Catalytic Hydrolysis of Peptides by Cerium(IV)

Tohru Takarada,[a] Morio Yashiro,[b] and Makoto Komiyama*[a]

Abstract: Oligopeptides are efficiently hydrolyzed by Ce^{IV} to the corresponding amino acids under mild conditions. The pseudo first-order rate constants for the hydrolysis of H-Gly-Phe-OH and H-Gly-Gly-OH at pH 7.0 and 50 °C are 3.5×10^{-1} and $2.8 \times 10^{-1}\ h^{-1}$, with $[Ce(NH_4)_2(NO_3)_6]_0 = 10\ mM$ (the half-lives are 2.0 and 2.5 h). The catalytic activity of the Ce^{IV} is far greater than those of other lanthanide ions and non-lanthanide ions. No oxidative cleavage was observed under the reaction conditions. Catalytic turnover of the Ce^{IV} was also evidenced. The hydrolysis is fast especially when the substrates have no metal-coordinating side chains. Tripeptides and tetrapeptides are hydrolyzed at the similar rates as the dipeptides. In the hydrolysis of tripeptides, the amide linkage near the N-terminus is preferentially hydrolyzed. Neither the *N*-carbobenzyloxy derivative nor the amide of H-Gly-Phe-OH is hydrolyzed to a measurable extent, showing that both the terminal amino group and the carboxylate are coordinated to the Ce^{IV} ion. This complexation is further confirmed by ^1H NMR spectroscopy. The Ce^{IV} ion is therefore one of the most active catalysts for peptide hydrolysis.

Keywords: catalysts · cerium · hydrolyses · lanthanides · peptides

Introduction

Preparation of artificial peptidases has been one of the most attractive subjects, since they are valuable tools for future biotechnology. However, amide bonds in proteins are extremely stable and resistant to hydrolysis. The intrinsic half-life of the amide linkage in Gly-Gly in neutral solutions at 25 °C, for example, is estimated to be 350 years.[1] Remarkably active catalysts are required to hydrolyze peptides under physiological conditions.

To date, Cu^{II}, Zn^{II}, Ni^{II}, and Co^{III} complexes for the hydrolysis of activated amides were prepared, and useful information on the catalytic mechanisms of natural enzymes was provided.[2–5] The N-terminal amide bond of peptide was selectively cleaved by Co^{III} complexes.[4] Recently, still greater catalytic activity and higher selectivity have been achieved.[6–15] Kostic et al. reported Pd^{II} and Pt^{II} complexes which hydrolyze peptides and proteins site selectively.[6] The scission occurs at the carboxyl sides of Met, His, or Trp residues, since they bind the metal complexes. A macrocyclic Cu^{II} complex for the hydrolysis of dipeptide and bovine serum albumin was presented by Burstyn et al.[7] At pH 8.1 and 50 °C, Gly-Gly (short for H-Gly-Gly-OH, unless stated otherwise) was hydrolyzed in 15% conversion within seven days. With Fe^{II}-EDTA,[9–13] Ni^{II}-(Gly-Gly-His),[14] or Cu^{I}-1,10-phenanthroline complexes[15] bound to the target position, proteins were cleaved site specifically. In spite of these remarkable progresses, further increase in the catalytic activity is desirable for versatile applications.

The present article reports that Ce^{IV} ion efficiently hydrolyzes oligopeptides under mild conditions.[16–18] In neutral solutions at 50 °C, the half-life of amide linkages is only a few hours. The overwhelming advantage of this metal ion over other metal ions is being substantiated in the following. The catalytic turnover of the Ce^{IV} is shown. Furthermore, the mechanism of Ce^{IV} catalysis is proposed on the basis of kinetic and spectroscopic evidence.

Results

$Ce(NH_4)_2(NO_3)_6$-induced hydrolysis of dipeptides: Figure 1 shows the HPLC spectra for the hydrolysis of Gly-Phe by $Ce(NH_4)_2(NO_3)_6$ at pH 7.0 and 50 °C. The dipeptide is rapidly cleaved, and the hydrolytic products (1:1 mixture of Gly and Phe) are formed (Figure 1a: the products are detected by the post-column *o*-phthalaldehyde method). Any by-products, assignable to oxidative cleavage of the substrates, can not be detected at all [this fact has been further confirmed by

