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States of Tryptophyl Residues and Stability of Recombinant Human Matrix Metalloproteinase 7 (Matrilysin) as Examined by Fluorescence[1]

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States of tryptophyl residues and stability of human matrilysin were studied. The activation energy for the thermal inactivation of matrilysin was determined to be 237 kJ/mol, and 50% of the activity was lost upon incubation at 69°C for 10 min. The activity was increased by adding NaCl, and was doubled with 3 M NaCl. Denaturation of matrilysin by guanidine hydrochloride (GdnHCl) and urea was monitored by fluorescence change of tryptophyl residues. Half of the change was observed at 2.2–2.7 M GdnHCl, whereas no change was observed even with 8 M urea. Half of the inactivation was induced at 0.8 M GndHCl and at 2 M urea. The presence of an inactive intermediate with the same fluorescence spectrum as the native enzyme was suggested in the denaturation. Matrilysin contains four tryptophyls, and their states were examined by fluorescence-quenching with iodide and cesium ions and acrylamide. No tryptophyls in the native enzyme were accessible to I^- and Cs^+, and 2.4 residues were accessible to acrylamide. Based on the crystallographic study, Trp154 is water-accessible, but it should be in a crevice not to contact with I^- and Cs^+. All tryptophyls in the GdnHCl-denatured enzyme were exposed to the quenchers, while a considerable part was inaccessible in the urea-denatured one.

Key words: denaturation, fluorescence quenching, matrilysin, matrix metalloproteinase, protein stability.

Matrix metalloproteinases (MMPs) comprise a family of zinc endopeptidases that degrade extracellular matrix and basement membrane components. These enzymes are believed to take part in processes involving physiological and pathological degradations, such as development, differentiation, tissue morphogenesis, wound healing, ovulation, rheumatoid arthritis, and tumor invasion (*1*, *2*). Matrilysin (MMP-7) [EC 3.4.24.23] is the smallest MMP family member, and lacks a carboxyl-terminal hemopexin-like domain conserved in common MMPs. The molecular mass of the latent pro-form of matrilysin is 28 kDa, and that of its mature form composed of 173 amino acids is 19 kDa (*3*, *4*). Human prepro-matrilysin is composed of 267 amino acid residues, including 17 residues in the signal peptide and 77 residues in the propeptide (*3*). The pro-enzyme is activated automatically by cleavage of the pro-peptide under physiological conditions. The pro-peptide domain contains a single cysteine residue that is involved in the cysteine-switch mechanism of activation (*5*). X-ray crystallographic analysis of recombinant human matrilysin produced from CHO cells demonstrated that matrilysin is composed of five-stranded β-sheet and three α-helices, and contains a zinc ion essential for enzyme activity, as well as a 2nd zinc ion and two calcium ions that are regarded as necessary for enzyme stability (*6*). Matrilysin degrades gelatins of type I, II, IV, and V, fibronectin, proteoglycan, laminin, and type IV basement membrane collagen (*4*, *7*). Human matrilysin is over-expressed in cancer cells of various organs including prostate (*8*), colorectum (*9*), brain (*10*), and stomach (*11*), and it is thought to play important roles in tumor invasion and metastasis. The matrilysin cDNA has been expressed in COS cells (*3*), NSO mouse myeloma cells (*12*), and CHO cells (*13*). High-level expression of the human pro-matrilysin cDNA was achieved in *Escherichia coli*, although the polypeptide expressed was formed as insoluble inclusion bodies and no activity was detected. Refolding of the pro-form from inclusion bodies has been reported (*14*, *15*). Recently, we have examined the effects of a non-ionic detergent, Brij-35, and charged amino acids, especially L-arginine, on the folding and recovery of human matrilysin from the inclusion bodies, and have proposed an effective procedure to prepare active matrilysin in good yield and at reasonable cost (*16*). Preparing a large quantity of matrilysin is indispensable for studying its structure–function relationship and for the development of inhibitors that could be useful for cancer therapy. The structure–function relationship of matrilysin in solution has not been well characterized. The structure and characteristics of the recombinant matrilysin should be examined in comparison with those of matrilysin isolated from the cancer cells.

In the present paper, we describe fluorescence properties, thermal stability, stability against denaturants, and states

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Abbreviations: GdnHCl, guanidine hydrochloride; MMP, matrix metalloproteinase; MOCAc-PLG, MOCAc-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.

of tryptophyl residues of the recombinant matrilysin.

MATERIALS AND METHODS

Materials—Recombinant *E. coli* cells expressing human prepro-matrilysin bearing the signal peptide of *E. coli* alkaline phosphatase were obtained according to the method reported by Kihira *et al.* (*15*). Prepro-matrilysin was overexpressed in inclusion bodies by the recombinant *E. coli*, and was converted to an active form of matrilysin (*16*). The concentrations of pro-matrilysin and matrilysin were determined using the molar absorption coefficients at 280 nm of 41.0 and 31.8 mM^{-1} cm^{-1}, respectively, calculated from amino acid compositions (*3*, *15*, *16*). Absorption was measured with a Shimadzu UV-2200 spectrophotometer (Kyoto).

