Substrate Specificity of the Human Matrix Metalloproteinase Stromelysin and the Development of Continuous Fluorometric Assays

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ABSTRACT: To probe the specificity of the metalloendoproteinase stromelysin toward peptide substrates, we determined k_c/K_m values for the stromelysin-catalyzed hydrolyses of peptides whose design was based loosely on the structure of a known SLN substrate, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$, hydrolysis at Gln-Phe, k_c/K_m = 1700 M^{-1} s^{-1}). Several noteworthy points emerge from this study: (i) Catalytic efficiency is dependent on peptide chain length with N-terminal truncation of substance P resulting in more pronounced rate-constant reductions than C-terminal truncation. These results suggest the existence of an extended active site for stromelysin. (ii) Preferences at positions P_3, P_2, P_1, P_1', and P_2' are for the hydrophobic amino acids Pro, Leu, Ala, Nva, and Trp, respectively. (iii) Investigation of specificity at P_3' supports our earlier hypothesis that SLN has a requirement for a hydrogen-bond donor at this position in its substrates. Based on these observations, we designed and had synthesized the fluorogenic substrate *N*-(2,4-dinitrophenyl)Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$, whose stromelysin-catalyzed hydrolysis can be monitored continuously (k_c/K_m = 45 000 M^{-1} s^{-1}).

Stromelysin 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–c; Stein, 1988).

An important mechanistic issue is the definition of SLN's substrate specificity. An earlier study (Teahan et al., 1989) from our laboratory reported data on the SLN-catalyzed hydrolyses of various analogues of substance P, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$. SLN cleaves this peptide exclusively at the Gln^6-Phe^7 bond with kinetics that allowed the development of a semicontinuous, HPLC-based assay (Harrison et al., 1989).

In this paper, we continue our exploration of the substrate specificity of SLN with the two goals of defining substrate structural requirements for efficient catalysis by SLN and laying the foundation for the development of a continuous assay for this enzyme. To determine SLN's requirements for its substrates, we measured values of k_c/K_m for SLN-catalyzed hydrolyses of peptides that were designed to probe the extended active site of SLN as well as subsites S_3 through S_3'.[2]

For the second goal of developing a continuous assay, our strategy was to use the specificity results to design an optimal peptide substrate for SLN and then incorporate this structure into a suitable format for continuous assay. Our studies led us to the optimal peptide, Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$, and its simple fluorogenic derivative DNP-Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$, whose hydrolysis can be followed continuously (Netzel-Arnett et al., 1991b; Stack & Gray, 1989).

MATERIALS AND METHODS

Materials. Peptide substrates were purchased from Multiple Peptide Systems (San Diego, CA) or Bachem (Philadelphia, PA) at a purity greater than 95%. Recombinant human prostromelysin (Whitman et al., 1986) was purchased from Celltech Ltd. (Slough, Berkshire, U.K.) 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 (Teahan et al., 1989; Lark et al., 1990a,b). Substrate specificity assays were performed in a buffer of 0.1 M HEPES and 0.01 M $CaCl_2$, pH 7.5.

Kinetic Experiments—Semicontinuous, HPLC-Based Assay. The general features of this assay have been described previously (Harrison et al., 1989; Teahan et al., 1989). The chromatographic aparatus consisted of a Waters 510 HPLC System (Division of Millipore, Millford, MA) 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). UV absorbance was measured at 215 nm using a Kratos Spectroflow 757 variable wavelength detector (ABI Analytical, Ramsey, NJ). Fluorescence emission was measured with an Hitachi F1200 fluorescence detector at λ_{ex} 280 nm and λ_{em} 345 nm. Peak integrations were performed on a PE Nelson Turbochrom Data Station (Cupertino, CA). Separations were performed on a Whatman Partisil 5 C8 analytical column (25 cm × 4.6 mm i.d.). The mobile phase was 0.1% TFA aqueous and CH_3CN. The flow rate was 1.5 mL/min.

Determination of k_c/K_m Values. In a typical kinetic measurement, 0.90 mL 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

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[1] Abbreviations: MMP, matrix metalloproteinase; SLN, stromelysin; CGase, collagenase; TLN, thermolysin; DNP, 2,4-dinitrophenyl; Ac, acetyl; Nva, norvaline; Abu, α-aminobutyric acid; SP, substance P.

