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Thermodynamic analysis of ionizable groups involved in the catalytic mechanism of human matrix metalloproteinase 7 (MMP-7)

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Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropane sulfonic acid; DMSO, dimethyl sulfoxide; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; $K_c$, proton dissociation constant; 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-diaminopropionyl]-L-Ala-L-Arg-NH$_2$.

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Keywords: ionizable group; matrix metalloproteinase; MMP-7; proton dissociation constant; thermodynamic analysis.
Abstract

Human matrix metalloproteinase 7 (MMP-7) exhibits a broad bell-shaped pH-dependence with the acidic and alkaline pKₐ (pKₑ₁ and pKₑ₂) values of about 4 and 10. In this study, we estimated the ionizable groups involved in its catalytic mechanism by thermodynamic analysis. pKₐ of side chains of L-Asp, L-Glu, L-His, L-Cys, L-Tyr, L-Lys, and L-Arg at 25-45°C were determined by the pH titration of amino-acid solutions, from which their enthalpy changes, ΔH°, of deprotonation were calculated. pKₑ₁ and pKₑ₂ of MMP-7 at 15-45°C were determined in the hydrolysis of (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N³-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH₂, from which ΔH° for pKₑ₁ and pKₑ₂ were calculated. The ΔH° for pKₑ₁ (-20.6 ± 6.1 kJ mol⁻¹) was similar to that for L-Glu (-23.6 ± 5.8 kJ mol⁻¹), and the ΔH° for pKₑ₂ (89.9 ± 4.0 kJ mol⁻¹) was similar to those for L-Arg (87.6 ± 5.5 kJ mol⁻¹) and L-Lys (70.4 ± 4.4 kJ mol⁻¹). The mutation of the active-site residue Glu198 into Ala completely abolished the activity, suggesting that Glu198 is the ionizable group for pKₑ₁. On the other hand, no arginine or lysine residues are found in the active site of MMP-7. We proposed a possibility that a protein-bound water is the ionizable group for pKₑ₂.
1. Introduction

Human matrix metalloproteinase 7 (MMP-7, Matrilysin) [EC 3.4.24.23] is the smallest matrix metalloproteinase (MMP), lacking a carboxyl terminal hemopexin-like domain conserved in common MMPs. It is believed to play an important role in tumor invasion and metastasis [1, 2]. The molecular mass of the latent pro-form is 28 kDa, and that of its mature form is 19 kDa [3]. MMP-7 is composed of a five-stranded β-sheet and three α-helices, and contains a zinc ion essential for activity and other zinc and calcium ions that are considered necessary for stability [4]. Like all other MMPs, it has the consensus sequence HEXXHXXGXXH, in which three histidine residues chelate a catalytic zinc ion, and a methionine-containing turn (Met-turn). Hence, it is grouped in clan MA(M) [5]. In recent years, target molecules through which MMP-7 exerts biological functions have become apparent, such as heparin [6], heparan sulfate [6], cholesterol sulfate [7-9], and ErbB4 receptor [10]. The inhibitions of MMP-7 activity by natural compounds [11,12], synthetic compounds [13], and detergents [14] were reported.

Generally, ionizable groups involved in the catalytic mechanisms of enzyme are estimated from the three-dimensional structure and pKₐ values. Figure 1A shows the structure of MMP-7-hydroxamate inhibitor complex [4]. In this study, the numbering of amino acid residues of pro-MMP-7 is according to the previous report [15], in which the mature MMP-7 begins at Tyr78. MMP-7 has three α helices and five β strands [4]. Tyr193 and Glu198 are located at the second α-helix (Leu192-Leu203), while Tyr216 and Tyr219 are located at the Met-turn (Pro211-Gly222). Sequence comparison of MMP-1 [16], MMP-2 [17], MMP-3 [18], MMP-7 [19], MMP-8 [20], MMP-9 [21],
MMP-10 [19], MMP-11 [22], MMP-12 [21], MMP-13 [21], and MMP-14 [23] revealed that Glu198 and Tyr219 are conserved in all MMPs, while Tyr216 is conserved in several MMPs, and Tyr193 is unique to MMP-7 [24]. Tyr219 and Tyr216 form the S1’ subsite. MMP-7 exhibits a broad bell-shaped pH-dependence with the acidic and alkaline $pK_a$ ($pK_{e1}$ and $pK_{e2}$) values of about 4 and 10 [13,25]. As a result, three ionization forms of MMP-7 and MMP-7-substrate complex are considered, respectively (Fig. 2). Glu198 and Tyr219 are believed to be the ionizable groups responsible for $pK_{e1}$ and $pK_{e2}$, respectively. However, Cha et al. proposed that the zinc-bound water might be the ionizable group responsible for $pK_{e1}$ [26]. We found that the MMP-7 whose tyrosyl residues were nitrated with tetranitromethane retained activity [27]. In addition, we recently demonstrated that all Tyr219 variants retained activity [28]. These results indicate that Tyr219 is not critical for catalytic activity. We also demonstrated that Tyr193 and Tyr216 variants retained activity [28].

