Mechanistic Studies on the Human Matrix Metalloproteinase Stromelysin

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ABSTRACT: To probe the mechanism of stromelysin (SLN)-catalyzed peptide hydrolysis, we determined the pH dependence of k_c/K_m and solvent deuterium isotope effects on k_c and k_c/K_m. pH dependencies of k_c/K_m were determined for the SLN-catalyzed hydrolysis of three peptides: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2, Arg-Pro-Ala-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH_2, and *N*-acetyl-Arg-Pro-Ala-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 (cleavage at Gln–Phe bond). The pH dependencies are all bell-shaped with shoulders that extend from pH 7.5 to 8.5. The existence of a shoulder indicates that the reaction mechanism involves at least two routes to products. These curves are governed by three proton ionizations with pK_a values of 5.4, 6.1, and 9.5. The solvent isotope effect measurements provided the following values: $^D(k_c/K_m) = 0.80 \pm 0.05$ and $^D(k_c) = 1.58 \pm 0.05$. That $^D(k_c/K_m)$ and $^D(k_c)$ are different suggests that the rate-limiting transition states for the processes governed by k_c/K_m and k_c cannot be the same. We use these results, together with analogy to thermolysin catalysis, to develop a mechanism for SLN catalysis.

Stromelysin (E.C. 3.4.24.17) is a member of the matrix metalloproteinase family (Emonard & Grimaud, 1990; Matrisian, 1990) and is thought to play a pathogenic role in arthritis (Emonard & Grimaud, 1990; Hasty, et al., 1990; Matrisian, 1990). As part of a program to develop therapeutic inhibitors of this and other MMP's,[1] we are studying mechanistic aspects of metalloproteinase catalysis and inhibition (Izquierdo & Stein, 1990; Izquierdo-Martin & Stein, 1992a,b; Stein, 1988).

In this paper, we report pH dependencies and solvent deuterium isotope effects for reactions of SLN. We determined pH dependencies of k_c/K_m for the SLN-catalyzed hydrolyses of three peptide substrates: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 (SP-Nle[11]), Arg-Pro-Ala-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 (SP-Ala[3],Nle[11]), and *N*-acetyl-Arg-Pro-Ala-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 (Ac-SP-Ala[3],Nle[11]) (cleavage at the Gln–Phe bond). These studies reveal the ionization state(s) of the catalytically active enzyme species and allow us to eliminate several mechanisms that are inconsistent with the observed ionization pattern for SLN. The isotope effect measurements that we performed were for both k_c and k_c/K_m and, by giving us insight into important proton reorganizations, allow us to begin to define the structures of rate-limiting transition states for the processes governed by these kinetic parameters.

MATERIALS AND METHODS

General. Peptide substrates were purchased from Bachem (Philadelphia, PA) at a purity greater than 98%. Water was purified through a Millipore Milli-Q deionization system (Waters, Millford, MA). HPLC grade acetonitrile and trifluroacetic acid (TFA) were from Fischer Scientific (Pittsburgh, PA).

Enzyme. Recombinant human proSLN (Whitman et al., 1986) was used in these experiments and was purchased from Celltech Ltd. (Slough, Berkshire, U.K.). The zymogen was activated by limited proteolysis by trypsin as previously described (Lark et al., 1990a,b; Teahan et al., 1989). Briefly, 25 nM trypsin was added to a 2.2 μM solution of proSLN in a pH 7.5 buffer containing 20 mM Tris, 10 mM $CaCl_2$, 0.05% brij, and 0.02% NaN_3, and the resultant solution was allowed to incubate at 37 °C for 30 min. The activation process was quenched by the addition of a 20-fold molar excess of soybean trypsin inhibitor bound to agarose (Sigma). Finally, the inhibitor–agarose was separated from active SLN by centrifugation.

Buffers. For studies of the pH dependence of SLN stability and catalysis, the following buffers were used: 0.10 M sodium acetate, pH 5.0; 0.10 M MES, pH's 5.5, 6.0, and 6.5; 0.10 M HEPES, pH's 7.0, 7.5, and 8.0; 0.10 M BICINE, pH 8.5; 0.10 M CHES, pH's 9.0 and 9.5; and 0.10 M CAPS buffers, pH's 10.0 and 10.5. In all cases, buffers contained 10 mM $CaCl_2$.

