J. Biochem. 129, 429–435 (2001)

Interactions of Human Matrix Metalloproteinase 7 (Matrilysin) with the Inhibitors Thiorphan and R-94138[1]

Hiroshi Oneda and Kuniyo Inouye[2]

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502

Received September 22, 2000; accepted December 19, 2000

The effects of the metalloproteinase inhibitors thiorphan and R-94138 on the matrilysin-catalyzed hydrolysis of (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2 [MOCAc-PLGL(Dpa)AR] were examined. The inhibitor constants (K_i) of thiorphan and R-94138 for matrilysin at pH 7.5, 25°C were determined to be 11.2 and 7.65 μM, respectively. From the temperature dependence of the K_i values at pH 7.5, the standard enthalpy change ($\Delta H°'$) values for the binding of matrilysin with thiorphan and R-94138 were determined to be –(18.2 ± 0.9) and (1.65 ± 1.07) kJ·mol^{-1}, respectively. The binding of matrilysin to thiorphan is exothermic and the free energy change in the complex formation depends mainly on the change in enthalpy, while the binding to R-94138 is endothermic and typically entropy-driven. Hydrophobic interactions are suggested to contribute significantly to the binding of matrilysin to R-94138 as well as to the substrate. The pH dependence of the K_i value suggests that at least two ionizing groups with pK_a values of 4.5 and 9.1–9.3 are involved in the binding. The matrilysin activity is regulated by ionizing groups with pK_a values of 4.3 and 9.6. Both inhibition and hydrolysis are suggested to be controlled by the same residues in matrilysin, most likely Glu 198 and Tyr 219, respectively.

Key words: inhibitor, matrilysin, matrix metalloproteinase, thermolysin, thiorphan.

Matrix metalloproteinases (MMPs) comprise a family of zinc endopeptidases that are believed to degrade extracellular matrix and take part in both normal and pathological processes, including development, reproduction, maintenance, tissue destruction, and fibrotic diseases (*1*, *2*).

Matrilysin (MMP-7) [EC 3.4.24.23] is the smallest MMP, consisting of a signal peptide, propeptide, and a catalytic domain; the molecular mass of the latent pro-form is 28 kDa and that of the mature form is 19 kDa (*3*, *4*). X-ray crystallographic analysis of recombinant human matrilysin in complex with inhibitors demonstrated that matrilysin is composed of a five-stranded β-sheet and three α-helices, and contains a zinc ion essential for enzyme activity, as well as another zinc ion and two calcium ions that are regarded as necessary for enzyme stability (*5*). Matrilysin has been detected in lesions in prostate (*6*), colon (*7*), brain (*8*), stomach (*9*), lung (*10*), and breast (*11*), and it degrades extracellular matrix components such as type I, III, IV, and V gelatins, type IV basement membrane collagen, fibronectin, vitronectin, proteoglycan, laminin, and elastin (*4*, *12–14*). This suggests that matrilysin may play a role in tumor invasion and metastasis. From this viewpoint, the development of matrilysin inhibitors is considered to be of therapeutic benefit.

Preparing large quantities of human matrilysin is indispensable for studying its structure–function relationships and for developing inhibitors that may be useful for cancer therapy. We have previously proposed an effective procedure to prepare active matrilysin from inclusion bodies expressed by *Eschericia coli* in good yield and at reasonable cost (*15*). We have studied the stability and denaturation of matrilysin, the states of the tryptophyl residues (*16*) and the effects of dimethyl sulfoxide, temperature, and sodium chloride on matrilysin activity (*17*), and reported the halophilic properties of matrilysin in comparison with those of thermolysin (*16*). The data suggest the significance of hydrophobic interactions at the active site of matrilysin with substrate and inhibitors (*17*).

In the present study, we demonstrate the inhibitory effects of the characteristic metalloproteinase inhibitors phosphoramidon, zincov, thiorphan, and a synthetic MMP inhibitor (R-94138) on matrilysin. The effects of temperature and pH on the binding of matrilysin to effective inhibitors, thiorphan, and R-94138, are further examined. Phosphoramidon and zincov are representative thermolysin in-

[1] This study was supported in part (K.I.) by Grants-in-Aid for Scientific Research (nos. 11460040 and 11167248) from the Ministry of Education, Science, Sports and Culture of Japan, and grants (nos. 9856 and 0049) from the Salt Science Foundation (Tokyo).

[2] To whom all correspondence should be addressed. Tel: +81-75-753-6266, Fax: +81-75-753-6265, E-mail: inouye@kais.kyoto-u.ac.jp

Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropane sulfonic acid; L(Dpa)AR, L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2; MES, 2-(*N*-morpholino)ethanesulfonic acid; MMP, matrix metalloproteinase; MOCAc-PLG, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly; MOCAc-PLGL(Dpa)AR, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2; phosphoramidon, *N*-(α-L-rhamnopyranosyloxyphospho)-L-Leu-L-Trp; R-94138, *N*-methyl-(3*S*)-2-[(2*R*)-2-hydroxyaminocarbonylmethyl-1-oxoundecyl]hexahydropyridazine-3-carboxamide; thiorphan, (DL-3-mercapto-2-benzylpropanoyl)-Gly; zincov, 2-(*N*-hydroxycarboxamide)-4-methylpentanoyl-L-Ala-Gly-NH_2.

hibitors and their inhibition has been studied spectrophotometrically and kinetically (*18*). Thiorphan was designed as a potent and specific enkephalinase (or neprilysin) inhibitor (*19*, *20*) and also inhibits thermolysin (*21*, *22*). It is a pseudopeptide and a so-called "right-hand side inhibitor" that binds on the right-hand side (the S′ subsites) of the catalytic site in the active center (*23*) with its thiol group positioned to bind the catalytic zinc (Fig. 1). R-94138 was designed as a gelatinase inhibitor based on matrystatin B (*23*). Structurally, it is also a right-hand side inhibitor, and its hydroxamic acid chelates the catalytic zinc (Fig. 1). It has been reported that R-94138 inhibits MMP-2 and MMP-9 strongly (*24*) and prevents the peritoneal dissemination of gastric cancer (*25*). The development of effective inhibitors against MMPs is eagerly desired for cancer therapy. Here, we provide new insights into the structural and functional characteristics of matrilysin and the significance of the hydrophobic interactions of effective inhibitors.

