Substrate Specificity of Human Fibroblast Stromelysin. Hydrolysis of Substance P and Its Analogues

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ABSTRACT: To probe the substrate specificity of the human metalloproteinase stromelysin (SLN), we determined values of k_c/K_m for the SLN-catalyzed hydrolysis of substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$; SP; k_c/K_m = 1790 ± 140 M^{-1} s^{-1}), 15 analogues of SP, and 17 other peptides. We found a remarkably narrow substrate specificity for SLN: while SP and its analogues could serve as substrates for SLN (hydrolysis occurred exclusively at the Gln^6–Phe^7 bond), peptides that were not direct analogues could not (k_c/K_m < 3 M^{-1} s^{-1}). From the study of the SLN-catalyzed hydrolysis of SP and its analogues, the following findings emerged: (1) Decreasing the length of SP results in decreases in k_c/K_m. (2) Conservative amino acid replacements near the scissle bond of SP decrease k_c/K_m. (3) The SP analogue in which Gly^9 is replaced with sarcosine (*N*-methylglycine) is not hydrolyzed by SLN (k_c/K_m < 3 M^{-1} s^{-1}). (4) Several SP analogues that are not hydrolyzed by SLN are inhibitors of the enzyme. The complexes formed from interaction of SLN with these peptides have dissociation constants that are similar to the K_m value for the complex of SLN and SP. Combined, these results suggest that SLN uses the energy that is available from favorable interactions with its substrate to stabilize catalytic transition states but not the Michaelis complex or other stable-state complexes.

The metalloproteinase stromelysin (Murphy et al., 1986, 1988; Okada et al., 1986, 1988) is thought to play a causative role in osteoarthritis and rheumatoid arthritis (Cawston et al., 1984; Ehrlich et al., 1987) and is therefore an attractive target for therapeutic intervention in these diseases. Inhibitors of SLN[1] should prevent the enzymatic destruction of connective tissue and, thus, retard the progress of arthritic diseases. A detailed catalytic mechanism is fundamental to the design of such inhibitors, and as a part of our program to develop inhibitors of SLN, we have undertaken studies to define this mechanism.

As a first step in the elucidation of the catalytic mechanism of SLN, we needed to determine the enzyme's substrate specificity.[2] In a previous paper from this laboratory (Harrison et al., 1989), we reported that substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$) is a substrate for SLN and is cleaved exclusively at the Gln^6–Phe^7 bond. In this paper, we extend this observation and report the first attempt to define SLN's substrate specificity. We have studied the SLN-catalyzed hydrolysis of 15 analogues of SP, as well as 17 other peptides, and find that only SP and its analogues are hydrolyzed by SLN. Furthermore, we find that the catalytic efficiency of SLN is acutely sensitive to structural features of the SP analogues.

Materials and Methods

Chemicals. Tris-HCl, sodium azide, and Brij 35 were obtained from Sigma (St. Louis, MO). Calcium chloride was purchased from Mallinckrodt (Paris, KY). House-distilled water was deionized by using a Milli-Q water purification system (Millipore, Millford, MA). HPLC grade acetonitrile and TFA were obtained from Fisher Scientific (Pittsburgh, PA).

Peptides. Peptides were purchased from Sigma (St. Louis, MO), Bachem (Philadelphia, PA), and Peninsula (Belmont, CA) and used without further purification. HPLC analysis of these peptides indicated that they were greater than 95% pure. For analysis as stromelysin substrates, peptides were dissolved directly in assay buffer (pH 7.5 buffer containing 20 mM Tris-HCl, 10 mM $CaCl_2$, 0.02% NaN_3, and 0.05% Brij 35).

Enzyme. Stromelysin was supplied by Dr. M. Lark of the Department of Biochemical and Molecular Pathology, Merck and Co. Dr. Lark and co-workers purified the enzyme as the zymogen from the media of cultured human gingival cells (Lark et al., 1988) and supplied it to us as a 240 μg/mL (4.4 μM; M_r = 54 000) solution in assay buffer containing 0.3 M NaCl. The zymogen was activated by incubation with 6 nM trypsin for 1 h at 37 °C. Following this incubation, trypsin was inactivated by a 20-min, 37 °C incubation with a 20-fold molar excess of soybean trypsin inhibitor bound to agarose (Sigma, St. Louis, MO). The final concentration of enzyme in these $[SLN]_{stock}$ solutions was 6.4 μg/mL or 0.14 μM, assuming a 90% conversion of pro-SLN to active SLN (M_r = 46 000). This level of activity is also supported by the stoichiometric inhibition of SLN by TIMP (Lark et al., 1989a). Details of pro-SLN purification and activation will be published elsewhere (Lark et al., 1989b).