One of the critical problems with using $pK_a$ values of free amino acids for estimation of charge states of amino acid residues is that little water (and hence protons) is available for the residues buried in a protein core, while large amounts of water (55 M water) is available for the residues facing bulk solution. In 1930-1960, enthalpy changes, $\Delta H^o$, of deprotonation of side chains of amino acid residues were determined by the measurement of pH- and temperature-dependences of electromotive force of the battery containing amino acids, dipeptides, or tripeptides in a cell [29-33]. It was demonstrated that in dipeptides and tripeptides, $\Delta H^o$ values of side chains of amino acid residues are not affected by the amino acid residues in their neighborhoods and are almost equal to the $\Delta H^o$ values of side chains of free amino acids [29-33]. Therefore, $\Delta H^o$ values can be used as a clue to estimate the ionizable groups in the catalytic mechanism of enzymes.
[29,34-36]. However, compared with the estimation of the ionizable groups with $pK_e$ values, the estimation with $\Delta H^\circ$ values is not commonly used. This might be due to that reliable $\Delta H^\circ$ values of side chains of amino acid residues have not been available. In this study, we determined the $\Delta H^\circ$ of side chains of amino acid residues by the pH-titration of amino-acid solutions and used them to estimate the ionizable groups involved in the catalytic mechanism of MMP-7.

2. Materials and methods

2.1. Materials

(7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N$^3$-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH$_2$ [MOCAc-PLGL(Dpa)AR] (Lot 491214, molecular mass 1093.2 Da) [37] and (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly (MOCAc-PLG) (Lot 510913, molecular mass 501.54 Da) were purchased from the Peptide Institute (Osaka, Japan). Their concentrations were determined by the denoted weight and the molecule weight. 3-[(1,1-Dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropane sulfonic acid (AMPSO, Lot 9355C, molecular mass 227.3 Da) and L-Glu (Lot TLE5153) were from Wako Pure Chemical (Osaka). L-Asp (Lot 115H0563) and N$^\alpha$-acetyl-L-Lys (Lot A1020) were from Sigma (St. Louis, MO, USA). L-His (Lot M2N7937), L-Cys (Lot M7H2081), L-Tyr, (Lot M7K3391), and L-Arg (Lot M7K6540) were from Nacalai Tesque (Kyoto, Japan). All other chemicals were from Nacalai Tesque.
2.2. Expression and purification of MMP-7

Expression in *Escherichia coli* and purification of recombinant MMP-7 were carried out, as described previously [31,39]. Briefly, mature MMP-7 (Met77-Lys250) was expressed in BL21(DE3) cells in the forms of inclusion bodies, solubilized with 6 M guanidine HCl, refolded with 1 M L-arginine, and purified by sequential ammonium sulfate precipitation and heparin affinity column-chromatography procedures of the refolded products. The concentration of MMP-7 was determined spectrophotometrically using the molar absorption coefficient at 280 nm, $\varepsilon_{280}$, of 31,800 M$^{-1}$ cm$^{-1}$ [38]. Site-directed mutagenesis was carried out using Quikchange$^\text{TM}$ site-directed mutagenesis kit (Stratagene, La Jolla, CA) for construction of E198A, A162G, and P217G. The nucleotide sequences of mutated MMP-7 genes were verified by a Shimadzu DNA sequencer DSQ-2000 (Kyoto).