[2] The nomenclature for the amino acid residues of the substrate (P_n-...-P_3-P_2-P_1-P_1'-P_2'-P_3'-...-P_n) and the corresponding protease subsites (S_n-...-S_3-S_2-S_1-S_1'-S_2'-S_3'-...-S_n) is that of Schecter and Berger (1967). Hydrolysis occurs at the P_1-P_1' bond.

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autosampler. A 0.90-mL aliquot of a diluted sample of $[SLN]_{stock}$ was then added to this vial to give final SLN and substrate concentrations of 0.035 and 50 μM, respectively. 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. Loss of substrate was quantitated either by absorbance at 215 nm or by tryptophan fluorescence. Values of k_c/K_m were calculated according to the expression $k_c/K_m = k_{obs}/[E]$, where k_{obs} is the observed first-order rate constant for loss of substrate obtained from progress curves recorded under the condition that $[S]_o = 50\ \mu M \ll K_m = 500\ \mu M$.

Kinetic Experiments—Continuous, Fluorescence-Quench Assay. This assay relies on the ability of the dinitrophenyl moiety (DNP) to internally quench the fluorescence emission of the tryptophan side chain of peptide substrates such as DNP-Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$. The intact peptide shows little fluorescence at 340 nm, since the fluorescence of the excited Trp is quenched by radiationless energy transfer to the 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 340 nm is observed. Similar assays have been described for other proteases (Netzel-Arnett et al., 1991b; Stack & Gray, 1989).

Determination of k_c/K_m Values. In a typical kinetic run, 2.96 mL of buffer and 0.020 mL of substrate in DMSO were added to a 3-mL cuvette, and the cuvette was 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 solution. Reaction progress was monitored by the increase in the fluorescence emission at 340 nm (λ_{ex} = 290 nm) that accompanies cleavage of DNP-blocked Trp-containing peptides. For each kinetic run, 1000 data points, corresponding to {time, F} pairs, were collected by a NEC Powermate 1 Plus microcomputer interfaced to the fluorescence spectrophotometer. Values of k_c/K_m were calculated from first-order progress curves for fluorescence increase with $[S]_o = 2\ \mu M \ll K_m$.

RESULTS AND DISCUSSION

The work described in this paper explores the substrate specificity of SLN toward peptides that are structurally related to the known SLN substrate, substance P (Harrison et al., 1989; Teahan et al., 1989). The measure of specificity that we chose to use in this study is the kinetic parameter k_c/K_m, which is the second-order rate constant for reaction of substrate and enzyme and reflects all enzymic reaction steps to and including the first irreversible step. Estimates of this parameter were determined from full reaction progress curves for the disappearance of substrate with $[S]_o = 50\ \mu M$. In all cases, these progress curves were strictly first-order, validating our assumption that $[S]_o \ll K_m \geq 500\ \mu M$ (Harrison et al., 1989; Teahan et al., 1989). The values of k_c/K_m that we report in Tables I–VIII are the means of not less than two independent determinations, and the error limits are either standard deviations ($n \geq 3$) or deviations from the mean ($n = 2$).

We performed these studies not only to probe an important mechanistic issue, that is, SLN's substrate structural requirements for efficient catalysis, but also to lay the foundation for the development of a continuous assay for this enzyme.

Table I: Effect of N-Terminal Truncation on k_c/K_m for the Stromelysin-Catalyzed Hydrolysis of Analogues of Substance P[a]

P_7- P_6- P_5 - P_4 - P_3 - P_2 - P_1 - P_1'- P_2'- P_3'-P_4' - P_5'	k_c/K_m[b]
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$[c]	1790 ± 140
Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$	800 ± 2
Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$	290 ± 84
Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$	<3
Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$	<3
Gln-Phe-Phe-Gly-Leu-MetNH$_2$	<3
Ac-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	1800 ± 92
Ac-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	840 ± 44
Ac-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	390 ± 26
Ac-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	500 ± 200
Ac-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	*d*
Ac-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	<3
Ac-Phe-Phe-Gly-Leu-NleNH$_2$	<3

[a] Reactions were conducted at pH 7.5 in a buffer containing 0.10 M HEPES and 10 mM $CaCl_2$ at 25 ± 0.1 °C. [b] Units: $M^{-1}\ s^{-1}$. [c] Cleavage sites are underlined. [d] This peptide disappears with a k_c/K_m value that is less than 100 $M^{-1}\ s^{-1}$. However, cleavage is not exclusively at the Gln–Phe bond. We are therefore unable to assign an accurate value of k_c/K_m for hydrolysis at the Gln–Phe position.