Chromatographic Conditions. The chromatographic apparatus consisted of a VISTA 54 automated HPLC system (Walnut Creek, CA) and a 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). Absorbance was measured at 215 nm by using a Kratos Spectroflow 757 variable-wavelength detector (ABI Analytical, Ramsey, NJ). Peak integrations were performed on a PE Nelson Turbochrom data

[1] Abbreviations: DNP, 2,4-dinitrophenyl; HPLC, high-performance liquid chromatography; k_c, first-order rate constant for the conversion of the most stable E·S complex to products, equal to $V_{max}/[E]$; SLN, stromelysin; SP, substance P; TFA, trifluoroacetic acid; TIMP, tissue inhibitor of metalloproteinases.

[2] We define substrate specificity as the correlation between substrate structure and catalytic efficiency. In this paper, we take k_c/K_m as the measure of catalytic efficiency.

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Table I: Peptides That Are Not Hydrolyzed by Stromelysin[a]

insulin chain B fragment 22–30	Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala
angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
ACTH fragment 1–10	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly
ACTH fragment 4–10	Met-Glu-His-Phe-Arg-Trp-Gly
dynorphin 1–7	Tyr-Gly-Gly-Phe-Leu-Arg-Arg
dynorphin 1–8	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile
human fibrinogen 1–21	
bradykinin	Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg
leucine-enkephalin	Tyr-Gly-Gly-Phe-Leu
kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2
eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
adipokinetic hormone	Pyr-Leu-Asn-Phe-Ser-Ala-Gly-Trp-NH_2
Tyr-CGRP	Tyr-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Gly-Trp-NH_2
somatostatin 14	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Cys
oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
	DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH_2
	DNP-Pro-Glu-Gly-Ile-Ala-Gly-Glu-Arg

[a] Underlined amino acids designate the anticipated site of cleavage.

station (Cupertino, CA). Separations were performed on a Whatman Partisil 5 C8 analytical column (25 cm × 4.6 mm i.d.). Mobile phase was 0.1% aqueous TFA and CH_3CN. The ratio of TFA to CH_3CN was adjusted for each peptide to optimize separation of parent peptide from hydrolysis products. Flow rate was 1.5 mL/min.

Screening of Peptides for Their Ability To Serve as SLN Substrates. In a typical assay, a 1 mM solution of the peptide was incubated with 35 nM SLN at 25 °C. After this solution had incubated for 20–24 h, the reaction solution was subjected to reverse-phase HPLC to separate unreacted substrate from products. Peak areas corresponding to unreacted substrate were then calculated and compared to control samples in which no enzyme had been added. To ensure a clean separation of the parent peptide from any hydrolysis products, the TFA/CH_3CN ratio of the HPLC mobile phase was adjusted so that the parent compound eluted at about 10 min on a chromatogram of about 20 min.

Kinetic Assays (Harrison et al., 1989). In a typical kinetic measurement, 900 μL of a buffered solution of substrate in a 2.0-mL glass autosampler vial was thermally equilibrated to 25 °C for 15 min in the thermostated compartment of the autosampler; 900 μL of $[SLN]_{stock}$ that had been thermally equilibrated to 25 °C was then added to this vial to give a final SLN concentration of 70 nM. The reaction solution was mixed by inversion and placed back in the autosampler. Immediately after the initiation of the reaction, and at predetermined time intervals thereafter, 20-μL aliquots were withdrawn from the reaction solution by the autosampler and injected onto the column. The sampling time was chosen to ensure that the progress curves contained no less than two data points per half-time of substrate disappearance.

