Role of His-224 in the Anomalous pH Dependence of Human Stromelysin-1†

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ABSTRACT: A plot of the pH dependence of k_{cat}/K_M for human stromelysin-1 (HS) exhibits a narrow range of maximal activity extending from pH 5.75 to 6.25 and a broad shoulder in the pH range of 7.5−8.5. In contrast, the pH profiles that have been reported for other members of the matrix metalloproteinase (MMP) family are bell-shaped and exhibit neutral pH optima. We hypothesized that the anomalous pH dependence of HS reflects the ionization of His-224, a residue located in a flexible loop that contributes to the S_1' binding pocket of the enzyme. HS is the only known MMP that has a histidine in this position. To test this hypothesis, the H224Q mutant of the short form (lacking the C-terminal hemopexin-like domain) of HS (sHS) has been prepared and studied. The pH profile of H224Q sHS is bell-shaped and similar to those reported for other MMPs. Although H224Q and wild-type sHS possess similar activities at pH <6, the k_{cat}/K_M of H224Q sHS is more than 5-fold greater than that of the wild-type enzyme at pH >7. These data strongly suggest that the deprotonation of His-224 attenuates the activity of HS, thereby accounting for its low pH optimum and the characteristic shoulder in its pH profile. This attenuation of activity appears to be predominantly a K_M effect, reflecting a decrease in the affinity of the enzyme for the peptide substrate.

Human stromelysin-1 (HS,[1] MMP-3, EC 3.4.24.17) is a member of the matrix metalloproteinase (MMP) family of zinc proteinases that is believed to play a major role in the degradation of macromolecular components of the extracellular matrix (*1*, *2*). Other members of the MMP family include stromelysins-2 and -3, matrilysin, collagenases-1, -2, and -3, gelatinases A and B, and four membrane-type MMPs (*3*). Individual MMPs have been implicated in the destruction of tissue that accompanies a number of pathological processes (*2*, *4*) and, as such, are prime targets for the development of novel chemotherapeutic agents (*5*). In particular, HS has been implicated in the destruction of articular cartilage in both rheumatoid and osteoarthritis. HS cleaves several matrix components of cartilage, including the core protein of aggrecan, link protein, and the telopeptides of type II collagen that contain the intermolecular cross-links to type IX collagen (*6*). The enzyme has also been implicated in the activation of the zymogens of other MMPs, including procollagenase-1, -2, and -3 (*6*). Thus, HS is a particularly attractive target for arthritis therapy (*7*).

The individual members of the MMP family exhibit distinct functional differences that are likely related to their diverse physiological roles. For example, they differ with respect to their mechanism of activation (*8*−*10*), catalytic efficiency, and peptide and protein substrate specificities (*11*−*14*). With respect to catalytic properties, HS exhibits a unique pH profile that differs markedly from the profiles of other MMPs (*15*). In particular, HS has a narrow acidic pH optimum extending from about pH 5.75 to 6.25, while other MMPs have broader pH optima ranging from neutral to basic pH. It is essential to attain a basic understanding of the kinetic properties of the MMP family members both to advance our ability to rationalize their physiological actions and to facilitate the preparation of potent, specific inhibitors.

Three-dimensional structures have been published for HS (*5*, *7*, *16*, *17*), collagenase-1 (MMP-1, EC 3.4.24.7) (*18*−*20*), collagenase-2 (MMP-8, EC 3.4.24.34) (*21*−*23*), and matrilysin (MMP-7, EC 3.4.24.3) (*24*). These structures are useful starting points with which to explain the pH dependence of these MMPs. In this paper, the source of the unusual pH dependence of k_{cat}/K_M for HS has been investigated. On the basis of several lines of evidence, including the overall conformation of the MMPs revealed in X-ray structures, we hypothesized that the titration of His-224 [numbering according to Nagase (*6*)], a residue not present in other MMPs, was responsible for the unusual pH dependence of HS. To test this theory, the H224Q mutant of the short form (e.g., the truncated form of the enzyme lacking the C-terminal hemopexin-like domain) of HS (sHS) has been prepared. The anomalous pH profile of wild-type (WT) sHS is transformed

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[1] Abbreviations: MMP, matrix metalloproteinase; HS, human stromelysin-1; sHS, short form of HS lacking the C-terminal hemopexin-like domain; H224Q, sHS mutant with His-224 replaced by Gln; WT, wild-type; LB_{amp}, Luria broth containing ampicillin; IPTG, isopropyl thiogalactopyranoside; APMA, 4-(aminophenyl)mercuric acetate; Hepes, 4-(2-hyroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Capso, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, *N*-3-(2,4-dinitrophenyl)-2,3-diaminopropionyl; Dnp, 2,4-dinitrophenyl; TIMP, tissue inhibitor of metalloproteinases.