MATERIALS AND METHODS

Materials—Recombinant human pro-matrilysin was prepared according to the method previously reported (*15*). Pro-matrilysin was incubated with 1 mM *p*-aminophenylmercuric acetate at pH 7.5, 37°C for 30 min, and dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 0.05% Brij-35 (buffer A) at 4°C. The preparation was subjected to metal chelation chromatography HPLC on a TSKgel Chelate-5PW [7.5 mm (inner diameter) × 75 mm] (Tosoh, Tokyo) equilibrated with buffer A containing 0.5 M NaCl at 25°C. After charging with 1 mM $ZnCl_2$, a linear gradient was generated from 0 to 0.5 M glycine over 30 min at a flow-rate of 1 ml/min at 25°C. Matrilysin was eluted at 0.24 M glycine, and the fractions were collected for further analysis. The concentration of matrilysin was determined using the molar absorption coefficient at 280 nm of 31.8 $mM^{-1}\cdot cm^{-1}$ calculated from the amino acid composition (*3*) with a Shimadzu UV-2200 spectrophotometer (Kyoto).

(7-Methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2 [MOCAc-PLGL(Dpa)AR, Lot 480429], MOCAc-L-Pro-L-Leu-Gly (MOCAc-PLG, Lot 471218), and phosphoramidon (Lot 220901) were purchased from the Peptide Institute (Osaka). MES (Lot 53H745) and DL-thiorphan (Lot 118H4063) were from Sigma (St. Louis, MO). AMPSO (Lot 7058A) was from Wako Pure Chemical (Osaka). Zincov (Lot 293085) was from Calbiochem (San Diego, CA). R-94138 was a generous gift from Sankyo (Tokyo). A substrate of matrilysin, MOCAc-PLGL(Dpa)AR, is known to be cleaved between glycine and leucine residues (*26*). The concentrations of MOCAc-PLGL(Dpa)AR and MOCAc-PLG were determined using the molar absorption coefficients ε_{410} = 7.5 $mM^{-1}\cdot cm^{-1}$ and ε_{324} = 12.9 $mM^{-1}\cdot cm^{-1}$, respectively (*26*). All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto). The HPLC apparatus consisted of a solvent-delivery system CCPM-II, a UV monitoring system UV-8020, a computer-control system PX-8020, a degasser SD-8020, a column oven CO-8020, and an integrator Chromatocorder 21 purchased from Tosoh.

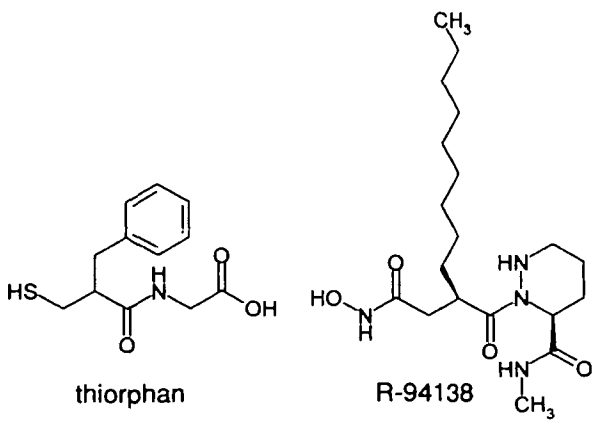

Fig. 1. **Molecular structures of thiorphan and R-94138.**

Effects of Inhibitors on the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—The matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was performed by mixing 1,232 μl of matrilysin solution (10.2 nM) in 50.7 mM HEPES buffer containing 10.1 mM $CaCl_2$, 10 μl of inhibitor (3.75 μM–12.5 mM) dissolved in DMSO, and 8 μl of MOCAc-PLGL(Dpa)AR (117–234 μM) dissolved in DMSO at pH 7.5, 25°C. The hydrolysis of MOCAc-PLGL(Dpa)AR was measured by following the increase in the fluorescence intensity at 393 nm with excitation at 328 nm with a JASCO FP-777 fluorescence spectrophotometer (Tokyo). The reaction was performed under pseudo-first order conditions, where the substrate concentration ([S]) is much lower than the Michaelis constant (K_m) in order to avoid absorptive quenching effects. Under these conditions, the reaction velocity, v, is expressed as $k_{cat}[E]_o[S]/K_m$, where k_{cat} is the catalytic constant, and $[E]_o$ is the initial enzyme concentration in the reaction mixture.

Thermodynamic Analysis of the Binding of Matrilysin to Thiorphan and R-94138—The effect of temperature on the inhibitor constants (K_i) of thiorphan and R-94138 against matrilysin was examined in 50 mM HEPES buffer (pH 7.5) plus 10 mM $CaCl_2$ and 1.4% DMSO. The standard enthalpy change ($\Delta H^{\circ\prime}$) for the binding of matrilysin to the inhibitors was determined from the slope ($-\Delta H^{\circ\prime}/R$) of the plot of $-\ln K_i$ against $1/T$ (Van't Hoff plot). The standard Gibbs free energy change ($\Delta G^{\circ\prime}$) and the standard entropy change ($\Delta S^{\circ\prime}$) for the binding of matrilysin to inhibitors were determined according to the following equations (*27*):

$$\Delta G^{\circ\prime} = -RT \ln (1/K_i) \quad (1)$$

$$\Delta S^{\circ\prime} = (\Delta H^{\circ\prime} - \Delta G^{\circ\prime})/T \quad (2)$$

where R and T are the gas constant and temperature in Kelvin, respectively.