Table II: Effect of C-Terminal Truncation on k_c/K_m for the Stromelysin-Catalyzed Hydrolysis of Analogues of Substance P[a]

P_6- P_5- P_4 - P_3 - P_2 - P_1- P_1'- P_2'- P_3'-P_4'- P_5'	k_c/K_m[b]
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$[c]	1700 ± 80
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-LeuNH$_2$	1850 ± 40
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-GlyNH$_2$	1480 ± 20
Arg-Pro-Lys-Pro-Gln-Gln-Phe-PheNH$_2$	1900 ± 380
Arg-Pro-Lys-Pro-Gln-Gln-PheNH$_2$	<3
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met	1100 ± 220
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu	1300 ± 91
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly	790 ± 120
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe	<3
Arg-Pro-Lys-Pro-Gln-Gln-Phe	<3

[a] Reactions were conducted at pH 7.5 in a buffer containing 0.10 M HEPES and 10 mM $CaCl_2$ at 25 ± 0.1 °C. [b] Units: $M^{-1}\ s^{-1}$. [c] Cleavage sites are underlined.

Our strategy was to first define an optimal peptide substrate for SLN and then to incorporate this structure into a suitable protease continuous assay format. As decribed below, we were successful in our development of a continuous assay (Table IX).

In the paragraphs that follows, we will describe the elements of SLN's substrate specificity that we were able to define in this work and compare these findings with recently reported specificity results for CGase, another MMP (Netzel-Arnett et al., 1991a). We will finish with a description of the continuous, fluorogenic assays that emerged from these studies.

Evidence for an Extended Active Site for Stromelysin. The results of Tables I and II demonstrate that catalytic efficiency is dependent on peptide chain length with N-terminal

Table III: P_2' and P_3' Specificities of Stromleysin[a]

P_6- P_5- P_4 - P_3 - P_2 - P_1- P_1'- P_2'- P_3'-P_4'- P_5'	k_c/K_m[b]
Arg-Pro-Lys-Pro-Gln-Gln-Phe-**PheNH$_2$**	1900 ± 380
Arg-Pro-Lys-Pro-Gln-Gln-Phe-**TrpNH$_2$**	4100 ± 130
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$[c]	1700 ± 80
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-**Sar-**Leu-MetNH$_2$	<3
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-**Ala-**Leu-NleNH$_2$	2700 ± 25
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-**Nle-**Leu-NleNH$_2$	3000 ± 400

[a] Reactions were conducted at pH 7.5 in a buffer containing 0.10 M HEPES and 10 mM $CaCl_2$ at 25 ± 0.1 °C. [b] Units: M^{-1} s^{-1}. [c] Cleavage sites are underlined. Residues in bold are changes relative to SP.

Table IV: P_1' Specificity of Stromelysin[a]

P_6- P_5 - P_4 - P_3 - P_2 - P_1 - P_1'- P_2'	k_c/K_m[b]
Arg-Pro-Lys-Pro-Leu-Ala-**Gly**-TrpNH$_2$[c]	<3
Arg-Pro-Lys-Pro-Leu-Ala-**Ala**-TrpNH$_2$	890 ± 130
Arg-Pro-Lys-Pro-Leu-Ala-**Abu**-TrpNH$_2$	9800 ± 710
Arg-Pro-Lys-Pro-Leu-Ala-**Nva**-TrpNH$_2$	31000 ± 1800
Arg-Pro-Lys-Pro-Leu-Ala-**Val**-TrpNH$_2$	5600 ± 230
Arg-Pro-Lys-Pro-Leu-Ala-**Nle**-TrpNH$_2$	9600 ± 2300
Arg-Pro-Lys-Pro-Leu-Ala-**Leu**-TrpNH$_2$	9300 ± 2400
Arg-Pro-Lys-Pro-Leu-Ala-**Phe**-TrpNH$_2$	12000 ± 1200
Arg-Pro-Lys-Pro-Leu-Ala-**Trp**-TrpNH$_2$	6000 ± 600
Arg-Pro-Lys-Pro-Leu-Ala-**Arg**-TrpNH$_2$	100 ± 67
Arg-Pro-Lys-Pro-Leu-Ala-**Lys**-TrpNH$_2$	930 ± 92
Arg-Pro-Lys-Pro-Leu-Ala-**Glu**-TrpNH$_2$	130 ± 47
Arg-Pro-Lys-Pro-Leu-Ala-**Pro**-TrpNH$_2$	<3

