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Mechanistic Studies on the Inhibition of Stromelysin by a Peptide Phosphonamidate

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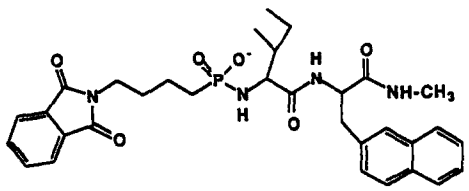
Abstract–We have investigated the inhibition of the human matrix metalloproteinase stromelysin (SLN) by the peptide phosphonamidate, phthaloyl-*N*-$(CH_2)_4$-$P(O_2^-)$-Ile-(β-naphthyl)Ala-NH-CH_3, and find that it is a potent, slow-binding inhibitor of SLN with k_{on} = 2.7 x 10^4 M^{-1} sec^{-1}, k_{off} = 1.9 x 10^{-4} sec^{-1}, and K_i = 7 nM (pH 5.0, 25°C). To probe the mechanism of inhibition, we determined pH-dependencies and solvent deuterium isotope effects. pH-dependencies of the kinetic parameters for inhibition are complex but reflect greater inhibitory potency at lower pH and suggest a mechanism for inhibition that involves the same active site groups as are involved in catalysis. The solvent isotope on k_{on} (k_{on},H_2O/k_{on},D_2O) is normal and equals 1.5 ± 0.1. Together with the pH-dependence of inhibition, this value suggests that k_{on} is rate-limited by a process that involves general-acid/general-base catalysis. We propose that k_{on} is rate-limited by general-acid catalyzed ligand exchange of inhibitor for the zinc-bound water molecule.

Introduction

Stromelysin is a member of the family of human matrix metalloproteinases (Matrisian, 1990). The principle members of this family are collagenase, gelatinase and SLN[1], and while their role in normal physiology is still unclear there is strong evidence implicating them in the pathogenesis of osteo- and rheumatoid arthritis (Pelletier, 1987). Inhibition of these enzymes is therefore an appealing strategy for pharmacologic intervention in these debilitating diseases.

As part of a program to develop inhibitors of these enzymes, we examined the mechanism of inhibition of SLN by the peptide phosphonamidate, Pht-Ape^P-Ile-Nal-MA.[2,3,4]



Phosphonamidates are a class of metalloproteinase inhibitor that combine with their target enzymes to form complexes that are stabilized by coordination of the active site zinc with one or both of the phosphonamidate oxygens (Bartlett & Marlowe, 1983; Bartlett & Marlowe, 1987; Holden *et al.*, 1987). In these complexes, the zinc-bound water molecule that exists in the active, uninhibited enzyme is absent, having been displaced by the inhibitor to bulk solvent. The peptide portions of the inhibitor interact with substrate subsites of the extended active site crevice (Holden *et al.*, 1987; Weaver *et al.*, 1977).

Peptide-derived phosphonamidates are frequently very potent inhibitors of metalloproteinases. For example, Bartlett and his co-workers (Bartlett & Marlowe, 1987) reported that Z-Phe^P-Leu-Ala is a potent inhibitor of TLN with K_i = 10^{-10} M (pH 7.0, 25°C). Their kinetic investigations further revealed that Z-Phe^P-Leu-Ala is a slow-binding inhibitor with k_{on} and k_{off} equal to 10^3 M^{-1} sec^{-1} and 10^{-7} sec^{-1}, respectively. k_{on} is at least five orders of magnitude smaller than the diffusion limit for combination of a protein and a small molecule. To explain this small value, Bartlett suggested that the inhibitor associates at the diffusion limit, but with a thermodynamically rare form of TLN. For such a mechanism, the experimentally determined association rate constant is an apparent value and has the form

$$k_{on,app} = \frac{k_{on}}{1 + K_{eq}} \quad (1)$$

In this equation, K_{eq} equals [E]/[E*], where E and E* are the major and minor form of the enzyme, respectively.

[1]ABBREVIATIONS: SLN, stromelysin; TLN, thermolysin; DNP, 2,4-dinitrophenyl; Pht, phthaloyl; Ape, 5-aminopentanoic acid; Nal, L-3-(β-naphthyl)-alanine; MA, methyl amide; FI, fluorescence intensity in arbitrary units; k_{on}, second-order rate constant for the association of enzyme and inhibitor; k_{off}, first-order rate constant for dissociation of the enzyme:inhibitor complex.

[2]A portion of this work has been presented as a communication (Izquierdo-Martin & Stein, 1992).

[3]Pht-Ape^P-Ile-Nal-MA was designed and synthesized by Dr Richard Galardy (U. of Kentucky) as an inhibitor of human collagenase and was purchased from Elastin Products, Pacific MO.

[4]The superscript P designates an amino acid in which the carboxylate moiety has been replaced with a phosphonate moiety.

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Bartlett goes on to propose that E* arises from the release to bulk solution of a specific, non-catalytic water molecule that was identified in an X-ray crystallography study of the complex of TLN and Z-Phe^P-Leu-Ala (Holden *et al.*, 1987).

While it is still uncertain if this proposal can account for the small rate constant for the association of TLN and Z-Phe^P-Leu-Ala, it is clear that the proposal cannot provide a general explanation for slow-binding inhibition of TLN and other metalloproteinases, since phosphoramidon is a slowbinding inhibitor of TLN [$k_{on} = 2.6 \times 10^4$ M^{-1} sec^{-1}; (Izquierdo-Martin & Stein, 1992; Kitagishi & Hiromi, 1984)] despite the fact that the specific water molecule is still in place (Holden *et al.*, 1987; Kester & Matthews, 1977). Clearly, additional studies need to be done to discover the mechanistic origins of slow-binding inhibition of metalloproteinases by phosphorous containing peptides.