2.3. Fluorometric analysis of hydrolysis of MOCAc-PLGL(Dpa)AR

The MMP-7-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was initiated by mixing 1222 µl of the reaction buffer, 20 µl of the MMP-7 solution (625 nM), and 8 µl of the substrate solution (234 µM) dissolved in DMSO. The initial concentrations of enzyme, MOCAc-PLGL(Dpa)AR, and DMSO were 10 nM, 1.5 µM, and 0.64% (v/v), respectively. The reaction buffers were 50 mM acetate-NaOH buffer at pH 3.6-5.8, 50 mM MES-NaOH buffer at pH 5.6-7.0, 50 mM HEPES-NaOH buffer at pH 6.8-8.6, and 50 mM AMPSO-NaOH buffer at pH 8.6-10.4, each containing 10 mM CaCl$_2$. The reaction 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, Japan). The peptide bond of Gly-L-Leu residues was cleaved by MMP-7, and the amount of the product MOCAc-PLG was estimated by the fluorescence intensity by comparison with the fluorescence intensity of an authentic MOCAc-PLG solution. The hydrolysis was carried out under pseudo-first order conditions, where the initial concentration of MOCAc-PLGL(Dpa)AR (1.5 μM) was much lower than $K_m$ (60 μM) [38]. The Michaelis-Menten equation is, then, expressed as Eq. 1.

$$v_o = \frac{(k_{cat}/K_m)[E]_o[S]_o}{1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]}}$$ (2)

In this equation, $(k_{cat}/K_m)_{obs}$ and $[H]$ mean the $k_{cat}/K_m$ value observed and the proton concentration, respectively, at the specified pH.

2.4. Thermodynamic analysis of $K_{e1}$ and $K_{e2}$
The enthalpy changes, $\Delta H^0$, of deprotonation were calculated from $pK_e$ shifts using Eq. 3, known as the van’t Hoff equation,

$$\frac{d (\ln K_e)}{dT} = \frac{\Delta H^0}{RT^2}$$  \hspace{1cm} (3)

where $T$ and $R$ mean the absolute temperature in degrees Kelvin and the gas constant (= 8.314 J K$^{-1}$ mol$^{-1}$), respectively. When this equation is integrated, it is expressed as Eq. 4,

$$-\log K_e = \frac{\Delta H^0}{2.303 RT} + A$$ \hspace{1cm} (4)

where $A$ means the constant of integration. Thus, a slope of a plot for $pK_e$ values versus $1/T$ gives $\Delta H^0$.

2.5. Titration of pH of amino-acid solution and thermodynamic analysis of proton dissociation constant of side chains of amino acids

Amino acid was dissolved in water to be 10 mM for L-Asp, L-Glu, L-His, L-Cys, and L-Arg and 1 mM for L-Tyr and $N^\alpha$-acetyl-L-Lys. Titration was made with 50 or 100 $\mu$l of 2 M HCl or 2 M NaOH for each amino-acid solution (500 ml) incubated at 25, 35, or 45ºC as follows: the L-Asp and L-Glu solutions were titrated with NaOH until pH reached 6.0. The L-His solution was titrated with HCl until pH reached 3.0. The L-Cys solution was titrated with HCl until pH reached 6.8. The L-Arg solution was titrated
with HCl until pH reached 9.8. The L-Tyr solution was titrated with HCl until pH reached 7.5. The N\textsuperscript{\textprime}-acetyl-L-Lys solution was titrated with HCl until pH reached 9.2.

The following is the case with L-Glu, as an example. Based on the previous reports that pK\textsubscript{a} values of \(\alpha\)-COOH and \(\gamma\)-COOH of L-Glu at 25\textdegree C are around 2.0 and 4.0, respectively [29-33], the course of a titration of L-Glu with NaOH from pH 2 to 6 can be represented in the following schemes.

\[
\begin{align*}
H_3N^+(CH(CH_2)_2COOH)COOH + OH^- &\rightarrow H_3N^+(CH(CH_2)_2COOH)COO^- + H_2O \\
H_3N^+(CH(CH_2)_2COOH)COO^- + OH^- &\rightarrow H_3N^+(CH(CH_2)_2COO^-)COO^- + H_2O
\end{align*}
\]

The degree of dissociation, \(\alpha\), is defined as the following equation,

\[
\alpha = \frac{2[H_3N^+(CH(CH_2)_2COO^-)COO^-] + [H_3N^+(CH(CH_2)_2COOH)COO^-]}{C}
\]  \hspace{1cm} (5)

where \(C\) means the initial concentration of L-Glu. The \(\alpha\) value increases up to 2 in the course of the titration. The electric balance is given by the following equation.