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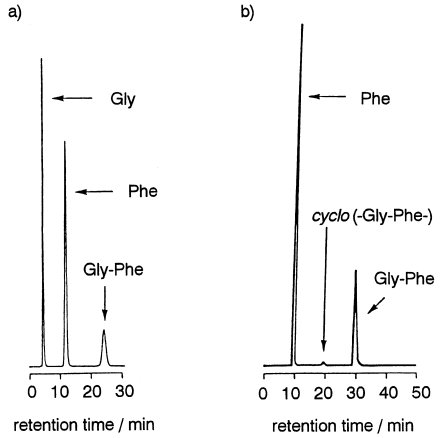
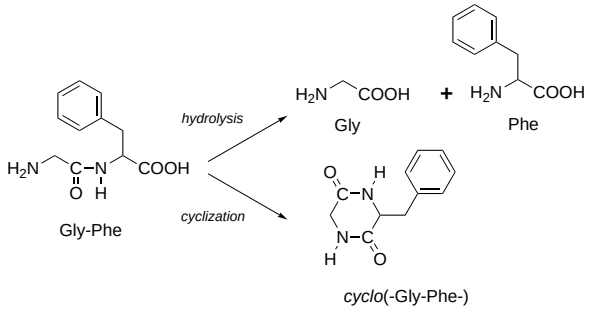


Figure 1. HPLC patterns for the hydrolysis of Gly-Phe by $Ce(NH_4)_2(NO_3)_6$ at pH 7.0 and 50 °C for 2.0 h: a) ion-exchange mode with the detection by the post-column *o*-phthalaldehyde method and b) reversed-phase mode with a UV detector (257 nm). $[Gly\text{-}Phe]_0 = [Ce(NH_4)_2(NO_3)_6]_0 = 10$ mM in 0.1 M HEPES buffer. In a), *cyclo*(-Gly-Phe-) without an amino group is not detectable, whereas in b) Gly is not detectable at 257 nm.

^{1}H NMR spectroscopy (data not shown)], although the Ce^{IV} is a well-known oxidation reagent. The cleavage proceeds via the hydrolysis of the amide linkage. In the reaction mixtures, most of the Ce^{IV} exist as the gel of metal hydroxide.

The only by-product of the present reactions is the cyclic dimer, which is formed by the intramolecular condensation between the carboxylate and the amino group (Scheme 1).[19]



Scheme 1. The hydrolysis of Gly-Phe and its cyclization to *cyclo*(-Gly-Phe-).

This cyclic dimer is detected by the UV detector (Figure 1b), since the post-column *o*-phthalaldehyde method is not applicable here. However, the formation of *cyclo*(-Gly-Phe-) is far slower than the hydrolysis of amide linkage (its yield is only 0.8 mol%, when 55 mol% of Gly-Phe is cleaved). The Ce^{IV} ion does not promote the cyclization (because in the absence of the Ce^{IV}, *cyclo*(-Gly-Phe-) is formed at almost the same rate). This is a great advantage of the Ce^{IV} for the peptide hydrolysis, since most of the other metal ions notably enhance the formation of the cyclic dimers (see below). According to control experiments, the cyclic dimers are not hydrolyzed by $Ce(NH_4)_2(NO_3)_6$ at measurable rates. Thus, the possibility that they are the reaction intermediates for the amide hydrolysis can be ruled out.

Other Ce^{IV} salts ($Ce(NH_4)_4(SO_4)_4$, $Ce(SO_4)_2$, and $Ce(OH)_4$) also hydrolyze the dipeptides at pH 7.0 and 50 °C, but are much less active than $Ce(NH_4)_2(NO_3)_6$ (the conversions at 2 h are 7.1, 0.3, and 4.5 mol%, respectively). The smaller activity of $Ce(NH_4)_4(SO_4)_4$ is ascribed to the competitive inhibition by SO_4^{2-}. Consistently, the catalysis by $Ce(NH_4)_2(NO_3)_6$ is notably suppressed by Na_2SO_4.[20] $Ce(SO_4)_2$ and $Ce(OH)_4$ are virtually insoluble in water. In all the following sections, $Ce(NH_4)_2(NO_3)_6$ is used as the Ce^{IV} salt.

Pseudo first-order kinetics and catalytic turnover of the Ce^{IV}: The reaction mixtures involve significant amounts of gel of Ce^{IV} hydroxide. Yet, all the reactions show fair pseudo first-order kinetics (the typical time-conversion curve is presented in Figure 1 in the Supporting Information). Catalytic turnover of the Ce^{IV} is clearly shown by the following experiments: At pH 7.0 and 50 °C, 10 mM Gly-Phe is treated with a 2 mM $Ce(NH_4)_2(NO_3)_6$ solution. After two days, 44 mol% of the substrate (2.2 equivalents with respect to the Ce^{IV}) is converted to Gly and Phe.

