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Resynthesis of Reactive Site Peptide Bond and Temporary Inhibition of *Streptomyces* Metalloproteinase Inhibitor

Sailaja S. Seeram, Kazumi Hiraga, and Kohei Oda[1]

Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Sakyo-ku, Kyoto 606

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***Streptomyces* metalloproteinase inhibitor (SMPI) is a small proteinaceous inhibitor which inhibits metalloproteinases such as thermolysin ($K_i = 1.14 \times 10^{-10}$ M). When incubated with the enzyme, it is gradually hydrolyzed at the Cys^{64}-Val^{65} peptide bond, which was identified as the reactive site by mutational analysis. To achieve a further understanding of the inhibition mechanism, we attempted to resynthesize the cleaved reactive site by using the enzyme catalytic action. The native inhibitor was resynthesized from the modified inhibitor ($K_i = 2.18 \times 10^{-8}$ M) by incubation with a catalytic amount of thermolysin under the same conditions as used for hydrolysis (pH 7.5, 25°C), suggesting that SMPI follows the standard mechanism of inhibition of serine proteinase inhibitors. Temporary inhibition was observed when the native inhibitor and thermolysin were incubated at a 1 : 100 (mol/mol) enzyme-inhibitor ratio at 37°C. SMPI showed temporary inhibition towards all the enzymes it inhibited. The inhibitory spectrum of SMPI was analyzed with various metalloproteinases based on the K_i values and limited proteolysis patterns. *Pseudomonas* elastase and *Streptomyces griseus* metalloproteinase II formed more stable complexes and showed much lower K_i values (~2 pM) than thermolysin. In the limited proteolysis experiments weak inhibitors were degraded by the enzymes. SMPI did not inhibit almelysin, *Streptomyces caespitosus* neutral proteinase or matrix metalloproteinases. SMPI specifically inhibits metalloproteinases which are sensitive to phosphoramidon.**

Key words: metalloproteinase, reactive site, resynthesis, SMPI, temporary inhibition.

The natural inhibitors existed in nature are a fascinating system and are mainly deviced to control inappropriate proteolytic activity. The requirements for a protein to act as an inhibitor instead of serving as a substrate are still obscure. Among the proteinaceous inhibitors of the four classes of proteolytic enzymes, many serine proteinase inhibitors have been identified, classified and studied in various aspects. The major group of serine proteinase inhibitors binds to the target enzymes in a substrate-like manner and forms stable complexes. These inhibitors are extremely stable to both denaturation and wanton proteolysis, and the inhibition mechanism is known as the "standard mechanism" (*1, 2*). Such inhibitors are cleaved at a specific peptide bond, which is called the reactive site. The cleavage is very slow and never goes to completion. The cleaved bond can be resynthesized by incubation with a catalytic amount of enzyme.

However, in spite of the wide occurrence of metalloproteinases, very few inhibitors of metalloproteinases have been identified. *Streptomyces* metalloproteinase inhibitor (SMPI) (*3*), isolated from *Streptomyces nigrescens* TK-23, is a novel metalloproteinase inhibitor. It is the only inhibitor which inhibits a wide range of metalloproteinases such as thermolysin. The amino acid sequence of SMPI was deduced (*4*), and the gene was cloned (*5, 6*) and expressed in *Escherichia coli* (*7*). SMPI is a proteinaceous inhibitor, having many characteristics similar to those of standard-mechanism inhibitors of serine proteinases and being cleaved enzymatically at the reactive site (*7*). In our previous work we identified the reactive site as Cys^{64}-Val^{65} by constructing many P1′ residue substitution mutants and analyzing their inhibitory activities against thermolysin (*7*).

The scope of the present work is as follows. (I) Resynthesis of the reactive site: in order to understand the inhibition mechanism of SMPI, taking into account the similarities of SMPI to serine proteinase inhibitors and limited proteolysis of the reactive site, we attempted to purify the modified inhibitor and resynthesize the cleaved bond by using catalytic action of the enzyme. (II) Temporary inhibition: since our previous mutational analysis (*7*) showed that the mutants which had less affinity for the enzyme were unstable and were degraded by the enzyme, we thought that native SMPI might also be degraded by the target enzyme, but at a slow rate, and might show tempo-

[1] To whom correspondence should be addressed: Tel: +81-75-724-7763, Fax: +81-75-724-7760, E-mail: bika@ipc.kit.ac.jp