[a] Reactions were conducted at pH 7.5 in a buffer containing 0.10 M HEPES and 10 mM $CaCl_2$ at 25 ± 0.1 °C. [b] Units: M^{-1} s^{-1}. [c] Cleavage sites are underlined. Residues in bold are changes relative to SP.

Table V: P_1 Specificity of Stromelysin[a]

P_6- P_5 - P_4 - P_3 - P_2 - P_1 - P_1'- P_2'- P_3'-P_4' - P_5'	k_c/K_m[b]
Arg-Pro-Lys-Pro-Gln-**Gly**-Phe-Phe-Gly-Leu-NleNH$_2$[c]	910 ± 40
Arg-Pro-Lys-Pro-Gln-**Ala**-Phe-Phe-Gly-Leu-NleNH$_2$	2700 ± 140
Arg-Pro-Lys-Pro-Gln-**Leu**-Phe-Phe-Gly-Leu-NleNH$_2$	780 ± 5
Arg-Pro-Lys-Pro-Gln-**Phe**-Phe-Phe-Gly-Leu-NleNH$_2$	1700 ± 60
Arg-Pro-Lys-Pro-Gln-**Asn**-Phe-Phe-Gly-Leu-NleNH$_2$	1500 ± 79
Arg-Pro-Lys-Pro-Gln-**Gln**-Phe-Phe-Gly-Leu-NleNH$_2$	1700 ± 80
Arg-Pro-Lys-Pro-Gln-**His**-Phe-Phe-Gly-Leu-NleNH$_2$	1300 ± 120
Arg-Pro-Lys-Pro-Gln-**Lys**-Phe-Phe-Gly-Leu-NleNH$_2$	550 ± 20

[a] Reactions were conducted at pH 7.5 in a buffer containing 0.10 M HEPES and 10 mM $CaCl_2$ at 25 ± 0.1 °C. [b] Units: M^{-1} s^{-1}. [c] Cleavage sites are underlined. Residues in bold are changes relative to SP.

truncation of the parent substrate, substance P, resulting in more pronounced rate-constant reductions than C-terminal truncation. Combined, these results provide kinetic evidence for an extended active site for SLN. While structural evidence that supports this is still lacking, structural evidence for an extended active site does exist for the mechanistically related metalloproteinase, thermolysin (Holden & Matthews, 1981,

Table VI: P_2 and P_3 Specificity of Stromelysin[a]

P_6- P_5 - P_4 - P_3 - P_2 - P_1 - P_1'- P_2'- P_3'-P_4' - P_5'	k_c/K_m[b]
Arg-Pro-Lys-Pro-Gln-**Ala**-Phe-TrpNH$_2$[c]	8900 ± 650
Arg-Pro-Lys-Pro-**Leu**-Ala-Phe-TrpNH$_2$	18000 ± 1300
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	1700 ± 80
Arg-Pro-Lys-**Ala**-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	460 ± 30
Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$	18000 ± 1300
Arg-Pro-Lys-**Nva**-Leu-Ala-Phe-TrpNH$_2$	75 ± 30
Arg-Pro-Lys-**Val**-Leu-Ala-Phe-TrpNH$_2$	530 ± 70
Arg-Pro-Lys-**Leu**-Leu-Ala-Phe-TrpNH$_2$	310 ± 180
Arg-Pro-Lys-**Asn**-Leu-Ala-Phe-TrpNH$_2$	160 ± 88

[a] Reactions were conducted at pH 7.5 in a buffer containing 0.10 M HEPES and 10 mM $CaCl_2$ at 25 ± 0.1 °C. [b] Units: M^{-1} s^{-1}. [c] Cleavage sites are underlined. Residues in bold are changes relative to SP.