Azocoll substrate (<50 mesh, Lot B10515), insoluble particles of collagen to which a bright-red azo-dye is attached, was purchased from Calbiochem (La Jolla, CA). (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] and MOCAc-L-Pro-L-Leu-Gly (MOCAc-PLG, Lot 471218) were purchased from the Peptide Institute (Osaka). The concentrations of MOCAc-PLGL(Dpa)AR and MOCAc-PLG were determined spectrophotometrically using the molar absorption coefficients ε_{410} = 7.5 mM^{-1} cm^{-1} and ε_{242} = 12.9 mM^{-1} cm^{-1} (*17*). All other reagents were of reagent grade and obtained from Nacalai Tesque (Kyoto).

Assays of Matrilysin—The collagenase activity of matrilysin was measured with azocoll (*18*, *19*). Azocoll (3.0 mg) was suspended in 2.0 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$ and 0.2 M NaCl (buffer A) and incubated at 37°C for 15 min before the hydrolysis. The matrilysin solution (2.0 ml, 0.17–0.50 μM in buffer A) was added to the azocoll suspension at 37°C, and the enzyme reaction was stopped at an appropriate time by removing insoluble azocoll by filtration with a Whatman paper filter. The hydrolysis at each azocoll concentration was performed in triplicate and monitored by measuring absorbance at 520 nm. The peptidase activity was measured with MOCAc-PLGL(Dpa)AR (*17*). It is known that the peptide bond between Gly and Leu residues is cleaved, and the amount of the product MOCAc-PLG was measured by the fluorescence intensity of the authentic MOCAc-PLG solution. The substrate was dissolved in dimethyl sulfoxide (DMSO) to 0.290 mM. The matrilysin-catalyzed hydrolysis of the substrate was performed by mixing 1,222 μl of buffer A containing 0.02% NaN_3 (buffer B), 8 μl of the substrate solution, and 20 μl of the matrilysin solution at 37°C. Concentrations of matrilysin, the substrate, and DMSO in the final conditions were 4.3 nM, 1.85 μM, and 0.6%, respectively. Hydrolysis of the substrate was measured by following the increase in the fluorescence intensity at 393 nm upon excitation at 328 nm with a JASCO FP-777 spectrofluorometer (Tokyo). The hydrolysis was carried out under pseudo-first order conditions, where the substrate concentration is lower than the Michaelis constant, K_m, because of the sparing solubility of the substrate and the large fluorescent intensity of the product. Thus, the enzyme activity was evaluated by the specificity constant, k_{cat}/K_m (*20*).

Thermal Inactivation of Matrilysin—Matrilysin (0.34 μM) was incubated in buffer A for a specified period at a temperature of 40–78°C. The solution was then kept for 3 min at 37°C, and the enzyme activity was measured with azocoll (*21*). The final concentration of azocoll in the reaction mixture was 1.5 mg/ml, and that of matrilysin was 0.17 μM. The apparent first-order rate constant of the thermal inactivation was determined by semi-log plots of the remaining activity against the incubation time at a specified temperature.

Effect of NaCl on the Matrilysin Activity—The matrilysin activity for azocoll hydrolysis was examined under the conditions described above, but instead of buffer A, 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$ (buffer C) was used in the presence of 0–4.0 M NaCl (*20–23*).

Denaturation of Matrilysin with Guanidine Hydrochloride (GdnHCl) and Urea—Equilibrium unfolding measurements were performed using stock solutions of GdnHCl or urea, both of ultrapure grade, purchased from ICN Biochemicals (Cleveland, OH) (*24*). One milliliter of 1.08 μM matrilysin was incubated with 3 ml of various concentrations of GdnHCl or urea in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$, 0.2 M NaCl, 0.02% NaN_3 (buffer B) at 25°C for 2 h, and fluorescence spectra were measured with excitation at 295 nm. The MOCAc-PLGL(Dpa)AR hydrolysis by the matrilysin treated with the denaturants was measured as described above.

The equilibrium unfolding experiments of matrilysin with GdnHCl, each repeated at least three times, were interpreted according to the simple two-state model:

$$N \longrightarrow U \tag{1}$$

where N and U represent the native and unfolded matrilysin species, respectively. The free energy values at each GdnHCl concentration were evaluated by:

$$\Delta G^0 = -RT\ln K \tag{2}$$

where the equilibrium constant K is calculated at each GdnHCl concentration from the ratio of the unfolded and folded fractional species, *i.e.* $K = f_u/f_n$, calculated in the denaturation experiments. In order to evaluate the total free energy change ($\Delta G^0_{H_2O}$) of unfolding, a linear fit of ΔG^0 was performed (*25*):

