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Transient Kinetic Studies of pH-Dependent Hydrolyses by Exo-type Carboxypeptidase P on a 27-MHz Quartz Crystal Microbalance

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pH-Dependent kinetic parameters (k_{on}, k_{off}, and k_{cat}) of protein (myoglobin) hydrolyses catalyzed by exo-enzyme (carboxypeptidase P, CPP) were obtained by using a protein-immobilized quartz crystal microbalance (QCM) in acidic aqueous solutions. The formation of the enzyme−substrate (ES) complex (k_{on}), the decay of the ES complex (k_{off}), and the formation of the product (k_{cat}) could be analyzed by transient kinetics as mass changes on the QCM plate. The K_d (k_{off}/k_{on}) value was different from the Michaelis constant K_m calculated from ($k_{off} + k_{cat}$)/k_{on} due to $k_{cat} > k_{off}$. The rate-determining step was the binding step (k_{on}), and the catalytic rate (k_{cat}) was faster than other k_{on} and k_{off} values. In the range of pH 2.5−5.0, values of k_{on} gradually increased with decreasing pH showing a maximum at pH 3.7, values of k_{off} were independent of pH, and k_{cat} increased gradually with decreasing pH. As a result, the apparent rate constant (k_{cat}/K_m) showed a maximum at pH 3.7 and gradually increased with decreasing pH. The optimum pH at 3.7 of k_{on} is explained by the optimum binding ability of CPP to the COOH terminus of the substrate with hydrogen bonds. The increase of k_{cat} at the lower pH correlated with the decrease of α-helix contents of the myoglobin substrate on the QCM.

Proteases have been widely studied as a typical hydrolytic enzyme for many years.[1] Recently, interactions between proteases and their substrates became more important in connection with curative for diseases. Kinetic studies of proteases should be needed for understanding these events.[2] When large proteins were used as a substrate instead of simple peptides, protease hydrolyses are known to often be affected by tertiary structures of large proteins and precise kinetic parameters have not been quantitatively studied.[3] Protease reactions have been conventionally followed by the produced peptides by using SDS-PAGE,[4] a colorimetric ninhydrin test,[5] fluorescent photometry (FERT),[6] and mass spectrometry.[7]

An enzyme mechanism of single-substrate reactions has been understood based on a simple Michaelis enzyme reaction represented as eq 1. The reaction process is presented as eqs 2 and 3 with a focus on each reaction rate such as binding rate constants (k_{on}), dissociation rate constants (k_{off}), and reaction rate constants (k_{cat}).

$$\mathrm{E} + \mathrm{S} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{cat}} \mathrm{E} + \mathrm{P} \tag{1}$$

$$\frac{\mathrm{d[ES]}}{\mathrm{d}t} = k_{on}[\mathrm{E}][\mathrm{S}] - (k_{off} + k_{cat})[\mathrm{ES}] \tag{2}$$

$$\frac{\mathrm{d[P]}}{\mathrm{d}t} = k_{cat}[\mathrm{ES}] \tag{3}$$

In general, kinetic parameters of enzyme reactions have been obtained using a Michaelis−Menten equation as eq 4 (steady-state kinetics), in which the concentration of the enzyme−substrate (ES) complex was hypothesized to be nearly constant during the reaction and detection of the ES complex was not required, because it was relatively difficult to detect the concentration of the ES complex.[1,8] The reaction rate was simply measured as the initial rate (v_0) of the product increase for a Michaelis−Menten equation (eq 4). From eq 4, only k_{cat} and K_m values could be obtained, where the Michaelis constant K_m is a complex value containing k_{cat}, k_{on}, and k_{off}, and resembles the dissociation constant (K_d) only when $k_{off} \gg k_{cat}$.

$$v_0 = \frac{k_{cat}[\mathrm{E}]_0[\mathrm{S}]_0}{[\mathrm{S}]_0 + K_m} \tag{4}$$

where

$$K_m = \frac{k_{off} + k_{cat}}{k_{on}}$$

However, the ES complex is obviously a key intermediate for analysis of enzyme kinetics (eqs 2 and 3). If the time-dependent formation and decay of the ES complex could be followed directly

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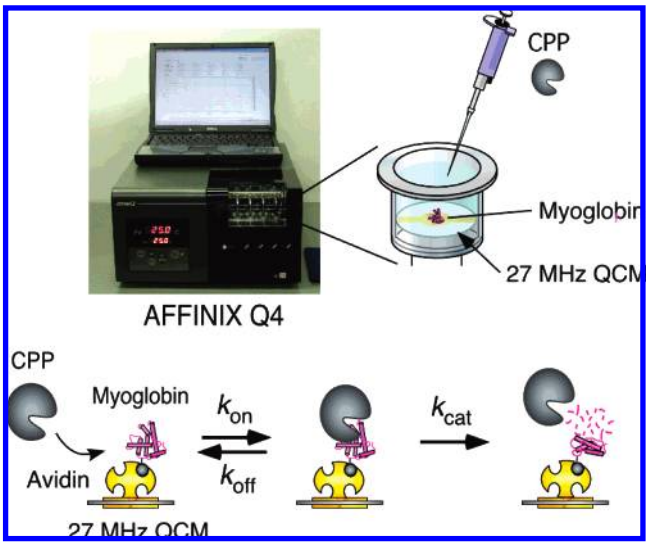