Substrate specificity: The rate constants (in 10^{-1} h^{-1}) for the hydrolysis of dipeptides at pH 7.0 and 50 °C are as follows: Phe-Gly (4.1) > Ala-Phe (3.9) > Gly-Phe (3.5) > Gly-Gly (2.8) > Gly-Ala (2.5) > Gly-Ser (2.3) > Gly-Trp (2.0) > Ser-Gly (1.9) = Leu-Phe (1.9) > His-Phe (1.4) > Lys-Phe (1.2) > Met-Phe (1.1) > Phe-Ser (0.9) > Phe-Phe (0.8) = Gly-Tyr (0.8) > Gly-Asp (0.6) > Arg-Phe (0.4) > Ser-Phe (0.3). In contrast to the efficient hydrolysis of Gly-Phe, *N*-(carbobenzyloxy)-Gly-Phe (Cbz-Gly-Phe), the amide of Gly-Phe (Gly-Phe-NH_2), and the amide of Cbz-Gly-Phe (Cbz-Gly-Phe-NH_2) are not hydrolyzed by the Ce^{IV} as can be determined within the experimental limits (Table 1). Both the amino group at the N-terminus and the carboxylate at the C-terminus are essential for the hydrolysis of this dipeptide with no metal-coordinating side chain.

Table 1. The rates of hydrolysis of dipeptide derivatives by Ce^{IV} at pH 8.0 and 50 °C.[a]

Substrate	Conversion [mol%]
Gly-Phe	62
Gly-Phe-NH_2	0
Cbz-Gly-Phe	0
Cbz-Gly-Phe-NH_2	0
Asp-Phe-NH_2	40
Asp-Phe	19

[a] The reaction time = 72 h. In 0.1 M TRIS buffer, $[peptide]_0 = [Ce(NH_4)_2(NO_3)_6]_0 = 10$ mM.

Quite interestingly, Asp-Phe-NH_2 is efficiently hydrolyzed by Ce^{IV}, even though the C-terminal carboxylate is protected (Table 1). Its hydrolysis rate is even greater than that of Asp-Phe. The carboxylate in the side chain of the Asp residue in Asp-Phe-NH_2 binds the Ce^{IV} in place of the carboxylate at the C-termini of unprotected dipeptides.

Hydrolysis of oligopeptides: Oligopeptides (Gly-Gly-Gly, Gly-Gly-Phe, Phe-Gly-Gly, and Gly-Gly-Gly-Gly) are hydro-

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lyzed by the Ce^{IV} at the similar rates (the rate constants for the disappearance of the substrates are 2.2×10^{-1}, 1.9×10^{-1}, 1.3×10^{-1}, and 1.9×10^{-1} h^{-1}, respectively). In the hydrolysis of Gly-Gly-Phe, the main products (in the early stage of the reaction) are Gly-Phe and Gly (Gly-Gly and Phe are the minor products). The amide linkage near the N-terminus is cleaved preferentially (a similar selectivity is observed in the hydrolysis of Phe-Gly-Gly). The rate constants for the scission of the amide linkages at the N-terminus of Gly-Gly-Phe and in its C-terminus are 1.7×10^{-1} and 0.2×10^{-1} h^{-1}, respectively (these values are determined by analyzing the time-course of the hydrolysis, as presented in Figure 2 in the Supporting Information).

Catalytic activities of various metal ions: The catalytic activities of metal ions for the hydrolysis of Gly-Phe are in the following order: $Ce^{IV} \gg Zr^{IV}$, $Hf^{IV} > Eu^{III} > Gd^{III}$, Tb^{III}, Tm^{III}, Yb^{III}, $Y^{III} > Pr^{III}$, Sm^{III}, Dy^{III}, Ho^{III}, Er^{III}, $Lu^{III} > Nd^{III} > Zn^{II} > La^{III}$, Fe^{II}, $Mn^{II} > Sc^{III}$, Ni^{II}, Sn^{II} (the open bars in Figure 2).[21] The activity of Ce^{IV} is overwhelmingly greater

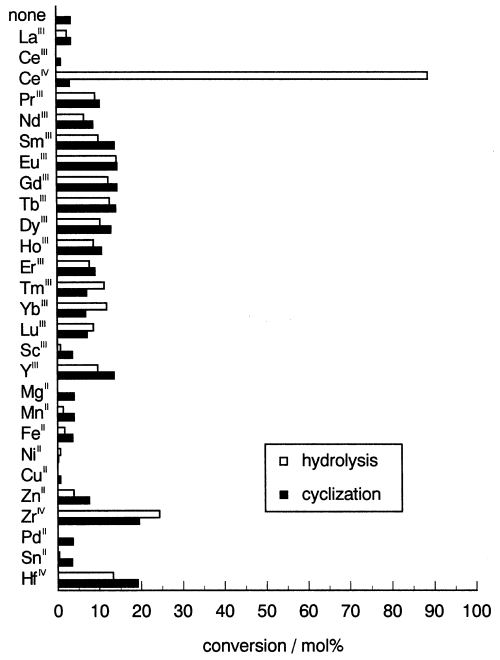

Figure 2. Catalytic activities of metal ions for the hydrolysis of Gly-Phe at pH 7.0 and 80 °C: $[Gly\text{-}Phe]_0 = [metal\ salt]_0 = 10$ mM in 0.1 M TRIS buffer. The open bars show the yields of the hydrolysis products at the reaction time 24 h. The activities for the formation of *cyclo*(-Gly-Phe-) are presented by the closed bars. The metal chlorides were used, except for $Ce(NH_4)_2(NO_3)_6$ (see ref. [21]). Experimental error in each of the yields is within 5%.