$$\Delta G^0 = \Delta G^0_{H_2O} - m[\text{GdnHCl}] \tag{3}$$

Fluorescence Quenching—Intrinsic fluorescence emission of tryptophyl residues in a protein provides a sensitive probe of structural properties of the protein microenvironment (*26*). Quenching of protein intrinsic fluorescence can be used to probe the relative exposure of fluorescent residues in proteins under a specified set of conditions. Quenching agents function through collision with the fluorophore, such that a change in quenching properties is reflective of a change in accessibility to the quenching agent. Fluorescence quenching of matrilysin or a tryptophan model compound, *N*-acetyl-tryptophan ethyl ester (Ac-Trp-OEt), was measured by addition of potassium iodide, cesium chloride, or acrylamide. Iodide and cesium ions are large polar anions and cations, respectively, with access to fluorophores (tryptophyl and tyrosyl residues) on the protein surface, whereas acrylamide is a polar nonionic quencher and can penetrate the protein surface (*27*). When an excited molecule is quenched on collision with a quencher, the fluorescence intensity might be expressed by the Stern-Volmer equation (*28*):

$$F_0/F = 1 + K_q[Q] \quad (4)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, Q, respectively. [Q] is the quencher concentration, and K_q is the quenching constant, which corresponds to the binding constant between the fluorophore and quencher. The accessibility of tryptophyl residues of matrilysin to the quencher was estimated by the ratio of the K_q value obtained with matrilysin to that obtained with the model compound. The fluorescence quantum yield of tryptophyl residues was determined according to the method of Parker and Rees by using L-tryptophan as the standard (*29*).

Steady-state fluorescence measurements were performed at 25°C with a JASCO FP-777 spectrofluorometer. An excitation wavelength of 295 nm was used to minimize interference from tyrosyl residues. The final concentrations of matrilysin and Ac-Trp-OEt were 2.0 and 8.0 μM, respectively, in buffer C containing acrylamide (0–67 mM), KI (0–0.67 M), and CsCl (0–0.67 M). The KI stock solution contained 0.1 M $Na_2S_2O_3$ to prevent formation of I_3^-, which absorbs at 290 nm. Excitation of fluorophores at 295 nm also prevents absorbance of exciting light by iodide (*28*). In the experiments with acrylamide, fluorescence intensities were corrected for the attenuation of the exciting light intensity due to absorption by acrylamide (*26*). With KI, CsCl, and acrylamide, no large change in the wavelength of maximal fluorescence was observed at any quencher concentration examined, indicating the absence of protein denaturation (*30*). The quenching experiments were also performed in the presence of 6 M GdnHCl or 8 M urea with matrilysin treated with 6 M GdnHCl or 8 M urea for 2 h at 25°C.

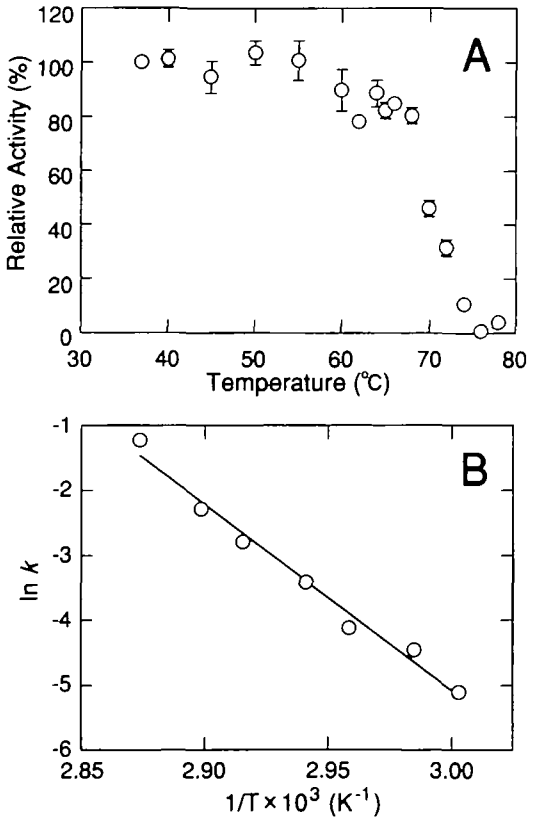

Fig. 1. **Effect of temperature on the stability of matrilysin.** Matrilysin was incubated in 50 mM Tris-HCl (pH 7.5) plus 10 mM $CaCl_2$ and 0.2 M NaCl (buffer A) for the specified time at the temperature indicated. Then it was incubated at 37°C for 3 min, and the collagenase activity was measured. The initial concentrations of matrilysin and azocoll in the reaction mixture were 0.17 μM and 1.5 mg/ml, respectively. (A) Thermal inactivation of matrilysin. Matrilysin was treated at the indicated temperature for 10 min. The activity (11 μg-azocoll ml^{-1} min^{-1}) of the native enzyme was taken as a relative activity of 100%. (B) Arrhenius plots of the apparent first-order rate constant, k, for thermal inactivation of matrilysin. The k (min^{-1}) value was determined from semi-log plots of the activity against the incubation time at the temperature indicated.