Figure 1. Schematic illustrations of enzymatic hydrolyses of myoglobin catalyzed by exo-type carboxypeptidase P (CPP) on a 27 MHz QCM (AFFINIX Q4). k_{on}, k_{off}, and k_{cat} are kinetic parameters obtained in this work.

during the reaction (transient kinetics), all kinetic values such as k_{cat}, k_{on}, and k_{off} could be separately obtained from eqs 2 and 3 and more detail enzymatic reaction mechanisms could be discussed. As a special case, Chance and co-workers had succeeded [8]to observe formations of the ES complex and the increase of the product from absorbance changes in the reaction of peroxidase including hematin iron with a substrate dye (leucomalachite green).[9] However, this technique could not apply for general protease reactions.

In this paper, we describe that all kinetic parameters (k_{cat}, k_{on}, and k_{off}) for hydrolyses of myoglobin as a substrate catalyzed by exo-type carboxypeptidase P (CPP) could be obtained quantitatively by using a myoglobin-immobilized 27 MHz quartz crystal microbalance (QCM) because the formation and decay of the ES complex and the formation of the product could be followed as mass changes on the QCM plate (Figure 1). QCMs are known to provide very sensitive mass measuring devices in aqueous solutions, and their resonance frequency is proved to decrease linearly upon the increase of mass on the QCM electrode at the nanogram level.[10−12] Although detection of an ES complex using QCM has shown interest and been performed previously, the results were not analyzed kineticaly.[13] In this study, we applied a curve fitting method based on differential equations (eqs 2 and 3, see Appendix) for single-substrate protease reactions to obtain the kinetic parameters. Thus, we could follow the time-dependent formation of the ES complex (k_{on}) as the frequency decreases (the mass increases), the decay of the ES complex (k_{off}) as the frequency increases (the mass decreases), and the formation of the product (k_{cat}) as the frequency increases (the mass decreases), because a QCM technique could detect amounts of both the ES complex and the hydrolysis product with the same physical signal (mass) that can be convertible into a unit on a molar basis.

We chose myoglobin (from Equine heart, MW, 17 kDa) as a substrate, which contains eight α-helix structures but no β-sheet structures and SS linkages in the molecule.[14] We chose an exopeptidase reaction of carboxypeptidase P (CPP from *Penicillium janthinellun*, optimum pH = 3.7, existing in lysosome, EC 3.4.16.1, MW, 51 kDa) that hydrolyzes from the C-terminus of proteins,[15−18] and release processes of amino acids can be followed as mass changes. Hydrolyses of myoglobin by CPP were carried out in acidic conditions (pH 2.0−6.0), and the results were explained by both the optimum pH of the enzyme and the conformational change of the substrate.

RESULTS AND DISCUSSION

AFFINIX Q4 was used as a QCM instrument (Initium Co. ltd, Tokyo, http://www.initium2000.com) having four 0.5 mL cells equipped with a 27 MHz QCM plate at the bottom of the cell and the stirring bar with the temperature-controlling system (see Figure 1).[11,12] Calibrations of the 27 MHz QCM in aqueous solutions are described in Experimental Section, and 1 Hz of frequency decrease was calibrated as an increase in 0.30 ng/cm^2 of myoglobin in aqueous solutions. One amino group of myoglobin (17 kDa) was biotinylated with a biotin-$(AC_5)_2$-Sulfo-OSu and anchored on an avidin-immobilized QCM plate according to the previous paper.[19] The immobilized amount of biotinylated myoglobin was maintained to be 60 ± 5 ng (3.5 ± 0.3 pmol) cm^{-2} indicating almost 30% surface coverage of the QCM electrode.

The curve a in Figure 2A shows typical frequency changes as a function of time of the myoglobin-immobilized QCM, responding to the addition of an exo-type protease of carboxypeptidase P (CPP, 3.2 μM) in the acidic aqueous solution at pH 3.7. In the addition of CPP, the frequency decreased (the mass increased) in the first several minutes, meaning the binding of enzymes to the protein substrate. Then the frequency gradually increased (the

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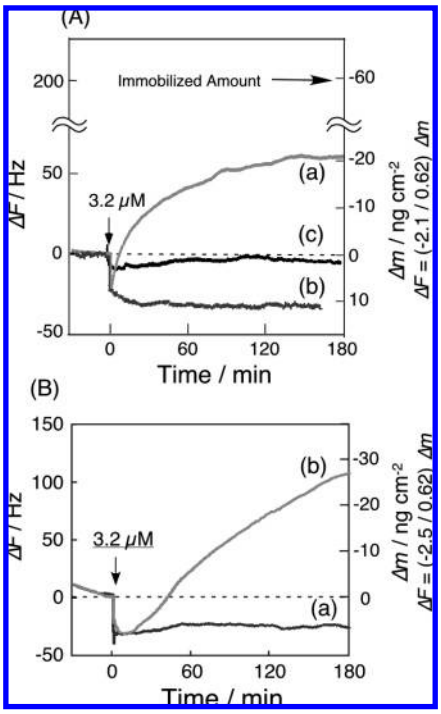

Figure 2. (A) Typical time courses of frequency changes of the myoglobin−avidin-immobilized QCM, responding to the addition of exo-type proteases of (a) CPP and (b) CPP inactivated with AEBSF (4-(2-aminoethyl)benzensulfonyl fluoride) and frequency changes of (c) the avidin-immobilized QCM, responding to the addition of CPP (50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C, [myoglobin] = 60 ng (3.5 pmol) cm^{-2} on the QCM, [CPP or inactivated CPP] = 3.2 μM). (B) Time courses of frequency changes of (a) the BSA−avidin-immobilized QCM and (b) the denatured BSA−avidin-immobilized QCM, responding to the addition of CPP (50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C, [BSA or denatured BSA] = 60 ng (3.5 pmol) cm^{-2} on the QCM, [CPP] = 3.2 μM).