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into a normal bell-shaped curve in the H224Q mutant, supporting the postulated effect of His-224.

MATERIALS AND METHODS

Construction of WT and H224Q sHS Expression Plasmids. A section of DNA encoding the Ala-0−Thr-255 portion of HS corresponding to the propeptide and catalytic domains (pro-HS) was ligated into an expression plasmid employing the T7 gene 10 promoter (C.-C. Kan and M. Gehring, unpublished results). The H224Q mutation was achieved with the method of Kunkel (*25*) using the oligonucleotide 5′-CCCACTCTATCAATCCTCACAGACCTG-3′, where the mutation-directing base is underlined. The mutation was verified by dideoxy sequencing of the entire coding region.

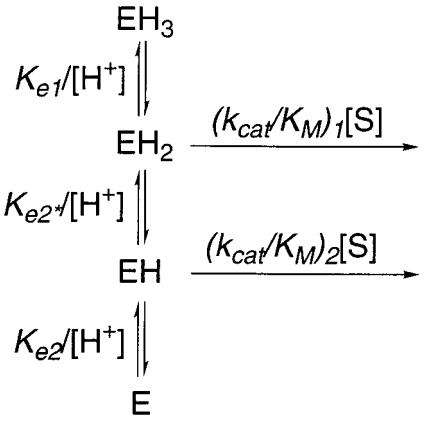
Protein Expression, Purification, and Activation. WT and H224Q pro-sHS were expressed in the *Escherichia coli* host BL21(DE3). Cells were grown to an OD_{600} of 1 in LB_{amp} at 37 °C and then induced for 3 h at 28−30 °C with 0.67 mM IPTG. The cells were harvested by centrifugation and purified as described previously (*26*). Briefly, the purification consisted of ammonium sulfate precipitation followed by Q-Sepharose and S-Sepharose ion-exchange chromatography steps.

WT and H224Q pro-HS were activated by treatment of 60 μM enzyme in 100 mM Tris (pH 7.5) with 1 mM APMA for 6 h at 30 °C. To monitor the progress of the activation, aliquots were removed at desired time points and boiled in 50 mM Tris (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 10% glycerol, and 0.1% bromophenol blue. The aliquots were run on a 14% SDS−PAGE gel (Novex) and stained with Coomassie blue. When activation was complete, APMA and small proteolysis products were removed by ultrafiltration using a Centriprep-30 (Amicon). The activated enzyme was stored at −20 °C in 20 mM Tris (pH 7.5), 5 mM $CaCl_2$, 0.5 mM $Zn(OAc)_2$, and 0.05% sodium azide.

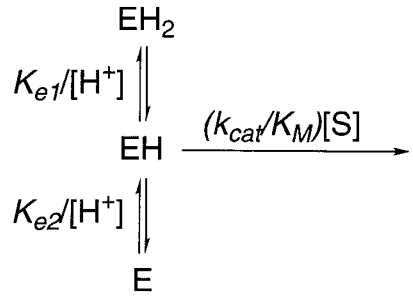
Enzymatic Assays and Kinetic Measurements. Two synthetic fluorescent peptides were used for kinetic studies: Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Mca-peptide) (*27*) and Dnp-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser (Dnp-peptide) (*28*). Assays with the Mca-peptide were carried out at a substrate concentration of 2.16 μM, at which $[S]_o \ll K_M$, and concentrations of sHS of 7.5 (H224Q) and 28 nM (WT). Under these conditions, the reaction is first-order in substrate, and pseudo-first-order rate constants (k_{obs}) were calculated from first-order plots. Values of k_{cat}/K_M were calculated from these data using the relationship $k_{obs} = (k_{cat}/K_M)[E]_o$. Assays with the Dnp-peptide were carried out at substrate concentrations in the 5−300 μM range at sHS concentrations of 4−17 (H224Q) and 12−27 nM (WT). The Dnp-peptide was developed earlier (*28*) to fill the need for a substrate with a low enough K_M value to allow evaluation of the individual kinetic parameters.