\[
[H^+] + [Na^+] + [H_3N^+(CH(CH_2)_2COOH)COOH] =
[OH^-] + [Cl^-] + [H_3N^+(CH(CH_2)_2COO^-)COO^-] \hspace{1cm} (6)
\]

Substituting Eq. 5 into Eq. 6 yields the following equation,
\[ \alpha = \frac{C - [H^+] + [Na^+] - K_w / [H^+] - [Cl^-]}{C} \]  

(7)

where \( K_w \) is ionic product. The \( \alpha \) at each pH was calculated from Eq. 7. \( K_{a1} \) and \( K_{a2} \), which are defined as Eqs. 8 and 9, respectively, were calculated from Eq. 10 by a non-linear least squares regression method with Kaleida Graph Version 3.5.

\[ K_{a1} = \frac{[H^+] [H_3N^+(CH(CH_2)_2COOH)COO^-]}{[H_3N^+(CH(CH_2)_2COOH)COOH]} \]  

(8)

\[ K_{a2} = \frac{[H^+] [H_3N^+(CH(CH_2)_2COO^-)COO^-]}{[H_3N^+(CH(CH_2)_2COOH)COO^-]} \]  

(9)

\[ \alpha = \frac{2 + \frac{[H^+]}{K_{a2}}}{1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]^2}{K_{a1} K_{a2}}} \]  

(10)

\( \Delta H^0 \) of deprotonation of side chains of L-Glu was calculated from p\( K_{a2} \) shift using Eq. 3.

3. Results

3.1. \( \Delta H^0 \) values of deprotonation of side chains of amino acids

The wild-type MMP-7 was prepared as described in Materials and methods section. Starting with 100 ml of \textit{E. coli} cultures, 2 mg purified enzyme was recovered. Upon SDS-PAGE under reducing conditions, each yielded a single band with a molecular
mass of 19 kDa (data not shown).

To determine $\Delta H^0$ values of deprotonation of side chains of amino acids, we made a titration of pH of L-Asp, L-Glu, L-His, L-Cys, L-Tyr, N$^a$-acetyl-L-Lys, and L-Arg solutions. The result with L-Glu is shown in Fig. 3, as an example. The degree of dissociation, $\alpha$, which is defined as Eq. 5, increased rapidly with an increase of pH from 1.9 to 2.5, gradually with the increase from 2.5 to 3.8, and rapidly again with the increase from 3.8 to 4.5. The $pK_{a1}$ and $pK_{a2}$ were calculated by Eq. 10 to be 2.1 $\pm$ 0.1 and 4.0 $\pm$ 0.1 for 25°C, 2.2 $\pm$ 0.1 and 4.1 $\pm$ 0.1 for 35°C, and 2.3 $\pm$ 0.1 and 4.2 $\pm$ 0.1 for 45°C, respectively. The $pK_{a1}$ was assigned to $\alpha$-carboxyl group, and the $pK_{a2}$ was assigned to the side chain of L-Glu, based on the previous reports [29-33]. $\Delta H^0$ for the $pK_{a2}$ was calculated by the van’t Hoff plot to be -23.6 $\pm$ 5.8 kJ mol$^{-1}$ (inset of Fig. 3). All results are summarized in Table 1. The $\Delta H^0$ of the side chains of L-Asp and L-Glu were negative, while those of the other five positive. The $\Delta H^0$ of the side chains of L-Asp, L-His, and L-Cys were almost the same as the ones previously reported, which were determined by measuring the pH- and temperature-dependences of electromotive force of the battery containing amino acids or dipeptides in the cell (-6 to 6 kJ mol$^{-1}$ for L-Asp [29], 29-32 kJ mol$^{-1}$ for L-His [31], and 24-26 kJ mol$^{-1}$ for L-Cys [33]). On the other hand, the $\Delta H^0$ of the side chains of the other four were substantially different from the ones previously reported (-6 to 6 kJ mol$^{-1}$ for L-Glu [29], 25-26 kJ mol$^{-1}$ for L-Tyr [31], 44-55 kJ mol$^{-1}$ for L-Lys [31], and 50-52 kJ mol$^{-1}$ for L-Arg [31]).