In this paper, we report that Pht-Ape^P-Ile-Nal-MA is a slow-binding inhibitor of SLN. pH-dependencies of the kinetic parameters for inhibition were determined and suggest mechanistic similarities between catalysis and inhibition. Taken together with a normal solvent deuterium isotope effect on the association rate constant, k_{on}, these studies suggest that k_{on} is rate-limited by general-acid catalyzed ligand exchange of inhibitor for the zinc-bound water molecule.

Experimental

General

Buffer salts and deuterium oxide were purchased from Sigma. Pht-Ape^P-Ile-Nal-MA was purchased from Elastin Products (Pacific, MO). DNP-Arg-Pro-Lys-Pro-Leu-Ala-PheTrpNH$_2$ was purchased from Bachem (Philadelphia, PA). Recombinant human prostromelysin (Whitman *et al.*, 1986) was used in all experiments of this paper and was purchased from Celltech Ltd. (Slough, Berkshire, UK) at a concentration of 100 μg/ml and a purity of 98% and in a buffer of 25 mM Tris-HCl, 10 mM $CaCl_2$, 0.1% sodium azide, and 0.05% Brij-35, pH 7.5. Trypsin-catalyzed activation of proSLN was according to published procedures (Lark *et al.*, 1990a; Lark *et al.*, 1990b; Teahan *et al.*, 1989) and furnished a 0.14 μM stock solution of SLN.

Buffers

For studies of the pH-dependence of inhibition, 0.10 M acetate buffer was used for pH 5.0; 0.10 M MES buffers were used for pHs 5.5, 6.0, and 6.5; 0.10 M HEPES buffers were used for pHs 7.0, 7.5, and 8.0; 0.10 M BICINE buffer was used for pH 8.5; 0.10 M CHES buffers were used for pHs 9.0 and 9.5; and, 0.10 M CAPS buffers were used for pHs 10.0 and 10.5. In all cases, buffers contained 10 mM $CaCl_2$. Buffers for the solvent isotope effect study were prepared as described previously (Stein *et al.*, 1983; Stein *et al.*, 1987).

Kinetic experiments

The assay that was used in these studies relies on the ability of the dinitrophenyl moiety (DNP) to internally quench the fluorescence emission of the tryptophan side chain of the peptide substrate, DNP-Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$ (cleavage at Ala-Phe). The intact peptide shows little fluorescence at 340 nm, since the emitted energy from the indole of Trp is absorbed by the nearby DNP group. When the peptide is hydrolyzed by SLN, the internal quenching of the peptide is released, and an increase in the fluorescence emission at 340nm is observed. Similar assays have been described for other proteases (Netzel-Arnett *et al.*, 1991; Stack & Gray, 1989).

In a typical kinetic run, 2.94 mL of buffer and 0.020 mL each of substrate and inhibitor in DMSO were added to a 3 mL cuvette and the cuvette placed in the jacketed cell holder of a Perkin-Elmer 650-40 fluorescence spectrophotometer. Reaction temperature was kept constant to ±0.02°C. After the reaction solution had reached thermal equilibrium, we initiated the reaction by addition of 0.020 mL of enzyme stock solution. Reaction progress was monitored by the increase in the fluorescence emission at 340 nm (λ_{ex} = 290 nm) that accompanies cleavage of DNP-Arg-Pro-LysPro-Leu-Ala-Phe-TrpNH$_2$ at the Ala-Phe bond. For each kinetic run, 1000 data points, corresponding to {time, FI) pairs, were collected by a NEC Powermate 1 Plus microcomputer interfaced to the fluorescence spectrophotometer.

Results

Slow-binding inhibition of stromelysin by Pht-Ape^P-Ile-Nal-MA

Figure 1 contains a reaction progress curve for the SLN-

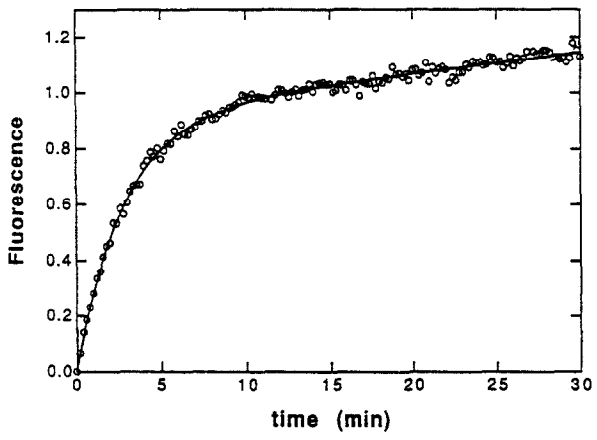


Figure 1 Progress curve for the slow-binding inhibition of SLN by Pht-Ape^P-Ile-Nal-MA. Fluorescence (λ_{em} = 290 nm, λ_{ex} = 340 nm) was recorded as a function of time for the SLN-catalyzed hydrolysis of DNP-Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$ in the presence of Pht-Ape^P-Ile-Nal-MA ($[E]_0$ = 0.015 μM, $[S]_0$ = 1.0 μM, $[I]_0$ = 0.5μM;pH 6.5, 25°C). The line through the data was drawn using eq. (2) and the best fit parameters: v_s=(8.9±0.2) x 10^{-3} FI/min; v_o=(8.99±0.04) x 10^{-1} FI/min; and, k_{obs} = 0.366±0.006 min^{-1}.