Abbreviations: A_2pr(Dnp), N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; FAGLA, 3-(2-furylacryloyl)glycyl-L-leucine amide; FPLC, fast protein liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOCAc, (7-methoxycoumarin-4-yl)acetyl; MMP, matrix metalloproteinase; Nva, norvaline; P. elastase, *Pseudomonas aeruginosa* elastase; PUMP, punctuated metalloproteinase; ScNP, *Streptomyces caespitosus* neutral proteinase; SGMP II, *Streptomyces griseus* metalloproteinase II; SMPI, *Streptomyces* metalloproteinase inhibitor. SMPI*, reactive site-cleaved (modified) inhibitor. Amino acid residues are represented by one-letter or three-letter abbreviations. Mutants are identified by giving the wild-type residue followed by the residue number and the substituted residue.

rary inhibition. Hence, the nature of inhibition by SMPI was analyzed by monitoring residual activity of enzyme and SDS-PAGE patterns. (III) Inhibition spectrum of SMPI: for the analysis of enzyme-inhibitor interactions by mutational, X-ray crystallographic or NMR analyses, stability of the inhibitor is necessary. Hence, to identify a better target enzyme, we analyzed the inhibition spectrum of SMPI with various metalloproteinases.

EXPERIMENTAL PROCEDURES

Materials—Thermolysin and native SMPI were kindly donated by Daiwa Kasei, Osaka. The fluorogenic substrate, MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2, was purchased from Peptide Institute, Osaka. All the mutant and wild-type proteins were purified as described previously (*7*). *Streptomyces griseus* metalloproteinase II (SGMP II) (*8*) and *Pseudomonas aeruginosa* elastase (P. elastase) (*9*) were kindly donated by Dr. Kumazaki, Hokkaido University. *Streptomyces caespitosus* neutral proteinase (ScNP) (*10*) was a generous gift from Prof. Kainosho, Tokyo Metropolitan University. Vimelysin (*11*) and almelysin (*12*) were purified as described previously. Type I collagenase (*13*), gelatinase A (*14*), and stromelysin (*15*) were purified as described elsewhere.

Purification of Modified SMPI—Native SMPI (3 mg) was incubated with thermolysin (0.15 mg) in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ buffer at 25°C for 48 h. Then the pH was adjusted to 2.5 by dialysis against 20 mM sodium acetate buffer, pH 2.5. The native and modified inhibitors were separated by FPLC using a Resource S, 1 ml column. Proteins were eluted under isocratic conditions using 20 mM sodium acetate (pH 2.5) and 250 mM NaCl buffer. The purified sample was dialyzed against distilled water and freeze-dried.

Hydrolysis or Limited Proteolysis and Resynthesis of Peptide Bond—Inhibitor (10 nmol) and enzyme (100 pmol) were incubated in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ buffer at 25°C. Samples were withdrawn at various intervals and analyzed by Tricine-SDS-PAGE (*16*) after reduction with mercaptoethanol. The gels were scanned with a CanoScan 300 scanner, then the protein concentration of native inhibitor at various intervals was calculated from the peak areas and the data were used for calculation of protein concentrations at equilibrium.

Temporary Inhibition—Native SMPI (40 μM) and thermolysin (0.4 μM), and enzyme alone (control) were incubated in 500 μl of 50 mM Tris-Cl (pH 7.5), 10 mM $CaCl_2$, 0.02% NaN_3 buffer, at 37°C. Aliquots of 10 μl were withdrawn at various incubation times and enzyme activity was monitored after 100-fold dilution. Residual enzyme activity (%) was calculated by considering the activity of the enzyme in the absence of inhibitor as 100%. Aliquots were withdrawn at intervals and analyzed by Tricine-SDS-PAGE. Similar experiments were conducted with vimelysin, P. elastase, and SGMP II at a 1:10 enzyme-inhibitor ratio.

Kinetic Analyses—All the reactions were carried out in 50 mM PIPES (pH 7.0), 10 mM $CaCl_2$, 0.005% Triton X-100 buffer at 25°C, and assays were done using MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2 as a substrate (*17*) to a final concentration of 1 μM at λ_{ex} 328 nm and λ_{em} 398 nm on a Hitachi model F-2000 fluorescence spectrophotometer. The kinetic parameters K_m, k_{cat}, and k_{cat}/K_m for the substrate (which has been used for matrix metalloproteinases) were calculated to be 8.2 μM, 55.36 s^{-1}, and 6.75×10^6 $M^{-1}\cdot s^{-1}$ for SGMP II, respectively; 7.14 μM, 17.53 s^{-1}, and 2.4×10^6 $M^{-1}\cdot s^{-1}$ for P. elastase, respectively; and 9.3 μM, 1.3 s^{-1}, and 1.4×10^5 $M^{-1}\cdot s^{-1}$ for vimelysin, respectively. Cleavage specificity of the fluorogenic substrate by various enzymes was analyzed by HPLC. SGMP II, P. elastase, thermolysin, vimelysin, and almelysin showed specific cleavage patterns, similar to that of PUMP (MMP) (*17*), while ScNP showed a different pattern of cleavage, a new major peak being observed along with the smaller specific peaks (data not shown).