RESULTS

Thermal Stability of Matrilysin—Figure 1A shows thermal stability of matrilysin as measured by the azocoll-hydrolysing activity. Upon incubation at temperatures ranging from 37 to 55°C, the relative activity remained constant at 100%. This decreased to zero as the incubation temperature was raised to 76°C. The temperature, T_{50}, giving 50% inactivation in a 10-min incubation was 69°C. Similarly, T_{50} for a 30-min incubation was 65.5°C. Thermal inactivation of 0.25 μM matrilysin was observed in the temperature range of 60–75°C. The inactivation followed pseudo-first order kinetics (data not shown), and the first-order rate constant, k, increased with increasing temperature: 6.02×10^{-3} min^{-1} at 60°C, 1.63×10^{-2} min^{-1} at 65°C, 6.07×10^{-2} min^{-1} at 70°C, and 0.294 min^{-1} at 75°C. The activation energy for the thermal inactivation was determined to be (237 ± 9) kJ/mol by Arrhenius plots (Fig. 1B).

Effect of NaCl on the Azocoll Hydrolysis by Matrilysin—The azocoll-hydrolysing activity of matrilysin was 11 μg-azocoll ml^{-1} min^{-1} (100% relative activity) in buffer A at 37°C. Upon addition of 0.2–0.5 M NaCl, the relative activity decreased to 40–50%, but with increasing [NaCl] from 0.5 to 3 M, it increased to 200%. The relative activity decreased again with increasing [NaCl] from 3 to 4 M, and the activity at 4 M NaCl was almost the same as that in the absence of NaCl (Fig. 2). The Michaelis constant (K_m) and catalytic

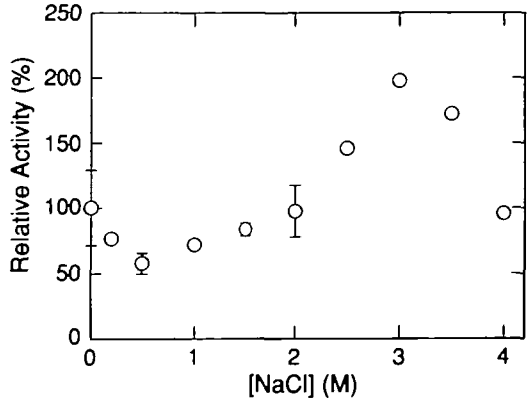

Fig. 2. **Effect of NaCl on the matrilysin-catalyzed hydrolysis of azocoll.** The activity of matrilysin was determined in 50 mM Tris-HCl (pH 7.5) plus 10 mM $CaCl_2$ (buffer C) with [NaCl] indicated at 37°C. The initial concentrations of matrilysin and azocoll in the reaction mixture were 0.17 μM and 1.5 mg/ml, respectively. The activity (14 μg ml^{-1} min^{-1}) obtained at 0 M NaCl was taken as a relative activity of 100%.

constant (k_{cat}) in the presence of NaCl was evaluated (Table I). With an increase in NaCl from 0.5 to 3 M, the K_m and k_{cat} values increased and the increment of k_{cat} was larger than that of K_m. At 4 M NaCl, the k_{cat}/K_m value was determined to be 6×10^4 M^{-1} min^{-1}, but the K_m value was not determined over the range of substrate concentration examined (0–6 mg/ml), and thus it was estimated to be >6 mg/ml. The k_{cat} value was thus estimated to be $>3.6 \times 10^5$ mg/ml min^{-1} M^{-1}.

Fluorescence Spectra of Matrilysin—Matrilysin contains four tryptophyl, eight tyrosyl, and nine phenylalanyl residues. Fluorescence spectra of matrilysin showed that the excitation and emission maxima were at 280 and 330 nm, respectively, in buffer A at 25°C. The emission spectra were also measured with excitation at 295 nm in order to observe the fluorescence spectra derived solely from tryptophyls with the emission maximum at 325 nm (Fig. 3). The quantum yield of four tryptophyl residues was estimated to be 0.52 per tryptophyl residue, which is 2.5 times larger than that (0.20) of free tryptophan, suggesting that the residues are located in hydrophobic and non-polar environments in matrilysin. The fluorescence spectrum of matrilysin treated with 6 M GdnHCl for 2 h at 25°C showed a considerably weaker intensity than the native enzyme, and the wavelength of the spectrum maximum was shifted to 355 nm. The quantum yield per tryptophyl residue was 0.20, the same as that of free tryptophan. The four tryptophyls in the denatured enzyme were suggested to be in polar environments.

TABLE I. **Effect of NaCl on the matrilysin-catalyzed hydrolysis of azocoll.** The activity of matrilysin was determined in 50 mM Tris-HCl (pH 7.5) plus 10 mM $CaCl_2$ at 37°C. The initial concentrations of matrilysin and azocoll in the reaction mixture were 0.17 μM and 1.5 mg/ml, respectively.