Buffers for Solvent Deuterium Isotope Effects. Buffered solutions of heavy water were prepared by lyophilization as follows. H_2O (25.0 mL) buffered at pH values of 6.0, 7.5, and 9.0 was lyophilized to dryness. D_2O (25.0 mL) was then added to dissolve the resultant residues. As theory predicts, the pH-meter reading of these buffered D_2O solutions were 0.1 pH units greater than the pH of the corresponding buffer in H_2O (Quinn & Sutton, 1991). Solutions prepared in this manner ensure that reactions are studied at equivalent positions on the pH-rate profiles in the isotopic solvents.

Chromatographic Conditions. The chromatographic apparatus consists of a Waters 510 HPLC System (Division of Millipore, Millford, MA) and an Hitachi model 655A-40 autosampler fitted with a temperature control option (E.M. Science, Cherry Hill, NJ). Temperature was maintained at 25.0 ± 0.1 °C by a Lauda RM6 refrigerated circulating water bath (Brinkmann, Westbury, NY). UV absorbance was measured at 215 nm using a Kratos Spectroflow 757 variable wavelength detector (ABI Analytical, Ramsey, NJ). Peak integrations were performed on a PE Nelson Turbochrom Data Station (Cupertino, CA). Separations were performed on a Whatman RAC II 5 C8 analytical column (10 cm × 4.6 mm i.d.). The mobile phase was 0.1% aqueous TFA and CH_3CN. The flow rate was 1.5 mL/min.

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[1] Abbreviations: MMP, matrix metalloproteinase; SLN, stromelysin; CGase, collagenase; GTase, gelatinase; PUMP, putative metalloproteinase; TLN, thermolysin; Ac, acetyl.

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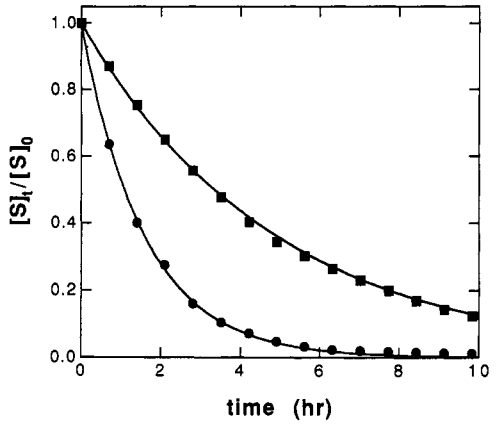


FIGURE 1: Progress curves for substrate disappearance for the SLN-catalyzed hydrolysis of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2. Filled circles, pH 6.5; filled squares, pH 9.5. Solid lines are drawn using the equation $[S]_t/[S]_0 = e^{-kt}$ and the best-fit parameters $k = 0.636 \pm 0.004$ h^{-1} (pH 6.5) and $k = 0.207 \pm 0.003$ h^{-1} (pH 9.5).

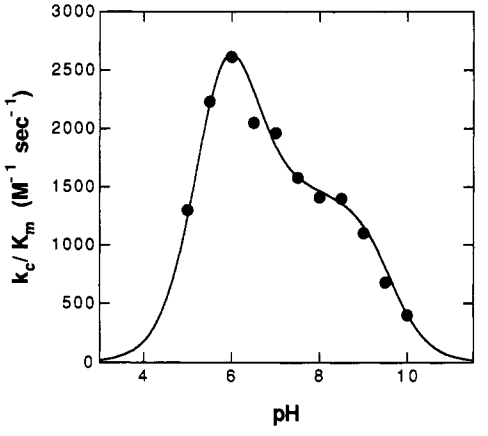


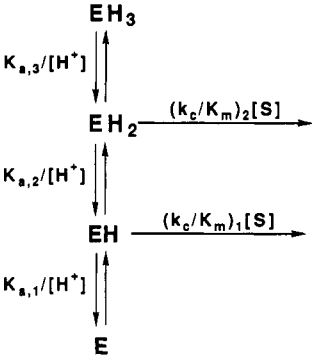
FIGURE 2: pH dependence of k_c/K_m for the SLN-catalyzed hydrolysis of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2. Each point represents the average of three independent experiments. See the text for details of experiment and analysis.

Kinetic Methods. The kinetic studies that are reported in this paper rely on our previously reported semicontinuous HPLC assay that is based on the ability of SLN to hydrolyze SP exclusively at the Gln^6–Phe^7 bond (Harrison et al., 1989).