Enzyme concentrations were determined by active site titration with the stoichiometric inhibitor phosphoramidon as described previously (*7*). For calculation of K_i values, SGMP II (92 pM) and various concentrations of inhibitor [wild-type, V65I, V65L, V65F, V65Y (20-200 pM), V65S (0.08-1.2 nM), V65G (0.2-3.0 nM), and SMPI* (0.2-3.0 nM)] were incubated for 14 h. For weak inhibitors, the enzyme (0.46 nM) and V65E (10-150 nM) or V65K (20-300 nM) were incubated for 2 h. P. elastase (109 pM) and inhibitor [wild-type, V65I, V65L, V65F, V65Y (20-200 pM), and SMPI* (80-800 pM)] were incubated for 14 h. In the case of weak inhibitors, enzyme (0.44 nM) and V65S (0.2-3 nM), V65G (0.2-3.0 nM), V65E (5-75 nM), and V65K (20-300 nM) were incubated for 2 h. Vimelysin (2.04 nM) and various concentrations of inhibitor [wild-type, V65I, V65L, V65F (0.2-3 nM), V65Y (1-10 nM), and SMPI* (1-15 nM)] were incubated for 15 min. Thermolysin (1 nM) and SMPI* (5-75 nM) were incubated for 15 min. K_i values were calculated by nonlinear least-squares analysis (*18*) as described previously (*7*).

MMP Assays—Partially purified human matrix metalloproteinases were activated using trypsin or 4-aminophenylmercuric acetate, and analyzed using wild-type inhibitor, V65L and V65I mutant SMPIs (1-4 μg/ml). Type I collagenase was analyzed using FITC collagen (0.025%) as a substrate in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 0.02% NaN_3 buffer, and enzyme activity was measured at λ_{ex} 495 nm and λ_{em} 520 nm on a Hitachi F-3010 fluorescence spectrophotometer. Progelatinase assay was conducted in 100 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 100 mM NaCl, 0.05% Brij 35 buffer. Enzyme and inhibitor were incubated for 20 min. After addition of a fluorogenic substrate, initial velocities were monitored at λ_{ex} 328 nm and λ_{em} 398 on a Shimadzu RF-5300PC spectrophotometer, and remaining enzyme activities (%) were calculated. Recombinant human prostromelysin (partially purified from *E. coli*) assay was conducted by incubating an inhibitor and fluorogenic substrate, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 (*19*) (20 μM) at 37°C for 5 min, then the activated enzyme was added and enzyme activity was observed at λ_{ex} 325 nm and λ_{em} 398 on a Labsystems Fluoroskan II.

Additional Methods—ScNP inhibition activity was analyzed using enzyme (137 pmol), inhibitor (native, V65F and V65Y; 100-1,000 pmol) and casein as a substrate according to the standard method as described elsewhere (*3*). For almelysin inhibition assay, enzyme (25 nM) and inhibitor (native and all the mutants; 500 nM) were incubated for 15 min at 25°C, and the fluorogenic substrate (1 μM) was added. Initial velocities were monitored at λ_{ex} 328

nm and λ_{em} 398 and remaining enzyme activities (%) were calculated.

RESULTS

I) Hydrolysis and Resynthesis of Reactive Site Bond—When SMPI was incubated with 1 mol% of thermolysin at pH 7.5 and 25°C, it underwent limited proteolysis at the reactive site (Cys^{64}-Val^{65}) by the enzyme, but the hydrolysis was not complete, and gradually reached equilibrium (Fig. 1A) after 48 h. To analyze the inhibition mechanism of SMPI, we attempted to resynthesize the cleaved reactive site peptide bond by utilizing the enzyme reaction. Modified SMPI (SMPI*) was purified by FPLC with a Resource S column. The inhibition curves obtained for native and modified inhibitors towards thermolysin are presented in Fig. 2. SMPI* was a weaker inhibitor than the native molecule, and to show 50% inhibition of thermolysin, 30 times more protein was required as compared with the native inhibitor. The K_i value (Table I) calculated for SMPI* ($K_i = 2.18 \times 10^{-8}$ M), was 191 times higher than that of the virgin inhibitor ($K_i = 1.14 \times 10^{-10}$ M). When SMPI* was incubated with thermolysin under the same conditions as used for the hydrolysis, *i.e.*, the same buffer, pH and enzyme concentration, resynthesis of virgin inhibitor was observed at 1 h incubation and reached equilibrium at around 6 h (Fig. 1B). The equilibrium was overwhelmingly in favor of the hydrolyzed form and at the equilibrium position only 7% of the inhibitor remained in the native form. The resynthesis of virgin inhibitor from SMPI* was also achieved by incubation with the other metalloproteinases, SGMP II, P. elastase and vimelysin (data not shown).