The hydrolysis of both substrates was monitored fluorimetrically using a Perkin-Elmer LS-50B luminescence spectrometer. Increases in fluoresence due to peptide cleavage were measured using excitation at 328 nm and emission at 393 nm for the Mca-peptide and excitation at 278 nm and emission at 358 nm for the Dnp-peptide. Initial rates were calculated from the change in fluorescence as described previously (*29*). Substrate concentrations were determined spectrophotometrically using an ϵ_{410} of 7500 M^{-1} cm^{-1} for the Mca-peptide (*27*) and an ϵ_{354} of 16 300 M^{-1} cm^{-1} for the Dnp-peptide (*28*). Enzyme concentrations were determined by active site titration using the tight binding inhibitor BB-94 (*30*). Assays with the Dnp-peptide were carried out in either 50 mM Hepes, 200 mM NaCl, 1 mM $CaCl_2$, and 0.01% Brij-35 (pH 7.5) or 50 mM Mes, 200 mM NaCl, 1 mM $CaCl_2$, and 0.01% Brij-35 (pH 6.0) at 30 °C to evaluate k_{cat} and K_M. This was achieved by fitting initial velocity data to the Michaelis−Menten equation using the nonlinear regression data analysis program ULTRAFIT (Elsevier-BIOSOFT).

Scheme 1



Scheme 2



The pH dependence of k_{cat}/K_M was determined for WT and H224Q sHS using the Mca-peptide in 50 mM Na^+X^-, 200 mM NaCl, 1 mM $CaCl_2$, and 0.01% Brij-35, where X is either Mes (pH 5.5−7.0), Hepes (pH 7.0−8.5), or Capso (pH 8.5−10.5). The concentration of Mca-peptide (2.16 μM) fulfilled the condition where $[S]_o \ll K_M$ throughout the entire pH range studied. The initial velocities were normalized for slight buffer and pH-dependent variations in the magnitude of the fluoresence change caused by substrate cleavage. The stability of the enzymes across the pH range investigated was assessed by incubating the enzyme for the length of time and at the concentration used in kinetic determinations and then initiating the assay by addition of substrate. pK_e values were determined for the enzymes by fitting the observed k_{cat}/K_M versus pH plot to either eq 1, in the case of H224Q sHS, or eq 2, in the case of WT sHS, using ULTRAFIT (Elsevier-Biosoft). Parameters are defined in Schemes 1 and 2.

$$(k_{cat}/K_M)_{obs} = \frac{(k_{cat}/K_M)}{\left(1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]}\right)} \quad (1)$$

$$(k_{cat}/K_M)_{obs} = \frac{(k_{cat}/K_M)_1}{\left(1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]} + \frac{K_{e2}K_{e2*}}{[H^+]^2}\right)} + \frac{(k_{cat}/K_M)_2}{\left(1 + \frac{[H^+]}{K_{e2*}} + \frac{K_{e2}}{[H^+]} + \frac{[H^+]^2}{K_{e2*}K_{e1}}\right)} \quad (2)$$

RESULTS AND DISCUSSION

HS consists of two domains, an N-terminal catalytic domain and a C-terminal hemopexin-like domain. sHS, a truncated form of HS lacking the C-terminal domain, was used in this study because it is more amenable to recombinant expression and structural determination than the full-length form. Deletion of the C-terminal domain has been shown to have little effect on the ability of HS to cleave synthetic peptides and macromolecular substrates (*26*, *31*, *32*), although the C-terminal domain does appear to play a role in the recognition of some macromolecular substrates and inhibitors (*16*).

The WT and H224Q forms of pro-sHS were expressed in *E. coli* and purified to greater than 95% homogeneity, as judged by Coomassie-stained SDS−PAGE. The behavior of H224Q sHS during the chromatographic steps in the purification was indistinguishable from that of WT sHS. Treatment of both the WT and H224Q proenzymes with 1 mM APMA at 30 °C resulted in autolytic processing to their active forms. SDS−PAGE was used to monitor the change in molecular mass of the enzyme upon cleavage of the propeptide. The processing of H224Q sHS is indistinguishable from that of WT sHS, both kinetically and in the apparent molecular mass of the proenzyme and mature forms of the enzyme, as determined by SDS−PAGE (results not shown).