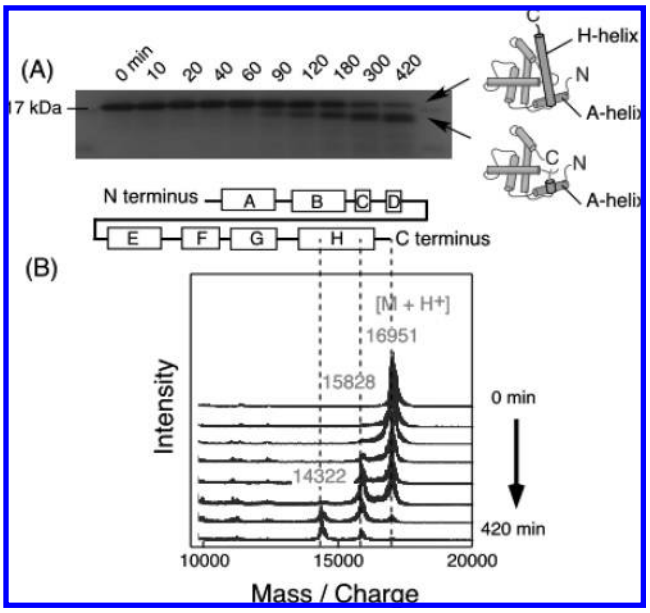

Figure 3. (A) SDS-PAGE and (B) MALDI TOF-MS analyses of the hydrolysis of myoglobin (17 kDa) catalyzed by CPP in the bulk solution 50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C, [myoglobin] = 100 μM, [CPP] = 1 μM).

mass decreased), corresponding to hydrolyses of the protein substrate on the QCM, and then it reached the constant value ($\Delta m = -20$ ng cm^{-2}). Because 60 ng cm^{-2} of myoglobin was immobilized in advance, the mass decrease of −20 ng cm^{-2} means that only 30% of the substrate was hydrolyzed. This indicates that the hydrolysis from the C-terminus is not completed even for myoglobin containing only α-helixes and no SS linkages. When the active Ser-OH group of CPP was inactivated covalently with 4-(2-aminoethyl)benzensulfonyl fluoride (AEBSF),[20] only the binding process (the initial frequency decrease) was observed and then the frequency did not increase (the hydrolysis did not occur) (curve b in Figure 2A). When CPP was injected to the avidin-immobilized QCM, the frequency hardly changed (curve c in Figure 2A). Thus, CPP could not bind to and hydrolyze avidin having SS linkages in the molecule. When biotinylated BSA was immobilized on the avidin surface of the QCM, CPP could bind to the substrate but not hydrolyze it, because BSA contains SS linkages (curve a in Figure 2B). On the contrary, when 17 SS linkages of BSA were reduced with α-iodoacetic acid forming $-SCH_2COO^-$ groups in order to denature the tertiary structure of BSA, CPP could bind and hydrolyze the denatured random coil structures of BSA on the QCM (curve b in Figure 2B). These results clearly indicate that the curve a in Figure 2A shows simply the hydrolysis of myoglobin on the QCM plate. Hydrolysis time courses of the denatured BSA shown in the curve b in Figure 2B were not constant depending on experimental runs, because random coil structures of the denatured BSA are not stable for hydrolysis reactions. Therefore, we chose the exo-type hydrolysis of myoglobin as a protease reaction.

Hydrolyses of myoglobin catalyzed by CPP were also carried out conventionally in the bulk solution at pH 3.7, in comparison with the QCM experiments. The reaction was followed by a SDS-PAGE and MALDI TOF-MS as shown in Figure 3. A new low-molecular weight band appeared with time in addition to the myoglobin band at 17 kDa in SDS-PAGE. Myoglobin (MW, 16 951, 153 amino acids) has eight helixes (A−H), where H- and A-helixes are located at the C- and N-terminus, respectively.[14] The intensity of M^+, 16 951 peak of myoglobin of the MALDI TOF-MS spectrum decreased, and peaks of M^+, 15 828 and 14 322 increased with reaction times. The MW = 14 322 corresponds to the cross point of the C-terminus H-helix and the N-terminus A-helix, where the hydrolysis will stop due to the steric hindrance (see illustrations in Figure 3A). Although the reaction could be roughly followed by band shifts of SDS-PAGE or MALDI TOF-MS, catalytic reaction rates were separately obtained by the ninhydrin test of the colorimetric analyses at 570 nm of produced amino acids at pH 3.7.[21,22] With the concentrations of the myoglobin substrate changed (25−100 μM) in the presence of CPP (1 μM) in the buffer solution (pH 3.7, 50 mM citrate, and 200 mM NaCl at 25 °C), the kinetic parameters of k_{cat} and K_m were obtained using the Michaelis−Menten equation (eq 4) and the results are summarized in Table 1, in comparison with the QCM results. Kinetic parameters obtained from SDS-PAGE were roughly consistent with those obtained from the ninhydrin test.

