

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in Fig. 1). In contrast, productive substrate binding is possible with either EH 2 or EH. It is especially notable that U24522, despite the extensive substrate-like interactions both along the substrate cleft and within the S1 pocket, differs significantly from substrate in its strict dependence on the presence of the His 224-sensitive substructure. All three inhibitors bind only on the primed side of the substrate cleft, whereas the substrate spans the cleft on both sides of the active site. Above the pKₐ of His 224, it appears that substrate binding on the unprimed side is sufficient to allow the subsequent, although somewhat limited, stabilization of this critical substructure, which permits productive substrate interaction with the active site. However, the significant increase in Kₐ under these conditions is an indication of the inefficiency of this process, and the resulting catalysis of the EH-S complex is represented by the broad shoulder of reduced catalytic efficiency above pH 7 in the pH profile of catalysis.

The importance of the S1’ substructure may derive from the

**FIG. 5.** Dixon-Webb plots describing the pH dependence of stromelysin-1 inhibitors. Closed circles, Compound I, an S₁′-binding carboxylate inhibitor; open circles, Compound II, an S₁'-binding hydroxamate; open squares, U24522, a peptidic hydroxamate. Error bars represent the S.D. of five Kᵢ determinations. A, a direct comparison of pH dependence of inhibitor potency, Kᵢ, for the three inhibitors. B, curve fit of 1/Kᵢ for Compound I. Fitted line was drawn according to Equation 1, where LₙH = 1/Kᵢ, Lₚ₃H = 0, Lₘ₃H = 0, Lₘₐ = 0. This model describes the pH-dependent behavior of an inhibitor that only binds to the Lₚ₃₃₃ form of the enzyme (Glu 202 is neutral and His 224 is protonated). pKₐ was set at 8.5, and data were solved for pKₐ₁ (5.7) and pKₐ₂ (6.3), r = 0.94. C, curve fit of 1/Kᵢ for Compound II. The line shown was fit to the data using Equation 1, where LₙH = 1/Kᵢ, Lₚ₃₃₃ = 0, Lₘ₃H = 0, and Lₘₐ = 0. This model describes an inhibitor that only binds to the Lₚ₃₃₃ form of the enzyme (Glu 202 is ionized and His 224 is protonated). pKₐ was set at 8.5, and data were solved for pKₐ₁ (5.4) and pKₐ₂ (6.2), r = 0.93. D, curve fit of 1/Kᵢ for U24522. The line shown was fit to the data using the model described for Compound II. Data were solved for pKₐ₁ (5.8) and pKₐ₂ (6.1), r = 0.93.
requirements imposed on matrixins by the natural substrates they digest. The complex matrix macromolecules are bulky and highly structured, requiring a shallow and solvent-exposed substrate-binding cleft on the surface of the enzyme. Because these substrates are extraordinarily resistant to proteolytic degradation, the matrixins have adapted by inducing a localized β-strand configuration in the substrate near the active site that exposes the scissile bond to proteolytic attack (see Ref. 17). This substrate interaction is held tightly in place through extensive antiparallel contacts to the enzyme β4-strand (see Fig. 6 for primed side interactions). The β-strand conformational change is probably facilitated to varying degrees by interactions with other domains, depending on the particular enzyme and substrate (10, 12, 45). At least in the case of collagen cleavage, productive substrate binding requires coordinate interactions with the collagenase C-terminal domain. It is possible that the critical S1’ substructure attributed to His224 in stromelysin-1 contributes to this induced β-strand substrate conformation and is maintained in other MMPs by nonionizing S1’ interactions.

During catalysis, the planar peptide bond passes through a tetrahedral intermediate that requires a significant spatial displacement of the residues on either side of the scissile bond. To accommodate this movement of bound substrate, the matrixins provide open and somewhat indistinct pockets on the unprimed side of the active site, whereas primed side interactions remain tight and relatively inflexible to preserve critical catalytic contacts with the zinc in the transition state. In crystal structures of MMP-3 CD with peptidic inhibitors, it is evident that primed side binding is maintained by important substrate interactions within the S1’ pocket, but it is enhanced by additional β-strand interactions with the tripeptide Pro221-Leu222-Tyr223 (“β-anchor”). In the model described in Fig. 6, the formation of the substrate-enzyme complex extends the five stranded β-sheet of the active enzyme by two strands on the primed side: (i) the induced β-strand of the substrate, and (ii) the β-anchor that forms the bottom wall of the substrate cleft. This structure locks the substrate in place and increases the hydrophobicity of the active site to enhance the Lewis acid potential of the catalytic zinc.