catalyzed hydrolysis of DNP-Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$ in the presence of Pht-Ape^P-Ile-Nal-MA. This curve is characterized by an initial velocity, v_o, that slowly decays to the final, steady-state velocity, v_s, with first-order rate constant, k_{obs}, and can be fit by nonlinear least-squares to the standard equation for slow-binding inhibition (Morrison & Walsh, 1988):

$$[\text{Product}] = v_s t + \left[\frac{v_o - v_s}{k_{obs}}\right][1 - \exp(-k_{obs}t)]. \quad (2)$$

For the progress curve of Figure 1, we determined the following best-fit parameters: $v_s = (8.9 \pm 0.2) \times 10^{-3}$ FI/min; $v_o = (8.99 \pm 0.04) \times 10^{-1}$ FI/min; and, $k_{obs} = 0.366 \pm 0.006$ min^{-1}. In this and all kinetic experiments reported in this paper, substrate consumption is never greater than 5%.

Progress curves similar to the one of Figure 1 were collected at various inhibitor concentrations and analyzed according to equation (2). We found that the dependencies of v_o, v_s, and k_{obs} on inhibitor concentration are unexceptional (data not shown) and show that: (i) v_o is independent of inhibitor concentration and equals the control velocity determined in the absence of inhibitor; (ii) v_s depends on inhibitor concentration according to eq. (3);

$$v_s = \frac{v_o}{1 + \frac{[I]}{K_i}} \quad (3)$$

and, (iii) k_{obs} is linearly dependent on inhibitor concentration. These observations support the simple mechanism of Scheme I.

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{:}I$$

Scheme I. Kinetic mechanism for slow-binding inhibition of stromelysin by Pht-Ape^P-Ile-Nal-MA.

According to this mechanism, the linear dependence of k_{obs} on inhibitor concentration is described by

$$k_{obs} = k_{on}[I] + k_{off} \quad (4)$$

and the inhibition constant, K_i, is given by

$$K_i = \frac{k_{off}}{k_{on}}. \quad (5)$$

Note that these experiments were all conducted under the condition: [S] = 10 μM << K_m = 120μM. This allows us to use the substrate concentration-independent equations (3) - (5).

K_i, k_{on}, and k_{off} were estimated from rearranged forms of equations (3) - (5) as shown below:

$$K_i = \frac{[I]}{\frac{v_o}{v_i} - 1} \quad (6)$$

$$k_{on} = \frac{k_{obs}}{[I] + K_i} \quad (7)$$

$$k_{off} = k_{on}K_i. \quad (8)$$

In practice, after a progress curve is obtained, best-fit values for v_o, v_s, and k_{obs} are determined, K_i is calculated from eq. (6), and then used together with eq. (7) to calculate k_{on}. Finally, k_{off} is estimated as $k_{on}K_i$. Replicate determinations under a given set of conditions (e.g., pH or solvent isotopic composition) are used to determine mean values. Standard deviations of the means were never greater than 10%.

pH-Dependence of the slow-binding inhibition of stromelysin by Pht-Ape^P-Ile-Nal-MA

To probe the mechanism of the slow-binding inhibition of SLN by Pht-Ape^P-Ile-NalMA, we determined the pH-dependence of K_{ass} (= $1/K_i$), k_{on} and k_{off} (see Figures 2 - 4). Viewed broadly, we see that inhibitory potency decreases with increasing pH. This is reflected in decreasing values of K_{ass} and k_{on} and increasing values of k_{off} with increasing pH (see below).

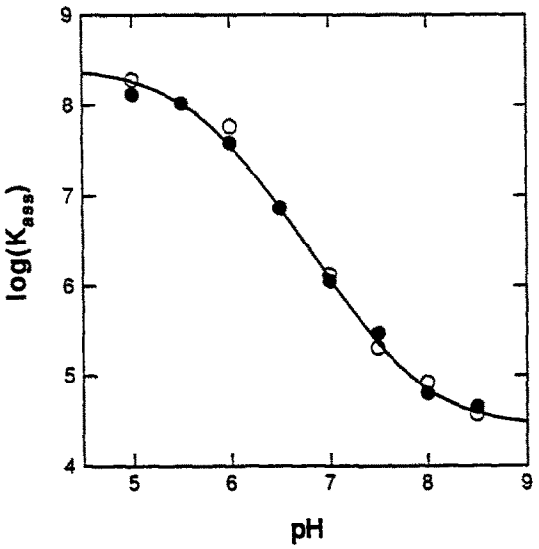
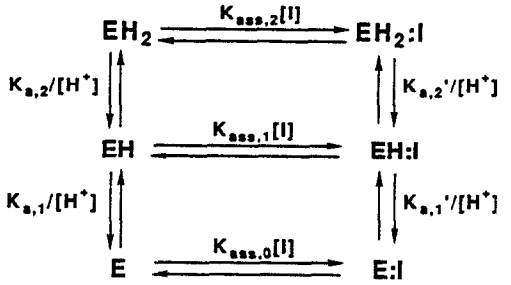


Figure 2 pH-Dependence of K_{ass} for the inhibition of SLN by Pht-Ape^P-Ile-Nal-MA. Values of K_{ass} were determined as described in the text as a function of pH. Filled and empty circles correspond to two independent experiments. The line through the data points was drawn using eq. (9) and the following parameters: $K_{ass,2}$=1.9x10^8 M^{-1}; $K_{ass,1}$ = 1x10^6 M^{-1}; $K_{ass,0}$=4.1x10^4 M^{-1}; $pK_{a,2}$=5.59; and, $pK_{a,1}$=6.22.

pH-Dependence of K_{ass}

In the pH range that we examined, K_{ass} decreases with increasing pH. Examination of the data in Figure 2 reveals that this dependence is steeper than predicted from a simple model involving two protonation states. This, together with the limiting value that K_{ass} approaches at high pH, suggests the mechanism of Scheme II.