Effect of pH on the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR and the Inhibitor Constants (K_i) of Thiorphan and R-94138—The following buffers were used to study the effect of pH on the enzyme activity and inhibition: 50 mM sodium acetate buffer at pH 3.6–5.8, 50 mM MES buffer at pH 6.0–6.8, 50 mM HEPES buffer at pH 6.8–8.5, and 50 mM AMPSO at pH 8.5–10.0, all of which contained 10 mM $CaCl_2$ at 25°C. The DMSO concentrations were 0.6 and 1.4% in the absence and presence of the inhibitors, respectively (*17*). The pK_a values of the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR were determined from the plot of $\log k_{cat}/K_m$ *versus* pH by fitting to the following equation (Eq. 3):

$$\log(k_{cat}/K_m) = \log(k_{cat}/K_m)_o - \log(1 + [H^+]/K_{e1} + K_{e2}/[H^+]) \quad (3)$$

where $(k_{cat}/K_m)_o$ is the intrinsic (k_{cat}/K_m) value that is independent of pH (*28*). The K_{e1} and K_{e2} values are the ionization constants (K_a) of the ionizing groups of matrilysin that are involved in the formation of the enzyme-substrate complex. Hereinafter, suffices 1 and 2 of K_{e1} and K_{e2} indicate the ionization constants (K_a) of the acidic and basic sides, respectively. The pK_a values of the inhibitor constants of thiorphan and R-94138 were determined from the plot of $-\log K_i$ against pH by fitting to the following equation (Eq. 4):

$$-\log K_i = -\log (K_i)_o - \log(1 + [H^+]/K_1 + K_2/[H^+]) \quad (4)$$

where $(K_i)_o$ is the intrinsic and pH-independent K_i value, and K_1 and K_2 are the ionization constants (K_a) of the ionizing groups that control the inhibition.

RESULTS

Effects of Phosphoramidon, Zincov, Thiorphan, and R-94138 on Matrilysin Activity—The inhibitory effects of the four inhibitors on the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR were examined (Fig. 2). No inhibition was observed with phosphoramidon in the concentration range examined ($3 \times 10^{-8} - 1 \times 10^{-4}$ M). The IC_{50} values (namely, the inhibitor concentration giving 50% inhibition) of zincov, thiorphan, and R-94138 were estimated to be 197, 10.9, and 7.76 μM, respectively. Thiorphan and R-94138 were shown to be 18 and 25 times stronger than the hydroxamate inhibitor, zincov, and the inhibitions by thiorphan and R-94138 were further examined. When $1/v$ is plotted against $[I]_o$ (Dixon plot for determining K_i) at two different substrate concentrations, the two lines cross apparently at a point on the $[I]_o$ axis (*27*, Fig. 3). The $1/v$ and $[I]_o$ values of the crossing point are, respectively, $1/V_{max}$ and $-K_i$ in the case of competitive inhibition, and zero and $-K_i$ in the case of non-competitive inhibition. Therefore, we can discriminate whether the inhibition is competitive or non-competitive from the position of the crossing point. However, it should be noted that competitive inhibition can not be discriminated from non-competitive inhibition when the v value observed is much smaller than the V_{max} value, or when the inhibitor constant (K_i) is much smaller than the substrate constant (K_s) that is close to the Michaelis constant (K_m) (*29*, *30*). Thiorphan and R-94138 are considered to be competitive inhibitors (*19*, *20*, *24*). Their concentrations in the present conditions were set to be lower than the K_m value (36 μM) because of their low solubilities (*17*). Accordingly, the v values observed were much lower than the V_{max} value, and the plots in Fig. 3 suggest the inhibition manner to be apparently non-competitive, although they could be competitive inhibitors. From the intersecting point of the two lines, the K_i values of thiorphan and R-94138 at pH 7.5, 25°C were determined to be 11.2 and 7.65 μM, respectively. In the same way, the K_i values at pH 7.5, 37°C were determined to be 15.0 and 7.65 μM, respectively.

Effects of Temperature on the Binding of Matrilysin to Thiorphan and R-94138—The temperature dependence of the K_i values of thiorphan and R-94138 were examined at pH 7.5 at temperatures between 20 and 45°C. From the slope of the van't Hoff plot, the standard enthalpy change ($\Delta H°'$) values for the binding of matrilysin to thiorphan and R-94138 were determined to be (-18.2 ± 0.9) and (1.65 ± 1.07) kJ·mol^{-1}, respectively (Fig. 4). The standard Gibbs free energy change ($\Delta G°'$) and the standard entropy change ($\Delta S°'$) values for the formation of matrilysin-thiorphan and matrilysin–R-94138 complexes are summarized in Table I. The thermodynamic parameters determined for the binding of matrilysin and the MOCAc-PLGL(Dpa)AR substrate (*17*) are also listed in the table. The binding of matrilysin to thiorphan gave large negative $\Delta H°'$ values, and the binding

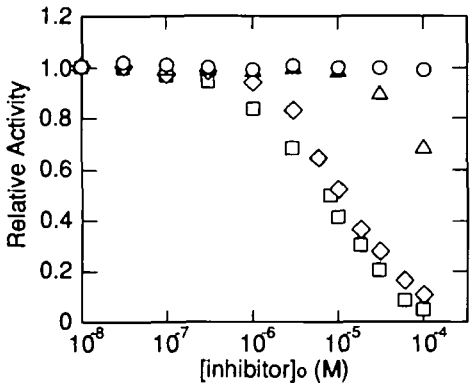

Fig. 2. **Effects of phosphoramidon, zincov, thiorphan, and R-94138 on matrilysin activity.** The reaction was performed in 50 mM HEPES buffer plus 10 mM $CaCl_2$ and 1.4% DMSO at pH 7.5, 25°C. The initial concentrations of matrilysin and MOCAc-PLGL-(Dpa)AR were 10.1 nM and 1.5 μM, respectively. The k_{cat}/K_m value of matrilysin was 0.13 μM^{-1}·s^{-1}, and this value was taken as 100% activity. Inhibitors: phosphoramidon, ○; zincov, △; thiorphan, ◇; R-94138, □. The IC_{50} values for zincov, thiorphan, and R-94138 were determined to be 197, 10.9, and 7.76 μM, respectively.