The stability of SMPI* was analyzed using different concentrations of thermolysin. As shown in Fig. 1B, when a 1 : 100 (mol/mol) enzyme-inhibitor ratio was used, the protein was stable and resynthesis of the virgin inhibitor reached equilibrium. But, with a 1:25 enzyme-inhibitor ratio at 25°C (Fig. 1C), after reaching the equilibrium, gradual degradation of SMPI* was observed. Because of the degradation of SMPI*, the resynthesized native inhibitor again converted to modified form to maintain the equilibrium, so that both forms of the inhibitor were gradually degraded and disappeared completely.

II) Temporary Inhibition—Reappearance of enzyme activity after formation of a stable enzyme-inhibitor complex has been described as temporary inhibition (*20*). To investigate the nature of inhibition by SMPI, native SMPI and thermolysin were incubated at a 1 : 100 (mol/mol) ratio at 37°C, and the enzyme activity was monitored at intervals. In addition aliquots were analyzed by Tricine-SDS-PAGE. As shown in Fig. 3, the enzyme was completely inhibited until 40 h, then the enzyme activity started reappearing and rose sharply to 100%. In Tricine-SDS-PAGE (Fig. 3 inset) the inhibitor band was almost undetectable from 43 h incubation. Temporary inhibition of vimelysin by native SMPI was also observed (E : I = 1 : 10) from 24 h (8 h onwards in the case of thermolysin under similar conditions), and 10 and 25 days for P. elastase and SGMP II, respectively.

III) Inhibition Spectrum—To find a better target enzyme

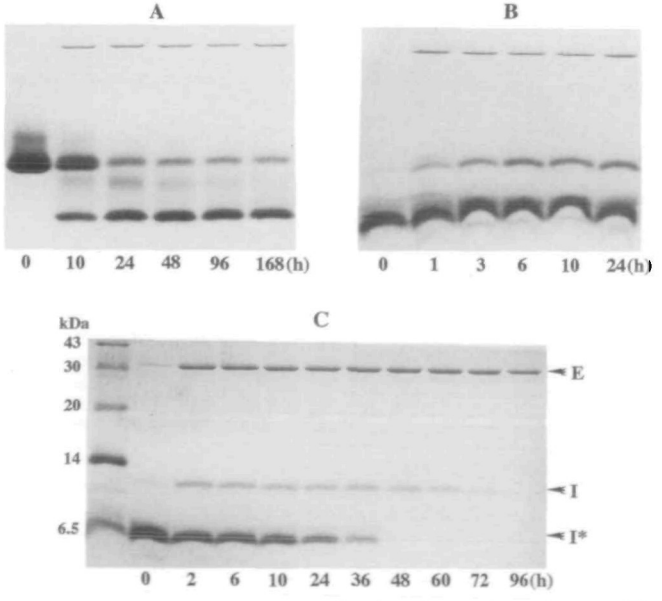

Fig. 1. **Hydrolysis and resynthesis of reactive site peptide bond and degradation of SMPI.** Native or SMPI* inhibitor and thermolysin were incubated in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ buffer at 25°C. Samples were withdrawn at intervals and analyzed by Tricine-SDS-PAGE after reduction with mercaptoethanol. Panel A, hydrolysis of reactive site. Enzyme (100 pmol) and native inhibitor (10 nmol) were incubated at a 1 : 100 (mol/mol) ratio. Panel B, resynthesis of reactive site. The enzyme (100 pmol) and SMPI* (10 nmol) were incubated at a 1 : 100 (mol/mol) ratio. Panel C, degradation of inhibitors after resynthesis. Enzyme (0.4 nmol) and partially purified SMPI* (10 nmol) were incubated at a 1 : 25 (mol/mol) ratio. E, enzyme; I, native form of inhibitor; I*, N-terminal half of the cleaved inhibitor. The protein band corresponding to the C-terminal half disappeared during destaining of the gels.