Although enzymes typically contain a large number of ionizing groups, plots of rate against pH usually take the form of simple single or double (bell-shaped) titration curves (*33*). This is because the only ionizations that are detectable are those of groups that are involved in catalysis or that are required for maintenance of the active conformation of the enzyme. The pK_a of these catalytically relevant functional groups on the free enzyme can often be extracted from the pH dependence of the kinetic parameter k_{cat}/K_M (*33*, *34*).[2] The pH profiles of a number of MMPs have been determined, including full-length HS (*15*), matrilysin (*35*), and porcine synovial collagenase-1 and gelatinase (*36*). With the exception of HS, all of these profiles are bell-shaped and possess a broad range of maximal activity extending from neutral to basic pH. Simple bell-shaped pH profiles typically reflect the titration of two catalytically relevant functional groups of the free enzyme. This mechanism is depicted in Scheme 1.

In contrast, the pH profile of HS is more complex, with a narrow range of maximal activity extending from pH 5.75 to 6.25 and a broad shoulder that extends from about pH 7.5 to 8.5 (*15*). This relatively complex profile reflects the titration of at least three catalytically relevant functional groups (*33*, *34*). The simplest mechanism consistent with this pH profile is depicted in Scheme 2. Here, both EH and EH_2 represent catalytically active forms of HS linked by pK_{e2*}. The activity of EH is attenuated relative to EH_2, explaining the relatively low pH optimum and the shoulder in the pH profile. HS is unique among the MMPs in that ionization pK_{e2*} is required to explain its pH profile.

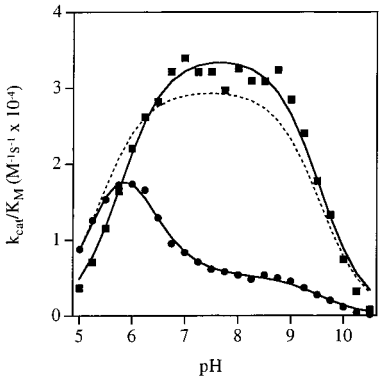


FIGURE 1: Dependence of k_{cat}/K_M on pH for (●) WT and (■) H224Q sHS obtained using the MCA substrate. The solid lines represent computer fits to the model shown in Scheme 1 for H224Q sHS and Scheme 2 for WT sHS. The dashed line represents the predicted pH dependence for WT sHS with pK_{e2*} removed.

Several lines of evidence pointed to His-224 as the residue responsible for pK_{e2*}. First, the predicted pK_a of the imidazole side chain of histidine is consistent with the observed value of pK_{e2*} (*37*). Second, amino acid sequence alignment shows that HS is the only known MMP with a histidine at position 224, which could explain the absence of a shoulder in the pH profiles of the other MMPs. Third, His-224 is located in a relatively flexible loop that forms part of its S_1' subsite [nomenclature of Schechter and Berger (*38*)] near the catalytic center of the enzyme (*5*, *7*). The S_1' subsite, a hydrophobic pocket, is structurally the most clearly defined binding site in the MMPs for which structures have been determined (*5*). While His-224 is located too far away from the scissile bond of the substrate to be directly involved in catalysis, the deprotonation of this residue might lead to a perturbation of the S_1' pocket and/or the catalytic center, resulting in the observed changes in catalytic efficiency.

To assess whether pK_{e2*} does indeed reflect the titration of His-224, the pH profiles of the WT and H224Q forms of sHS have been determined. Glutamine was chosen for the mutation because it is sterically similar to histidine and shares its ability to act as both a hydrogen bond donor and acceptor, but lacks any ionizations in the physiologically relevant pH range that could give rise to a pK_{e2*}. The pH profiles obtained for WT and H224Q sHS obtained with the Mca-peptide are shown in Figure 1. The pH dependence of WT sHS is characterized by a narrow range of maximal activity extending from pH 5.75 to 6.25 and a broad shoulder in the pH 7.5−8.5 range. This agrees very well with the results for the nonrecombinant, full-length HS obtained with three different synthetic peptide substrates (*15*). This supports the assumption that the pH dependence determined for WT sHS is neither substrate-specific nor affected by deletion of the C-terminal domain.