NaCl (M)	K_m (mg ml^{-1})	$k_{cat} \times 10^{-5}$ (mg ml^{-1} M^{-1} min^{-1})	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})
0	3.7 ± 0.3	1.7 ± 0.2	4.7 ± 0.5
0.5	5.5 ± 0.5	1.7 ± 0.3	3.0 ± 0.4
1.0	4.7 ± 0.4	1.8 ± 0.3	3.8 ± 0.5
2.0	5.0 ± 0.5	2.8 ± 0.4	5.7 ± 0.5
3.0	5.9 ± 0.5	5.1 ± 0.4	8.6 ± 0.7
4.0	> 6	> 3.6	6.0 ± 0.6

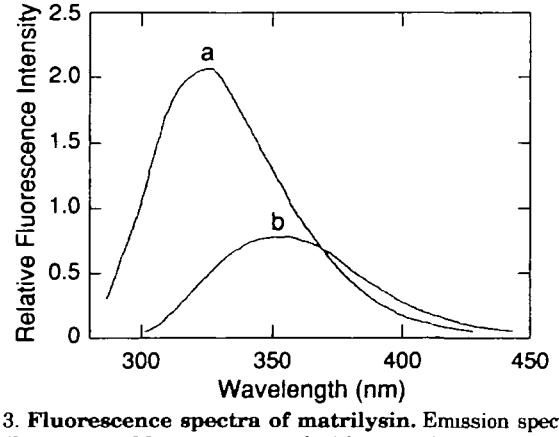

Fig. 3. **Fluorescence spectra of matrilysin.** Emission spectra of matrilysin (0.27 μM) were measured with excitation at 295 nm in 50 mM Tris-HCl (pH 7.5) plus 10 mM $CaCl_2$, 0.2 M NaCl, and 0.02% NaN_3 (buffer B) at 25°C. a. native matrilysin; b: matrilysin treated with 6 M GdnHCl for 2 h at 25°C.

Denaturation of Matrilysin with GdnHCl and Urea—The emission intensity and the wavelength of the emission maximum of the fluorescence spectrum of matrilysin observed by excitation at 295 nm changed depending on the GdnHCl concentration used for the denaturation of matrilysin, and the denaturation process was separated into three distinct phases (Fig. 4A). The relative value of the intensity increased from 100 to 125% with increasing [GdnHCl] from 0 to 1.5 M (phase I); it sharply decreased to 30% when [GdnHCl] increased from 1.5 to 3.5 M (phase II); and it increased slightly from 30 to 40% with an increase of [GdnHCl] from 3.5 to 6 M (phase III). The fluorescence intensity of free tryptophan increased linearly from 20 to 40% when [GdnHCl] increased from 0 to 6 M. This increase may be derived from a perturbation effect on the solution properties due to the high added concentration of GdnHCl. The increase in the fluorescence intensity from 100 to 125% observed in phase I may be the result of more hydrophobic states of the tryptophyl residues, due to the perturbation of the water structure and/or the conformational change of the enzyme induced by GdnHCl. Half of the total change in the fluorescence intensity was attained at 2.2 M GdnHCl.

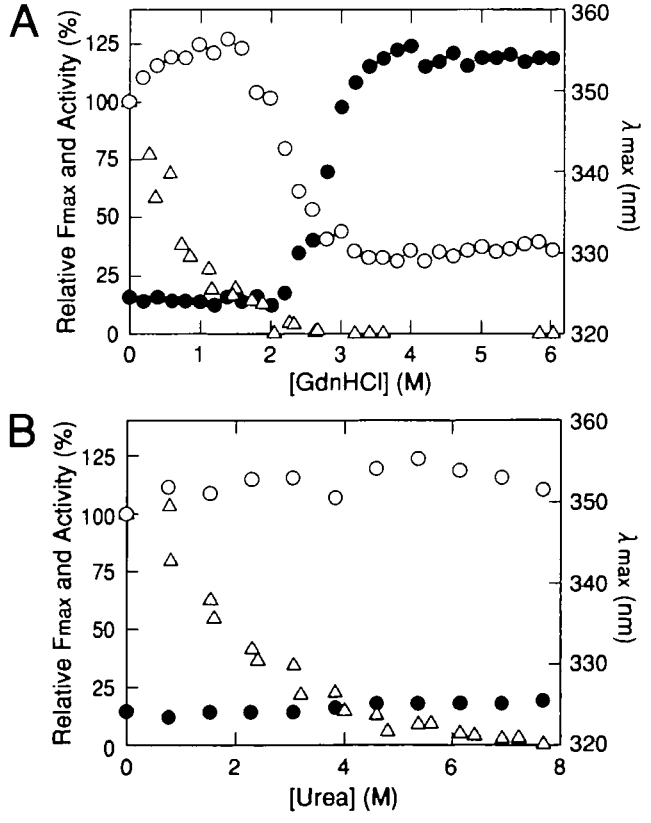

Fig. 4. **Denaturation of matrilysin by GdnHCl and urea.** Matrilysin (0.27 μM) was treated with GdnHCl or urea as described in the legend of Fig. 3, and the fluorescence was measured with excitation at 295 nm. A portion of the matrilysin solution was mixed with the MOCAc-PLGL(Dpa)AR solution containing the same concentration of the denaturant. The concentrations of matrilysin and the substrate were 4.3 nM and 1.85 μM, respectively. The k_{cat}/K_m value of matrilysin was 0.13 μM^{-1} s^{-1}, and this was taken as a relative activity of 100%. ○, the maximal fluorescence intensity (F_{max}); ●, the wavelength (λ_{max}) giving F_{max}, and △, the relative activity of matrilysin. Denaturants: A, GdnHCl; and B, urea.