Scheme II. pH-Dependence of K_{ass} for the interaction of SLN and Pht-Ape^P-Ile-Nal-MA.

The rate expression for this model appears in equation (9)

$$K_{ass} = K_{ass2}\left\{\frac{1 + \frac{K_{a2}'}{[H^+]} + \frac{K_{a2}'}{[H^+]}\frac{K_{a1}'}{[H^+]}}{1 + \frac{K_{a2}}{[H^+]} + \frac{K_{a2}}{[H^+]}\frac{K_{a1}}{[H^+]}}\right\} \quad (9)$$

According to this mechanism,

$$\frac{K_{a2}K_{a1}}{K_{ass2}} = \frac{K_{a2}'K_{a1}'}{K_{ass0}} = \frac{K_{a2}'K_{a1}}{K_{ass1}} \quad (10)$$

Thus

$$K_{ass,0} = K_{ass,2}\left(\frac{K_{a2}'K_{a1}'}{K_{a2}K_{a1}}\right) \quad (11)$$

and

$$K_{ass,1} = K_{ass,2}\left(\frac{K_{a2}'K_{a1}}{K_{a2}K_{a1}}\right) \quad (12)$$

Attempts at fitting the data of Figure 2 to eq. (9) failed due to non-convergence. However, when we constrained $pK_{a,2}$ and $pK_{a,1}$ to 5.4 and 6.2, respectively, we obtained the following: $K_{ass,2} = (2.6 \pm 0.2) \times 10^8$ M^{-1}; $pK_{a,2}' = 7.48 \pm 0.15$; and, $pK_{a,1}' = 8.10 \pm 0.23$. The numerical values to which $pK_{a,2}$ and $pK_{a,1}$ were constrained were chosen for their similarity to values obtained from the pH-dependencies of k_c/K_m (Harrison *et al.*, 1992) and k_{on} (see below). From the best-fit values of $K_{ass,2}$, $pK_{a,2}'$, and $pK_{a,1}'$ we calculate: $K_{ass,0} = 2.7 \times 10^4$ M^{-1} and $K_{ass,1} = 2.2 \times 10^6$ M^{-1}.

We see that as the pH is raised, potency decreases by four orders of magnitude; that is, the K_i value changes from a limiting value at low pH of 5 nM to 50 μM at high pH. This latter value is similar to K_m values measured for the reaction of SLN with several of its substrates (unpublished data) and suggests that at high pH, Pht-Ape^P-Ile-Nal-MA may only form a simple, Michaelis-like complex with SLN.

pH-Dependence of k_{on}

In the pH range examined, k_{on} also decreases with increasing pH (Figure 3). These data are consistent with the mechanism of Scheme III and when fitted to the rate expression of eq. (13) by non-linear least squares yields: $k_{on,2} = 34 \pm 2$ mM^{-1} sec^{-1}; $k_{on,1} = 8.3 \pm 1.9$ mM^{-1} sec^{-1}; $k_{on,0} = 0.24 \pm 0.01$ mM^{-1} sec^{-1}; $pK_{a,2} = 5.44 \pm 0.11$; and, $pK_{a,1} = 6.42 \pm 0.18$.

$$k_{on} = \frac{k_{on2}}{1 + \frac{K_{a2}}{[H^+]} + \frac{K_{a2}K_{a1}}{[H^+]^2}} + \frac{k_{on1}}{\frac{[H^+]}{K_{a2}} + 1 + \frac{K_{a1}}{[H^+]}} + \frac{k_{on0}}{\frac{[H^+]^2}{K_{a2}K_{a1}} + \frac{[H^+]}{K_{a1}} + 1} \quad (13)$$

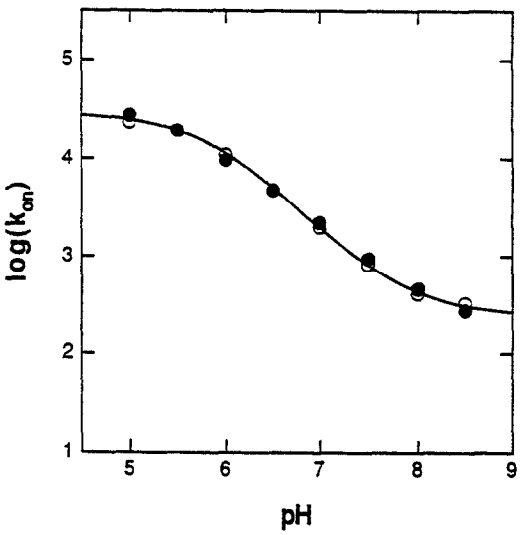
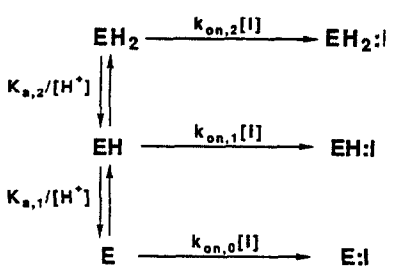


Figure 3 pH-Dependence of k_{on} for the inhibition of SLN by Pht-Ape^P-Ile-Nal-MA. Values of k_{on} were determined as described in the text as a function of pH. Filled and empty circles correspond to two independent experiments. The line through the data points was drawn using eq. (13) and the following parameters: $k_{on,2}$=34 mM^{-1} sec^{-1}; $k_{on,1}$=8.3 mM^{-1} sec^{-1}; $k_{on,0}$=0.24 mM^{-1} sec^{-1}; $pK_{a,2}$=5.44; and, $pK_{a,1}$=6.42.