The pH profile for H224Q sHS differs dramatically from that of WT sHS in that it exhibits the simple bell shape

[2] If the substrate is ionized in the pH range studied, such ionizations can also be reflected in the pH dependence of k_{cat}/K_M. However, in this study, the substrates lack functional groups that titrate in the pH range investigated and this possibility can be disregarded.

Table 1: Parameters Describing the pH Dependence of WT and H224Q sHS

parameter	WT	H224Q
pK_{e1}	5.4 ± 0.1	5.8 ± 0.1
pK_{e2*}	6.2 ± 0.1	N/A[a]
pK_{e2}	9.5 ± 0.2	9.5 ± 0.1
$(k_{cat}/K_M)_1$	29700 ± 4000 M^{-1} s^{-1}	N/A
$(k_{cat}/K_M)_2$	5260 ± 410 M^{-1} s^{-1}	N/A
(k_{cat}/K_M)	N/A	34200 ± 1200 M^{-1} s^{-1}

[a] Not available.

characteristic of the other MMPs. There is no apparent shoulder in the pH 6−7 region and no trace of the pK_{e2*} function-altering ionization. This result is consistent with the hypothesis that the complexity in the pH profile of WT sHS reflects the titration of His-224. The impact of eliminating pK_{e2*} in H224Q sHS is to markedly increase the value of k_{cat}/K_M above pH 6 compared to that of WT sHS. The range of maximal activity is also broader and extends to a much higher pH. It is apparent that the deprotonation of His-224 in WT sHS attenuates, but does not completely eliminate, catalytic activity. This attenuation of activity with increasing pH is responsible for the low pH optimum of the enzyme.

Crystal structures have been reported for WT sHS bound to a number of different synthetic inhibitors (*5*, *7*). His-224, as part of the flexible loop, has been observed to assume dramatically different orientations depending upon the identity of the bound inhibitor. The inhibitors include P_1' moieties (hydrophobic groups intended to bind to the S_1' subsite) that vary substantially in size and shape. This flexibility of His-224 and the flexible loop allow the S_1' binding pocket to accommodate these structurally diverse moieties. Significantly, in a structure where the P_1' moiety of the inhibitor is leucine (the same P_1' moiety as in the peptide substrates), His-224 is observed to form a hydrogen bond with a main chain carbonyl group (*7*). It is reasonable to speculate that in solution this hydrogen bond is present when WT sHS binds to peptide substrates at relatively low pH, and that it is disrupted by the deprotonation of His-224 with increasing pH. The loss of the hydrogen bond apparently makes catalysis less efficient. In H224Q, the amide nitrogen of the Gln-224 could serve as a hydrogen bond donor throughout the pH range investigated, resulting in its enhanced activity at higher pH.

To determine the kinetic parameters defined in Schemes 1 and 2 for WT and H224Q sHS, the pH dependences of $(k_{cat}/K_M)_{obs}$ shown in Figure 1 were fitted to eq 1 (H224Q) or eq 2 (WT), respectively. The resulting pK_e and k_{cat}/K_M values for WT and H224Q sHS are listed in Table 1. The kinetic parameters for WT sHS are virtually identical to those determined previously for full-length HS (*15*). The value of pK_{e2*} of 6.2 ± 0.1 is reasonable for histidine. The expected pK_a of a histidine side chain in proteins has been estimated to be 6.2 on the basis of the pK_a values of small model compounds, though the pK_a of any given amino acid residue is strongly influenced by the protein microenvironment in which it resides (*37*).

The identity of the groups responsible for pK_{e1} and pK_{e2} in the MMP family remains unsettled. Cha and co-workers originally assigned pK_{e1} in matrilysin to Glu-202 (*39*). This residue is analagous to Glu-270 in carboxypeptidase A and Glu-143 in thermolysin, both of which had been assigned to similar acidic pK_e values in these enzymes (*40*, *41*). Mutation of Glu-202 to other residues is known to markedly affect activity for collagenase-1 (*42*) and gelatinase A (*43*). More recent studies, however, indicate that pK_{e1} in matrilysin cannot be attributed to Glu-202, since this ionization is unaffected by mutation of this residue (*44*). Moreoever, the assignments of the equivalent acidic pK_e in carboxypeptidase A to Glu-270 and in thermolysin to Glu-143 have been reevaluated and assigned instead to the ionization of zinc-bound water (*45*, *46*). Thus, it now seems likely that pK_{e1} in the MMP family is also due to zinc-bound water (*44*). The alkaline pK_{e2} could be due to Tyr-223, a residue conserved in the MMPs (*44*).