than those of the other metal ions. A high reaction temperature (80 °C) was chosen, since otherwise these metal ions (except for the Ce^{IV}) hardly catalyze the peptide hydrolysis at all. The superiority of the Ce^{IV} is still more explicit at lower temperatures. The possibility that the formation of a gel is the main reason for the remarkable activity of Ce^{IV} is unlikely, since Zr^{IV}, Hf^{IV}, Pd^{II}, and Sn^{II} also form gels under the reaction conditions but even though are far less active than Ce^{IV}.

More significantly, Ce^{IV} does not catalyze the formation of the cyclic dimers (see the closed bars in Figure 2). At the reaction time of 24 h (92 mol% of the Gly-Phe is consumed by then), the yield of *cyclo*(-Gly-Phe-) is only 3.2 mol%. This value is almost identical with that (3.5 mol%) in the absence of the metal ion. In contrast, Zr^{IV}, Hf^{IV}, and Eu^{III}, which are rather active in peptide hydrolysis, notably promote the cyclization. The rates of formation of the cyclic dimers are almost the same as (or even greater than) those of hydrolysis of amide bond. With respect to both the activity and the selectivity, the Ce^{IV} ion is superb for dipeptide hydrolysis in neutral solutions.

Kinetic study on the hydrolysis of Gly-Phe by $Ce(NH_4)_2(NO_3)_6$: The rate constant (k_{obs}) increases monotonously with increasing $[Ce(NH_4)_2(NO_3)_6]_0$ concentration, when the initial concentration of Gly-Phe is kept constant at 10 mM (Figure 3).

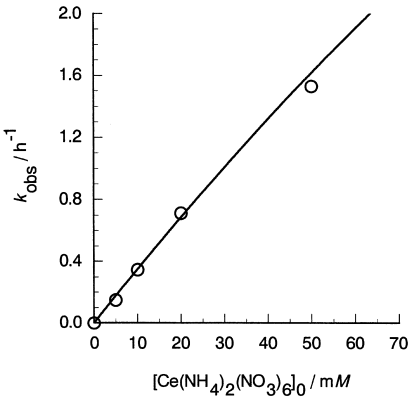

Figure 3. Plot of the rate constant for the hydrolysis of Gly-Phe versus $[Ce(NH_4)_2(NO_3)_6]_0$ at pH 7.0 and 50 °C in 0.1 M HEPES buffer: $[Gly\text{-}Phe]_0 = 10$ mM. The solid line represents the theoretical relationship, which has been calculated by taking the formation constant of the substrate-Ce^{IV} complex as 2.1 M^{-1}.

According to Lineweaver–Burk analysis, the formation constant of the Ce^{IV}-substrate complex is 2.1 M^{-1}.[22] The pH rate constant profile (the open circles in Figure 3, Supporting Information) is composed of the pH-dependent region at pH 4.5–7.0 and the pH-independent region at pH 7.0–8.5. All the experimental points fit nicely the theoretical line, which has been obtained under the assumption that the reaction is governed by a species with pK_a 6.2.

The activation energy, determined at 40–70 °C and pH 7.0 in 0.1 M HEPES buffer, is 15 kcal mol^{-1}. By using this value, the rate constant at pH 7.0 and 25 °C is estimated to be 4.7×10^{-2} h^{-1} (the half-life is 15 h). The acceleration by the $Ce(NH_4)_2(NO_3)_6$ (10 mM) is more than 10^5 fold (the rate constant for uncatalyzed hydrolysis of Gly-Gly is 2.2×10^{-7} h^{-1} at pH 6.8 and 25 °C).[1]

Pr^{III}-catalyzed hydrolysis of Gly-Phe (^1H NMR spectroscopy on the complex formation and kinetic study): In order to shed light on the reaction mechanism, the complex formation between Gly-Phe (and also of other dipeptides) and Pr^{III} ion has been analyzed by ^1H NMR spectroscopy. Furthermore, the pH dependence of the rate of Gly-Phe hydrolysis has been

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determined. Pr^{III} is useful in this experiment, since it is active in the dipeptide hydrolysis, and, in addition, provides totally homogeneous reaction mixtures (the Ce^{IV} system is more complex, because of gel formation).