Scheme III. pH-Dependence of k_{on} for the interaction of SLN and Pht-Ape^P-Ile-Nal-MA.

The data of Figure 3 can also be fit to a simpler mechanism involving a single pK_a and only two parallel pathways. However, to produce an internally consistent picture for the inhibition of SLN by Pht-Ape^P-Ile-Nal-MA, we must use a mechanism for k_{on} that is analogous to the mechanism for K_{ass}. Thus, we choose to use the more complex model of Scheme III.

pH-Dependence of k_{off}

The pH-dependence of k_{off} (Figure 4) suggests the model of Scheme IV.

$$k_{off} = \frac{k_{off,2}}{\frac{K_{a2}'K_{a1}'}{[H^+]^2} + \frac{K_{a1}'}{[H^+]} + 1} + \frac{k_{off,1}}{\frac{K_{a1}'}{[H^+]} + 1 + \frac{[H^+]}{K_{a2}'}} + \frac{k_{off,0}}{1 + \frac{[H^+]}{K_{a1}'} + \frac{[H^+]^2}{K_{a2}'K_{a1}'}} \quad (14)$$

The data are not of sufficient quality to allow fitting directly to eq. (14), but if we constrain $pK_{a,2}'$ and $pK_{a,1}'$ to 7.5 and 8.1, respectively, we obtain the following best fit parameters: $k_{off,2} = (0.14 \pm 0.02) \times 10^{-3}$ sec^{-1}; $k_{off,1} = (5.5 \pm 0.7) \times 10^{-3}$ sec^{-1}; and, $k_{off,0} = (8.1 \pm 1.8) \times 10^{-3}$ sec^{-1}.

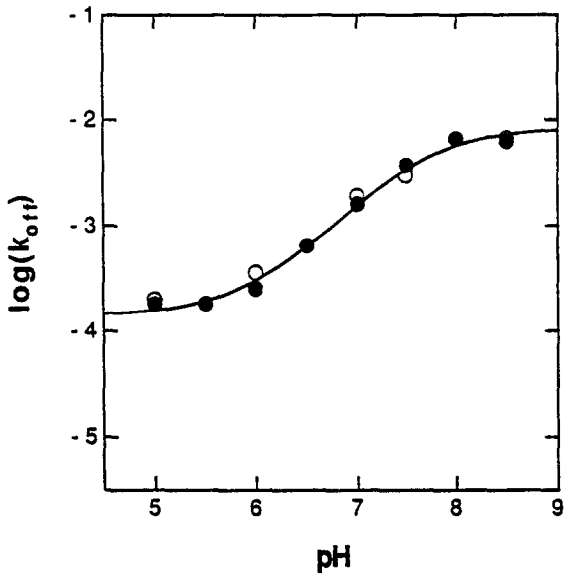
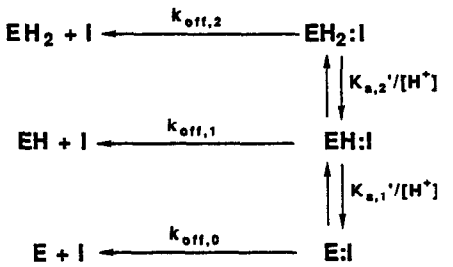


Figure 4 pH-Dependence of k_{off} for the inhibiton of SLN by Pht-Ape^P-Ile-Nal-MA. Values of k_{off} were determined as described in the text as a function of pH. Filled and empty circles correspond to two independent experiments. The line through the data points was drawn using eq. (14) and the following parameters: $k_{off,2}$-0.17×10^{-3} sec^{-1}; $k_{off,1}$=0.4×10^{-3} sec^{-1}; $k_{off,0}$=7.1×10^{-3} sec^{-1}; $pK_{a,2}'$=6.75; and $pK_{a,1}'$=7.38.



Scheme IV. pH-Dependence of k_{off} for the interaction of SLN and Pht-Ape^P-Ile-Nal-MA.

These are reasonable values, since the ratios, $k_{on,n}/k_{off,n}$, are nearly identical to the corresponding $K_{ass,n}$ values: $k_{on,2}/k_{off,2}$ = 34 mM^{-1} sec^{-1}/0.14×10^{-3} sec^{-1} = 2.4×10^8 M^{-1}, $K_{ass,2} = 2.6 \times 10^8$ M^{-1}; $k_{on,1}/k_{off,1}$ = 8.3 mM^{-1} sec^{-1} /5.5×10^{-3} sec^{-1} = 1.5×10^6 M^{-1}, $K_{ass,1} = 2.2 \times 10^6$ M^{-1}; and, $k_{on,0}/k_{off,0}$ = 240 M^{-1} sec^{-1}/8.1×10^{-3} sec^{-1} = 3.0×10^4 M^{-1}, $K_{ass,0} = 2.7 \times 10^4$ M^{-1}.

Solvent deuterium isotope effect on k_{on} for the inhibition of stromelysin by Pht-Ape^P-Ile-Nal-MA

Solvent deuterium isotope effects on k_{on}, $^Dk_{on}$, were determined at pH 5.0 and pD-equivalent for the inhibition of SLN by Pht-Ape^P-Ile-Nal-MA. In these experiments, [E] = 17 nM, [S] = 10 μM, and [I] = 0.2 μM. Under these conditions, $k_{on}[I] = 5.4 \times 10^{-3}$ sec^{-1} >> $k_{off} = 0.2 \times 10^{-3}$ sec^{-1}. Therefore, $k_{obs}/[I] = k_{on}$ and $^Dk_{obs} = {}^Dk_{on}$. In two separate experiments, $^Dk_{on}$ was determined to be 1.51 ± 0.14 and 1.52 ± 0.08, respectively (Table 1).