Inhibition Assay. SP (0.3 mM), 35 nM SLN, and various concentrations of inhibitor were incubated for 60 min at 25 °C in a final volume of 200 μL of assay buffer. At the end of this time, the reaction was terminated by the addition of 200 μL of 0.15 M H_3PO_4 (final pH 2). The samples were then subjected to HPLC analysis, outlined above, to separate unreacted substrate from the reaction product, SP^{7-11}. Peak areas corresponding to the product were then plotted vs [I]. Since under these experimental conditions product accumulation is linear with time, peak area is directly proportional to the steady-state reaction velocity, v_{ss}. We can, therefore, fit the raw data, by nonlinear least-squares, to the expression for competitive inhibition, $v_{ss} = V_m[S]/\{K_m(1 + [I]/K_i) + [S]\}$, to arrive at values for K_i.

Oxidation of SP. SP (1.2 mM) was reacted with 2 mM *N*-chlorosuccinimide in assay buffer for 5 min at 25 °C. At the end of this time, a 20-μL aliquot of the reaction solution was injected onto a Whatman C8 reverse-phase HPLC column (25 cm × 4.6 mm i.d.) and eluted with an aqueous solution of 0.1% TFA and acetonitrile at a volume ratio of 72/28. Detection was at 215 nm.

Results

Screening of Peptides as Substrates for Stromelysin. In attempts to find a low molecular weight substrate for SLN, we tested a number of commercially available peptides as SLN substrates. These peptides are listed in Table I. None of these were hydrolyzed to an extent greater than 3% under the conditions of the assay (35 nM SLN, 20 h, 25 °C). This corresponds to a rate of SLN-catalyzed hydrolysis of less than 0.024 μM/min and should be compared to the rate of SP hydrolysis of 2.1 μM/min.

Several of the negative results of Table I are noteworthy. In contrast to the results reported by Shaw and co-workers (1988), neither the ACTH fragments, the dynorphins, nor Leu-enkephalin was found to be substrate for SLN. We should note, however, that Shaw used a partially purified rabbit chondrocyte enzyme, not the human fibroblast enzyme used in the present work. None of the peptides that contain Gln, His, or Asn at P_1[3] were hydrolyzed, as may have been anticipated on the basis of the enzyme's ability to hydrolyze SP. Similarly, none of the peptides with Phe or Ile at P_1' served as substrates for SLN. Note that these peptides include kassinin and eledoisin, which are tachykinins and structurally related to SP. To date, only SP and certain analogues have been found to be substrates for SLN. Combined, these results indicate that SLN has a remarkably narrow specificity toward peptide substrates, where SP displays the highest activity as a substrate, thus far.

Hydrolysis of Substance P Analogues by Stromelysin. During our search for SLN substrates, we discovered that SP and a number of its analogues were hydrolyzed by the enzyme. In all cases, we found that peptide bond cleavage occurs exclusively at the Gln–Phe bond. In preliminary investigations of the SLN-catalyzed hydrolyses of these peptides, we found that at an SLN concentration of 35 nM many of the peptides

[3] The nomenclature for the amino acid residues of the substrate (P_1, P_2, P_3, ..., P_n) and the corresponding protease subsites to which they bind (S_1, S_2, S_3, ..., S_n) is that of Schecter and Berger (1967).

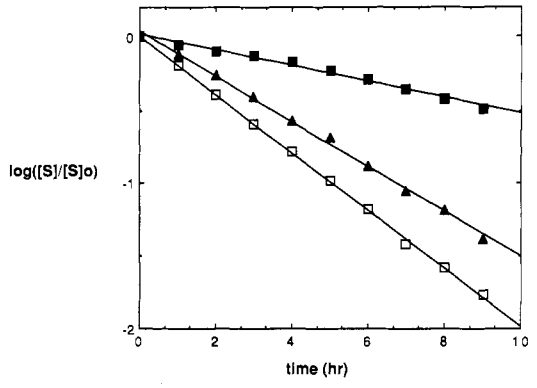

FIGURE 1: First-order rate plots of $\log[S]_t/[S]_0$ vs time for the SLN-catalyzed hydrolyses of SP^{3-11} (■), $SP(^{NO2}Phe\text{-}7)$ (▲), and SP(Nle-11) (□). Reactions were conducted at pH 7.5 in a buffer containing 20 mM Tris-HCl, 10 mM $CaCl_2$, 0.02% NaN_3, and 0.06% Brij. [SLN] = 70 nM; temp = 25 ± 0.1 °C.

were hydrolyzed at rates that are appropriate for HPLC-based kinetic studies, that is, between 0.2 and 2 μM/min. These results encouraged us to undertake a study of the substrate specificity of SLN using this series of SP analogues.