Enzyme Stability. The pH dependence of the stability of SLN over 16 h was determined by using the following protocol. Fifty-microliter aliquots of stock SLN (2.2 μM in pH 7.5 buffer containing 50 mM HEPES and 10 mM $CaCl_2$) was added to 450 μL of buffers that ranged in pH from 4.5 to 10.5. At time zero and at 2-h increments thereafter, the amount of active enzyme that remained in these solutions was determined. This was done by adding 50-μL aliquots of these solutions to assay solutions (0.35 mM SP-Nle^{11} in pH 7.5 buffer containing 20 mM Tris, 10 mM $CaCl_2$, 0.05% brij, and 0.02% NaN_3) and determining initial velocities for SP-Nle^{11} hydrolysis. Initial velocities are of course directly proportional to the concentration of catalytically active enzyme.

Initial velocities were determined with our semicontinuous assay (Harrison et al., 1989) in which 30-μL aliquots of the solution of SLN and SP-NLE^{11} are removed by the autosampler at 3-min intervals and injected onto the HPLC which separates intact peptide from the hydrolysis products. Formation of the hydrolysis product Phe-Phe-Gly-Leu-$NleNH_2$ is monitored by UV absorbance at 215 nM. Finally, the initial velocity is calculated as the slope of the plot of product area vs time.

Scheme I: Kinetic Mechanism for the pH Dependence of Stromelysin Catalysis



pH Dependence of k_c/K_m. To determine the pH dependence of k_c/K_m, full reaction progress curves were monitored as a function of pH at low substrate concentration. In a typical experiment, 50 μL of a 1 mM solution of substrate was added to 1.7 mL of buffer, and the resultant solution was allowed reach thermal equilibrium at 25 °C. Fifty microliters of a 2.2 μM solution of SLN was then added to initiate the reaction ($([S])_0 = 28$ μM; $[E]_0 = 60$ nM). At time zero and at 30-min intervals, 40-μL aliquots of the reaction solution were removed by the autosampler, injected onto the column, and eluted isocratically. The intact peptide was separated from the two hydrolysis products, and the loss of the intact peptide was monitored by UV absorbance at 215 nm. The temporal dependence of substrate peak area was fit to the first-order rate law, $[S]_t = [S]_0 \exp(-k_{obs}t)$, to calculate values of k_{obs}.

Solvent Deuterium Isotope Effects. Each isotope effect that we report represents the mean for the combined results of two or three independent isotope effect experiments (standard deviations ≤15% of the mean). Each isotope effect experiment involved 3–5 individual isotope effect measurements, where each isotope effect measurement is the ratio of two kinetic runs: $^{H}k_{obs}/^{D}k_{obs}$.

RESULTS

pH Dependence of the Stability of Stromelysin. To ensure that the activity seen in the pH dependence of catalysis is not compromised by loss of activity at extremes of pH due to SLN degradation or denaturation, the stability of SLN was determined over the pH range 4.5–10.5. At pH values of 4.5 and 10.5, SLN loses more then 80% of the original activity by 15 h. However, the enzyme loses no more than half its activity at pH values of 5.0 and 10, and complete activity is retained at pH values ranging from 5.5 to 9.5.

pH Dependence of Stromelysin Catalysis. Values of k_c/K_m were determined by observing the first-order disappearance of substrate at low substrate concentrations where $[S]_0 = 50$ μM $\ll K_m$ and $k_{obs} = (k_c/K_m)[E]_0$ (see Figure 1). In independent experiments, we demonstrated that, at pH values of 6.5, 7.5, and 9.5, 0.4 mM $< K_m <$ 1.2 mM (data not shown).