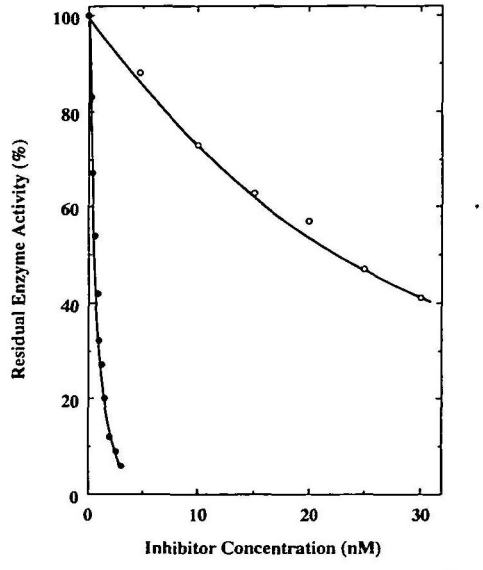

Fig. 2. **Inhibition curves of thermolysin obtained for native and modified inhibitors.** Filled circles represent native inhibitor, open circles represent SMPI*. Thermolysin (1 nM) and various concentrations of native (0.2-3 nM) or SMPI* inhibitors (5-30 nM) were incubated in 50 mM PIPES (pH 7.0), 10 mM $CaCl_2$, 0.005% Triton X-100 buffer at 25°C for 15 min, then the enzyme activity was measured using a fluorogenic substrate. Residual enzyme activities (%) were calculated and plotted against inhibitor concentrations.

for SMPI, we analyzed the inhibition capabilities of SMPIs with various enzymes, based on K_i values and limited proteolysis patterns. We expected that the inhibitors isolated from *Streptomyces* to have strong interactions with enzymes of *Streptomyces* origin, compared to other sources, based on the interactions of SSI with *S. griseus* proteases A and B (SGPA and SGPB) (*21*), and the interactions of SSI family inhibitors with *S. griseus* metalloproteinase, SGMP II (*22, 23*). Hence, SGMP II (which is a constituent of pronase) and ScNP (*10, 24, 25*) (which is the smallest metalloproteinase ever reported, containing only 132 amino acid residues) were selected for analysis. P. elastase was used because it has a similar substrate specificity to thermolysin (*26-28*), it is well characterized compared to other metalloproteinases and also its three-dimensional structure is known (*29*). Vimelysin, an alcohol-resistant metalloproteinase isolated from *Vibrio* sp. T1800, and almelysin, a psychrophilic enzyme from *Alteromonas* sp. no. 3696 (which is not inhibited by phosphoramidon), were also used. Inhibitory capability towards matrix metalloproteinases, type I collagenase (MMP-1), gelatinase A (MMP-2), and stromelysin (MMP-3), was also analyzed.

The calculated K_i values are presented in Table I. For convenience, we classified all the inhibitors into two groups as follows: strong inhibitors (wild-type, V65I, V65L, V65F, V65Y, SMPI*) and weak inhibitors (V65S, V65G, V65E, V65K). SGMP II and P. elastase showed strongest interactions with the lowest K_i values among all the enzymes tested. The K_i values were found to be in the range of pM-nM. The inhibitory capabilities were as follows: for SGMP II, V65F > V65I, V65L > wild-type > V65Y > SMPI* > V65S > V65G > V65E > V65K; P. elastase, V65F, V65I > wild-type > V65Y > V65L > SMPI* > V65S > V65G > V65E > V65K; and vimelysin, V65I, V65F > wild-type > V65L > V65Y > SMPI*. The weak inhibitors showed higher K_i values by 2-5 orders of magnitude than the strong inhibitors towards SGMP II and P. elastase, and the K_i values for weak inhibitors could not be calculated in the case of vimelysin.

No inhibition of ScNP by native inhibitor, V65F and V65Y, or of almelysin by wild-type and mutant SMPIs was found. The assays for inhibitory capabilities towards type 1 collagenase (MMP-1), gelatinase A (MMP-2), and stromelysin (MMP-3) were carried out using wild- type inhibitor, V65I and V65L mutants, and no inhibition was observed.

Limited proteolysis: Stability of inhibitors to the en-

TABLE I. **K_i values for SMPIs against SGMP II, P. elastase, vimelysin, and thermolysin.**

Inhibitors	K_i (M)			
	SGMP II	P. elastase	Vimelysin	Thermolysin[a]
WT	2.32×10^{-12}	2.54×10^{-12}	9.90×10^{-11}	1.14×10^{-10}
V65I	1.55×10^{-12}	1.29×10^{-12}	6.58×10^{-11}	5.46×10^{-11}
V65L	1.58×10^{-12}	7.09×10^{-12}	4.22×10^{-10}	7.75×10^{-10}
V65F	0.97×10^{-12}	1.05×10^{-12}	5.88×10^{-11}	9.80×10^{-11}
V65Y	6.94×10^{-12}	4.10×10^{-12}	1.06×10^{-9}	6.06×10^{-9}
V65S	2.70×10^{-10}	5.26×10^{-10}	ND	3.54×10^{-6}
V65G	7.75×10^{-10}	2.08×10^{-9}	ND	ND
V65E	4.35×10^{-8}	2.24×10^{-8}	ND	ND
V65K	6.29×10^{-8}	6.02×10^{-8}	ND	ND
SMPI*	1.26×10^{-11}	3.11×10^{-11}	2.19×10^{-9}	2.18×10^{-8}[b]

ND, not determined. [a]Values published previously (*7*). [b]Value calculated in the present study.