To investigate the substrate specificity of SLN, we determined values of k_c/K_m for each peptide. Values of k_c/K_m were calculated from reaction progress curves of substrate disappearance at $[S]_0 = 50$ μM and $[E]_0 = 70$ nM. A substrate concentration of 50 μM was chosen on the assumption that 50 μM is much less than K_m. This is a reasonable assumption and is based on the K_m value of 380 μM for SP (Harrison et al., 1989) and K_i values of 400 μM for SP(Sar-9), SP^{4-11}, and SP^{6-11} (see below). This assumption is supported experimentally below by the observation of first-order kinetics for the the SLN-catalyzed hydrolysis of substrates at a concentration of 50 μM.

Analysis of progress curves that are run at low substrate concentration is a convenient way of determining the kinetic parameter k_c/K_m. If the condition is met that $[S] \ll K_m$, the Michaelis–Menten equation

$$v_{ss} = -d[S]/dt = k_c[E][S]/(K_m + [S]) \quad (1)$$

simplifies to

$$-d[S]/dt = (k_c/K_m)[E][S] \quad (2)$$

or

$$-d[S]/dt = k[S] \quad (3)$$

where k is a pseudo-first-order rate constant equaling $(k_c/K_m)[E]$. Integration of eq 3 yields

$$[S]_t = [S]_0 e^{-kt} \quad (4)$$

where $[S]_t$ and $[S]_0$ are the substrate concentrations at times t and zero. We see then that the time-dependent disappearance of substrate at $[S]_0 \ll K_m$ is a simple first-order process.

The data we obtain from reaction progress curves are pairs of SP peak areas and their corresponding reaction times. Since SP peak area is directly proportional to SP concentration (Harrison et al., 1989), the data can be fit directly to eq 4 by nonlinear least-squares to arrive at the kinetic parameter k. Division of k by [E] then yields k_c/K_m. Alternately, estimates of k can be obtained from plots of log $([S]_t/[S]_0)$ vs time. Examples of these plots are shown in Figure 1 for the SLN-catalyzed hydrolysis of SP^{3-11}, $SP(^{NO2}Phe\text{-}7)$, and SP(Nle-11). The plot for the hydrolysis of SP(Nle-11) is linear for more than 6 half-lives ($t_{1/2}$ = 1.5 h), or greater than 98% of the reaction, and indicates strict adherence of this reaction to a first-order rate law. This supports our assumption that $[S]_0 \ll K_m$ for these substrates. These two methods of calculating k yield values that are identical within a few percent. For example, for SP(Nle-11), k can be estimated by nonlinear regression of $[S]_t/[S]_0$ vs time and linear regression of log $([S]_t/[S]_0)$ vs time to be 1.25×10^{-4} and 1.27×10^{-4} s^{-1}, respectively. Similarly, k was estimated as 0.91×10^{-4} and 0.96×10^{-4} s^{-1} for $SP(^{NO2}Phe\text{-}7)$ and 0.32×10^{-4} and 0.34×10^{-4} s^{-1} for SP^{3-11} by the nonlinear and linear methods.

Table II: Hydrolysis of Substance P Analogues by Stromelysin[a]