3.2. $\Delta H^0$ of deprotonation of ionizable groups responsible for $pK_{e1}$ and $pK_{e2}$ of MMP-7

According to Fig. 2 and Eq. 2, the pH dependence of $k_{cat}/K_m$ results from the
association and dissociation of proton in the free MMP-7, but not the MMP-7 combined with substrate. The $k_{cat}/K_m$ values of MMP-7 in the hydrolysis of MOCAc-PLGL(Dpa)AR in the pH range of 3.6-10.4 at 15, 25, 35, and 45°C were determined by Eq. 1 and are shown in Fig. 4. All plots showed bell-shaped curves. The plot at 15°C showed the widest active pH-range while that at 45°C showed the narrowest. The intrinsic $k_{cat}/K_m$, $(k_{cat}/K_m)_o$, and the pK$_e$ values were determined by Eq. 2, which are summarized in Table 2. The $(k_{cat}/K_m)_o$ value was the lowest at 15°C and the highest at 45°C. Figure 5 shows van’t Hoff plot for pK$_{e1}$ and pK$_{e2}$. $\Delta H^o$ of deprotonation were calculated to be -20.6 ± 6.1 kJ mol$^{-1}$ for pK$_{e1}$ and 89.9 ± 4.0 kJ mol$^{-1}$ for pK$_{e2}$.

3.3. Estimation of the ionizable groups responsible for pK$_{e1}$ and pK$_{e2}$ of MMP-7

By comparison with the $\Delta H^o$ of MMP-7 (-20.6 ± 6.1 kJ mol$^{-1}$ for pK$_{e1}$ and 89.9 ± 4.0 kJ mol$^{-1}$ for pK$_{e2}$) with the $\Delta H^o$ of side chains of amino acids (Table 1), glutamate residue was thought to be the ionizable group responsible for pK$_{e1}$, and arginine or lysine residue for pK$_{e2}$. This suggested that Glu198 is the ionizable group for pK$_{e1}$ (Fig. 1), as previously pointed out [4]. However, no arginine or lysine residues are found in the active site (Fig. 1). We therefore hypothesized that a protein-bound water is the ionizable group for pK$_{e2}$.

3.4. Water molecules as the candidate for ionizable group responsible for pK$_{e2}$

The hydroxamate (R-CO-NH-OH) peptide mimetic inhibitor, which binds covalently to the active-site zinc ion, is the first MMP inhibitor [40]. In the
MMP-7-hydroxamate inhibitor complex (Protein Data Bank no. 1MMQ) [4], we noticed two water molecules (W1 and W2) as the possible candidates for the ionizable group responsible for pK_e2 (Fig. 1B), based on the following reasons: (i) W1 and W2 are located 2.7 Å far from each one of the two oxygen atoms of hydroxamate bound to the active site zinc ion; (ii) W1 binds to the main-chain nitrogen atom of Ala162, and W2 binds to the main-chain carbonyl oxygen atom of Pro217. Ala162 is located at the fourth β-sheet (Ala162-Ala164) of MMP-7, and Pro217 is located at the Met-turn (Pro211-Gly222). Both Ala162 and Pro217 are conserved among MMPs; and (iii) In the X-ray crystallographic structure of MMP-7 complexed with carboxylate inhibitor (1MMP) and sulfodiimine inhibitor (1MMR), the water molecules corresponding to W1 and W2 are found [4].

3.5. Kinetic analysis of the MMP-7 variants

To see if the ionizable group responsible for pK_e1 is Glu198, we constructed the MMP-7 variant, E198A. To explore the possibility that either W1 or W2 is the ionizable group responsible for pK_e2, we constructed the variants, A162G and P217G, assuming that the main-chain structure of MMP-7 could be changed by mutating Ala162 or Pro217 into glycine, which is the most flexible amino acid residue, and that such mutation alters pK_e2 if the ionizable group responsible for pK_e2 is located in the neighborhood of the site of mutation. The variants were produced in the E. coli expression system [38]. The pH-dependence of the k_cat/K_m of the wild-type MMP-7, E198A, A162G, and P217G in the hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C is shown in Fig. 6, and the kinetic parameters are summarized in Table 3. E198A
completely lacked the activity. To see if it retained small MOCA-PLGL(Dpa)AR-hydrolyzing activity, we made HPLC analysis [27]. No activity was detected in E198A even with the enzyme and substrate concentrations of 1 μM and 140 μM, respectively, and the reaction time of 120 min (data not shown). On the other hand, the activity was detected in the wild-type enzyme with the enzyme and substrate concentrations of 1 nM and 140 μM, respectively, and the reaction time of 10 min (data not shown). This indicated that the activity of E198A, if any, was less than 0.01% of that of the wild-type enzyme, suggesting that E198A completely lost the activity and that Glu198 is not the ionizable group responsible for pK_{e1}. A162G and P217G retained the activity with the (k_{cat}/K_m) values of 57% and 78% of that of the wild-type enzyme, respectively (Table 3). The pK_{e1} of A162G and P217G were 4.9 ± 0.1 and 5.3 ± 0.1, being higher by 0.3 ± 0.2 and 0.7 ± 0.2 unit, respectively, than that of the wild-type enzyme (4.6 ± 0.1). The pK_{e2} of A162G and P217G were 10.3 ± 0.1 and 10.0 ± 0.1, being higher by 0.6 ± 0.2 and 0.3 ± 0.2 unit, respectively, than that of the wild-type enzyme (9.7 ± 0.1). These results indicated that the mutations of Ala162→Gly and Pro217→Gly affected the electrostatic environment of the ionizable groups responsible for not only pK_{e2} but also pK_{e1}.