Transient Kinetic Analyses on the QCM. The ladle-shaped curve a in Figure 2A indicates total hydrolysis steps of myoglobin

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Table 1. Kinetic Parameters of the Exo-Type Hydrolysis of Myoglobin by CPP[a]

methods	k_{on} /10^3 M^{-1} s^{-1}	k_{off} /s^{-1}	K_d[b] /10^{-6} M	K_m[c] /10^{-6} M	k_{cat} /s^{-1}	k_{cat}/K_m[d] /10^3 M^{-1} s^{-1}
transient kinetics CPP on the QCM[e]	10	0.20	20	130	1.1	8.5
Michaelis−Menten kinetics in the bulk solution[f]				100	0.30	3.0

[a] All kinetic parameters contain ±10% of experimental errors. [b] Calculated from $K_d = k_{off}/k_{on}$. [c] Calculated from $K_m = (k_{off} + k_{cat})/k_{on}$ of eq 4 in the text. [d] Apparent second-order rate constants. [e] [myoglobin] = 60 ng (3.5 pmol) cm^{-2} on the QCM, [CPP] = 1.0−4.0 μM, 50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C. [f] [myoglobin] = 25−100 μM, CPP = 1 μM, 50 mM citrate buffer, pH 3.7, 200 mM NaCl at 25 °C.

from the C-terminus catalyzed by CPP according to eq 1. The time dependence of ΔF reflects the difference between the formation of the ES complex (ΔF_{ES}) and the catalytic hydrolysis (the formation of the product, ΔF_P), as shown in eq 5, where MW_E and MW_P are the molecular weight of the enzyme and the released product, respectively. Concentrations of ES and P at the time t are shown in eqs 6 and 7, respectively (see Appendix).

$$\Delta F = \Delta F_{ES} - \Delta F_P = -MW_E[ES] - (-MW_P[P]) \quad (5)$$

$$[ES] = Z(e^{-Xt} - e^{-Yt}) \quad (6)$$

$$[P] = k_{cat}Z\left(\frac{1 - e^{-Xt}}{X} - \frac{1 - e^{-Yt}}{Y}\right) \quad (7)$$

where

$$X = \frac{1}{2}(A - \sqrt{A^2 - 4B})$$

$$Y = \frac{1}{2}(A + \sqrt{A^2 - 4B})$$

$$Z = \frac{k_{on}[E]_0[S]_0}{Y - X}$$

$$A = k_{on}[E]_0 + k_{off} + k_{cat}$$

$$B = k_{on}k_{cat}[E]_0$$

Theoretical curves of ΔF_{ES}, ΔF_P, and ΔF calculated from eqs 5−7 with a least-square approach using KaleidaGraph 4.0 (Synergy Software) are shown as curves a, b and c in Figure 4, respectively. The experimental curve d shown as a blue line of the myoglobin hydrolysis by CPP was well fitted with the theoretical curve c shown as a red line. Since this is the multiple hydrolysis from the C-terminus, the obtained $k_{cat}^{(apparent)}$ value shows the single step of the hydrolysis and the real k_{cat} value of multiple attacks is calculated as $k_{cat} = nk_{cat}^{(apparent)}$, where n is a turnover number of enzymes per one binding to substrate. The turnover number was calculated to be $n = 51$ from 30% of the hydrolysis amount (20 ng cm^{-2}) for the immobilized amount (60 ng cm^{-2}) (see Figure 2A) and the number of amino acids of myoglobin (153 amino acids) (153 × (20/60)). The kinetic parameters of $k_{on} = (10 \pm 0.9) \times 10^3$ M^{-1} s^{-1}, $k_{off} = 0.20 \pm 0.02$ s^{-1}, and $k_{cat} = 1.1 \pm 0.1$ s^{-1} were obtained by curve fitting methods and the results are summarized in Table 1, together with the calculated $K_d = k_{off}/k_{on}$ and $K_m = (k_{off} + k_{cat})/k_{on}$ (from eq 4) values. When the immobilized amount of the myoglobin substrate was changed in the range of 20−120 ng cm^{-2} on the QCM, and the concentration of CPP was changed from 1.0 to 4.0 μM, all kinetic parameters (k_{on}, k_{off}, and k_{cat}) hardly changed within ±10% experimental errors.

Comparison of Transient and Steady-State Kinetics. In the transient kinetics on the QCM, kinetic parameters for each step such as the formation (k_{on}) and decomposition (k_{off}) of the ES complex and the catalytic hydrolysis process (k_{cat}) could be obtained. In the conventional Michaelis−Menten kinetics (steady-state kinetics) in the bulk solution, however, only k_{cat} and K_m values are obtained, in which the Michaelis constant of K_m contains three parameters of k_{on}, k_{off}, and k_{cat} (eq 4). The K_m (calculated from $(k_{off} + k_{cat})/k_{on}$) and k_{cat} values obtained by the QCM method were relatively consistent with those obtained by Michaelis−Menten kinetics in the bulk solution (see Table 1). Thus, the immobilization of substrates hardly affects hydrolysis reactions in this experimental condition.