Abbreviation	P_6- P_5 -P_4- P_3- P_2- P_1- P_1'- P_2'-P_3'-P_4'-P_5'-P_6'-P_7'-P_8'	k_c/K_m[b] (M^{-1} sec^{-1})
SP	Arg-Pro-Lys-Pro-Gln-Gln- Phe-Phe-Gly-Leu-$MetNH_2$	1790 ± 40
SP^{2-11}	Pro-Lys-Pro-Gln-Gln- Phe-Phe-Gly-Leu-$MetNH_2$	800 ± 2
SP^{3-11}	Lys-Pro-Gln-Gln- Phe-Phe-Gly-Leu-$MetNH_2$	290 ± 84
SP^{4-11}	Pro-Gln-Gln-Phe-Phe-Gly-Leu-$MetNH_2$	<3
SP^{5-11}	Gln-Gln- Phe-Phe-Gly-Leu-$MetNH_2$	<3
SP^{6-11}	Gln- Phe-Phe-Gly-Leu-$MetNH_2$	<3
SP^{1-9}	Arg-Pro-Lys-Pro-Gln-Gln- Phe-Phe-GlyOH	785 ± 115
SP^{1-7}	Arg-Pro-Lys-Pro-Gln-Gln- PheOH	<3
SP(Met-11)	Arg-Pro-Lys-Pro-Gln-Gln- Phe-Phe-Gly-Leu-MetOH	1090 ± 220
SP(Nle-11)	Arg-Pro-Lys-Pro-Gln-Gln- Phe-Phe-Gly-Leu-$NleNH_2$	1630 ± 74
SP(Gly-12,Lys-13)	Arg-Pro-Lys-Pro-Gln-Gln- Phe-Phe-Gly-Leu-Met-Gly-Lys	1840 ± 400
SP(Gly-12,Lys-13,Arg,14)	Arg-Pro-Lys-Pro-Gln-Gln- Phe-Phe-Gly-Leu-Met-Gly-Lys-Arg	1660 ± 20
SP(Sar-9)	Arg-Pro-Lys-Pro-Gln-Gln- Phe-Phe-Sar-Leu-$MetNH_2$	<3
SP(Tyr-8)	Arg-Pro-Lys-Pro-Gln-Gln- Phe-Tyr-Gly-Leu-$MetNH_2$	1510 ± 98
$SP(^{NO2}Phe\text{-}7)$	Arg-Pro-Lys-Pro-Gln-Gln-^{NO2}Phe-Phe-Gly-Leu-$MetNH_2$	1090 ± 32
$SP(^{Cl}Phe\text{-}7,8)$	Arg-Pro-Lys-Pro-Gln-Gln-^{Cl}Phe-^{Cl}Phe-Gly-Leu-$MetNH_2$	1250 ± 300

[a] Reactions were conducted at pH 7.5 in a buffer containing 20 mM Tris-HCl, 10 mM $CaCl_2$, 0.02% NaN_3, and 0.06% Brij; temp = 25 ± 0.1 °C. [b] Corrected for oxidation of Met ($k_{ox} \sim 7 \times 10^{-6}$ s^{-1}). For the hydrolysis of SP, the mean and standard deviation are based on a total of 14 determinations. For the other peptides, n = 2 or 3.

Values of k_c/K_m were determined for a number of SP analogues and are summarized in Table II. Several points that relate to the substrate specificity of SLN are illustrated in this table: (1) Decreasing the length of the substrate by one or two amino acids from the N-terminal end of SP results in dramatic decreases in k_c/K_m. Truncation by three or more amino acid residues completely eliminates the peptide's ability to serve as a substrate for SLN. (2) Decreasing the length of SP from the C-terminus also results in decreases in k_c/K_m. For valid comparisons in these cases, k_c/K_m must be compared to k_c/K_m for the free acid form of SP (k_c/K_m for the free acid of SP is 60% the value of k_c/K_m for SP amide). It is clear from this comparison that the sensitivity of k_c/K_m to C-terminal truncaion is much less than the sensitivity of k_c/K_m to N-terminal truncation. (3) Peptides in which the C-terminus is lengthened from $P_5'^3$ to P_7' and P_8' are hydrolyzed by SLN no faster than is SP. (4) Although conservative amino acid replacements made within SP are tolerated by SLN, they are accompanied by decreases in k_c/K_m. (5) SP(Sar-9) is not hydrolyzed by SLN.