The proline and tyrosine residues in the β-anchor are absolutely conserved in the MMP family, and both are required to position and maintain this strand as part of the substrate cleft.

*Fig. 6.* Proposed β-sheet structure of enzyme-substrate complex emphasizing the critical pH-sensitive, His224 dependent S1’ substructure. Extended β-strand conformation within the substrate cleft is induced in the macromolecular matrix substrate upon binding to the enzyme (top). This in turn induces the β-configuration in the β-anchor (the Pro221-Leu222-Tyr223 tripeptide in stromelysin-1), which extends and strengthens the overall enzyme β-sheet structure in the enzyme-substrate complex (bottom).

### Table I

| Profile                  | $pK_a^1$ | $pK_a^2$ | $R$ |
|-------------------------|---------|---------|-----|
| Thiopeptolide cleavage  |         |         |     |
| $k_{cat}/K_M$            | 5.8     | 6.0     | 0.99|
| Inhibition              |         |         |     |
| Compound I              | 5.7     | 6.3     | 0.94|
| Compound II             | 5.4     | 6.2     | 0.93|
| U24522                  | 5.8     | 6.1     | 0.93|
| Ionizable group         | Glu-202 · H2O · zinc | His-224 |
rigid constraints of its cyclic structure. The firmly anchored Pro221 carboxyl may also form indirect hydrogen bonds with the scissile carboxyl and amino groups through a tightly bound water molecule (15), an interaction that would lend additional stabilization to the β-anchor and the tetrahedral intermediate.

Tyr223 lies in a hydrophobic pocket near the beginning of the active site helix (αB), surrounded by the side chains of three highly conserved residues (Leu164, Trp196, and Val198). A fairly weak hydrogen bond between Tyr223 and a backbone amide (Asn194) augments this binding interaction. Leu225 apparently acts as a spacer with the side chain extending into the bulk solvent and makes little contact with the main chain atoms of the substrate. Not surprisingly, there is little conservation of this residue among the matrixins.

In human stromelysin-1, the His224 hydrogen bond provides additional and apparently essential stabilization of this β-strand substructure. A recent MMP-3-thiadiazole inhibitor crystal structure illustrates what happens to the β-anchor when the S1’ hydrogen bond is disrupted (46). This inhibitor binds on the unprimed side of the active site and demonstrates amazing selectivity for human stromelysin-1 over collagenase and gelatinase. The enzyme-inhibitor complex was formed at pH 7.5, where the His224 hydrogen bond is dis favored, and was subsequently crystallized at pH 7.0. Without the stabilizing S1’ hydrogen bond, His224 has rotated into the substrate cleft, and Tyr223 has moved 7 Å toward the active site to bind firmly within the S1’ pocket. This conformation would interfere with the proposed β-strand substrate interactions and may be largely responsible for the reported stromelysin-1 selectivity of this class of inhibitors. In addition, the presence of the intramolecular Tyr223 in the S1’ pocket would effectively block the primed side binding of inhibitor or substrate. Apparently, at pH > 6.3, substrate binding that initiates on the unprimed side of the stromelysin-1 active site is sufficient to allow a reorientation of the β-anchor with the ensuing (but much reduced) catalytic activity characteristic of this enzyme.

The studies described here illustrate the value of relating empirically determined biochemical properties of enzymes with the corresponding atomic relationships deduced from crystal structures. The pH dependence of inhibition and catalysis of human stromelysin-1 support a model of general base catalysis characteristic of this enzyme.

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A Rationalization of the Acidic pH Dependence for Stromelysin-1 (Matrix Metalloproteinase-3) Catalysis and Inhibition

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