The calculated value of $K_m = 130 \times 10^{-6}$ M on the QCM (or $K_m = 100 \times 10^{-6}$ M obtained from Michaelis−Menten kinetics in the bulk solution) was largely different from $K_d = 20 \times 10^{-6}$ M. K_m means a ratio of ($k_{off} + k_{cat}$) and k_{on}, and K_d means a ratio of k_{off} and k_{on}. As a mathematical presentation, the K_m value consists of the dissociation constant K_d only when $k_{off} \gg k_{cat}$ (eq 4). In enzymatic kinetics, the case of $k_{off} \gg k_{cat}$ means that an interaction between enzyme−substrate should be rapid equilibrium, while an enzyme reaction with $k_{off} \ll k_{cat}$ means an almost irreversible reaction. The classification of an enzymatic reaction model requires comparison between values of k_{off} and k_{cat}. The results of $k_{cat} = 1.1$ $s^{-1} \gg k_{off} = 0.2$ s^{-1} in this study indicates an almost irreversible reaction. Thus, in the case of the exo-type hydrolysis of myoglobin by CPP, the value of K_m does not reflect the dissociation constant (K_d). The K_d value obtained directly from k_{off}/k_{on} by the QCM method, which could not be obtained from Michaelis−Menten kinetics in bulk solution, reflects the real dissociation constant. It is reasonable that k_{cat} is larger than k_{off} for the exo-type enzyme (an almost irreversible reaction), because the enzyme should continuously hydrolyze the terminus unit before the release from the substrate for efficient hydrolyses. The apparent first-order binding rate constant can be calculated as k_{on}-[CPP] = 0.032 s^{-1} when [CPP] = 3.2 μM, which can be compared with $k_{off} = 0.20$ s^{-1} and $k_{cat} = 1.1$ s^{-1} as the same first-order rate constant. Thus, the rate-determining step of CPP is the binding step to the substrate (k_{on}[CPP]), and the catalytic hydrolysis step (k_{cat}) is faster than the binding (k_{on}[CPP]) and release (k_{off}) processes. Therefore, it is important to grasp all kinetic parameters such as k_{on}, k_{off}, and k_{cat} on the one device to precisely understand the enzyme reactions.

pH-Dependences of Kinetic Parameters. Although CPP from *Penicillium janthinellum* is a serine protease, it usually exists

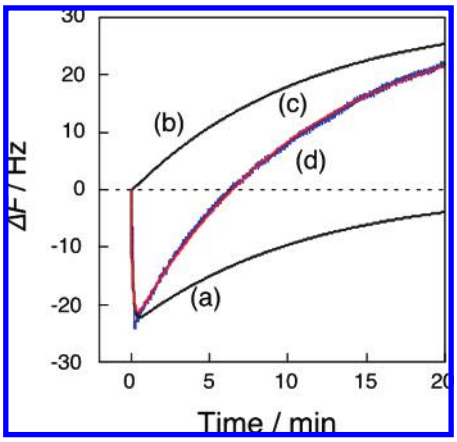


Figure 4. (a) The theoretical time-dependence of [ES] (ΔF_{ES}), (b) the theoretical time dependence of [P] (ΔF_P), and (c) ($\Delta F_{ES} - \Delta F_P$) as a red line (eq 5 in the text), and (d) the initial experimental curve a of Figure 2A as a blue line. The correlation coefficient of the fitting curve c was 0.998 58.

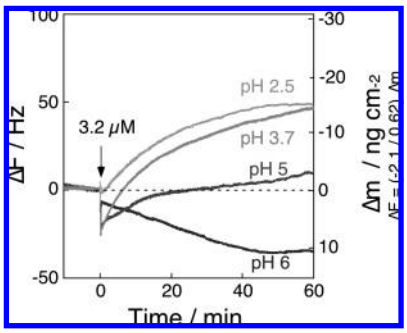


Figure 5. Effect of pH on frequency changes of the myoglobin–avidin-immobilized QCM, responding to the addition of CPP (50 mM citrate buffer, pH 2.5–6.0, 200 mM NaCl at 25 °C, [myoglobin] = 60 ng (3.5 pmol) cm^{-2} on the QCM, [CPP] = 3.2 μM).