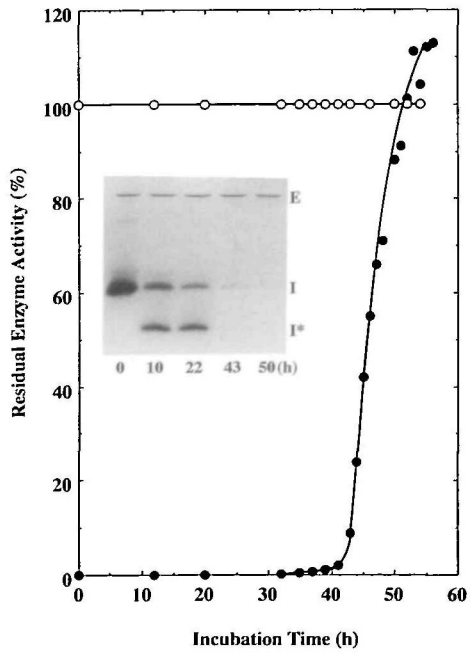

Fig. 3. **Temporary inhibition of thermolysin by native SMPI.** Native SMPI (40 μM) and thermolysin (0.4 μM) or enzyme without inhibitor were incubated in 500 μl of 50 mM Tris-Cl (pH 7.5), 10 mM $CaCl_2$, 0.02% NaN_3 buffer, at 37°C. Aliquots of 10 μl were withdrawn at various incubation times and enzyme activity was monitored after 100-fold dilution. Residual enzyme activities (%) were plotted against incubation time. The inset figure shows Tricine-SDS-PAGE patterns at various intervals. E, enzyme; I, native form of inhibitor; I*, N-terminal half of the cleaved inhibitor.

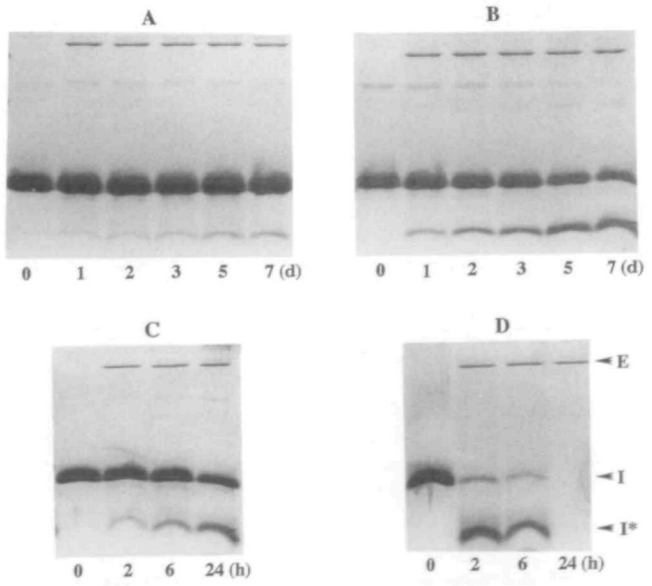

Fig. 4. **Limited proteolysis patterns of wild-type inhibitor and V65K mutant with SGMP II and P. elastase.** Conditions were as explained in the legend to Fig. 1. Enzyme and inhibitor were incubated at a 1 : 100 (mol/mol) ratio, and samples were withdrawn at intervals and analyzed by Tricine-SDS-PAGE. (A) Wild-type SMPI and SGMP II; (B) wild-type inhibitor and P. elastase; (C) V65K and SGMP II; (D) V65K and P. elastase. E, enzyme; I, native form of inhibitor; I*, N-terminal half of the cleaved inhibitor.

zymes was analyzed by limited proteolysis using 1 : 100 (mol/mol) enzyme-inhibitor ratio. All the inhibitor proteins were most stable to SGMP II and then to P. elastase (Fig. 4). Using SGMP II and wild-type inhibitor, as shown in Fig. 4A, the inhibitor was stable and very little cleaved protein (less than 1-2%) was seen even up to 1 week of incubation. With P. elastase (Fig. 4B), cleavage could be seen from 24 h, and the cleaved protein increased gradually, but equilibrium was not attained even by 1 week. In the case of V65K mutant, the weakest inhibitor, cleavage by SGMP II could be observed from 2 h incubation and the protein was stable at least up to 24 h (Fig. 4C), but it underwent degradation after 48 h (data not shown). With P. elastase the cleavage was faster and the protein band disappeared after 6 h incubation (Fig. 4D). Limited proteolysis patterns with vimelysin (data not shown) were similar to those with thermolysin (*7*). Almelysin and ScNP degraded the protein without generating specific cleavage products.