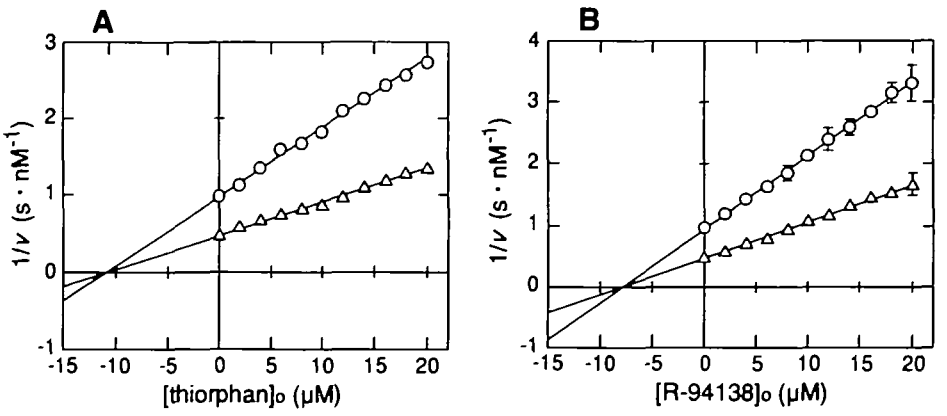

Fig. 3. **Dixon plot of the inhibition of matrilysin activity by thiorphan (A) and R-94138 (B).** The reaction was performed in 50 mM HEPES buffer plus 10 mM $CaCl_2$ and 1.4% DMSO at pH 7.5, 25°C. The initial concentration of matrilysin was 10.1 nM, and those of MOCAc-PLGL(Dpa)AR were 0.75 (○) and 1.5 (△) μM. The inhibitor constants (K_i) for thiorphan and R-94138 were determined to be 11.2 and 7.65 μM, respectively.

was found to be exothermic. The $\Delta G^{\circ\prime}$ value was mainly contributed by the $\Delta H^{\circ\prime}$ value, and the binding of matrilysin to thiorphan is considered to be enthalpy-driven. On the other hand, the binding of matrilysin to R-94138 was slightly endothermic with a large positive $\Delta S^{\circ\prime}$ value, suggesting that the binding is driven by a large increase in entropy. It is noted that the binding of matrilysin to the substrate differs from binding to inhibitors, and is slightly exothermic and entropy-driven. The binding modes of matrilysin to R-94138 and substrate might be similar, because the free energy change for the binding in both cases is contributed mostly by a large increase in entropy. This large increase in entropy suggests that hydrophobic interactions play a significant role in the binding of matrilysin to R-94138 and MOCAc-PLGL(Dpa)AR.

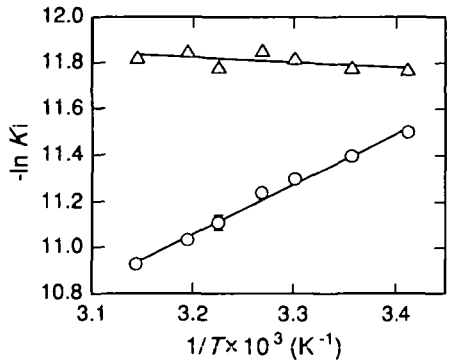

Fig. 4. **Effect of temperature on the inhibitor constants (K_i) of thiorphan and R-94138.** The reaction was performed in 50 mM HEPES buffer plus 10 mM $CaCl_2$ and 1.4% DMSO at pH 7.5. The initial concentration of matrilysin was 10.1 nM, and those of MOCAc-PLGL(Dpa)AR were 0.75 and 1.5 μM. Standard enthalpy change ($\Delta H^{\circ\prime}$) for the formation of the matrilysin-thiorphan (○) and matrilysin–R-94138 (△) complexes were determined to be (−18.2 ± 0.9) and (1.65 ± 1.07) kJ·mol^{-1}, respectively.

Effects of pH on the Matrilysin-Catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR—Figure 5A shows the pH dependence of the specificity constant (k_{cat}/K_m) for the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR in the pH range of 3.6 to 10.0, at 25°C. The maximal k_{cat}/K_m value [or intrinsic k_{cat}/K_m, $(k_{cat}/K_m)_o$] was observed over a wide pH range, pH 5.5–8.5, and was determined to be 1.5×10^5 $M^{-1}\cdot s^{-1}$. The plot of log k_{cat}/K_m *versus* pH (Dixon plot for determining pK_a) was fitted to Eq. 3, and the pK_a values (pK_{e1} and pK_{e2}) were determined to be 4.3 and 9.6. This suggests that the enzyme activity is controlled by at least two ionizing groups of pK_a 4.3 and 9.6 that locate on the enzyme molecule. Because K_m and k_{cat} cannot be determined separately under the present conditions, it is not clear whether the ionizing groups control K_m or k_{cat}.