Table 1. Solvent deuterium isotope effect on k_{on} for the inhibition of stromelysin by Pht-Ape^P-Ile-Nal-Ma[a]

Expt. #1				Expt. #2			
Run	L_2O	k_{obs} (mM^{-1} sec^{-1})	$^Dk_{on}$[b]	Run	L_2O	k_{obs} (mM^{-1} sec^{-1})	$^Dk_{on}$[b]
1	H	39.1	1.72	1	H	32.2	1.51
2	D	27.7		2	D	21.3	
3	H	32.1	1.40	3	H	35.2	1.56
4	D	22.9		4	D	22.5	
5	H	31.1	1.38	5	H	35.6	1.55
6	D	22.5		6	D	23.0	
7	H	37.8	1.55	7	H	34.4	1.59
8	D	21.8		8	D	21.6	
9	H	31.0	1.48	9	H	32.1	1.39
10	D	20.9		10	D	23.1	
			1.51 ± 0.14				1.52 ± 0.08

(*a*) Reactions were run at pH 5.0 and pD equivalent with [E] = 17 nM, [S] = 10 μM, [I] = 0.2 μM.
(*b*) At [I] = 0.2 μM, $k_{on}[I] = 5.4 \times 10^{-3}$ sec^{-1}>>$k_{off} = 0.2 \times 10^{-3}$ sec^{-1}. Therefore, $k_{obs}/[I] = k_{on}$ and $^Dk_{obs} = {}^Dk_{on}$.

Discussion

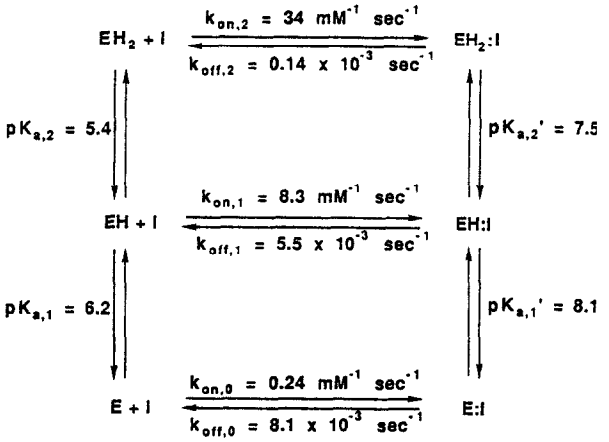
A principle finding of this study is that Pht-Ape^P-Ile-Nal-MA is a slow-binding inhibitor of SLN. This extends the list of enzymes that are subject to slow-binding inhibition (Morrison & Walsh, 1988) and supports the generality of the slow-binding phenomenon for phosphorous containing inhibitors of metalloproteinases. Pht-Ape^P-Ile-Nal-MA is also a potent inhibitor with $K_{i,limit}$ equal to 5 nM and, thus, may form the basis for the design of *in vivo* active metalloproteinase inhibitors.

The limiting value for k_{on} at low pH is only 3×10^4 M^{-1} sec^{-1}, several orders of magnitude smaller than the diffusion controlled limit for association of a protein and small

molecule. The goal of this study was to understand why this value is so small. To this end, we determined pH-dependencies and solvent deuterium isotope effects for the inhibition kinetic parameters.

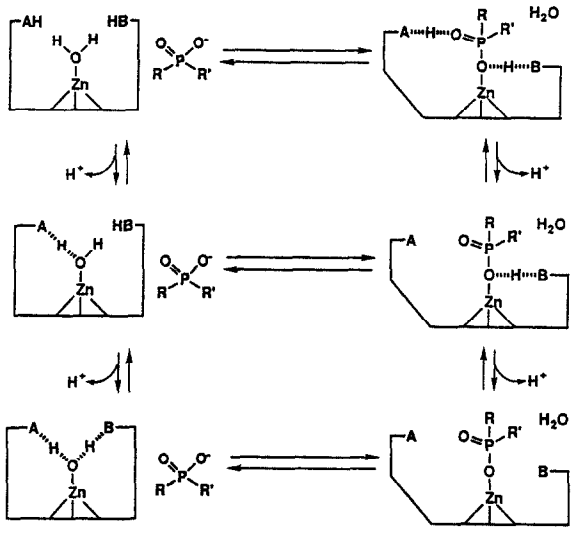
pH-Dependence of inhibition of stromelysin by Pht-Ape^P-Ile-Nal-MA

The kinetic parameters together with the kinetic mechanism for the pH-dependence of inhibition of SLN by Pht-Ape^P-Ile-Nal-MA are summarized in Scheme V. An interesting feature of this pH-dependence is the existence of parallel pathways for combination of enzyme and inhibitor. This indicates that the reduced ability of EH and E to bind Pht-Ape^P-Ile-Nal-MA is not absolute. We believe that the progressive reduction in k_{on} for EH_2, EH, and E reflects the successive elimination of active site groups that can donate hydrogen bonds to the inhibitor in the transition states for these three processes. This inability to donate stabilizing hydrogen bonds is also apparent in the progressive instability of EH_2:I, EH:I, and E:I as reflected in the increasing values of k_{off} for these complexes.



Scheme V. Kinetic mechanism for the pH-dependence of inhibition of SLN by Pht-Ape^P-Ile-Nal-MA.