Table VII: Importance of the N-Terminal Amino Acids for the Hydrolysis of Substance P[a]

P_6- P_5 - P_4 - P_3 - P_2 - P_1 - P_1'- P_2'- P_3'-P_4' - P_5'	k_c/K_m[b]
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$[c]	1700 ± 80
Ala-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	1000 ± 30
Arg-Pro-**Ala-**Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	1700 ± 220
Ala-Pro-**Ala-**Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	1000 ± 10
Arg-**Ala-**Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	1200 ± 50
Arg-Pro-Lys-**Ala-**Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	460 ± 30
Arg-**Ala-**Lys-**Ala-**Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	190 ± 5
Ala-Ala-Ala-Ala-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$	220 ± 90

[a] Reactions were conducted at pH 7.5 in a buffer containing 0.10 M HEPES and 10 mM $CaCl_2$ at 25 ± 0.1 °C. [b] Units: M^{-1} s^{-1}. [c] Cleavage sites are underlined. Residues in bold are changes relative to SP.

1988; Kester & Matthews, 1977; Monsingo & Matthews, 1984; Weaver et al., 1977).

In the series of substrates that explore the effect of N-terminal truncation on k_c/K_m, we studied peptides with both free and acetylated N-termini. Studies of the acetylated peptides were performed to differentiate rate effects caused by subsite occupation from rate effects caused by nonspecific electrostatic effects originating from proximity of the postively charged N-terminus to the catalytic machinery of SLN. Table I indicates similar trends for both series. We believe that reductions in k_c/K_m that accompany N-terminal truncation of SP are caused by the inability of the shorter peptides to interact at remote, but catalytically important, subsites. The means by which these subsites interactions are translated into catalytic power is discussed in the last section of this paper.

A notable pair of peptides from Table I is Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$ and its acetylated derivative. While the former has a k_c/K_m value that is less than 3 M^{-1} s^{-1}, the acetylated derivative is hydrolyzed with a k_c/K_m value that is equal to 500 M^{-1} s^{-1}. These results suggest that either SLN is intolerant to a positive charge at P_3 or SLN requires a carbonyl moiety at P_3, possibly to serve as a hydrogen-bond acceptor. We can exclude the latter reason since, as we show below, DNP-Pro-Leu-Ala-Phe-TrpNH$_2$ is a substrate for SLN

Table VIII: Additivity of Free Energy Differences for Hydrolyses of Stromelysin Substrates

position	amino acid change	$(k_c/K_m)/(k_c/K_m)$SP	$\Delta\Delta G^{\ddagger}$ [a] (kcal/mol)
P_6	Arg to Ala[b]	0.59	−0.32
P_5	Pro to Ala[b]	0.71	−0.21
P_4	Lys to Ala[b]	1.00	0.00
P_3	Pro to Ala[b]	0.27	−0.79
		$\Pi = 0.11$	$\Sigma = -1.32$
	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$		
	$k_c/K_m = 1700$ M^{-1} s^{-1}		
	Ala-Ala-Ala-Ala-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$		
	$k_c/K_m = 220$ M^{-1} s^{-1}		
	Factor = 0.13		
	$\Delta\Delta G^{\ddagger} = -1.22$ kcal/mol		
P_2	Gln to Leu[c]	2.02	0.42
P_1	Gln to Ala[d]	1.59	0.28
P_1'	Phe to Nva[e]	2.58	0.57
P_2'	Phe to Trp[f]	2.16	0.46
		$\Pi = 17.9$	$\Sigma = 1.73$
	Arg-Pro-Lys-Pro-Gln-Gln-Phe-PheNH$_2$		
	$k_c/K_m = 1900$ M^{-1} s^{-1}		
	Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$		
	$k_c/K_m = 31000$ M^{-1} s^{-1}		
	Factor = 16.3		
	$\Delta\Delta G^{\ddagger} = 1.67$ kcal/mol		

[a] $\Delta\Delta G^{\ddagger} = RT\ln\{(k_c/K_m)/(k_c/K_m)_{SP}\}$; $T = 298$ K. [b] Table VII. [c] Table IV. [d] Table III. [e] Table V. [f] Table VI.