Effects of pH on the Binding of Matrilysin to Thiorphan and R-94138—Dixon plots of the K_i values of thiorphan and R-94138 show a good fit to Eq. 4 (Fig. 5, A and B), suggesting that the binding between matrilysin and the inhibitors is also controlled by at least two ionizing groups as in the binding of matrilysin to substrate. The minimal K_i values [or intrinsic K_i, $(K_i)_o$] for thiorphan and R-94138 were determined to be 11.2 and 10.5 μM, respectively. The pK_a values of the K_i value of thiorphan were determined to be 4.5 and 9.3, and those of R-94138 were 4.5 and 9.1. This

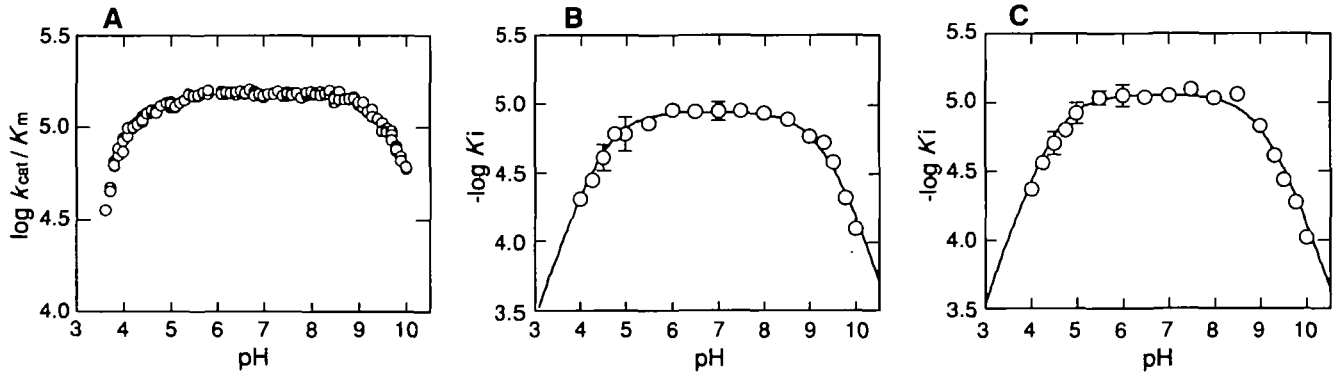

Fig. 5. **Effect of pH on matrilysin activity and the inhibitor constants (K_i) of thiorphan and R-94138.** The reaction buffers used were 50 mM sodium acetate buffer at pH 3.6–5.8, 50 mM MES buffer at pH 6.0–6.8, 50 mM HEPES buffer at pH 6.8–8.5, and 50 mM AMPSO at pH 8.5–10.0 plus 10 mM $CaCl_2$ at 25°C. A: Effect of pH on the specificity constant (k_{cat}/K_m). The reaction was performed in reaction buffer plus 0.6% DMSO at 25°C. The initial concentrations of matrilysin and MOCAc-PLGL(Dpa)AR were 6.3 nM and 1.5 μM, respectively. The pK_1 and pK_2 values were determined to be 4.3 and 9.6, respectively. B: Effect of pH on the inhibitor constant (K_i) of thiorphan. The reaction was performed in reaction buffer plus 1.4% DMSO at 25°C. The initial concentration of matrilysin was 10.1 nM, and that of MOCAc-PLGL(Dpa)AR were 0.75. The pK_1 and pK_2 values were determined to be 4.5 and 9.3, respectively. C: Effect of pH on the inhibitor constant (K_i) of R-94138. The reaction conditions were the same as described for panel B. The pK_1 and pK_2 values were determined to be 4.5 and 9.1, respectively.

TABLE I. **Thermodynamic parameters for the binding of matrilysin to thiorphan and R-94138 at pH 7.5, 25°C.** $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$, and $\Delta S^{\circ\prime}$ are standard free energy change, standard enthalpy change, and standard entropy change, respectively.

	$\Delta G^{\circ\prime}$ (kJ·mol^{-1})	$\Delta H^{\circ\prime}$ (kJ·mol^{-1})	$\Delta S^{\circ\prime}$ (J·mol^{-1}·K^{-1})
Thiorphan	−28.2 ± 0.1	−18.2 ± 0.9	33.6 ± 3.0
R-94138	−29.2 ± 0.1	1.65 ± 1.07	104 ± 4
MOCAc-PLGL(Dpa)AR[a]	−26.1 ± 0.1	−1.90 ± 2.43	80.9 ± 8.6

[a] Values are cited from Ref. *17*.

suggests that ionizing groups with pK_a values of 4.5 and 9.1–9.3 are involved in the inhibition of matrilysin by both inhibitors. It is noteworthy that the acidic and alkaline pK_a values obtained for the pH-dependence of the inhibitor constant are similar to those for the enzyme activity, suggesting that the ionizing groups involved in inhibition might be the same as those controlling the hydrolysis of MOCAc-PLGL(Dpa)AR.

DISCUSSION

Inhibition Potency of the Examined Inhibitors against Matrilysin—The effects of four metalloproteinase inhibitors on matrilysin activity have been examined. Phosphoramidon is a transition state analogue of thermolysin (*31*) and inhibits thermolysin with a K_i value of 10 nM, while zincov inhibits thermolysin with a K_i value of 180 nM at pH 7.5, 25°C (*18*). Phosphoramidon does not inhibit matrilysin, even at a concentration of 100 μM, while zincov inhibits matrilysin with an IC_{50} value 1,000 times higher than that for thermolysin. Phosphoramidon at a concentration of 25 μM does not inhibit rat matrilysin, while zincov at concentrations of 50 and 500 μM inhibits it by 32 and 65%, respectively (*4*). This indicates that rat and human matrilysins have a rather similar geography around the active site.

Thiorphan inhibits neprilysin with a K_i value of 2.4 nM at pH 7.4, 37°C, and also inhibits thermolysin with K_i = 1.6 μM at pH 6.8, 37°C (*21*). The K_i value of thiorphan against matrilysin is 15.0 μM at pH 7.5, 37°C, indicating that thiorphan inhibits neprilysin at least 6,000 more strongly than matrilysin. Accordingly, the standard free energy change in the binding of thiorphan to neprilysin is larger than that to matrilysin by 22.5 $kJ \cdot mol^{-1}$ at 37°C, suggesting that one or two hydrogen bonds or 110–140 $Å^2$ of hydrocarbonaceous surface area involved in the binding of thiorphan to neprilysin might be lost upon binding to matrilysin (*32*).