Inhibition of Stromelysin by Substance P Analogues. It was of some interest to determine if peptides that are not substrates for SLN are able to bind to the enzyme. The ability of peptides to bind to SLN was determined in inhibition experiments in which the peptides were treated as inhibitors of SLN. K_i values that are determined in such experiments can then be interpreted as if they are K_m values. Such experiments were performed for the inhibition of SLN by SP(Sar-9), SP^{4-11}, SP^{6-11}, and SP^{1-7} and provide K_i values ranging from 0.38 to 1 mM (see Table III; each value is the average of at least two independent experiments). We should note here that

Table III: Steady-State Kinetic Parameters for the Stromelysin-Catalyzed Hydrolysis of Substance P Analogues[a,b]

P_6-P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'-P_5'	K_m (mM)	k_c (sec^{-1})	k_c/K_m ($M^{-1}sec^{-1}$)
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-$MetNH_2$[c]	0.38	1.1	2,900
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Sar-Leu-$MetNH_2$	0.45	<0.001	<3
Pro-Gln-Gln-Phe-Phe-Gly-Leu-$MetNH_2$	0.38	<0.001	<3
Gln-Phe-Phe-Gly-Leu-$MetNH_2$	0.55	<0.001	<3
Arg-Pro-Lys-Pro-Gln-Gln-PheOH	1.1	<0.003	<3

[a] Reactions conducted at pH 7.5 in a buffer containing 20 mM Tris-HCl, 10 mM $CaCl_2$, 0.02% NaN_3, and 0.06% Brij; temp. = 25 ± 0.1 °C. [b] Error limits for K_m values are 20% or less in all cases. [c] Data from Harrison et al. (1989).

SP^{5-11} does *not* inhibit SLN; that is, $K_i > 10$ mM. Given the K_i values for SP^{4-11} and SP^{6-11}, we are at a loss to explain this result for SP^{5-11}.

To obtain an upper limit for k_c for the reaction of these peptides with SLN, their K_i values, which are equivalent to K_m values, can be multiplied by 3 M^{-1} s^{-1}, the upper limit for k_c/K_m. This value is near 0.001 s^{-1} and should be compared with a k_c value of 1 s^{-1} for the SLN-catalyzed hydrolysis of SP (see Table III).

Oxidation of Substance P. In the course of our kinetic experiments, it became clear that SP and its hydrolysis product, SP^{7-11}, are slowly oxidized at Met^{11}. In aqueous buffer at pH 7.5, both SP and SP^{7-11} disappear with an apparent first-order rate constant, k_{ox}, of 7×10^{-6} s^{-1}. The disappearance of either SP or SP^{7-11} is accompanied by the appearance of new peaks in the chromatogram. These materials chromatograph with retention times that are identical with the retention times of the products of the *N*-chlorosuccinimide-promoted oxidation of SP (Figure 2) and SP^{7-11} (data not shown). Oxidation of SP by *N*-chlorosuccinimide produces the major peak of Figure 2B, peak e. This material is present in all commercial samples of SP and increases in concentration in aqueous solutions of SP that are allowed to age. Much smaller amounts of peaks i and j also appear when SP is treated with *N*-chlorosuccinimide. The identities of peaks e, i, and j are unknown, although we suspect that i and e are the sulfone and the sulfoxide of SP, respectively. The elution pattern observed here for SP and its oxidation products is consistent with observations of Higa and Desiderio (1988). For substrates that contain Met^{11}, it was obvious that the observed first-order rate constant for the disappearance of SP, k_{obs}, is equal to the sum of k, the first-order rate constant for the enzymatic hydrolysis of SP, and k_{ox}, the first-order rate constant for the oxidative loss of SP (see eq 5). True values of

$$k_{obs} = k + k_{ox} \quad (5)$$

$$k_c/K_m = k/[E] = (k_{obs} - k_{ox})/[E] \quad (6)$$

k_c/K_m, corrected for the oxidative loss of substrate, were calculated according to eq 6. It is these corrected values that appear in Tables II and III.