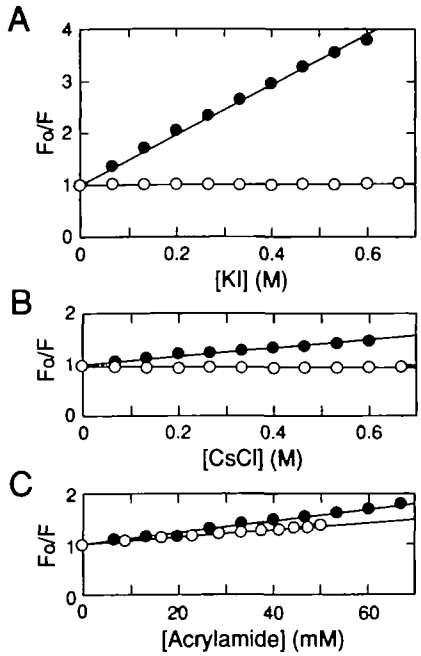

Fig. 5. **Stern-Volmer plots for the fluorescence-quenching.** Emission spectra of 2.0 μM matrilysin (○) and 8.0 μM Ac-Trp-OEt (●) were measured in the presence of various concentrations of quenchers in buffer C (pH 7.5) at 25°C. Quenchers: A, KI; B, CsCl; and C, acrylamide.

The emission maximum was at 325 nm in phase I, and shifted to 355 nm in phase II. It stayed at 355 nm in phase III, and half of the total change in the wavelength shift was observed at 2.7 M GdnHCl. The relative activity of matrilysin decreased with increasing [GdnHCl]. Half of the activity observed in the absence of GdnHCl was lost at 0.8 M GdnHCl, and the activity was completely lost at 3.2 M GdnHCl. It is noted that 80% of the activity was lost in phase I, and the remaining 20% was lost in phase II. Matrilysin may be in a stable state but with 20% activity at 1.5 M GdnHCl, and in partially and fully denatured states in phases II and III, respectively.

The denaturation of matrilysin induced by GdnHCl showed a sigmoid behavior with a slight fluorescence increase observed in phase I. Thus, a two-state unfolding model (Eq. 1) appears to be applicable to analyze the experimental data, assuming that a simple equilibrium is established between the folded and unfolded species. According to Eq. 3, the GdnHCl-dependence of the matrilysin stability (the m value) and $\Delta G^0_{H_2O}$ values were determined to be (7.3 ± 0.4) kJ mol^{-1} M^{-1} and (15.6 ± 0.9) kJ mol^{-1}, respectively.

When matrilysin was treated with [GdnHCl] < 1.5 M for 2 h, and then the solution was diluted to 20 volumes with buffer B, the activity and the fluorescence spectrum recovered immediately to those of the native matrilysin. However, those of matrilysin treated with [GdnHCl] > 2 M recovered only incompletely. It is plausible that the zinc ion essential for the activity may be retained in the polypeptide at [GdnHCl] < 1.5 M, but removed from it at [GdnHCl] > 2 M.

The effect of urea on denaturation of matrilysin was also examined (Fig. 4B). The maximal intensity of the fluorescence emission spectrum and the wavelength of the maximal intensity were constant at urea concentrations up to 8 M. The activity, on the other hand, decreased with increasing urea concentration. At 2.0 M urea, the activity was half of that observed in the absence of urea, and the enzyme was fully inactivated at 7.9 M urea.

TABLE II. **Quenching constants (K_q) of tryptophan fluorescence of matrilysin.**

	K_q[b] (M^{-1})		
	KI	CsCl	Acrylamide
Ac-Trp-OEt	4.6 ± 0.2	0.77 ± 0.02	11.8 ± 0.5
Native matrilysin	0.0 (0%)[a]	0.0 (0%)	7.2 ± 0.3 (61%)
Matrilysin treated with 6 M GdnHCl	3.9 ± 0.2 (84%)	0.78 ± 0.03 (102%)	11.6 ± 0.7 (98%)
Matrilysin treated with 8 M urea	2.3 ± 0.2 (51%)	0.61 ± 0.04 (79%)	9.0 ± 0.5 (77%)

[a] The value in parenthesis is obtained by dividing the K_q value for matrilysin by that for Ac-Trp-OEt. It represents the relative accessibility of the tryptophyls in matrilysin to the quencher, assuming that the tryptophyl residue in Ac-Trp-OEt is fully accessible.
[b] The K_q values were estimated by the Stern-Volmer plot (Eq. 4).

Quenching of the Tryptophan Fluorescence of Matrilysin—The fluorescence intensity of a tryptophyl model compound, Ac-Trp-OEt, decreased depending on the added concentration of a quencher, KI, CsCl, or acrylamide, and the fluorescence change was plotted according to the Stern-Volmer equation (Fig. 5). The plots were linear over the concentration ranges of quenchers examined, and the quenching constant, K_q, was determined from the slope of the plots (Table II). Judging from the K_q values, the binding affinity of the quenchers to Ac-Trp-OEt was in the order: acrylamide > I^- > Cs^+. On the other hand, the effect of the quenchers on the matrilysin fluorescence was considerably different from that on Ac-Trp-OEt. No quenching was observed with KI or CsCl; and with acrylamide, the degree of the quenching was 61% of that observed with Ac-Trp-OEt. This result suggests that all tryptophyl residues of matrilysin are buried in states that do not allow contact with I^- and Cs^+, but 2.4 residues are accessible to acrylamide. Acrylamide may penetrate the surface of matrilysin (*27*). Charged groups may lie around the acrylamide-accessible tryptophyls so that I^- and Cs^+ are unable to make contact with them.