A logical extension of the kinetic mechanism of Scheme V is the chemical mechanism of Scheme VI. This scheme incorporates active site features that we believe SLN must possess; that is, a zinc bound water molecule and two catalytically essential amino acid residues (Harrison *et al.*, 1992). According to Scheme VI, at pH values less than 5, the ionizable groups at the active site are protonated and donate two, stabilizing hydrogen bonds to the bound inhibitor in the complex, EH_2I. This hydrogen-bonding pattern has been observed in inhibitor complexes of TLN (Holden *et al.*, 1987; Tronrud *et al.*, 1986; Weaver *et al.*, 1977) and a similar mechanism can be used to explain the pH-dependence of inhibition of TLN by phosphoramidon (unpublished data of authors) in which case A and B would correspond to Glu_{143} and His_{231}, respectively. As the pH increases, AH and BH become unprotonated and can no longer stabilize enzyme:inhibitor complexes. Thus, this scheme explains why Pht-Ape^P-Ile-Nal-MA displays its greatest potency as an inhibitor of SLN at low pH.



Scheme VI. Mechanistic proposal for the pH-dependence of the inhbition of SLN by Pht-Ape^P-Ile-Nal-MA.

Solvent isotope effects for the association of stromelysin with Pht-Ape^P-Ile-Nal-MA

To explore transition state structural features for the association of SLN with Pht-Ape^P-Ile-Nal-MA, we determined the solvent deuterium isotope effect for k_{on}. The pH-independent value for $^D(k_{on})$ is 1.5 ± 0.1 and can be interpreted in terms of the general expression of eq. (15),

$$^Dk_{on} = (Z^{-1})^n \frac{\Pi\Phi_{reactant}}{\Pi\Phi_{\neq}} \qquad (15)$$

which expresses $^Dk_{on}$ as the ratio of products of reactant state and transition state fractionation factors (Quinn & Sutton, 1991) multiplied by a term, Z, that reflects medium effects (Stein, 1983, 1985a, b; Kurz *et al.*, 1992). For the reactions under consideration here, we expand eq. (15) to:

$$^Dk_{on} = (Z^{-1})^n \frac{(\Phi_{Zn\text{-}OH2})^2[\Pi\Phi_{inhibitor}][\Pi\Phi_{stromelysin}]}{\Pi\Phi_{\neq}} \qquad (16)$$

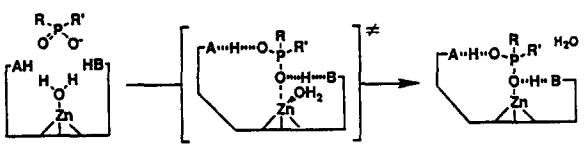
where $\Phi_{Zn\text{-}OH_2}$ is the fractionation factor for one of the two hydrogens of the zinc-bound water molecule and $\Pi\Phi_{inhibitor}$ and $\Pi\Phi_{stromelysin}$ are the products of fractionation factors for the inhibitor and enzyme, respectively.

Z is the product of many small fraction factors that originate from the solvent reorganization that accompanies binding of substrate or inhibitor (Schowen & Schowen, 1982; Venkatasubban & Schowen, 1985; Stein & Matta,

1985) and reflects the greater solubility of these substances in light water (Julin & Kirsch, 1981). Z is similiar in magnitude to equilibrium solvent isotope effects on dissociation constants for E:S or E:I complexes when the processes governed by K_s or K_i do not contain chemical steps (Stein, 1983, 1985a, b; Kurz *et al.*, 1992). We do not know the magnitude of Z for the interaction of SLN and Pht-Ape^P-Ile-Nal-MA. ^DK_m for the SLN-catalyzed hydrolysis of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH_2 is 2.0 ± 0.2 (Harrison *et al.*, 1992), but for this reaction K_m is thought to reflect chemistry and thus is not the dissociation constant for E:S. Since values of Z that are larger than 1.0 will only serve to enlarge the observed isotope effect, we will take the conservative position and, in the arguments that follow, assume that Z is unity.

Having discussed the Z term, we are now in a position to calculate a value for $\Pi\Phi_{\neq}$. If we make the reasonable assumptions that the fractionation factors for the substrate and enzyme are unity (Quinn & Sutton, 1991; Venkatasubban & Schowen, 1985) and set $\Phi_{Zn\text{-}OH_2}$ equal to 0.85 (Kassebaum & Silverman, 1989; Schmidt *et al.*, 1979; Izquierdo-Martin & Stein, 1992b), we can calculate a value for $\Pi\Phi_{\neq}$ of 0.48. The inverse of this value, 2.1, reflects the transition state contribution to the overall isotope effect contribution. We see then that the observed isotope effect of 1.5 is the transition state contribution of 2.1 offset by a ground state contribution of 0.74 (0.85^2). Note that the transition state contribution to the isotope effect increases as Z increases since $(\Pi\Phi_{\neq})^{-1} = Z^D k_{on}(\Pi\Phi_{reactant})^{-1} = Z(2.1)$.

To explain the normal isotope effect on k_{on} of 1.5, we formulate a transition state structure that involves proton bridging of the sort observed in general-acid/general-base catalysis. One possible proposal for this transition state appears in Scheme VII and shows the displacement of the zinc-bound water accompanied by formation of two proton bridges. If these are of the sort described by Kreevoy (Kreevoy *et al.*, 1977), they will manifest themselves in transition state fractionation factors that are less than unity and produce normal isotope effects. A similar mechanism was used to explain the slow-binding inhibition of TLN by phosphoramidon [$k_{on} = 1.1 \times 10^5\ M^{-1}\ sec^{-1}$, $k_{off} = 4 \times 10^{-5}\ sec^{-1}$, pH 5.5; (Izquierdo-Martin & Stein, 1992)]. In this case, we observed an even larger isotope effect on k_{on} of 1.8 (Izquierdo-Martin & Stein, 1992).