R-94138 is a highly potent MMP inhibitor, but does not inhibit thermolysin even at a concentration of 260 μM (*24*). Assuming that the K_i value is close to the IC_{50} value, the K_i value (7.65 μM) of R-94138 against matrilysin at pH 7.5, 37°C is much higher than those for MMP-2 (IC_{50} = 38 nM) or MMP-9 (IC_{50} = 1.2 nM) (*24*, *25*), and the matrilysin–R-94138 complex is considered to be less stable than the MMP-2– and MMP-9–R-94138 complexes by 13.7 and 22.6 $kJ \cdot mol^{-1}$, respectively. In the development of specific inhibitors for individual MMPs, the depth of the S1′ pocket often appears to be a target. R-94138 was designed for the nonyl group at the P1′ position to fit successfully into the S1′ pocket of MMP-2 and MMP-9. Matrilysin has a tyrosyl residue at position 193, which forms the bottom of the S1′ pocket and makes it shallower than those of other MMPs (*33*), suggesting that the nonyl group at the P1′ position of R-94138 is too bulky to fit into the S1′ pocket of matrilysin.

Thermodynamic Parameters for the Binding of Matrilysin to Inhibitors—Negative $\Delta H°'$ and positive $\Delta S°'$ values were observed for the binding of matrilysin to thiorphan. On the other hand, positive $\Delta H°'$ and $\Delta S°'$ values were observed for the binding of matrilysin to R-94138, suggesting that the binding involves mainly hydrophobic interactions. As for the zinc binding groups, a hydroxamate group providing a bidentate ligand to the zinc is more potent than a group providing only a monodentate ligand, such as sulfodiimine (*5*). When comparing the region except for the zinc binding group, thiorphan is expected to show a higher binding affinity than R-94138, especially due to favorable fitting at the S1′ pocket of matrilysin.

Ionizing Residues Involved in the Binding of Matrilysin to Substrate and Inhibitors—MMPs from mammalian tissues are known to share some amino acid sequence homology with thermolysin around thermolysin residue Glu 143 (*34*). This residue is thought to be involved in thermolysin catalysis by a general base mechanism and to polarize the water molecule that attacks the scissile bond during catalysis; His 231 of thermolysin is considered to play a role in stabilizing the tetrahedral intermediate during catalysis by donating a hydrogen bond to the hydrated peptide (*35*, *36*). An equivalent of thermolysin residue His 231 cannot be readily identified in matrilysin (*34*), and confirmation of the catalytic mechanism must await further structural and kinetic studies. The reaction mechanism of thermolysin, however, has given rise to much controversy (*35*, *37–39*).

The pK_{e1} and pK_{e2} values were determined to be 4.3 and 9.6, respectively, by the pH-dependence of k_{cat}/K_m for the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR (Fig. 5A). These values suggest that a carboxyl group might be responsible for pK_{e1} and either of a tyrosyl, an amino group, or a zinc-bound water molecule might be responsible for pK_{e2}. Glu 198 in human matrilysin [the numbering of amino acid residues of pro-matrilysin (27.9 kDa) is applied to matrilysin beginning at 78 for the N-terminal tyrosyl residue (*40*)] is a conserved residue among MMPs (*41*) and is regarded to play an important role in catalysis on the basis of mechanistic proposals for thermolysin and other zinc proteinases (*34*, *35*). Site-directed mutagenesis studies of Glu 198 have implied that the ionization of this residue is not responsible for pK_{e1}, and thus the ionization of the zinc-bound water may be responsible instead (*42*). The role of Glu 198 in catalysis may be to stabilize the transition state or to act as a general acid catalyst after the rate-determining step. Matrilysin has Tyr 219 in the Met-turn, and is located in the S1′ substrate binding subsite (*5*). This tyrosyl residue is conserved in all MMPs (*41*, *43*), and is the most likely group responsible for the pK_{e2} value (9.6).

The pK_a values evaluated from the pH-dependence of the K_i values of thiorphan and R-94138 are in reasonable agreement with the pK_a values for matrilysin activity (k_{cat}/K_m), suggesting that the same ionizing groups may be concerned in the binding of matrilysin to both inhibitors and substrate. In general, enzyme reactions are expressed by the Michaelis-Menten equation. The rate constants for the processes from (E + S) to the ES complex, and the ES complex to (E + S), and the ES complex to (E + P) are expressed by k_1, k_{-1}, and k_2, respectively. The Michaelis constant [$K_m = (k_{-1} + k_2)/k_1$], which is assumed to be equal to the substrate constant [$K_s = k_{-1}/k_1$], and the inhibitor constant (K_i) might be controlled by the same ionizing groups. The X-ray crystallographic analysis of matrilysin bound to a hydroxamate inhibitor has demonstrated that the hydroxamate group forms two hydrogen bonds with the ε-oxygen atoms of Glu 198 (*5*). The ionization of Glu 198 may be favorable for matrilysin to bind R-94138, and the hydroxamate group (pK_a = 9) is protonated simultaneously (*44*). Therefore, the pK_{a1} value of the K_i value of R-94138 is considered to be attributable to Glu 198. If the pH-dependence of the K_i and K_m values is assumed to be controlled by the

same residues with pK_a 4.3 and 9.1–9.6, then the pH-dependence of k_{cat}/K_m of the hydrolysis of MOCAc-PLGL-(Dpa)AR is suggested to be derived from only the pH-dependence of K_m. In this case, the k_{cat} value, which could not be determined separately from the K_m under the present conditions, should be constant in a pH-range from 4.3–9.6, and be controlled by a group with pK_a >9.6 or <4.3. The most plausible candidates are a zinc-bound water molecule and a carboxyl group, respectively, and the former is considered to be more likely than the latter from the results so far reported. Examination of the roles of Glu 198 and Tyr 219 in the catalysis is currently under way by site-directed mutagenesis and chemical modification.