Table IX: Fluorogenic Substrates for the Continuous Assay of Stromelysin[a]

P_7-P_6-P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'	k_c/K_m[b]
Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$	12000
DNP-Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$	17000
Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$	31000
DNP-Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$	45000
DNP-Pro-Leu-Ala-Phe-TrpNH$_2$	2400
DNP-Pro-Leu-Ala-Phe-Trp-Ala-ArgNH$_2$	3200

[a] Reactions were conducted at pH 7.5 in a buffer containing 0.10 M HEPES and 10 mM $CaCl_2$ at 25 ± 0.1 °C. [b] Units: M^{-1} s^{-1}. [c] Cleavage sites are underlined.

with a k_c/K_m value of 2400 M^{-1} s^{-1}. Unlike Ac-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$, DNP-Pro-Leu-Ala-Phe-TrpNH$_2$ does not have a carbonyl group at P_3.

In the series of substrates exploring the effect of C-terminal truncation on k_c/K_m, we also studied both blocked and free peptides. In contrast to the results discussed above, Table II shows that a mechanistically significant decrease in k_c/K_m does not occur until SP is truncated to Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe, which does not undergo detectable hydrolysis by SLN. Significantly, the amide of this peptide is as at least as active as longer analogues. An explanation for this dramatic rate constant difference between Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe and its amide is offered below.

In Table X, we compare these results for SLN with results for CGase (Netzel-Arnett et al., 1991a). To probe the existence of an extended active site for CGase, Van Wart used the parent substrates Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln

Table X: Comparison of the Substrate Specificities of the Human Matrix Metalloproteinases Stromelysin and Collagenase[a]

specificity		SLN[b]	CGase[c]
extended active site[d]	P_4–P_m'	100	100
	P_3–P_m'	<1	160
	P_3–P_m'	<1	7
	P_1–P_m'	<1	<3
	P_n–P_5'	100	100
	P_n–P_4'	120	120
	P_n–P_3'	72	84
	P_n–P_2'	<0.3	16
	P_n–P_1'	<0.3	<3
P_1	Gly	100	100
	Ala	300	640
	Leu	86	27
	Phe	190	95
	Gln	190	37
	His	140	160
P_2	Gln	100	100
	Leu	260	150
P_3	Pro	100	100
	Ala	27	50
	Leu	17	14
	Asn	9	17
P_1'	Leu	100	100
	Val	60	7
	Phe	130	<3
	Glu	2	<3
	Pro	<0.3	<3
P_2'	Phe	100	100
	Trp	240	170
P_3'	Gly	100	100
	Ala	160	220

[a] Values are k_c/K_m relative to k_c/K_m for the first member of the series. [b] This work; pH 7.5, 25 °C. [c] Collagenase data are from the work of Netzell-Arnett et al. (1991a); pH 7.5, 25 °C. [d] For SLN, m and n are 5 and 6, respectively; while for CGase, m and n are both 4.

($k_c/K_m = 60$ M^{-1} s^{-1}) and Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg ($k_c/K_m = 50$ M^{-1} s^{-1}) for N-terminal and C-terminal truncation, respectively. From the comparison shown in Table X, it appears that subsite interactions are more important for SLN catalysis than for CGase catalysis. While the N-terminally truncated peptide Pro-Gln-Gly-Ile-Ala-Gly-Gln is an even more efficient substrate for CGase than the parent peptide Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln, the truncated SLN substrate Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$ is completely inactive as a substrate. Note that both truncated peptides can potentially interact at subsites S_3–S_1 of their respective enzymes. Likewise, while peptides as short as Pro-Gln-Gly-Ile-Ala are still hydrolyzed by CGase, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe is not hydrolyzed by SLN. The requirement for extended substrates that SLN and CGase display is not universal among metalloendoproteinases. For example, TLN can hydrolyze simple N-blocked tripeptides with k_c/K_m values that exceed 10^6 M^{-1} s^{-1} (Fukuda & Kunugi, 1984).

P_2' and P_3' and Specificities of Stromelysin. Our exploration of P_2' specificity is very limited and was motivated by practical concerns of assay development. In an attempt to increase the sensitivity of our HPLC-based assay, we substituted Trp for Phe at P_2' in the peptide Arg-Pro-Lys-Pro-Gln-Gln-Phe-PheNH$_2$ and found that the Trp-substituted substrate not only increased assay sensitivity by allowing us to use fluorescence detection but also was hydrolyzed twice as fast as the parent substrate (see Table III).