Scheme VII. Proposed transition state structure for the interaction of SLN and Pht-Ape^P-Ile-Nal-MA at low pH.

Conclusions

The results reported herein together with similar results for the inhibition of TLN by phosphoramidon (Izquierdo-Martin & Stein, 1992) effectively eliminate "rare-form" inhibition and protein conformational changes as general causes of slow-binding inhibition of metalloproteinases. pH-Dependencies for inhibition reveal pK_a values that are identical to those observed for catalysis. This would only occur if the protein conformational change or production of the "rare" form of the enzyme are pH-independent processes. Furthermore, neither of these mechanisms would be expected to produce large, normal solvent isotope effects on k_{on}. However, ligand exchange on zinc to displace a water molecule may represent a general mechanism for slow-binding inhibition of metalloproteinases.

References

Bartlett, P.A. and Marlowe, C.K. (1983) *Biochemistry* **22**, 4618-4624.

Bartlett, P.A. and Marlowe, C.K. (1987) *Biochemistry* **26**, 8553-8561.

Harrison, R.K., Chang, B., Niedzwiecki, L. and Stein, R.L. (1992) *Biochemistry* **31**, 10757-10762.

Holden, H.M., Tronrud, D.E., Monzingo, A.F., Weaver, L.H. and Matthews, B.W. (1987) *Biochemistry* **26**, 8542-8553.

Izquierdo, M. and Stein, R.L. (1990) *J. Amer. Chem. Soc.* **112**, 6054-6062.

Izquierdo-Martin, M. and Stein, R.L. (1992) *J. Amer. Chem. Soc.* **114**, 1527-1528.

Izquierdo-Martin, M. and Stein, R.L. (1992b) *J. Amer. Chem. Soc.* **114**, 325-331.

Julin, D. and Kirsch, J.F. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **40**, 1657.

Kassebaum, J.W. and Silverman, D.N. (1989) *J. Amer. Chem. Soc.* **111**, 2691-2696.

Kester, W.R. and Matthews, B.W. (1977) *Biochemistry* **16**, 2506-2516.

Kitagishi, K. and Hiromi, K. (1984) *J. Biochem.* **95**, 529-534.

Kreevoy, M.M., Liang, T. and Chang, K.C. (1977) *J. Amer. Chem. Soc.* **99**, 5207-5209.

Kurz, L.C., Moiz, L., Riley, M.C., and Frieden, C. (1992) *Biochemistry* **31**, 39-48.

Lark, M.W., Saphos, C.A., Walakovits, L.A. and Moore, V.L. (1990a) *Biochem. Pharmacol.* **39**, 2041-2049.

Lark, M.W., Walakovits, L.A., Shah, T.K., VanMiddlesworth, J., Cameron, P.M. and Lin, T.Y. (1990b) *Connective Tissue Res.* **25**, 49-65.

Matrisian, L.M. (1990) *Trends Genetics* **6**, 121-126.

Morrison, J.F. and Walsh, C.T. (1988) *Adv. Enzym.* **61**, 201-301.

Netzel-Arnett, S., Mallya, S.K., Nagase, H., Birkedal-Hansen, H. and Van Wart, H.E. (1991) *Anal. Biochem.* **195**, 86-92.

Pelletier, J.-P. (1987) *J. Rheumatology, Special Issue*, **14**, 1-138.

Quinn, D.M. and Sutton, L.D. (1991) In P. F. Cook (Ed.), *Enzyme Mechanism from Isotope Effects* (pp. 73-126). Boston: CRC Press.

Schowen, K.B. and Schowen, R.L. (1982) *Meth. Enzymol.* **87**, 551-606.

Schmidt, J., Chen, J., DeTraglia, M., Minkel, D. and McFarland, J.T. (1979) *J. Amer. Chem. Soc.* **101**, 3634-3640.

Stack, M.S. and Gray, R.D. (1989) *J. Biol. Chem.* **264**, 4277-4281.

Stein, R.L. (1983) *J. Amer. Chem. Soc.* **105**, 5111-5116.

Stein, R.L. (1985a) *J. Amer. Chem. Soc.* **107**, 6039-6042.

Stein, R.L. (1985b) *J. Amer. Chem. Soc.* **107**, 7768-7769.

Stein, R.L. (1988) *J. Amer. Chem. Soc.* **110**, 7907-7908.

Stein, R.L. and Matta, M.S. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **44**, 1055.

Stein, R.L., Elrod, J.P. and Schowen, R.L. (1983) *J. Amer. Chem. Soc.* **105**, 2446-2452.

Stein, R.L., Strimpler, A.M., Hitoshi, H. and Powers, J.C. (1987) *Biochemistry* **26**, 1305-1314.

Teahan, J., Harrison, R., Izquierdo, M. and Stein, R.L. (1989) *Biochemistry* **28**, 8497-8501.

Tronrud, D.E., Monzingo, A.F. and Matthews, B.W. (1986) *Eur. J. Biochem.* **157**, 261-268.

Venkatasubban, K.S. and Schowen, R.L. (1985) *CRC Crit. Rev. Biochem.* **17**, 1-41.

Weaver, L.H., Kester., W.R. and Matthews, B.W. (1977) *J. Mol. Biol.* **114**, 119-132.

Whitman, S.E., Murphy, G., Angel, P., Rahmsdorf, H.J., Smith, B., Lyons, A., Harris, T.J.R., Herrlich, P. and Docherty, A.J.P. (1986) *Biochem. J.* **240**, 913-916.