Values of k_c/K_m are plotted as a function of pH in Figure 2. We see that the pH dependence of k_c/K_m is bell-shaped with a shoulder that extends from about pH 7.5 to 8.5. The existence of a shoulder tells us that the observed values of k_c/K_m at these pH values are larger than they would be in the absence of the shoulder. This indicates that the reaction mechanism must involve at least two routes to product. The simplest kinetic mechanism that can account for our results

Table I: Kinetic Parameters for the pH Dependence of the SLN-Catalyzed Hydrolysis of R-Arg-Pro-Xaa-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2

R	Xaa	$(k_c/K_m)_2$ (mM^{-1} s^{-1})	$(k_c/K_m)_1$ (mM^{-1} s^{-1})	$pK_{a,3}$	$pK_{a,2}$	$pK_{a,1}$
H–	Lys	3.8 ± 0.6	1.5 ± 0.1	5.3 ± 0.1	6.2 ± 0.2	9.5 ± 0.1
H–	Ala	4.9 ● 0.3	0.7 ± 0.1	5.8 ± 0.1	6.0 ● 0.1	9.9 ● 0.1
Ac–	Ala	17 ± 4	3.8 ± 0.6	5.2 ± 0.2	6.1 ± 0.3	8.7 ± 0.4

is shown in Scheme I,[2] in which EH_2 and EH are the catalytically active forms of SLN, and EH_3 and E are the catalytically inactive forms that predominant at acid and alkaline pH values, respectively.

The steady-state rate expression that describes the mechanism of Scheme I was derived using rapid equilibrium assumptions and is shown in eq 1. The line that is drawn

$$(k_c/K_m)_{obs} = \frac{(k_c/K_m)_2}{\frac{[H^+]}{K_{a3}} + 1 + \frac{K_{a2}}{[H^+]} + \frac{K_{a1}K_{a2}}{[H^+]^2}} + \frac{(k_c/K_m)_1}{\frac{[H^+]^2}{K_{a3}K_{a2}} + \frac{[H^+]}{K_{a2}} + 1 + \frac{K_{a1}}{[H^+]}} \quad (1)$$

through the data points of Figure 2 is based on the nonlinear least-squares fit of the data to eq 1 (see Table I for parameter estimates).

In general, pK_a values that are extracted from the pH dependence of the kinetic parameter k_c/K_m reflect catalytically essential ionizations of free enzyme and/or substrate. In the present case, we needed to eliminate the possibility that ionization of substrate leads to the observed pK_a values of Figure 2. To this end, we studied pH dependencies of k_c/K_m for the SLN-catalyzed hydroyses of two additional substrates: SP-Ala[3],Nle[11] and Ac-SP-Ala[3],Nle[11]. The shapes of these pH dependencies are identical to that of Figure 2 (data not shown). The data from these experiments were readily fit to eq 1, and the best-fit pK_a values are similar to those obtained for the SLN-catalyzed hydrolysis of SP-Nle[11] [3] (see Table I). Combined, these results indicate that the pK_a values determined from titration of k_c/K_m reflect catalytically essential ionizations of SLN.

Solvent Deuterium Isotope Effects. To determine the solvent deuterium isotope effects on k_c and k_c/K_m, we examined the substrate concentration dependence of the observed solvent isotope effect on steady-state velocity. The results of these experiments are shown in Figure 3, where we see that $^Dv_{ss}$ increases from an inverse value at low $[S]_0$, to large, normal values at high $[S]_0$.

To determine $^D(k_c/K_m)$ and $^D(k_c)$, we fit the data Figure 3 to eq 2 which describes the dependence of $^Dv_{ss}$ on $[S]_0$.

$$^Dv_{ss} = {}^D(k_c/K_m) + [{}^D(k_c) - {}^D(k_c/K_m)]\left(\frac{[S]_0}{K_m + [S]_0}\right) \quad (2)$$

[2] A trivial explanation for the shoulder is that there is more than a single protease in our enzyme preparation. We exclude this on the basis of the following: (i) The second enzyme would have to specifically hydrolyze SP and its derivatives at the Gln–Phe position. We believe that this is highly unlikely. (ii) The same results are obtained with native and recombinant enzyme (data not shown). (iii) The enzyme is pure by a number of criteria, including N-terminal sequence analysis, isoelectric focusing gels, and SDS–PAGE (Lark et al., 1990a,b).

[3] The considerable variation in $pK_{a,1}$ values among the three substrates results from our inability to extend the pH range to values higher than 10. Thus, values of $pK_{a,1}$ are poorly defined.