4. Discussion

4.1. Estimation of ionizable groups involved in the catalytic mechanism of MMP-7

Browner et al. proposed that, based on the crystal structure of the complex of MMP-7 and its inhibitor, Glu198 is the ionizable group responsible for pK_{e1}: it functions
both as a base and an acid, deprotonating the zinc-bound water and transferring the proton to the leaving amine [4]. Cha et al. proposed that, based on the pH-dependence of the activity, Tyr219 is the ionizable group responsible for $pK_{e2}$: the ionized side chain of Tyr219 makes the active site of MMP-7 hydrophilic [25]. They also proposed that the zinc-bound water, but not Glu198, is the ionizable group responsible for $pK_{e1}$: the ionized zinc-bound water molecule attacks the carbonyl carbon of the scissile bond as a nucleophile [26]. We demonstrated that, based on the results with chemical modification [27] and site-directed mutagenesis [28], Tyr219 is not the ionizable group responsible for $pK_{e2}$.

 Thermodynamic analysis in this study suggested that glutamate residue is the ionizable group for $pK_{e1}$, and that arginine or lysine residue is that for $pK_{e2}$ (Tables 1 and 2). The mutation of Glu198→Ala completely abolished activity (Table 3). We think that E198A has similar three-dimensional folds to the wild-type MMP-7 because the expression level and stability of E198A were similar to those of the wild-type MMP-7: starting from 100-ml culture, 2 mg purified E198A was obtained by the denaturation and refolding processes. Purified E198A was stable on storage at 4°C. On the other hand, some MMP-7 variants precipitated during the refolding process (K. Y. and K. I., unpublished data). Although it is difficult to exclude a possibility that the loss of activity by the mutation Glu198→Ala does not result from the structural change, our result suggests that Glu198 is the ionizable group responsible for $pK_{e1}$. This agrees well with the previous reports that in MMP-1 [41], MMP-3 [42], and MMP-9 [43], the glutamate residue corresponding to Glu198 of MMP-7 is catalytically important. On the other hand, arginine and lysine residue were declined for the ionizable group for $pK_{e2}$ because there are no lysine or arginine residues in the active site. We therefore proposed that a
protein-bound water is the ionizable group for $pK_{e2}$. We noticed two water molecules, Ala162-bound water (W1) and Pro217-bound water (W2), as the candidates for the ionizable group for $pK_{e2}$. The mutations of Ala162→Gly and Pro217→Gly altered not only $pK_{e2}$ but also $pK_{e1}$ values. This indicated that the mutations affected the electrostatic environment of the active site. To further explore our hypothesis, extensive site-directed mutagenesis study is required.

4.2. Catalytic mechanism of MMP-7

Based on the results in this study, we propose the following catalytic mechanism of MMP-7 (Fig. 7). In free enzyme, Glu198 must be in its deprotonated state and the protein-bound water must be in its unionized state for catalysis (Fig. 7A). The Michaelis complex is formed when the carbonyl oxygen of the scissile bond binds the zinc ion. Zinc ion polarizes the carbonyl group of the scissile bond. Glu198 accepts a proton from the zinc-bound water (Fig. 7B). The tetrahedral complex is formed when the ionized zinc-bound water attacks the carbonyl carbon of the scissile bond, and then stabilized by the interaction between the carbonyl oxygen of the scissile bond and the oxygen of the protein-bound water in its unionized state (Fig. 7C). This stabilization does not occur when this protein-bound water is negatively charged. The amino product is released when Glu198 transfers the proton to the nitrogen of the scissile bond (Fig. 7D).