The peptides that we examined with substitutions at P_3' were designed to extend our earlier observation that the peptide with sarcosine (i.e., *N*-methyl-Gly) substituted for Gly at P_3'

is not a substrate for SLN (Teahan et al., 1989). We hypothesized in our earlier paper that this lack of reactivity was due to SLN's requirement for a hydrogen-bond donor at this position in its substrates. Another possibility is that SLN is very sensitive to steric bulk at this position in its substrates. Given the efficiency with which SLN hydrolyzes both SP-Ala^9-Nle^{11} and SP-Nle^9-Nle^{11} (see Table III), this argument cannot be defended.

The importance of hydrogen-bonding interactions at this position is also supported by the observation that while Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe is not a substrate for SLN (k_c/K_m < 3 M^{-1} s^{-1}, Table I), Arg-Pro-Lys-Pro-Gln-Gln-Phe-PheNH$_2$ is a substrate for SLN (k_c/K_m = 1900 M^{-1} s^{-1}, Table I). Note that while the latter peptide can donate a hydrogen bond at the P_3' position, the former peptide cannot.

P_1' Specificity of Stromelysin. The data of Table IV indicate that SLN prefers hydrophobic residues at the P_1' position of its substrates; charged residues and Pro are not well tolerated.

Upon inspection of the results of Table IV, one sees a curious and interesting feature of SLN's P_1' specificity. If we consider only the naturally occurring hydrophobic amino acids, we see that Phe is preferred. However, if we also include the unnatural amino acids that we studied, we see that the smaller amino acid Nva is clearly the amino acid of choice at P_1'. Equally curious is fact that the substrate with Abu at P_1' is hydrolyzed just as rapidly as the substrates with Nle or Leu at this position and almost as rapidly as the Phe-containing substrate. These apparently contradictory results do not lead to a clear structural picture of SLN's specificity pocket at S_1'; this will have to await the solution of the three-dimensional structure for this enzyme.

The P_1' specificity of CGase is markedly different from that of SLN (see Table X). While SLN readily hydrolyzes substrates with Leu, Val, and Phe at P_1', CGase will only hydrolyze substrates with Leu at this position. In fact, it is at P_1' where the specificities of SLN and CGase differ the most.

P_1 Specificity of Stromelysin. The results of Table V indicate that SLN does not have a well-defined P_1specificity. Of the many SP-based peptides that SLN hydrolyzes, there is a slight preference for Ala at this position. Lys is not well tolerated at P_1. In contrast, CGase has a much defined P_1 specificity with a clear preference for Ala at this position (Table X).

P_2 and P_3 Specificities of Stromelysin. A limited number of peptides were prepared to examine the P_2 and P_3 specificities of SLN (Table VI). We see that at P_2 Leu is preferred over Gln and that at P_3 Pro is preferred. Similar results were obtained for CGase (Table X).

Our first indication that Pro might be important at P_3 came with the observation of a 4-fold decrease in k_c/K_m when the P_3-Pro of SP-Nle^{11} is replaced by Ala (Table VI). A much larger decrease in reactivity is observed when the P_3-Pro of Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$ is replaced by the Nva. This 240-fold decrease in k_c/K_m occurs even though Nva is isosteric with Pro. Other substitutions at P_3 were also ineffective as Pro replacements.

Importance of the N-Terminal Residues for Stromelysin Catalysis. The observations that are summarized in Table I that N-terminal truncation leads to dramatic reductions in catalytic efficiency prompted us to investigate the importance of the individual residues at these remote positions. We are interested in answering the following question: Due these rate constant reductions result from the loss of general backbone interactions between the peptide substrate and enzyme or from the loss of subsite interactions that are specific to a given residue? This question was addressed by replacing the P_2–P_6 amino acid residues by Ala. The results are summarized in Table VII.

We will first consider the cationic groups at positions P_6 and P_4. When the P_6 Arg is replaced by Ala, we observe a decrease in k_c/K_m of nearly 2-fold. This contrasts with replacement of the P_4 Lys with Ala where there is no change in reactivity. This result suggests that the positive charge of Arg's guanidinium group influences the reactivity of SP toward SLN while the positive charge Lys's ϵ-amino group has no catalytic influence. Substitution of both cationic residues by Ala has the predicted effect if the indivudual effects are additive (see below).

When we replace the Pro residues with Ala, we find the Pro at P_3 exerts a larger effect on k_c/K_m than does the Pro at P_5 (see above). Again, when these residues are replaced simultaneously, the effect is additive.