The pH dependence of the Pr^{III}-induced changes in the chemical shifts of Gly-Phe is summarized in Figure 4. At pD 7.4, the protons of both the Gly and the Phe units show

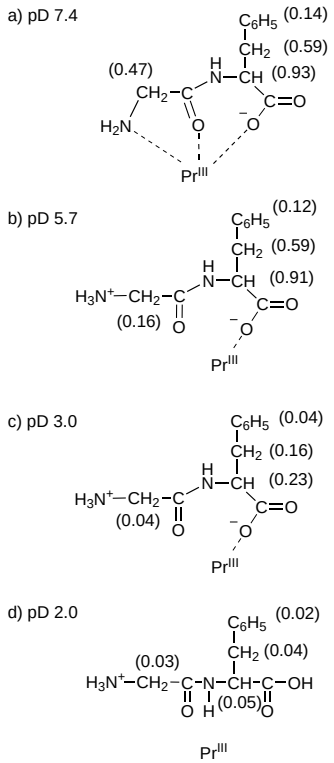

Figure 4. Pr^{III}-induced ^1H NMR shift changes of Gly-Phe in D_2O. The numbers show the chemical shifts [ppm] toward the lower magnetic field. $[Gly\text{-}Phe]_0 = [PrCl_3]_0 = 20\,mM$.

marked downfield shifts. With decreasing pD, the shift for the Gly unit significantly decreases ($\delta = 0.47$ at pD 7.4, but $\delta =$ 0.16 at pD 5.7). The shift for the α-proton of the Phe unit, however, is kept almost constant in this pH region ($\delta = 0.93$ at pD 7.4 and $\delta = 0.91$ at pD 5.7), and notably decreases only when the pD is lowered to 3.0. The methylene protons and the phenyl protons in the Phe show almost the same pH dependence. Apparently, the Gly-Phe/Pr^{III} complex involves the coordination of both the amino group at the N-terminus and the carboxylate at the C-terminus. The coordination of the amino group to the Pr^{III} is largely suppressed when it is protonated below around pD 6. On the other hand, the carboxylate retains its deprotonated form (and is efficiently coordinated to the Pr^{III}), as long as the pD is greater than three.

The chemical shift of the methylene protons of the Gly significantly changes when the pD decreases from 7.4 to 5.7, since the carbonyl oxygen of the substrate is effectively coordinated to the Pr^{III} at pD 7.4 but not at pD 5.7. For a coordination of this carbonyl residue to Pr^{III}, the terminal amino group must be in its neutral form and has to be coordinated to the Pr^{III} together with the carboxylate ion (this activated amide residue is hydrolyzed by the Pr^{III}, as described in the Discussion). If the carbonyl coordination was to be taking place at pD 5.7, the lanthanide-induced shift should be kept almost constant from pD 7.4 to 5.7 (under these conditions, the mutual position of this methylene residue, with respect to the Pr^{III}, does not dependent very much on whether the amino group is coordinated to the Pr^{III} or not). The pH dependence of the rate for the Pr^{III}-catalyzed hydrolysis of Gly-Phe is depicted by the closed circles in Supporting Information Figure 3. This profile is similar in shape to the Ce^{IV}-catalyzed reaction (the open circles), and shows that the catalysis is governed by a group with pK_a 7.1.

The chemical shifts for Gly-Phe, Ala-Phe, and Leu-Phe are almost identical with each other, whereas Arg-Phe shows considerably smaller shifts (Table 2). The positive charge in the Arg residue is unfavorable for the complex formation with Pr^{III}.

Table 2. Pr^{III}-induced ^1H NMR shift changes of the α-protons in dipeptides at pD 7.4.[a]

	Chemical-shift change [ppm]	
Dipeptide	N-terminus side	C-terminus side
Gly-Phe	0.47	0.93
Ala-Phe	0.40	1.01
Leu-Phe	0.39	1.11
Arg-Phe	0.26	0.58

[a] $[Dipeptide]_0 = [PrCl_3]_0 = 20\,mM$. All the shift changes are toward the lower magnetic field.

Discussion

Substrate specificity: In Figure 5, the rate constants of hydrolysis of dipeptides X-Phe a) and Gly-X b) are plotted as the functions of molecular volume of the amino acid X.[23] In Figure 5a, the amino acid in the C-terminal side is fixed to Phe, and the other is varied. The rate constant decreases monotonically with increasing molecular volume of X, as long as the X has no metal-coordinating side chain (Gly, Ala, Leu, and Phe: open circles). The activity of the sequential isomer Phe-Gly also fits the straight line (closed circle). The steric factor is dominant, when one of the components of the dipeptides is Phe and is rather bulky. In Figure 5b, however, one of the components is fixed to Gly. Quite interestingly, the bulkiness of X causes no notable effect here. Although Phe is considerably more bulky than Gly, Gly-Phe is hydrolyzed at the comparable rate as Gly-Gly. To achieve an efficient catalysis, at least one of the amino acids in dipeptides has to be less bulky. When the X has a metal-coordinating side chain (His, Lys, Met, Tyr, and Ser), however, this relationship is not observed (open triangles in Figure 5). Some of the dipeptides are then hydrolyzed considerably slower than the dipeptides composed of two non-coordinating amino acids, whereas the others are hydrolyzed with comparable rates.