Strategy for the Development of Effective Inhibitors against Matrilysin—The interactions of R-94138 and the substrate MOCAc-PLGL(Dpa)AR with matrilysin are entropy-driven, and are mainly derived from the hydrophobic interaction at the S1′ pocket of the active site (Table I). Therefore, a better P1′ group on the inhibitor would give a larger entropy change. On the other hand, the inhibition by thiorphan is enthalpy-driven. The origin of the enthalpy change is not known, but one possibility is hydrogen bonding. From these lines of evidence. it is suggested that an effective matrilysin inhibitor could be designed to bind matrilysin through hydrophobic interactions and as many hydrogen bonds as possible. The observations reported in this paper may lead to the development of effective inhibitors useful for cancer therapy.

We thank Dr. K. Tanzawa (Sankyo Co. Ltd., Tokyo) for providing the R-94138 inhibitor and for helpful discussion.

REFERENCES

1. Woessner, J.F., Jr. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* **5**, 2145–2155
2. Matrisian, L.M. (1992) The matrix-degrading metalloproteinases. *BioEssays* **14**, 455–463
3. Quantin, B., Murphy, G., and Breathnach, R. (1989) Pump-1 cDNA codes for a protein with characteristics similar to those of classical collagenase family members. *Biochemistry* **28**, 5327–5334
4. Woessner, J.F., Jr. and Taplin, C.J. (1988) Purification and properties of a small latent matrix metallo-proteinase of the rat uterus. *J. Biol. Chem.* **263**, 16918–16925
5. Browner, M.F., Smith, W.W., and Castelhano, A.L. (1995) Matrilysin-inhibitor complexes: common themes among metalloproteases. *Biochemistry* **34**, 6602–6610
6. Pajouh, M.S., Nagle, R.B., Brethnach, R., Finch, J.S., Brawer, M.K., and Bowden, G.T. (1991) Expression of metalloproteinase genes in human prostate cancer. *J. Cancer Res. Clin. Oncol.* **117**, 114–150
7. Yoshimoto, M., Itoh, F., Yamamoto, H., Hinoda, Y., Imai, K., and Yachi, A. (1993) Expression of MMP-7 (PUMP-1) mRNA in human colorectal cancers. *Int. J. Cancer* **54**, 614–618
8. Nakano, A., Tani, E., Miyazaki, K., Yamamoto, Y., and Furuyama, J. (1995) Matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gliomas. *J. Neurosurg.* **83**, 298–307
9. Adachi, Y., Itoh, F., Yamamoto, H., Matsuno, K., Arimura, Y., Kusano, M., Endo, T., Hinoda, Y., Oohara, M., Hosokawa, M., and Imai, K. (1998) Matrix metalloproteinase matrilysin (MMP-7) participates in the progression of human gastric and esophageal cancers. *Int. J. Oncol.* **13**, 1031–1035
10. Muller, D., Breathnach, R., Engelmann, A., Millon, R., Bronner, G., Flesch, H., Dumont, P., Eber, M., and Abecassis, J. (1991) Expression of collagenase-related metalloproteinase genes in human lung or head and neck tumours. *Int. J. Cancer* **48**, 550–556
11. Happner, K.J., Matrisian, L.M., Jensen, R.A., and Rodgers, W.H. (1996) Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. *Am. J. Pathol.* **149**, 273–282
12. Miyazaki, K., Hattori, Y., Umenishi, F., Yasumitsu, H., and Umeda, M. (1990) Purification and characterization of extracellular matrix-degrading metalloproteinase, matrin (pump-1), secreted from human rectal carcinoma cell line. *Cancer Res.* **50**, 7758–7764
13. Imai, K., Shikata, H., and Okada, Y. (1995) Degradation of vitronectin by matrix metalloproteinases-1, -2, -3, -7 and -9. *FEBS Lett.* **369**, 249–251
14. Murphy, G., Cockett, M.I., Ward, R.V., and Docherty, A.J.P. (1991) Matrix matalloproteinase degradation of elastin, type IV collagen, and proteoglycan: A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2, and punctuated metalloproteinase (PUMP). *Biochem. J.* **277**, 277–279
15. Oneda, H. and Inouye, K. (1999) Refolding and recovery of recombinant human matrix metalloproteinase 7 (matrilysin) from inclusion bodies expressed by *Escherichia coli*. *J. Biochem.* **126**, 905–911
16. Inouye, K., Tanaka, H., and Oneda, H. (2000) States of tryptophyl residues and stability of recombinant human matrix metalloproteinase 7 (matrilysin) as examined by fluorescence. *J. Biochem.* **128**, 363–369
17. Oneda, H. and Inouye, K. (2000) Effects of dimethyl sulfoxide, temperature, and sodium chloride on the activity of human matrix metalloproteinase 7 (matrilysin). *J. Biochem.* **128**, 785–791
18. Inouye, K., Kuzuya, K., and Tonomura, B. (1994) A spectrophotometric study on the interaction of thermolysin with chloride and bromide ions, and the state of tryptophyl residue 115. *J. Biochem.* **116**, 530–535
19. Roques, B.P., Fouri-Zaluski, M.C., Soroca, E., Lecomte, J.M., Malfroy, B., Llorens, C., and Schwartz, J.C. (1980) The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. *Nature* **288**, 286–288
20. Roques, B.P., Noble, F., Crine, P., and Fouri-Zaluski, M.C. (1995) Inhibitors of neprilysin: design, pharmacological and clinical applications in *Methods in Enzymology* (Barrett, A.J., ed.) Vol. 248, pp. 263–283, Academic Press, San Diego
21. Marie-Claire, C., Ruffet, E., Antonczac, S., Beaumont, A., O'Donohue, M.J., Roques, B.P., and Fouri-Zaluski, M.C. (1997) Evidence by site-directed mutagenesis that arginine 203 of thermolysin and arginine 717 of neprilysin (neutral endopeptidase) play equivalent critical roles in substrate hydrolysis and inhibitor binding. *Biochemistry* **36**, 13938–13945
22. Marie-Claire, C., Ruffet, E., Tiraboschi, G., and Fouri-Zaluski, M.C. (1998) Difference in transition state stabilization between thermolysin (EC 3.4.24.27) and neprilysin (EC 3.4.24.11). *FEBS Lett.* **438**, 215–219
23. Brown, P.D. (1998) Synthetic inhibitors of matrix metalloproteinases in *Matrix Metalloproteinases* (Parks, W.C. and Mecham, R.P., eds.) pp. 243–261, Academic Press, New York
24. Tamaki, K., Tanzawa, K., Kurihara, S., Oikawa, T., Monma, S., Shimada, K., and Sugimura, Y. (1995) Synthesis and structure-activity relationship of gelatinase inhibitors derived from matrystatins. *Chem. Pharm. Bull.* **43**, 1883–1893
25. Igarashi, N., Kubota, T., Otani, Y., Matsuzaki, S.W., Watanabe, M., Teramoto, T., Kumai, K., Tamaki, K., Tanzawa, K., Kobayashi, T., and Kitajima, M. (1999) Preventive effect of matrix metalloproteinase inhibitor, R-94138, in combination with mitmycin C or cisplatin on peritoneal dissemination of human gastric cancer cell line TMK-1 in nude mice. *Jpn. J. Cancer Res.* **90**, 116–121
26. Knight, C.G., Willenbrock, F., and Murphy, G. (1992) A novel coumarin-labelled peptide for sensitive continuous assay of matrix metalloproteinases. *FEBS Lett.* **296**, 263–266