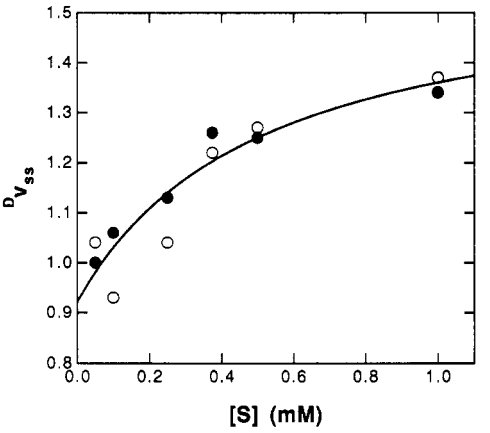


FIGURE 3: Dependence of observed solvent deuterium isotope effect on substrate concentration for the SLN-catalyzed hydrolysis of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2. Experiments were run at pH 6.0 and pD equivalent. Filled and empty circles represent two independent experiments. The line through the data was drawn using eq 2 and the best fit parameters $^D(k_c/K_m) = 0.92 \pm 0.03$ and $^D(k_c) = 1.58 \pm 0.05$.

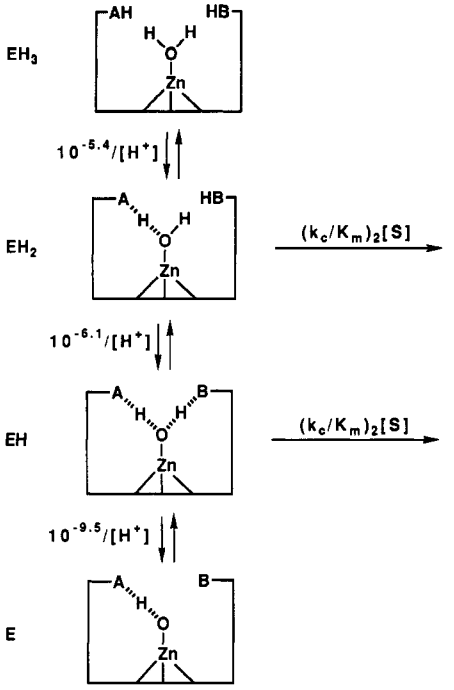
Constraining K_m to its known value of 0.5 mM (Harrison et al., 1989), we find $^D(k_c/K_m) = 0.92 \pm 0.03$ and $^D(k_c) = 1.58 \pm 0.05$.

Because precise initial velocity measurements at low substrate concentrations are difficult to measure using our semicontinuous assay, we sought an independent determination of $^D(k_c/K_m)$. This was accomplished by monitoring full progress curves at low substrate concentration (i.e., $[S]_0 = 50$ $\mu M \ll K_m$) in H_2O and D_2O. $^D(k_c/K_m)$ determined in this way was measured at three pH values, 6.0, 7.5, and 9.5, and equal 0.80 ± 0.03, 0.85 ± 0.6, and 0.75 ± 0.02, respectively. We see that $^D(k_c/K_m)$ is independent of pH and equals 0.80. This value, which is in fair agreement with the value for $^D(k_c/K_m)$ described above, reflects a more accurate measure of the solvent isotope effect on k_c/K_m.

DISCUSSION

pH Dependence of k_c/K_m. pH dependencies of k_c/K_m for the SLN-catalyzed hydrolyses of three peptide substrates give a consistent picture in which hydrolysis occurs by the mechanism of Scheme I involving four important ionization states of the enzyme and two pathways to product. This is an unusual pH dependence and is more complex than the simple bell-shaped pH dependencies of k_c/K_m for other metalloendoproteinases. For the mechanistically related enzyme TLN, k_c/K_m titrates with pK_a values of 5.1 and 8.2 (Izquierdo-Martin & Stein, 1992a), while for the MMP's CGase and GTase, the pH dependencies are broader with pK_a values of 6 and 10 (Stack & Gray, 1991). In the latter two cases, the data are not of sufficient precision to rule out the existence of a shoulder. Interestingly, for a fourth matrix metalloproteinase, PUMP, careful study of the pH dependence of k_c/K_m for the hydrolysis of DNP-Pro-Leu-Gly-Leu-Trp-Ala-Arg-NH_2 (cleavage at Gly–Leu) reveals a shape that is identical to the one we observe here with a clear shoulder around pH 8 (personal communication from Dr. Frances Willenbrock, Celltech, Ltd.).