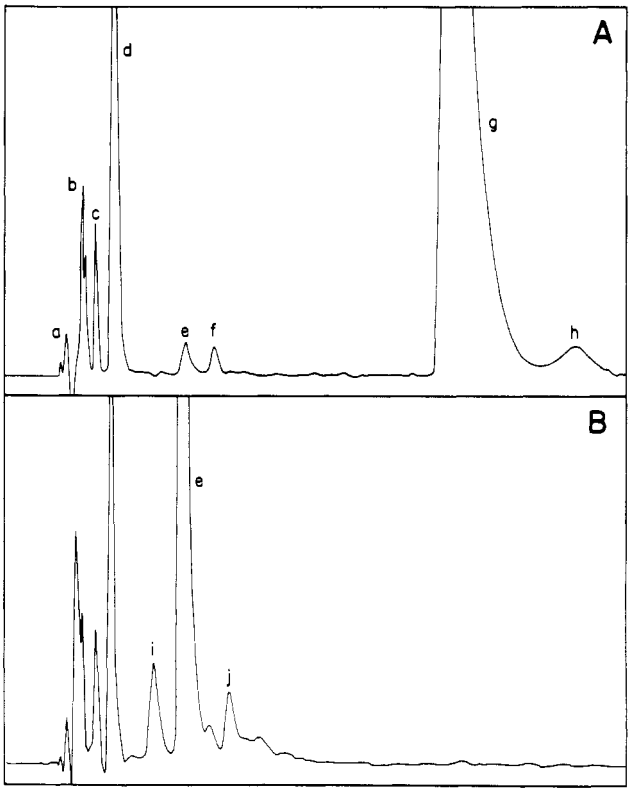

FIGURE 2: Chromatograms of SP (A) and oxidized SP (B). Peak identity: a, Tris; b, solvent front; c, SP impurity; d, azide; e, SP_{ox-1}; f, SP impurity; g, SP; h, SP impurity; i, SP_{ox-2}; j, SP_{ox-3}. See text for experimental conditions.

Discussion

Two intriguing observations emerge from this study of stromelysin's substrate specificity: the dramatic dependence of catalytic activity on peptide chain length and the complete abolition of activity when Gly^9 of SP is replaced with sarcosine. In this section, we will discuss plausible mechanisms that can account for these observations and propose a general hypothesis for the mechanistic origin of stromelysin's substrate specificity.

We can offer three mechanistic alternatives for the dependence of k_c/K_m on peptide chain length: (1) Stromelysin has a substrate binding region that includes at least 11 subsites, S_6 to S_5', and can therefore accommodate peptides that are equal in length to SP. If binding at all of these subsites is required for either binding or turnover, then shorter substrates will be hydrolyzed more slowly by SLN. (2) Of the several distinct but rapidly interconvertible conformations that SP can assume in aqueous solution (Chassaing et al., 1986), only a few are bound by SLN. If these conformational states are unavailable to shorter analogues of SP, then these peptides will not be bound and hydrolyzed by SLN. (3) Finally, SLN recognizes the high density of positive charge on the N-terminus of SP. If this charge density is essential for either binding or turnover, then SP analogues that are truncated at the N-terminus will not be hydrolyzed by this enzyme.

We can exclude the second alternative, since peptides of various length are able to bind to SLN with similar affinity[4] (Table III). If recognition of a small set of aqueous conformers of SP was important, this would be manifested in binding. Unfortunately, we are currently unable to narrow the mechanistic choices any further; alternatives 1 and 3 are equally plausible. However, it is clear from the data of Table III that SLN manifests its specificity at the stage of turnover and not during the initial binding process, which, therefore, allows us to restate alternatives 1 and 3 entirely in terms of turnover. This will be discussed in more detail below.

We now consider the inability of SP(Sar-9) to be hydrolyzed by SLN. This result clearly suggests that a catalytically

[4] Implicit in our discussion is the assumption that K_m and K_i are simple dissociation constants for the initially formed encounter complex of enzyme and peptide and do not reflect the accumulation of intermediates that are formed subsequent to these encounter complexes. We believe this assumption is justified by the relatively large magnitudes of the observed K_m and K_i values and the similarity between K_m values for hydrolysis of SP by SLN and K_i values for the inhibition SLN by SP analogues that are resistant to SLN-calyzed hydrolysis.

essential hydrogen bond is associated with the N–H of Gly[9] of SP. What is not so clear is the location and catalytic function of this hydrogen bond. Two possibilities exist: (1) The hydrogen bond is intermolecular and bridges the substrate and enzyme; the role of such a hydrogen bond would be to stabilize catalytic transition states. (2) The hydrogen bond is intramolecular within SP (Chassaing et al., 1986); the role of this hydrogen bond would be to stabilize a population of substrate conformations that can be bound by SLN. The results of Table III suggest that the hydrogen bond associated with Gly[9] is not involved in stabilizing catalytically essential solution-phase conformations since binding constants for SP and SP(Sar-9) are identical. Rather, this hydrogen bond must be involved in specific interactions between the enzyme and substrate in the transition state.