Table 2: Kinetic Parameters Describing the Hydrolysis of the Dnp-Peptide by WT and H224Q sHS

enzyme	pH	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
WT	6.0	1.4 ± 0.1	47 ± 8	30 000
WT	7.5	1.1 ± 0.1	95 ± 17	11 000
H224Q	6.0	0.86 ± 0.05	26 ± 6	32 500
H224Q	7.5	0.75 ± 0.04	13 ± 4	59 000

Table 3: Ratios of Kinetic Parameters Determined for WT and H224Q sHS at pH 6 and 7.5

enzyme	substrate	$(k_{cat})_{pH6.0}/(k_{cat})_{pH7.5}$	$(K_M)_{pH6.0}/(K_M)_{pH7.5}$	$(k_{cat}/K_M)_{pH6.0}/(k_{cat}/K_M)_{pH7.5}$
WT	Mca	N/A[a]	N/A	2.76
WT	Dnp	1.27	0.49	2.73
H224Q	Mca	N/A	N/A	0.64
H224Q	Dnp	1.15	2.06	0.55

[a] Not available.

The value of $(k_{cat}/K_M)_1$ for WT sHS is very close to the k_{cat}/K_M for H224Q. These values represent the potential maximum activity of the enzymes with all ionizing functional groups in their "active" protonation state. At neutral pH, the $(k_{cat}/K_M)_{obs}$ of WT sHS (Figure 1) is much lower than its $(k_{cat}/K_M)_1$ due to the deprotonation of His-224 which attenuates its activity before it can achieve its maximum. The dashed curve shows the predicted profile for WT sHS if pK_{e2*} were absent. It is very similar to the profile observed for H224Q sHS. Thus, HS has the potential to be a significantly more active at neutral pH were it not for the effect of His-224.

The low solubility of the Mca-peptide relative to its K_M renders it a poor substrate for the independent determination of k_{cat} and K_M. Therefore, the more soluble Dnp-peptide has been used to determine k_{cat} and K_M for WT and H224Q sHS at pH 6 and 7.5 (Table 2). For both enzymes, increasing the pH from 6.0 to 7.5 results in a slight decrease in k_{cat}, giving ratios of $(k_{cat})_{pH6.0}/(k_{cat})_{pH7.5}$ for WT and H224Q sHS that are both slightly greater than unity (Table 3). In contrast, the K_M for WT sHS doubles as the pH is increased from 6.0 to 7.5, while the K_M for H224Q sHS is halved. Thus, the decrease in k_{cat}/K_M observed for WT sHS as the pH is increased from 6 to 7.5 is due mostly to a 4-fold increase in K_M relative to that of H224Q sHS (Table 3) caused by the deprotonation of His-224. This increase in K_M probably reflects decreased substrate affinity, since it has been shown, using pre-steady-state kinetics, that K_M for sHS is approximately equal to K_d (M. Brenner and H. E. Van Wart, unpublished data). The sensitivity of K_M to the protonation state of His-224 is probably due to the contribution this

residue makes to the S_1' subsite, and thus to the ability of the enzyme to bind peptide substrates.

The anomalous pH dependence of HS activity could be physiologically relevant. Indeed, the low pH optimum for HS activity has been observed using natural substrates, such as aggrecan and fibronectin (*47*, *48*). Although studies using synthetic peptide substrates have shown the attenuation in HS activity with increasing pH to be modest, the effect might be larger under physiological conditions. Even among synthetic peptide substrates, there appears to be some substrate-dependent variation in the magnitude of the pH effect (*15*). In addition to the effect of pH on catalysis, it has been demonstrated that TIMP-1, one of a family of proteins known to inhibit MMPs, is less effective at inhibiting HS at lower pHs (*49*). These observations have led to the suggestion that the physiological activity of HS might, at least to some extent, be regulated by pH (*47*). His-224, a residue unique to HS, could play a physiological role by targeting the activity of this MMP to low-pH environments.

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