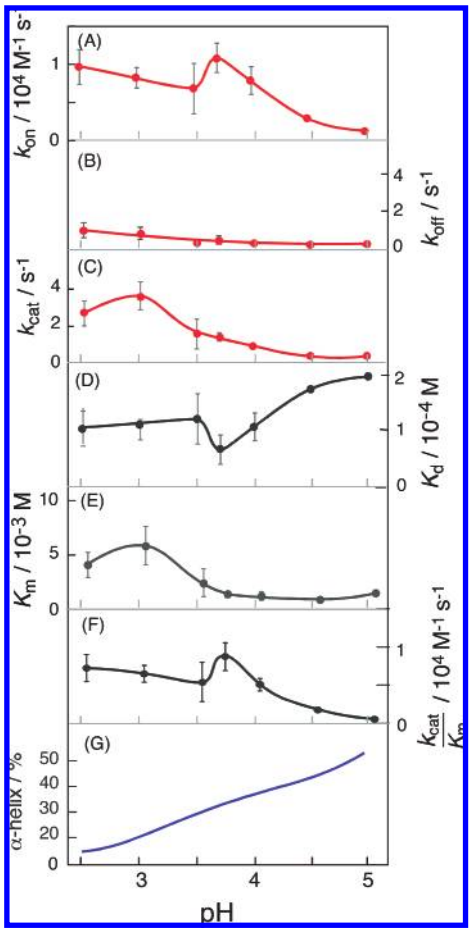


Figure 6. pH Dependences of all kinetic parameters of hydrolysis of myoglobin by CPP on the QCM. Values of (A) k_{on}, (B) k_{off}, and (C) k_{cat} were obtained by eqs 5–7 at the different pH. Values of (D) $K_d = k_{off}/k_{on}$, (E) $K_m = (k_{off} + k_{cat})/k_{on}$, and (F) k_{cat}/K_m were calculated from k_{on}, k_{off}, and k_{cat} values at the different pH. (G) pH Dependences of α-helix contents (%) of myoglobin obtained by CD spectra (at 222 nm) in the bulk solution (10 mM citrate, pH 2.5–5.0, 40 mM NaCl, at 25 °C).

in lysosomes and shows the hydrolytic activity in acidic conditions.[16] Myoglobin contains only α-helix structures and its conformation changes easily to the loose structure in the acidic condition. Therefore we expect that hydrolysis rates depend on pH changes due to both the optimum pH of the enzyme and the conformational change of the protein substrate.

Figure 5 shows typical time courses of hydrolyses of myoglobin immobilized on the QCM catalyzed by CPP in the range of pH = 2.5–6.0. Both the frequency decrease at first (the ES complex formation) and the following frequency increase (the catalytic hydrolysis) changed complicatedly depending on pH. CPP could bind to the substrate but hardly hydrolyzed it at pH 6.0. Each curve showing transient kinetics in the range of pH 2.5–5.0 was analyzed according to eqs 5–7 and obtained k_{on}, k_{off}, and k_{cat} values at each pH. They were plotted against pH in Figure 6, together with calculated vales of $K_d = k_{off}/k_{on}$, $K_m = (k_{off} + k_{cat})/k_{on}$, and k_{cat}/K_m. Helix contents of myoglobin in the range of pH = 2.5–5.0 were obtained from CD spectra at 222 nm and plotted in Figure 6G.

As shown in Figure 6, k_{on} and k_{cat}/K_m values showed a maximum at pH 3.7 and k_{cat} and K_m values increased with decreasing pH with a small peak at pH 3.0. The value of k_{off}, however, was independent of pH (Figures 6B). These complicated pH dependences must include both the optimum pH of the enzyme and the conformational change of the C-terminus of proteins. As shown in Figure 6G, the α-helix content of myoglobin decreases with decreasing pH, where enzymes can easily attack the substrate. The binding rate of CPP to myoglobin (k_{on}, the formation of the ES complex) showed a maximum at pH 3.7 with a gradual increase at the lower pH. This indicates that CPP itself shows a large affinity to myoglobin at pH 3.7, and the affinity gradually increases with the denaturation of the substrate at the lower pH. The release rate of CPP from the ES complex (k_{off}), however, did not depend on pH. As a result, the dissociation constant ($K_d = k_{off}/k_{on}$) showed a minimum at pH 3.7 (Figure 6D). The catalytic hydrolysis rates (k_{cat}) were gradually increased with decreasing pH due to the denaturation of the substrate. Since k_{cat} is larger than k_{off} in this reaction, the pH dependence of $K_m = (k_{off} + k_{cat})/k_{on}$ is mainly determined by k_{cat} (see Figure 6E). The apparent enzyme activity (second-order rate constant) is shown by $k_{cat}/K_m = k_{cat}(k_{on}/(k_{off} + k_{cat}))$, which is mainly determined by k_{on} when $k_{cat} > k_{off}$ (Figure 6F).

The apparent activity of CPP for peptide hydrolyses has been reported to show a maximum around pH 3.5 in the bulk solution.[15–18] Carboxypeptidase A (from Porcine pancreas, EC 3.4.17.1) and carboxypeptidase B (from Porcine pancreas, EC

3.4.17.2) recognize the terminus $-COO^-$ group of peptide substrates by the electrostatic interactions with the cationic Arg side chain in the enzyme and show the maximum activity around pH 7−9.[17,18] On the contrary, CPP recognizes the terminus −COOH group by a hydrogen bond network with Asn51 and Glu145 in the enzyme and can work in the acidic condition of lysosome.[18] Thus, the apparent maximum activity of CPP at pH 3.7 is explained by the maximum binding ability to the substrate (k_{on}) even in acidic conditions, but both k_{off} and k_{cat} are almost independent of pH. The gradual increase of k_{cat} at the lower pH is simply explained by the denaturation of the protein substrate. Therefore, in order to understand the complicated pH-rate profiles of protein hydrolyses by CPP, it is important to grasp all kinetic parameters in each enzyme reaction step (k_{on}, k_{off}, and k_{cat}) by using the QCM technique.