The results of Table III and the foregoing discussion allow us to formulate a proposal for the mechanistic origin of SLN's substrate specificity. As we have pointed out, Table III indicates that SP, SP(Sar-9), SP^{4-11}, SP^{6-11}, and SP^{1-7} all form complexes of similar stability with SLN ($K_m = K_i \sim 0.4$ mM). But only SP is hydrolyzed. These results indicate that SLN manifests its substrate specificity in catalysis and not in substrate binding. SLN uses the energy that is available from favorable interactions with its substrate to stabilize catalytic transition states and does not use this energy to stabilize Michaelis complexes (Fersht, 1974; Jencks, 1975; Schowen, 1978).

References

Cawston, T., Mercer, E., deSilva, M., & Hazelman, B. (1984) *Arthritis Rheum. 27*, 285–290.

Chassaing, G., Convert, O., & Lavielle, S. (1986) *Eur. J. Biochem. 154*, 77–85.

Ehrlich, M., Armstrong, A., Treadwell, B., & Mankin, H. (1987) *J. Rheumatol. 14*, 30–33.

Fersht, A. R. (1974) *Proc. R. Soc. London B187*, 397–407.

Harrison, R., Teahan, J., & Stein, R. (1989) *Anal. Biochem. 180*, 110–113.

Higa, T., & Desiderio, D. M. (1988) *Anal. Biochem. 173*, 463–468.

Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219–410.

Lark, M. W., VanMiddlsworth, J., Walakovits, L. A., & Azzolina, T. (1988) *Proceedings of the Fourth International Conference of the Inflammation Research Association*, Abstract 145, McNeil Pharmaceuticals, Spring House, PA.

Lark, M. W., Walakovits, L. A., & Moore, V. L. (1989a) *FASEB J.*, Abstract A911.

Lark, M. W., Walakovits, L. A., VanMiddlsworth, J., Shah, T., & Lin, T. Y. (1989b) *J. Biol. Chem.* (submitted for publication).

Murphy, G., Gavrilovic, J., & McAlpine, C. (1986) *Proteinases in Inflammation and Tumor Invasion*, de Gruyter, New York.

Murphy, G., Nagase, H., & Brinckerhoff, C. (1988) *Collagen Relat. Res. 8*, 389–391.

Okada, Y., Nagase, H., & Harris, E. D. (1986) *Trans. Assoc. Am. Physicians 99*, 143–153.

Schecter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Schowen, R. L. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 77–114, Plenum Publishing, New York.

Shaw, A., Roberts, R., & Wolanin, D. J. (1988) *New Perspectives in Anti-Inflammatory Therapies* (Lewis, A., Ed.) Vol. 12, pp 67–80, Raven Press, New York.

Tropomyosin Stabilizes the Pointed End of Actin Filaments by Slowing Depolymerization†

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ABSTRACT: Tropomyosin is postulated to confer stability to actin filaments in nonmuscle cells. We have found that a nonmuscle tropomyosin isolated from the intestinal epithelium can directly stabilize actin filaments by slowing depolymerization from the pointed, or slow-growing, filament end. Kinetics of elongation and depolymerization from the pointed end were measured in fluorescence assays using pyrenylactin filaments capped at the barbed end by villin. The initial pointed end depolymerization rate in the presence of tropomyosin averaged 56% of the control rate. Elongation from the pointed filament end in the presence of tropomyosin occurred at a lower free G-actin concentration, although the on rate constant, k_p^+, was not greatly affected. Furthermore, in the presence of tropomyosin, the free G-actin concentration was lower at steady state. Therefore, nonmuscle tropomyosin stabilizes the pointed filament end by lowering the off rate constant, k_p^-.

The polymerization of monomeric actin in vitro occurs rapidly after a slow nucleation step above the critical G-actin concentration [for reviews, see Frieden (1985), Korn (1982), and Pollard and Cooper (1986)]. The ends of the linear actin polymer are kinetically distinct. The barbed, or fast-growing, end elongates at a greater rate and at a lower monomer concentration than the pointed, or slow-growing, end of the filament. In vivo, actin filament assembly must be tightly regulated since the cellular pool of unpolymerized actin is much greater than the critical concentration for polymerization in vitro. The regulation of actin polymerization and organization

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