Proposed mechanism of peptide hydrolysis by the Ce^{IV}: The mechanism of the hydrolysis of dipeptides (without a metal-coordinating side chain) by the Ce^{IV} is depicted schematically in Figure 6. First, the dipeptide forms a complex with the Ce^{IV} in the hydroxide gel. The coordinating sites are 1) the amino

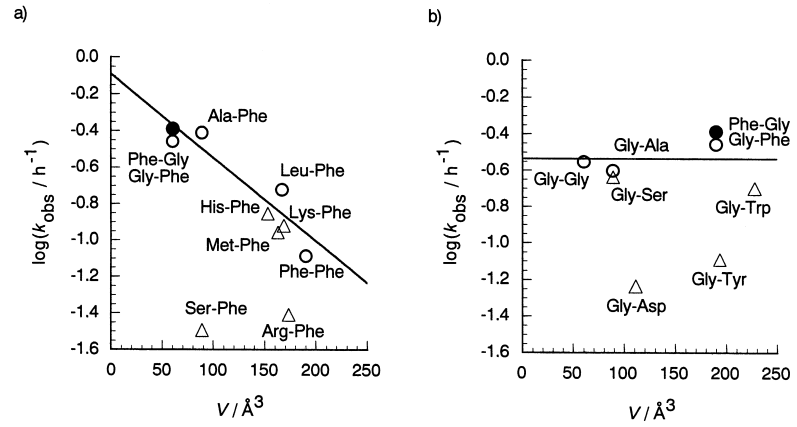

Figure 5. Dependence of the rate constant for the hydrolysis of a) X-Phe and b) Gly-X on the molecular volume V of the amino acid X. The open circles are for the dipeptides without metal-coordinating side chains, whereas the triangles are for the dipeptides with them. The results for the hydrolysis of Phe-Gly are also presented (the closed circles).

group at the N-terminus, 2) the carboxylate at the C-terminus, and 3) the carbonyl oxygen of the amide bond cleaved. The essential roles of both the N-terminal amino group and the C-terminal carboxylate are obvious from Table 1. Through the assistance of the coordination of these two terminal groups, the carbonyl group can be efficiently coordinated to the Ce^{IV}. The ^1H NMR analysis on the complex formation between Gly-Phe and Pr^{III} (Figure 4) is totally consistent with these arguments. Due to this complex formation, the electrophilicity of the carbonyl carbon of the amide linkage is enhanced. Thus, this carbon is efficiently attacked by hydroxide ion, forming a tetrahedral intermediate.[24] When the highly unstable leaving group (R-NH^-) is removed from the carbonyl carbon, the water bound to the Ce^{IV} ion acts as an acid catalyst and stabilizes it.[25] In the hydrolysis of tripeptides, the terminal amino group and carboxylate are also coordinated to the Ce^{IV}. The amide linkage near the N-terminus is preferably hydrolyzed, because a stable five-membered ring is formed when the corresponding carbonyl group is coordinated to the Ce^{IV} (together with the terminal amino group). Note that this carbonyl-coordination requires the simultaneous coordination of the amino group (see Figure 4). If the carbonyl group near the C-terminus is coordinated to the Ce^{IV}, however, a less stable seven-membered ring must be formed.

The proposed mechanism is consistent with the pH-rate constant profile (the open circles in Figure 3 in the Supporting Information). A pK_a 6.2 corresponds to the protonation of the amino group at the N-terminus (for the catalysis, this group must be unprotonated and coordinated to the Ce^{IV}). This pK_a is significantly smaller than its intrinsic value (8.08),[26] because the protonation of this particular amino group is suppressed by the positive charges of the Ce^{IV} clusters. At physiological pH, most of the substrate exists as the active monoanion. The carboxylate of the dipeptide is in its anionic form throughout the pH range investigated. According to potentiometric titration, the Ce^{IV} hydroxide releases three protons up to pH 3 and shows no ionization at pH 4–9. All these arguments are completely consistent with the kinetic and spectroscopic results on the Pr^{III}-catalyzed hydrolysis of Gly-Phe.

It is noteworthy that the deprotonation of N–H group in the amide bond, which makes the amide insusceptible to hydrolysis,[27] hardly takes place up to pH 8 (the highest pH investigated). The coordination of the Ce^{IV} ion to the nitrogen atom is unfavorable, since this metal ion intrinsically prefers oxygen ligands to nitrogen ligands.[28] Thus, the present amide hydrolysis is successfully achieved at around pH 7. In the amide hydrolysis by Cu^{II}, Ni^{II}, and Pd^{II} ions, however, the N–H deprotonation is dominant above pH 6.[29] For example, Cu^{II} is almost completely inactive in this pH region, although it is somewhat active in acidic solutions.