Scheme II: pH Dependence of Catalysis



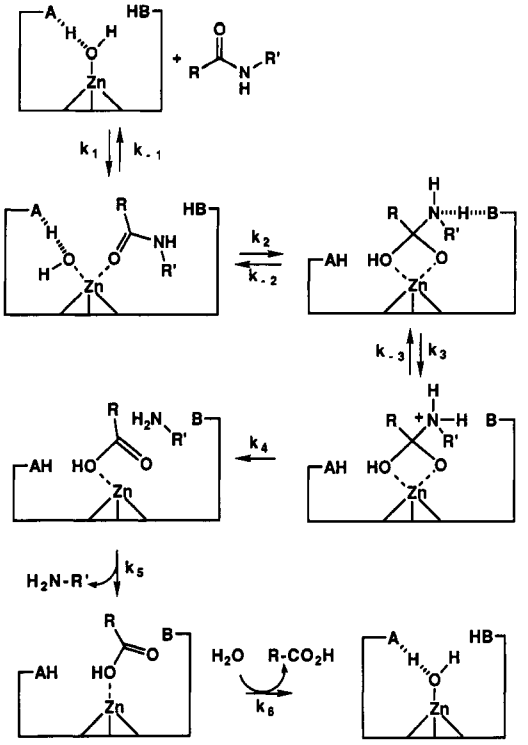
We interpreted the pH dependencies of k_c/K_m for TLN catalysis in terms of two active site ionizations (Izquierdo-Martin & Stein, 1992a). We proposed that the pK_a of 5 reflects ionization of the active site pair [Glu_{143}–COOH HO(H)–Zn] to [Glu_{143}–COO···H···O(H)–Zn], while the pK_a of 8 corresponds to ionization of His_{231}–ImH^+. By analogy to TLN, we propose the active site ionizations of Scheme II for SLN involving a zinc-bound water and two ionizable residues, AH and BH. Since structural data has not yet been reported for SLN, we are currently unable to identify these residues.

To provide a mechanistic framework for our discussion of both the pH dependence of catalysis and the roles of A and B, we propose the chemical mechanism of Scheme III for SLN-catalyzed amide hydrolysis. This mechanism is similar to mechanisms that have been proposed previously for metalloproteinase catalysis (Fukuda & Kunugi, 1984; Hanguer et al., 1984; Izquierdo-Martin & Stein, 1992a; Kunugi et al., 1982). In the first step of this mechanism, SLN and peptide substrate, R–C(O)–NH–R′, combine to form a Michaelis complex in which the carbonyl oxygen of the substrate is loosely coordinated to the active site zinc and the zinc-bound water is displaced toward residue A (Hanguer et al., 1984). This step is followed by attack of the zinc-bound water on the carbonyl carbon of the substrate to form a tetrahedral addition adduct. This intermediate then collapses with expulsion of the amine hydrolysis product, H_2N–R′. Finally, the carboxylate product, R-CO_2^-, is displaced from the zinc by water to regenerate active enzyme.

In the mechanism of Scheme III, we see that residues A and B participate in the proton reorganization that is required for amide bond hydrolysis. According to our mechanism, A acts as a general base to promote nucleophilic attack of zinc-bound water on the substrate and, therefore, must be unprotonated to fulfill its role. B, on the other hand, donates a proton to the departing amine product and must be protonated to fulfill its role. Proton donation from BH is probably complete before H_2N–R′ is expelled from the tetrahedral intermediate (Satterthwaite & Jencks, 1974).

With Scheme III providing our chemical mechanistic framework, we can now explain the pH-dependence of catalysis

Scheme III: Mechanistic Proposal for Peptide Bond Hydrolysis by Stromelysin, EH_2



as illustrated in Scheme II. At low pH, the active site is fully protonated and SLN exists as the inactive species, EH_3. As the pH is raised above 5.4, the active site unit, [AH HO(H)–Zn], ionizes to the hydrogen-bonded species [A···H···O(H)–Zn], and the enzyme exists as active EH_2. As we mentioned above, in EH_2, A acts as a general base and promotes the attack of the zinc-bound water on the carbonyl carbon of substrates. As the pH is raised even more, [BH HO(H)–Zn] ionizes to [B···H···O(H)–Zn], and the enzyme exists as EH. This is evidently not a crucial ionization, since EH is only slightly less active than EH_2. Finally, at high pH, the enzyme exists as the inactive species, E. Here B exists as the conjugate base and is no longer able to donate a proton to the departing amine product.