$pK_a$ of free water is 15.7. The above mechanism is consistent with the pH dependence of MMP-7 activity with $pK_{e1}$ and $pK_{e2}$ values of 4.0 and 9.8 if the $pK_a$ of the protein-bound water greatly decreases. The zinc-bound water should be released in the MMP-7-hydroxamate inhibitor complex (Protein Data Bank no. 1MMQ) [4].
because hydroxamate (R-CO-NH-OH) binds to the zinc ion as the bidentate ligand.

When the substrate coordinates to the zinc ion through the mono dentate ligand such as the carbonyl oxygen of the scissile bond, it still binds to the zinc ion. Therefore, it is thought that the pKₐ of the zinc-bound water also greatly decreases. Considering the mechanism that the ionized zinc-bound water attacks the carbonyl carbon of the scissile bond (Fig. 7), it is thought that the ionizable group responsible for pKₑ₂ is the protein-bound, but not zinc-bound, water.

It should be mentioned that in MMP-3, the protein-bound water involved in the catalytic mechanism was in its protonated state for catalysis, stabilizing the tetrahedral intermediate by coordinating the carbonyl oxygen of the scissile bond of the substrate [44]. In carboxypeptidase A, which belongs to Clan MC of zinc metalloproteinase, the ionizable group for pKₑ₁ of 7 was assigned to the active-site glutamate residue, and that for pKₑ₂ of 10 was assigned to the zinc-bound water molecule [45]. It was thought that the active-site glutamate residue functions as a general base while the zinc-bound water is in its unionized state, stabilizing the tetrahedral intermediate [45].

4.3. Estimation of the ionizable groups involved in the catalytic mechanism of enzymes by ΔH° of deprotonation

pKₐ of active-site residues can vary depending on their microenvironment. To our knowledge, the highest pKₐ of the active-site glutamate residue is 8.4 in xylanase [46], suggesting that it is difficult to estimate the ionizable groups of enzymes only by their pKₐ values. In this study, we determined pKₐ of side chains of L-Asp, L-Glu, L-His, L-Cys, L-Tyr, L-Lys, and L-Arg at 25-45°C by the pH titration of amino-acid solutions,
from which we calculated $\Delta H^o$ of deprotonation. In the previous reports [29], $\Delta H^o$ of
side chains of L-Asp and L-Glu were the same (in the range of -6 to 6 kJ mol$^{-1}$). In this
study, they were different (-6.4 ± 3.5 kJ mol$^{-1}$ for L-Asp and -23.6 ± 5.8 kJ mol$^{-1}$ for
L-Glu) (Table 1). Considering that the ionizable group for $pK_{e1}$ can be assigned to
Glu198 by the $\Delta H^o$ value (-20.6 ± 6.1 kJ mol$^{-1}$), the $\Delta H^o$ of side chains of amino acids
presented in this study might be a powerful tool to estimate the ionizable groups
involved in the catalytic mechanism of various enzymes.

In conclusion, we propose that Glu198 and unidentified protein-bound water are
the ionizable groups involved in the catalytic mechanism of MMP-7. To identify the
protein-bound water, site-directed mutagenesis study of MMP-7 is currently underway.

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Figure Legends

Fig. 1. Structure of MMP-7. The MMP-7-hydroxamate inhibitor complex (Protein Data Bank no. 1MMQ) [4] was drawn using Swiss-Pdb Viewer 4.0. The active-site zinc ion is shown as a sphere. (A) Overall structure. Peptide chain is represented by a ribbon. The side chains of Glu198, Tyr193, Tyr216, and Tyr219 and the hydroxamate inhibitor are shown as a stick. (B) Close-up view of the active site. The side chains of Glu198 and Tyr219, the main and side chains of Ala162 and Pro217, and the two water molecules (W1 and W2) are shown as a ball and stick. The hydroxamate inhibitor is shown as a wire with the nearest two oxygen atoms to the active-site zinc ion as a ball. The number indicates that of the amino acid residues.