DISCUSSION

Enzyme hydrolysis and resynthesis of peptide bonds in substrates is possible with all four classes of proteolytic enzymes (*30*). Hence, the hydrolysis and resynthesis of reactive sites of inhibitors which bind to enzymes in a way similar to that of substrates should also be possible, theoretically. Almost all proteinaceous inhibitors of serine proteinases are modified by the cognate enzymes. In contrast, metalloproteinase inhibitors are not modified by complex formation with an enzyme (*31-34*), except SMPI. SMPI is the only metalloproteinase inhibitor of its kind. In order to understand the nature of SMPI in detail, we conducted various experiments. Here, we describe three important aspects of SMPI: (I) resynthesis of the reactive site peptide bond, (II) temporary inhibition, and (III) the inhibition spectrum.

I) In this study we showed that virgin SMPI is regenerated from the modified inhibitor. This is the first demonstration of resynthesis of the cleaved reactive site bond by enzyme action for a metalloproteinase inhibitor or of resynthesis using a metalloproteinase. The resynthesis was achieved under the same conditions as used for the hydrolysis, and indeed, it occurred faster than the hydrolysis. This observation confirms our previous identification of the reactive site as Cys^{64}-Val^{65} based on mutational analysis (*7*). The resynthesis of the virgin inhibitor was seen with various metalloproteinases, confirming that SMPI inhibits all the enzymes at the same reactive site. This strongly suggests that SMPI follows the standard mechanism of inhibition of serine proteinase inhibitors, which can be written simply as follows: $E+I=EI=EI^*=E+I^*$.

II) In general, serine proteinase inhibitors are very stable to degradation by the enzyme. Temporary inhibition has not been reported for inhibitors with very large K_i values (10^{-6}-10^{-8}) or in prolonged incubation. However, temporary inhibition of trypsin was observed with a few serine proteinase inhibitors of kazal family, the PSTI and ovomucoids (*20*). In the case of SSI, various modifications, such as deletion of the four C-terminal residues (*35*), removal of the cystine bridge near the reactive site (*36*), substitution of Trp-36 to His (*37*), and deletions in the reactive site loop (*38*) resulted in temporary inhibition. Temporary inhibition has been observed for many serpins (*39*), which follow a different mechanism of inhibition (*40*).

However, in spite of its many similarities to serine proteinase inhibitors, SMPI showed wanton proteolysis and temporary inhibition. When native SMPI was incubated with various enzymes at 37°C at a 1 : 10 E : I ratio, reappearance of enzyme activity was observed from 8 and 24 h for thermolysin and vimelysin, respectively, and from 10 and 25 days for P. elastase and SGMP II, respectively. The release times from temporary inhibition, *i.e.*, 8 h, 24 h, 10 d, and 25 d as mentioned above, are in good agreement with the K_i values (strength of inhibition) (Table I) of SMPI* towards the corresponding enzymes. That is, the largest K_i value (weaker inhibition) and fastest reappearance of enzyme activity were observed with thermolysin, where as the lowest K_i value (stronger inhibition) and slowest reappearance of enzyme activity were observed with SGMP II. The time of reappearance of activity can be regarded as a measure of the specificity of SMPI for the enzymes. This idea is supported by a previous report of faster degradation of SMPI by *Bacillus subtilis* var. *amylosaccharitic*us neutral proteinase (*41*), which was more weakly inhibited than thermolysin. Degradation of native inhibitor occurs through a reactive site-cleaved (modified) inhibitor as an intermediate (Fig. 1C). In all cases, the same peptide bond Cys^{64}-Val^{65} was both responsible for inhibition of the enzyme and the target of degradation by the enzyme. Such temporary inhibition is very unusual when the K_i values are so low (to pM). But temporary inhibition seems to be the intrinsic nature of SMPI. Structural alterations, denaturation, decreased specificity towards enzyme, *etc.*, which generally account for degradation of and temporary inhibition by mutant inhibitors, could be ruled out here. The reasons for and the mechanism of the temporary inhibition remain to be elucidated.