States of Tryptophyl Residues in Matrilysin Treated with Denaturants—Matrilysin was treated with 6 M GdnHCl or 8 M urea for 2 h at 25°C, and the fluorescence quenching in the denatured matrilysin was examined (Fig. 5 and Table II). Following treatment with GdnHCl, the four tryptophyl residues of matrilysin, which are totally inaccessible to I^- and Cs^+ in the native enzyme, became fully accessible to the quenchers. A considerable fraction of the tryptophyls were, however, still inaccessible to the quenchers in the urea-denatured matrilysin, suggesting that matrilysin is not in the fully unfolded state after treatment with 8 M urea for 2 h.

DISCUSSION

It has been shown that the collagenase activity of matrilysin is enhanced by the addition of NaCl (Fig. 2). This halophilic behavior is similar to that reported with a well-studied metalloproteinase, thermolysin (*20–23*). The activa-

tion of thermolysin is brought about solely by increase in k_{cat} (*20*, *22*). On the other hand, the activity of matrilysin is dependent on the changes in K_m and k_{cat} in a complex manner (Fig. 2 and Table I). This complex manner might be derived in part from the use of a proteinous substrate, azocoll. An increase in K_m with increasing [NaCl] from 0 to 1 M can be due to neutralization of electrostatic interactions between the substrate and matrilysin. The molecular activity increases with an increase in [NaCl] from 0 to 3 M, but it is decreased at 4 M NaCl, probably due to denaturation of the enzyme. The salt effect on matrilysin should be examined more precisely with synthetic substrates and various salts, as has been done with thermolysin. The halophilic properties would provide a clue to examine the molecular aspects of matrilysin. Based on the halostability of matrilysin, the fluorescence quenching experiments with KI and CsCl were performed. The tryptophyls of matrilysin must be in highly hydrophobic and non-polar environments (Fig. 3). The emission maximal wavelength (325 nm) and quantum yield (0.52) suggest that the state of tryptophyls might be close to that in *p*-dioxane, the dielectric constant of which is 2–3 (Inouye, K., unpublished data). This result seems to be consistent with the evidence that no tryptophyls are accessible to I^- and Cs^+. Their water-accessibility was evaluated by X-ray crystallographic analysis (*6*). Four sets of structural data on matrilysin, determined using matrilysin complexed with three different inhibitors, are available. Their relative water-accessibility was determined by comparing their water-accessibility to that of a tripeptide, Ala-Trp-Ala. The relative water-accessibility of Trp-109, Trp141, Trp154, and Trp203 was (12.2 ± 1.0)%, (0.5 ± 0.1)%, (75.5 ± 8.3)%, and (4.7 ± 0.5)%, respectively, indicating that almost one out of four tryptophyls is accessible to water of radius 1.4 Å. In particular, Trp154 has fairly high water-accessibility based on the crystallographic data, although the fluorescence spectrum (Fig. 3) and the fluorescence-quenching with I^- and Cs^+ (Fig. 5 and Table II) suggest that all of the tryptophyls are buried or located in a crevice. This discrepancy may suggest that Trp154 is in a strained state in solution in comparison with the state in the crystal. The binding of the inhibitor to matrilysin may cause Trp154 to move to a more water-accessible position. We are currently investigating the states of tryptophyls in matrilysin complexed with inhibitors. It has been pointed out that acrylamide penetrates the protein surface because of its polarity (*27*). In this study, 2.4 tryptophyl residues were shown to be accessible to acrylamide, unlike I^- and Cs^+, but this value could be overestimated as the number of solvent-accessible residues.

Inactivation of matrilysin with GdnHCl, especially from 20 to 0%, was accompanied with a drastic change in fluorescence (phase II), whereas the enzyme was inactivated by urea without a change in the fluorescence spectrum (Fig. 4). This suggests that the four tryptophyls are totally in polar environments in the matrilysin inactivated with 4 M GdnHCl, but still in non-polar environments in matrilysin inactivated with 8 M urea. By denaturation of matrilysin with 6 M GdnHCl, the four tryptophyl residues, which are totally inaccessible to I^- and Cs^+ in the native enzyme, became fully accessible to the quenchers. A considerable fraction of the tryptophyls was, however, still inaccessible to the quenchers in the urea-denatured matrilysin, suggesting that matrilysin is not in a fully unfolded state after treatment with 8 M urea for 2 h (Table II). This accords with the finding that the fluorescence spectrum of the urea-denatured matrilysin is the same as that of the native one (Fig. 4B). These lines of evidence strongly suggest that the denaturation process by GdnHCl and urea involves an inactive intermediate with the same fluorescence spectrum as the native enzyme but higher accessibility to the quenchers. The intermediate may appear at [GdnHCl] < 1.5 M or [urea] = 1–8 M. It is noteworthy that matrilysin is in the intermediate form at 8 M urea, without the activity but with a similar structure to the native enzyme. The denaturation model of matrilysin should be improved by considering the presence of the intermediate (*I*) as below:

$$N \longrightarrow I \longrightarrow U \qquad (5)$$

The states of tryptophyl residues could be a suitable probe for investigating local conformation changes and interactions between functional residues.