27. Segel, I.H. (1975) *Enzyme Kinetics*, pp. 929–931, John Wiley and Sons, New York
28. Dixon, M. and Webb, E.C. (1979) *Enzymes* 3rd ed., pp. 138–164, Academic Press, New York
29. Dixon, M. (1953) The determination of enzyme inhibitor constants. *Biochem. J.* **55**, 170–171
30. Inouye, K., Tonomura, B., Hiromi, K., Sato, S., and Murao, S. (1977) The stoichiometry of inhibition and binding of a protein proteinase inhibitor from *Streptomyces* (*Streptomyces* subtilisin inhibitor) against subtilisin BPN'. *J. Biochem.* **82**, 961–967
31. Weaver, L.H., Kester, W.R., and Matthews, B.W. (1977) A crystallographic study of the complex of phosphoramidon with thermolysin. A model for the presumed catalytic transition state and for the binding of extended substrates. *J. Mol. Biol.* **114**, 119–132
32. Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., pp. 293–310, Freeman, New York
33. Welch, A.R., Holman, C.M., Huber, M., Brenner, M.C., Browner, M.F., and Van Wart, H.E. (1996) Understanding the P1' specificity of the matrix matalloproteinases: Effect of S1' pocket mutations in matrilysin and stromelysin-1. *Biochemistry* **35**, 10103–10109
34. Vallee, B.L. and Auld, D.S. (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* **29**, 5647–5659
35. Matthews, B.W. (1988) Structural basis of the action of thermolysin and related zinc peptidases. *Acc. Chem. Res.* **21**, 333–340
36. Beaumont, A., O'Donohue, M.J., Paredes, N., Rouseelet, N., Assicot, M., Bohuon, C., Fouri-Zaluski, M.-C., and Roques, B.P. (1995) The role of histidine 231 in thermolysin-like enzymes. A site-directed mutagenesis study. *J. Biol. Chem.* **270**, 16803–16808
37. Inouye, K., Lee, S.-B., Nambu, K., and Tonomura, B. (1997) Effects of pH, temperature, and alcohols on the remarkable activation of thermolysin by salts. *J. Biochem.* **122**, 358–364
38. Mock, W.L. and Aksamawati, M. (1994) Binding to thermolysin of phenolate-containing inhibitors necessitates a revised mechanism of catalysis. *Biochem. J.* **302**, 57–68
39. Mock, W.L. and Stanford, D.J. (19969 Arazoformyl dipeptide substrates for thermolysin. Confirmation of a reversed protonation catalytic mechanism. *Biochemistry* **35**, 7369–7377
40. Crabbe, T., Willenbrock, F., Eaton, D., Hynds, P., Carne, A.F., Murphy, G., and Docherty, A.J.P. (1992) Biochemical characterization of matrilysin. Activation conforms to the stepwise mechanisms proposed for other matrix metalloproteinases. *Biochemistry* **31**, 8500–8507
41. Sang, Q.A. and Douglas, D.A. (1996) Computational sequence analysis of matrix metalloproteinases. *J. Protein Chem.* **15**, 137–160
42. Cha, J. and Auld, D.S. (1997) Site-directed mutagenesis of active site glutamate in human matrilysin: Investigation of its role in catalysis. *Biochemistry* **36**, 16019–16024
43. Cha, J., Pedersen, M.V., and Auld, D.S. (1996) Metal and pH dependence of heptapeptide catalysis by human matrilysin. *Biochemistry* **35**, 15831–15838
44. Johnson, L.L., Pavlovsky, A.G., Johnson, A.R., Janowicz, J.A., Man, C.-F., Ortwine, D.F., Purchase, C.F., II, White, A.D., and Hupe, D.J. (2000) A rationalization of the acidic pH dependence for stromelysin-1 (matrix metalloproteinase-3) catalysis and inhibition. *J. Biol. Chem.* **275**, 11026–11033