Solvent Deuterium Isotope Effects. Our isotope effect experiments reveal that while $^D(k_c/K_m)$ is inverse and equal to 0.8, $^D(k_c)$ is normal and equal to 1.6. This difference is large enough to suggest that it is mechanistically significant. In the discussion that follows, we argue that the difference in observed isotope effects reflects a difference in the structure of the rate-limiting transition states for k_c/K_m and k_c.

We start with eq 3, which expresses the solvent isotope

$$^Dk = \frac{\prod \Phi_{\text{reactant}}}{\prod \Phi_{\neq}} \tag{3}$$

effect as the ratio of products of reactant-state and transition-state fractionation factors (Quinn & Sutton, 1991). For k_c/K_m and k_c, the related eqs 4 and 5 hold:

$$^D(k_c/K_m) = \frac{\prod \Phi_E}{\prod \Phi_{\neq\alpha}} \tag{4}$$

$$^D(k_c) = \frac{\prod \Phi_{ES}}{\prod \Phi_{\neq\beta}} \tag{5}$$

We see that, by definition, k_c/K_m and k_c will always reflect different reactant states. For k_c/K_m, enzyme is uncomplexed and the reactant state is enzyme- and substrate-free in solution. In contrast, for k_c, the enzyme is bound up entirely by substrate and exists as the most stable enzyme-substrate complex. This complex is the reactant state for the process that is governed by k_c. However, depending on the kinetic situation, the transition states for the two processes may or may not be the same, and thus $\prod\Phi_{\neq\alpha}$ may or may not equal $\prod\Phi_{\neq\beta}$.

Thus, isotope effects on k_c/K_m and k_c can differ as a result of differences between reactant-state fractionation factors or differences between transition-state fractionation factors or both. In the present case, however, it is unlikely that $^D(k_c/K_m)$ and $^D(k_c)$ differ due to differences solely in reactant-state fractionation factors. This point can be demonstrated if we first assume that $\prod\Phi_{\neq\alpha}$ equals $\prod\Phi_{\neq\beta}$. Given that $^D(k_c/K_m)$ equals 0.8 and $^D(k_c)$ equals 1.6, we can use eqs 4 and 5 to calculate values of $\prod\Phi_{ES}$ and $\prod\Phi_{\neq}$ for any assumed value of $\prod\Phi_E$. For example, if we set $\prod\Phi_E$ equal to 0.75 (Izquierdo & Stein, 1990; Stein, 1988), we can calculate values for $\prod\Phi_{ES}$ and $\prod\Phi_{\neq}$ of 1.5 and 0.94, respectively. While we can rationalize a $\prod\Phi_{\neq}$ value of 0.94, a ready explanation for a $\prod\Phi_{ES}$ value of 1.5 is much more difficult. In fact, at no value of $\prod\Phi_E$ between 0.6 and 1.2 do we calculate reasonable values for both $\prod\Phi_{ES}$ and $\prod\Phi_{\neq}$. We must conclude that the difference between $^D(k_c/K_m)$ and $^D(k_c)$ results from differences in transition-state properties for the processes governed by k_c/K_m and k_c.

Clearly, for k_c/K_m and k_c to reflect different transition states, certain kinetic conditions must be met. We illustrate this with two limiting cases that emerge from consideration of Scheme III.

Case I. Here, we propose that all reversible steps are in rapid equilibrium (i.e., $k_{-1} \gg k_2$; $k_{-2} \gg k_3$; and $k_{-3} \gg k_4$) and that release of the final product is very slow relative to all other forward steps. These conditions lead to the following rate expressions:

$$\frac{k_c}{K_m} = \frac{k_1 k_2 k_3}{k_{-1}k_{-2}k_{-3}}k_4 \tag{6}$$

$$k_c = k_6 \tag{7}$$

Under these conditions, the process governed by k_c/K_m is rate-limited by the transition state corresponding to k_4, or decomposition of the protonated tetrahedral intermediate, while k_c equals k_6, the rate constant for exchange of product for water and generation of active enzyme.

We can now interpret our observed isotope effects in the context of case I. We first expand eq 4 to

$$^D(k_c/K_m) = \frac{(\Phi_{Zn\text{-}OH_2})^2[\prod\Phi_{substrate}][\prod\Phi_{SLN}]}{\prod\Phi_{\neq}} \tag{8}$$

where $\Phi_{Zn\text{-}OH_2}$ is the fractionation factor for one of the two hydrogens of the zinc-bound water molecule and $\prod\Phi_{substrate}$ and $\prod\Phi_{SLN}$ are the products of fractionation factors for the substrate and enzyme, respectively.