CONCLUSION

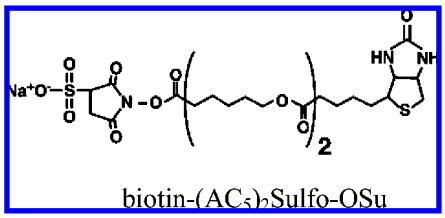
It is important to know kinetic parameters of each step of protease reactions in order to understand the precise reaction mechanism. The QCM technique will become a new tool to generally analyze enzyme reaction mechanisms as well as protease reactions, because each step of the formation and decay of the ES complex and the catalytic hydrolysis reaction could be directly observed as mass changes at the nanogram level. This technique is also useful to find effective inhibitors for protease as curatives.

EXPERIMENTAL SECTION

Reagents. Carboxypeptidase P (from *Penicillium janthinellum*, 51 kDa, EC 3.4.16.1, lot no. 1921, 2001, 2031) was purchased from TaKaRa Bio Inc. (Shiga, Japan). Myoglobin (from Equine heart, 17 kDa, lot no. 044K7006) and BSA (bovine serum albumin, lot no. 17F-0150) were purchased from Sigma-Aldrich Japan, Co. (Tokyo, Japan). Biotin-$(AC_5)_2$Sulfo-OSu and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) were purchased from DOJINDO, Co. (Kumamoto, Japan). *N*-Hydroxysuccinimide (NHS) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were purchased from Nacalai Tesque Co. (Kyoto, Japan) and used without further purification.

Preparation of Denatured BSA. S−S linkages of BSA were reduced by incubating in 0.35 M mercaptoethanol containing buffer solution (0.5 M Tris-HCl, 7 M GnHCl, 10 mM EDTA, pH 8.7) under bubbling with N_2 gas at 37 °C for 4 h.[23,24] Then reduced SH groups were carboxymethylated by the addition of iodoacetic acid (final concentration 18.7 mM) at 25 °C for 2 h. The denatured BSA was recovered by precipitation in acetone 3 times.

Biotinylation of Myoglobin, BSA, and Denatured BSA. Proteins (60 μM) were reacted with biotin-$(AC_5)_2$Sulfo-OSu (30 μM) in 200 μL of a buffer solution (10 mM HEPES, pH 8.0, 200 mM NaCl) for 30 min at room temperature. After addition of Tris buffer (250 mM Tris-HCl, pH 7.4) to quench the reaction, the solution was dialyzed to remove unreacted biotin compounds in a buffer solution (20 mM HEPES, pH 7.4, 200 mM NaCl). It was confirmed by MALDI TOF-MS (AXIMA CFR, Shimadzu, Co. Ltd, Kyoto) that one biotin group was reacted with an amino group of myoglobin (a biotinylated myoglobin, $M^+ = 17\,404.4$ and a native myoglobin, $M^+ = 16\,951.3$) or BSA (a biotinylated BSA, $M^+ = 67\,071.35$ and a native BSA, $M^+ = 66\,944.4$).



Hydrolysis of Myoglobin in the Bulk Solution. Hydrolyses of myoglobin (25−100 μM) catalyzed by CPP (1 μM) were carried out at pH 2.5−5.0 in 50 mM citrate buffer, 200 mM NaCl at 25 °C. A small sample solution was picked, and the unreacted protein was analyzed by the conventional SDS-PAGE method and MALDI TOF-MS (AXIMA CFR, Shimadzu, Co. Ltd, Kyoto, Japan) at every appropriate period. The amount of the produced amino acid was determined by the colorimetric ninhydrin test at 570 nm.[5,21]

CD Spectra Measurement of Myoglobin. CD spectra of myoglobin (1 mM) were measured in the buffer solution of 10 mM citrate, 40 mM NaCl, at 25 °C (J-720WI spectropolarimeter, JASCO, Co., Tokyo).

Immobilization of Avidin on a 27 MHz QCM Plate. Avidin (68 kDa) was covalently immobilized on the QCM plate as follows.[11] To the cleaned bare Au electrode, 3,3′-dithiodipropionic acid was immobilized and then carboxylic acids were activated as *N*-hydroxysuccinimidyl esters on the surface. Avidin was reacted with activated esters by mounting aqueous solutions on the QCM plate.

27 MHz QCM (AFFINX Q4) Setup and Its Calibration in Aqueous Solutions. AFFINIX Q4 was used as a QCM instrument (Initium Co. Ltd, Tokyo, Japan: http://www.initium2000.com) having four 0.5 mL cells equipped with a 27 MHz QCM plate (60 μm thickness and 8.7 mm diameter of a quartz plate and an area of 5.7 mm^2 of the Au electrode) at the bottom of each cell and the stirring bar with the temperature controlling system.[11,12] The Sauerbrey's equation (eq 8) was obtained for the AT-cut shear mode QCM in the air phase,[25]

$$\Delta F_{air} = -\frac{2F_0^2}{A\sqrt{\rho_q \mu_q}}\Delta m \qquad (8)$$

where ΔF_{air} is the measured frequency change in the air phase [in Hz], F_0 the fundamental frequency of the quartz crystal prior to a mass change [27×10^6 Hz], Δm the mass change [in g], A the electrode area [5.7 mm^2], ρ_q the density of quartz [2.65 g cm^{-3}], and μ_q the shear modulus of quartz [2.95×10^{11} dyn cm^{-2}]. In the air phase, a 0.62 ng cm^{-2} mass increase per 1 Hz of frequency decrease is expected. However, when the QCM is employed for binding of biomolecules in aqueous solutions, one must consider effects of hydration and/or viscoelasticity of biomolecules (eq 9).[19]