Fig. 2. Reaction scheme for the pH-dependence of MMP-7 activity with two ionizable groups involved in enzyme activity. E, S, H, and P denote MMP-7, the substrate, proton, and the product, respectively. $K_{e1}$ and $K_{e2}$ are proton dissociation constants of the ionizable groups of the free MMP-7, respectively, and $K_{es1}$ and $K_{es2}$ are proton dissociation constants of the MMP-7 combined with substrate, respectively [27,34].

Fig. 3. Titration curve of pH of L-Glu solution. The titration was carried out at 25 (open circle), 35 (open square), and 45°C (open triangle). The degree of dissociation of L-Glu at each pH was calculated by Eq. 7. The $pK_{a1}$ and $pK_{a2}$ were calculated by Eq. 10, which were $2.1 \pm 0.1$ and $4.0 \pm 0.1$ for 25°C, $2.2 \pm 0.1$ and $4.1 \pm 0.1$ for 35°C, and $2.3 \pm 0.1$ and $4.2 \pm 0.1$ for 45°C, respectively. Inset shows van’t Hoff plot for $pK_{a2}$ of L-Glu. Enthalpy change, $\Delta H^\circ$, of deprotonation was calculated to be $-23.6 \pm 5.8$ kJ mol$^{-1}$. One of the representative data is shown.
Fig. 4. Effect of pH on the wild-type MMP-7-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR. The reaction was carried out at 15 (open circle), 25 (open square), 35 (open triangle), and 45°C (open diamond), each with the initial enzyme and substrate concentrations of 10 nM and 1.5 μM, respectively. The relative $k_{cat}/K_m$ is defined as the ratio of the $k_{cat}/K_m$ at the indicated pH to that at the optimal pH (1.63 × $10^{-4}$ M⁻¹ s⁻¹ at pH 6.8 for 15°C, 4.01 × $10^{-4}$ M⁻¹ s⁻¹ at pH 6.8 for 25°C, 5.39 × $10^{-4}$ M⁻¹ s⁻¹ at pH 6.6 for 35°C, and 7.77 × $10^{-4}$ M⁻¹ s⁻¹ at pH 6.0 for 45°C). Error bars indicate SD values. One of the representative data is shown.

Fig. 5. van’t Hoff plot for $pK_e$ of MMP-7. $pK_{e1}$ (A) and $pK_{e2}$ (B) values of MMP-7 were plotted against the reciprocal of the absolute temperature. Error bars indicate SD values. Enthalpy change, $\Delta H^o$, of deprotonation was calculated from the slope: (A) -20.6 ± 6.1 kJ mol⁻¹; (B) 89.9 ± 4.0 kJ mol⁻¹. One of the representative data is shown.

Fig. 6. Effect of pH on the MMP-7 variants-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR at 25°C. The reaction was carried out with the wild-type MMP-7 (open circle), A162G (open square), and P217G (open triangle). The initial enzyme and substrate concentrations were 10 nM and 1.5 μM, respectively. The relative $k_{cat}/K_m$ is defined as the ratio of the $k_{cat}/K_m$ at the indicated pH to that at the optimal pH (4.01 × $10^{-4}$ M⁻¹ s⁻¹ at pH 6.8 for the wild-type MMP-7, 2.46 × $10^{-4}$ M⁻¹ s⁻¹ at pH 6.8 for A162G, and 3.18 × $10^{-4}$ M⁻¹ s⁻¹ at pH 7.0 for P217G). Error bars indicate SD values. One of the representative data is shown.
Fig. 7. Proposed mechanism for the MMP-7-catalyzed cleavage of peptides. See the text for details.
Fig. 1

A

[Tyr219] [Glu198] [Tyr193] [Zn$^{2+}$] [hydroxamate inhibitor]

[Tyr216] [Zn$^{2+}$] [Zn$^{2+}$] [Zn$^{2+}$]
Fig. 1
\[
\begin{align*}
E & \rightleftharpoons \text{ES} \\
\text{EH} & \rightleftharpoons \text{EHS} \xrightarrow{k_{\text{cat}}} \text{EH} + P \\
\text{EH} & \rightleftharpoons \text{EH}_2S
\end{align*}
\]
Relative $k_{cat}/K_m$ vs. pH

Fig. 4
Fig. 5
Fig. 6
**Fig. 7**

**A**  Free enzyme

**B**  Michaelis complex

**C**  Transition state

**D**  Enzyme-product complex