When a metal-coordinating side chain in the substrate, if any, is coordinated to the Ce^{IV}, the coordination of carbonyl residue of the substrate to the Ce^{IV} is suppressed and/or the structure of complex is perturbed. The magnitudes of these factors are notably dependent on the structure of substrate. The slow hydrolysis of Arg-Phe is ascribed to the poor binding of this positively charged dipeptide to the Ce^{IV}, as indicated by the ^1H NMR spectroscopy (see Table 2). In the hydrolysis of Gly-Asp, both the C-terminal carboxylate and the carboxylate in the side chain of Asp are coordinated to the Ce^{IV} and the resultant decrease in the net positive charge on the Ce ion diminishes the activation of the substrate. These arguments are supported by the fact that Asp-Phe-NH_2, in which only the carboxylate in the side chain binds the Ce^{IV}, is hydrolyzed efficiently (Table 1).[30] The hydrolysis of Ser-Phe is slower

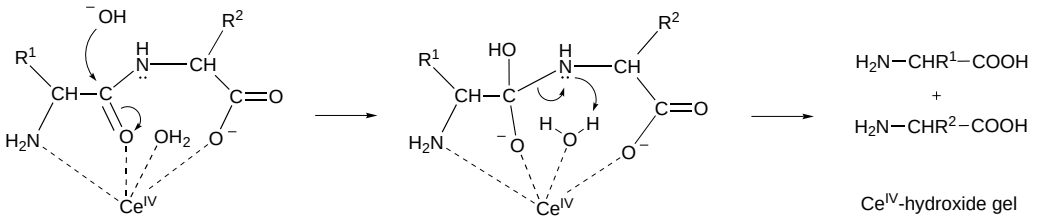

Figure 6. Proposed mechanism for the Ce^{IV}-catalyzed hydrolysis of the dipeptide without a metal-coordinating side chain. The hydroxide ion can be replaced by the metal-coordinated hydroxide (see ref. [24]). For clarity, the several Ce^{IV} ions adjacent to the catalytic center in the gel, which assist the catalysis, are omitted.

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than expected from the straight line in Figure 5a. Probably, the OH group of Ser interacts with the Ce^{IV} (or with its coordination water) and is fixed near the carbonyl residue. Notable steric hindrance is induced around the reactive center, since the other amino acid residue Phe is bulky. In Gly-Ser, however, the counterpart Gly is small and thus the effect is marginal (Figure 5b). A similar argument has been made on the hydrolysis of dipeptides with no metal-coordinating side chain (see above). Consequently, Ser-Gly is hydrolyzed at a similar rate ($k_{obs} = 1.9 \times 10^{-1}\,h^{-1}$) as Gly-Ser ($2.3 \times 10^{-1}\,h^{-1}$), whereas the hydrolysis of Phe-Ser is slow ($0.9 \times 10^{-1}\,h^{-1}$).

Anatomy of the enormous catalytic activity of the Ce^{IV} for peptide hydrolysis: In Figure 7, the catalytic activities of various metal ions for the hydrolysis of Gly-Phe are plotted as

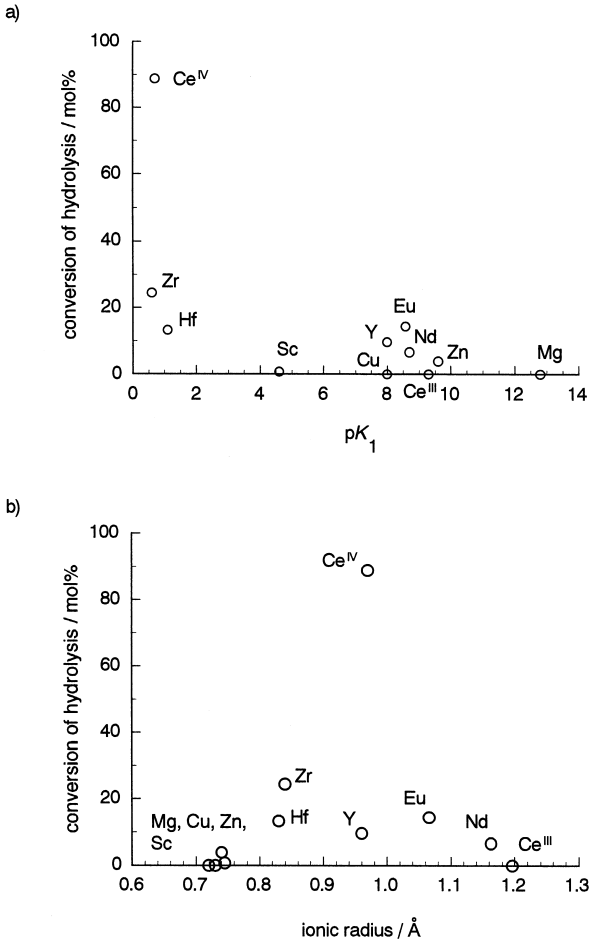

Figure 7. Plots of the catalytic activities for Gly-Phe hydrolysis of various metal ions against a) the pK_a of the metal-bound water and b) the ionic radius of the metal ion. The data in Figure 2 are used (some of the metal ions are removed to avoid making the figures too complicated).