We conclude from these results that the loss of reactivity toward SLN when SP is truncated from its N-termini is due to both the loss of backbone interactions as well as the loss of interactions specific to SP's P_2–P_6 amino acid residues. Whether these are subsite interactions or interactions that are dependent on the conformation of SP is not clear.

Free Energy Additivity. To determine if the cumulative effect of substitutions in SP are additive or synergistic in their influence on k_c/K_m for SLN-catalyzed hydrolysis, we calculated free energy differences for substitutions at positions P_6–P_3 and positions P_2–P_2' (see Table VIII). For substitutions at positions P_6–P_3, k_c/K_m values are smaller relative to k_c/K_m values for SP hydrolysis, and therefore values of $\Delta\Delta G^{\ddagger}$ are negative. In contrast, for substitutions at positions P_2–P_2', k_c/K_m values are larger relative to k_c/K_m for SP hydrolysis, and therefore values of $\Delta\Delta G^{\ddagger}$ are positive. In both cases, the cumulative effect of these changes [i.e., $\sum(\Delta\Delta G^{\ddagger})$] equals the experimental value of $\Delta\Delta G^{\ddagger}$ for the parent and maximally substituted substrate. This indicates that the cumulative effects are additive. There is no hint of synergy. This point is made more dramatic if we consider the transformation of Ala-Ala-Ala-Ala-Gln-Gln-Phe-Phe-Gly-Leu-NleNH$_2$ (k_c/K_m = 200 M^{-1} s^{-1}) into Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$ (k_c/K_m = 31 000 M^{-1} s^{-1}). The sum of $\Delta\Delta G^{\ddagger}$ values is 3.05 kcal/mol while the observed is 2.97 kcal/mol. These values are identical within experimental error and demonstrate the lack of synergy for substitution within the peptide substrate. For example, changes at P_5 will have no influence on the change brought about by substitution at P_2'. This will clearly be important in the design of inhibitors for SLN.

Development of Continuous Assays for Stromelysin. A goal of this study was to apply insights from specificity studies to the design of substrates that are suitable for the continuous assay of SLN. The studies that are summarized in Tables I–VII led us to the two most reactive substrates, Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$ (k_c/K_m = 12 000 M^{-1} s^{-1}) and Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$ (k_c/K_m = 31 000 M^{-1} s^{-1}). These substrates allowed us to test a strategy used by two other groups (Netzel-Arnett et al., 1991b; Stack & Gray, 1989) in which the N-terminus of peptide substrate is blocked by DNP, which quenches the fluorescence of the Trp. DNP-Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$ and DNP-Arg-Pro-Lys-Pro-Leu-Ala-Nva-TrpNH$_2$ were synthesized, and HPLC analysis indicated that both are hydrolyzed at the indicated sites and with values of k_c/K_m (data not shown) that are identical to those obtained from monitoring the

continuous fluorescence change. Both peptides are hydrolyzed faster than the corresponding parent substrate.

DNP-Pro-Leu-Ala-Phe-TrpNH$_2$ and DNP-Pro-Leu-Ala-Phe-Trp-Ala-ArgNH$_2$ were also synthesized but offer no advantage over DNP-Arg-Pro-Lys-Pro-Leu-Ala-Phe-Trp-NH$_2$. In fact, DNP-Pro-Leu-Ala-Phe-TrpNH$_2$ is so insoluble in aqueous buffer as to make it useless as an SLN substrate.

Finally, the utility of a continuous, fluorogenic assay for mechanistic studies of SLN has already been demonstrated (Izquierdo-Martin & Stein, 1992c) with DNP-Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$.

Mechanistic Origins of Substrate Specificity. The results of this and other studies of protease substrate specificity consistently show that substrate structural changes at positions that are remote from the scissile bond can have dramatic effects on catalytic efficiency. This can be attributed to an enzyme's ability to utilize free energy derived from favorable interactions between enzyme and substrate for transition-state stabilization. As true as this statement may be, it is not mechanistically informative: it gives us no indication how interactions at remote subsites are transmitted to the catalytic apparatus and used for more efficient peptide bond cleavage. The challenge in the protease field will be to couple detailed mechanistic studies with structural probes to obtain a more accurate picture of the mechanistic origins of substrate specificity.

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