III) To select a better target enzyme, the inhibition spectrum of SMPI was analyzed. Among the enzymes used, SMPI inhibited only thermolysin, vimelysin, P. elastase, and SGMP II. In the kinetic analysis, the K_i values towards SGMP II and P. elastase were of pM order for strong inhibitors. The strong interactions of these enzymes with the inhibitors enabled calculation of K_i values for the weak inhibitors also. In contrast to thermolysin, V65Y showed nearly as strong inhibition as the wild-type inhibitor of SGMP II and P. elastase, and vimelysin was inhibited more strongly than thermolysin by V65Y, showing that these enzymes can accommodate large hydrophobic residues in their substrate-binding pockets. As regards limited proteolysis, the inhibitors were much more stable to SGMP II and P. elastase than thermolysin and vimelysin. However, the weaker inhibitors were degraded by the enzymes during prolonged incubation. The enzymes SGMP II and P. elastase should be useful for various studies which require stable enzyme-inhibitor complexes. Even though all the inhibitors were most stable to SGMP II (Fig. 4), P. elastase is preferable from the viewpoint that it is well characterized and its structure is known. The other enzymes, almelysin, ScNP, and type I collagenase, gelatinase A and stromelysin were not inhibited by SMPIs, despite the similarities in the substrate specificities to the other enzymes, suggesting that they represent a different class of enzymes.

Many researchers have classified metalloproteases into

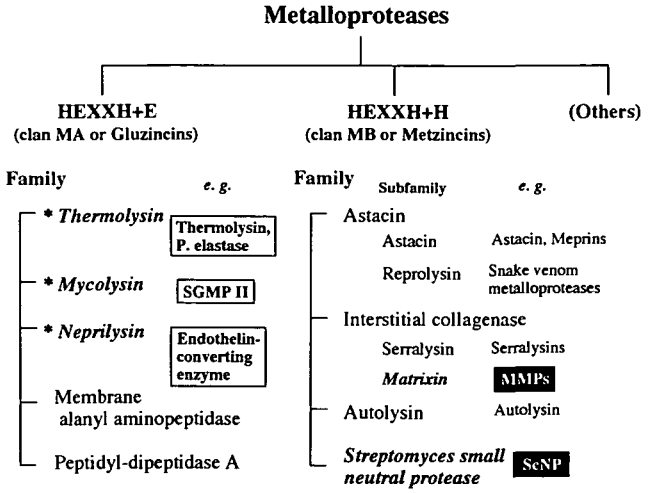

Fig. 5. **Inhibition spectrum of SMPI in relation to the classification of metalloproteases.** The presentation of the figure is according to the classification of Rawlings and Barrett (*42*). Asterisks indicate phosphoramidon-sensitive families. The families of the enzymes used in this study are represented with bold italics. The light shaded boxes represent enzymes which are inhibited by SMPI and the black-colored boxes represent the enzymes which are not inhibited.

different categories on the basis of the zinc ligands. Most of the zinc metalloproteases contain the consensus sequence His-Glu-Xaa-Xaa-His (HEXXH). The two His residues act as Zn chelators and Glu participates in the catalysis. Depending on the third Zn-ligand and surrounding sequences, the HEXXH-containing enzymes were subdivided into HEXXH+E and HEXXH+H groups. Rawlings and Barrett (*42*) named them clans "MA and MB (metzincins)," respectively and Hooper (*43*) called them "gluzincins and metzincins," respectively. The clans "MA and MB" are well characterized and classified further into families and subfamilies. All of the families belonging to a clan are interrelated in some respects. The members of clan MA show strong inhibition by phosphoramidon, while the members of clan MB do not.

Based on the classification of Rawlings and Barrett, the enzymes used in this study can be presented as shown in Fig. 5. The enzymes which are inhibited by SMPI belong to the following families: thermolysin and P. elastase come under the thermolysin family, SGMP II comes into the mycolysin family, and we have some preliminary and unpublished data which show that SMPI inhibits endothelin converting enzyme, which is a member of the neprilysin family. All these families are members of clan MA or gluzincins, and the enzymes are inhibited by phosphoramidon too. The enzymes which are not inhibited by SMPI belong to the following families: Type I collagenase, gelatinase A and stromelysin belong to the matrixin subfamily and ScNP to the *Streptomyces* small neutral protease family. Both come into clan MB or the metzincins group and are insensitive to phosphoramidon as well. ScNP is placed in the family of *Streptomyces* small neutral proteases, based on its amino acid sequence (*10*). Vimelysin and almelysin could not be definitively placed in any family because of the lack of necessary data. Even so, vimelysin can be placed in clan MA, based on its sensitivity to phosphoramidon and SMPI. Based on these observations, we surmise that SMPI specifically inhibits the enzymes of clan MA, or those which show strong inhibition by phosphoramidon.

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