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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. Van Wart, H.E. and Birkedal-Hansen, H. (1990) The cysteine switch: A principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. USA* **87**, 5578–5582
6. Browner, M.F., Smith, W.W., and Castelhano, A.L. (1995) Matrilysin-inhibitor complexes: Common themes among metalloproteinases. *Biochemistry* **34**, 6602–6610
7. 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
8. Pajouh, M.S., Nagle, R.B., Brethnach, R., Finch, J.S., Brawer, M.K., and Bawden, G.T. (1991) Expression of metalloproteinase genes in human prostate cancer. *J. Cancer Res. Clin. Oncol.* **117**, 114–150
9. 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
10. 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
11. 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 gastrix and esophageal cancers. *Int. J. Oncol.* **13**, 1031–1035
12. Murphy, G., Cockett, M.I., Ward, R.V., and Docherty, A.J.P. (1991) Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. *Biochem. J.* **277**, 277–279
13. Barnett, J., Straub, K., Nguyen, B., Chow, J., Suttman, R., Thompson, K., Tsing, S., Benton, P., Schatzman, R., and Chen,

M. (1994) Production, purification, and characterization of human matrilysin (PUMP) from recombinant Chinese hamster ovary cells. *Protein Exp. Purif.* **5**, 27–36
14. Ye, Q.Z., Johnson, L.L., and Baragi, V. (1992) Gene synthesis and expression in *E. coli* for pump, a human matrix metalloproteinase. *Biochem. Biophys. Res. Commun.* **186**, 143–149
15. Kihira, Y., Mori, K., Miyazaki, K., and Matsuo, Y. (1996) Production of recombinant human matrix metalloproteinase 7 (matrilysin) with potential role in tumor invasion by refolding from *Escherichia coli* inclusion bodies and development of sandwich ELISA of MMP-7. *Urol. Oncol.* **2**, 20–26
16. 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
17. Knight, C.G., Willenbock, F., and Murphy, G. (1992) A novel coumarin-labelled peptide for sensitive continuous assays of matrix metalloproteinases. *FEBS Lett.* **296**, 263–266
18. Chavira, R., Jr., Burnett, T.J., and Hageman, J.H. (1984) Assaying proteinases with azocoll. *Anal. Biochem.* **136**, 446–450
19. Woessner, J.F., Jr. (1995) Matrilysin in *Methods in Enzymology* (Barrett, A.J., ed.) Vol. 248, pp. 485–495, Academic Press, San Diego, CA
20. Inouye, K., Lee, S.-B., and Tonomura, B. (1996) Effect of amino acid residues at the cleavable site of substrates on the remarkable activation of thermolysin by salts. *Biochem. J.* **315**, 133–138
21. Inouye, K., Kuzuya, K., and Tonomura, B. (1998) Sodium chloride enhances markedly the thermal stability of thermolysin as well as its catalytic activity. *Biochim. Biophys. Acta* **1388**, 209–214
22. Inouye, K. (1992) Effects of salts on thermolysin: Activation of hydrolysis and synthesis of *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester, and a unique change in the absorption spectrum of thermolysin. *J. Biochem.* **112**, 335–340
23. 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
24. Tanaka, A., Okuda, K., Senoo, K., Obata, H., and Inouye, K. (1999) Guanidine hydrochloride-induced denaturation of *Pseudomonas cepacia* lipase. *J. Biochem.* **126**, 382–386
25. Pace, C.N., Shirley, B.A., and Thompson, J.A. (1989) Measuring the conformational stability of a protein in *Protein Structure, a Practical Approach* (Creighton, T.E., ed.) pp. 311–330, IRL Press, New York, NY
26. Eftink, M.R. and Ghiron, C.A. (1981) Fluorescence quenching studies with proteins. *Anal. Biochem.* **114**, 199–227
27. Stack, M.S., Itoh, Y., Young, T.N., and Nagase, H. (1996) Fluorescence quenching studies of matrix metalloproteinases (MMPs): Evidence for structural rearrangement of the pro-MMP-2/TIMP-2 complex upon mercurial activation. *Arch. Biochem. Biophys.* **333**, 163–169
28. Lehrer, S.S. (1971) Solute perturbation of protein fluorescence. The quenching of the tryptophan fluorescence of model compounds and lysozyme by iodide ion. *Biochemistry* **10**, 3254–3263
29. Parker, C.A. and Rees, W.T. (1962) Fluorescence spectrometry. *Analyst* **87**, 83–111
30. Burnstein, E.A., Vedenkina, N.S., and Ivkova, M.N. (1973) Fluorescence and the location of tryptophan residues in protein molecules. *Photochem. Photobiol.* **18**, 263–279