If we make the reasonable assumptions that the fraction factors for the substrate (Quinn & Sutton, 1991; Venkatasubban & Schowen, 1985), enzyme (Quinn & Sutton, 1991; Venkatasubban & Schowen, 1985), and transition state (Izquierdo & Stein, 1990; Stein, 1988) are unity and set $\Phi_{Zn\text{-}OH_2}$ equal to 0.85 (Kassebaum & Silverman, 1989; Schmidt et al., 1979), we can calculate a value for $^D(k_c/K_m)$ of 0.72. This calculation agrees reasonably well with our experimental value of 0.8 and supports a mechanism in which the zinc–bound water molecule of uncomplexed SLN undergoes reaction with substrate to become part of a tetrahedral addition adduct with a fractionation factor of unity (Izquierdo & Stein, 1990; Stein, 1988). This mechanism is identical to the mechanism we suggested for TLN where we observe $^D(k_c/K_m)$ values of 0.75 (Izquierdo & Stein, 1990; Stein, 1988).

For k_c, case I proposes that release of the carboxylate product is rate-limiting. Note, however, that this is not a simple dissociation process but rather involves a ligand exchange reaction; that is, k_6 involves the attack of water on zinc to displace the carboxylate product. This process is the reverse of the association of inhibitors with metalloproteinases. For these reactions, inhibitor displaces zinc-bound water to form the final enzyme–inhibitor complex and generates solvent isotope effects on k_{on} between 1.6 and 1.9 (Izquierdo-Martin & Stein, 1992b). In these cases, we propose that the large normal $^D(k_{on})$ values arise from proton catalytic bridges that form in the transition states for k_{on}. If similar proton bridging occurs in the transition state for k_6, a normal solvent isotope would also be observed on k_c.

Case II. In this limiting case, we assume the Briggs–Haldane kinetic mechanism in which association of enzyme and substrate rate-limits k_c/K_m and

$$\frac{k_c}{K_m} = k_1 \tag{9}$$

We also assume here that k_2 is slower than all other forward steps and, thus,

$$k_c = k_2 \tag{10}$$

In this situation, k_c/K_m is rate-limited by the transition state of k_1 and k_c reflects attack of zinc-bound water on the substrate from the stage of the Michaelis complex.

This mechanism can easily explain our observed isotope effects. Given that $\prod\Phi_{reactant}$ is around 0.8 for k_c/K_m, an inverse isotope effect on k_c/K_m will result if $\prod\Phi_{\neq}$ is near unity. This in not unreasonable since the physical processes that might rate-limit k_1 (e.g., protein conformational changes or diffusion-limited binding of substrate) are likely to have $\prod\Phi_{\neq}$ near unity (Quinn & Sutton, 1991). The normal isotope effect on k_c is readily explained by the general-base catalysis that we propose for k_2.

However, there is a problem with this mechanism. We are left trying to identify the nature of the process that is governed by k_1. It is unlikely that k_1 reflects diffusional collision of enzyme and substrate since the diffusion-controlled limit is no less than 10^7 M^{-1} s^{-1}, and k_c/K_m values for SLN-catalyzed reactions are only as large as 10^4 M^{-1} s^{-1} (see Table I). We can of course propose that k_1 is rate-limited by a conformation isomerization of an initially formed encounter complex of enzyme and substrate, but this is unsatisfying since there is no independent evidence to support a conformational change for SLN.

Summary: Mechanistic Proposal for Stromelysin Catalysis. The pH dependence of SLN catalysis is complex and suggests no less than three important ionizing groups at the enzyme's active site as well as at least two pH-dependent pathways to product. Viewed broadly, the pH dependence supports a mechanism of catalysis that has previously been proposed for other metalloproteinases involving an active site residue that acts as a general base to activate a zinc-bound water for nucleophilic attack on substrate and another active site residue that donates a proton to the departing amine

hydrolysis product. The solvent isotope effects are also consistent with mechanisms previously described for metalloproteinase catalysis and inhibition. While our observed isotope effects are consistent with at least two mechanisms, we favor one in which k_c/K_m is rate-limited by the transition state corresponding to collapse of a protonated tetrahedral intermediate and k_c reflects dissociation of the carboxylate product.

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