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(24) Kumar, N.; Kella, D.; Kinsella, J. E. *J. Biochem. Biophys. Methods* **1985**, *11*, 251−263.
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$$\Delta F_{water} = -\frac{\Delta F_{water}}{\Delta F_{air}} \frac{2F_0^{\,2}}{A\sqrt{\rho_q \mu_q}} \Delta m \quad (9)$$

We therefore directly calibrated the relationship between ΔF_{water} and Δm in aqueous solutions by changing the immobilized amount of proteins including avidin, biotinylated myoglobin, and biotinylated BSA on the Au electrode of the 27 MHz QCM. In the case of avidin, it covalently immobilized with the activated carboxyl groups on the QCM. When biotinylated proteins were injected onto the avidin-covered Au electrode of the QCM in aqueous solutions, frequency decreases (ΔF_{water}) due to the avidin−biotin interaction were followed. After binding had reached equilibrium, the QCM surface was dried in the air phase with silica gel, and ΔF_{air} reflecting a real mass change (Δm) was obtained. There was a good linear correlation between ΔF_{water} and ΔF_{air}(Δm) with a slope of 2.8 ± 0.1 for the immobilization of avidin, and 2.1 ± 0.2, 2.5 ± 0.2, and 4.7 ± 0.4 for the immobilization of biotinylated myoglobin, biotinylated BSA, and biotinylated denatured-BSA to the avidin-immobilized surface, respectively. Thus, frequency decreases (ΔF_{water}) due to the immobilization of proteins were 2.1−4.7 times larger than those in the air phase (ΔF_{air}), because hydrodynamic water vibrates with proteins. We assumed that the enzyme bound to the substrate vibrates similarly to the substrate on the QCM plate. Therefore, $\Delta F_{water}/\Delta F_{air}$ values for the combination of myoglobin or BSA and CPP were determined to be 2.1 ± 0.2 or 2.5 ± 0.2, respectively, and the factor of Sauerbrey's equation (eq 9) for the myoglobin hydrolysis or the BSA hydrolysis in aqueous solutions was obtained as 2.1/0.62 = −3.4 Hz per 1 ng cm^{-2} (0.30 ng cm^{-2} per −1 Hz) or 2.5/0.62 = −4.0 Hz per 1 ng cm^{-2} (0.25 ng cm^{-2} per −1 Hz), respectively (see vertical axes of Figure 2).

The noise level of the 27 MHz QCM was ±1 Hz in buffer solutions at 25 °C, and the standard deviation of the frequency was ±2 Hz for 1 h in buffer solutions at 25 °C. A sensitivity of 0.25−0.30 ng/cm^2 per −1 Hz is sufficiently large to sense the binding of enzymes.

Enzyme Reactions on the QCM Plate. A protein-immobilized QCM cell was filled with 0.5 mL of assay buffer (50 mM citrate buffer, pH 2.0−6.0, 200 mM NaCl at 25 °C) until the resonance frequency was held constant (±1 Hz for 30 min). Frequency changes in response to the addition of the enzyme were followed with time. The solution was vigorously stirred to avoid any effect from the slow diffusion of enzymes. The stirring did not affect the stability and magnitude of frequency changes.

APPENDIX

To obtain a theoretical curve (curve c in Figure 4 and eqs 5−7), we applied extensive Michaelis theory represented by Briggs and Haldane (eqs 1−3).[9,26] In the QCM experimental condition, [E] is excessively large compared with [S], [ES], and [P], which are concentrations at time t of the enzyme, the substrate, the enzyme−substrate complex, and the product, respectively. [E] is nearly equal to $[E]_0$ and [S] is represented by $[S]_0 - [ES] - [P]$, where $[E]_0$ and $[S]_0$ are the initial concentrations of the enzyme and the substrate, respectively. The formation rate of ES (eq 2) is revised to be eq 10.

$$\frac{d[ES]}{dt} = k_{on}[E]_0([S]_0 - [ES] - [P]) - (k_{off} + k_{cat})[ES] \quad (10)$$

From eqs 3 and 10, the second-dimensional linear differential equation is obtained as eq 11.

$$\frac{d^2[ES]}{dt^2} + (k_{on}[E]_0 + k_{off} + k_{cat})\frac{d[ES]}{dt} + k_{on}k_{cat}[E]_0[ES] = 0 \quad (11)$$

The solution of the differential equation (eq 11) can be given by eqs 6 and 7, considering [ES] = 0 and $d[ES]/dt = k_1[E]_0[S]_0$ at time $t = 0$.

When an experimental curve is fitted with eqs 5−7 as a theoretical curve, parameter values of X, Y, Z, and k_{cat} can be determined. Then, values of k_{on} and k_{off} are obtained from eqs 12 and 13.

$$X + Y(= A) = k_{on}[E]_0 + k_{off} + k_{cat} \quad (12)$$

$$XY(= B) = k_{on}k_{cat}[E]_0 \quad (13)$$

Thus, all kinetic values of k_{on}, k_{off}, and k_{cat} can be given by only one experimental curve.

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