the function of either a) the pK_1 of the corresponding metal-bound water[31] or b) the ionic radius of the metal ion.[32] In both of these plots, there is no explicit correlation, and only the Ce^{IV} is far beyond the other lanthanide ions and non-lanthanide ions. The superiority of this metal ion cannot be interpreted simply in terms of these physicochemical properties. The formation constant of the Gly-Phe/Ce^{IV} complex ($2.1\,M^{-1}$) is considerably smaller than the values of the Gly-Gly complexes of Cu^{II}, Zn^{II}, and Ni^{II} (400000, 1300, and $10000\,M^{-1}$),[29a] which are much less active than is the Ce^{IV}.

Presumably, the Ce^{IV} withdraws electrons from the amide linkage (and promotes the electrophilicity of the carbonyl carbon) much more efficiently than the other metal ions. This is mainly due to the stability of the trivalent state of Ce.[33, 34] The trivalent states of Zr and Hf, as well as divalent states of lanthanide metals, are too unstable, and thus the electron-withdrawal by Zr^{IV}, Hf^{IV}, and the lanthanide(III) ions is not as effective. This argument is also supported by the fact that the Ce^{III} ion is inactive in the hydrolysis of dipeptides (Figure 2). The large coordination number of the Ce^{IV} (usually 8)[28] is favorable in the present catalysis as well. At least one of the coordination-water molecules is oriented appropriately for the catalysis. In the gel of Ce^{IV} hydroxide, a number of metal ions are accumulated so that their positive charges suppress the protonation of the terminal amino group of substrate by electrostatic repulsion. Furthermore, the transition state of the reaction, which is negatively charged, is stabilized by the positive charges. Finally, the cyclization of the dipeptides to cyclic dimers is minimized, since the Ce^{IV} ions adjacent to the catalytic center in the gel also interact with the terminal amino group of the dipeptides and decrease the nucleophilicity of this amino group.

Conclusion

The Ce^{IV} ion efficiently catalyzes the hydrolysis of oligopeptides under mild conditions. The half-lives of Gly-Phe, Gly-Gly-Phe, and Gly-Gly-Gly-Gly at pH 7.0 and 50 °C (with 10 mM $Ce(NH_4)_2(NO_3)_6$) are only 2.0, 3.7, and 3.7 h, respectively. The hydrolysis is fast especially when the substrates have no metal-coordinating side chains. In the hydrolysis of tripeptides, the amide linkage near the N-terminus is hydrolyzed preferentially. The Ce^{IV}-catalyzed hydrolysis of peptides proceeds via the coordination of the terminal amino group and the carboxylate. The deprotonation of the amide residue hardly takes place at physiological pH so that the amide bond is efficiently hydrolyzed. The Ce^{IV} ion is one of the most active catalysts for peptide hydrolysis, and promising as the active sites of artificial peptidases.

Experimental Section

Materials: Gly-Phe was obtained from Nacalai, Gly-Gly-Gly and Gly-Gly-Gly-Gly from Tokyo Kasei, *cyclo*(-Gly-Phe-) from Bachem, and all other peptides from Sigma. $Ce(NH_4)_2(NO_3)_6$ and $Ce(NH_4)_4(SO_4)_4$ (from Nacalai), $Ce(SO_4)_2$ (from Kojima), and $Ce(OH)_4$ (from Soekawa) were used without further purification. The lanthanide(III) salts (in the forms of chloride) were obtained from Soekawa, except for $LaCl_3$ from Nacalai. All other metal salts were purchased from Nacalai. D_2O (99.9 atom % D) and DCl (99.5 atom % D) were obtained from Aldrich. Highly purified and deionized water was used for the kinetic studies. Throughout the present study, a great care was taken to avoid contamination by natural enzymes; their absence was confirmed by careful control experiments.

Kinetic analysis of peptide hydrolysis by $Ce(NH_4)_2(NO_3)_6$: In a typical run, $Ce(NH_4)_2(NO_3)_6$ (0.05 mmol) and oligopeptide (0.05 mmol) were mixed in 0.1 M HEPES pH 7.0 buffer or 0.1 M TRIS-HCl buffer (5 mL). The

hydrolysis of oligopeptide (or its derivative) was carried out at 40–80 °C. At an appropriate interval, a 50 μL aliquot of the reaction solution was taken out and passed through a disposable pretreatment filter (Tosoh, W-3–2). Then, the compounds were analyzed by the HPLC using a Merck LiChrospher RP-18(e) ODS column (the typical pattern is depicted in Figure 1b). The eluent used for the HPLC analysis was water/acetonitrile mixture 90:10 *v*/*v* at pH 3.5, which contained sodium pentanesulfonate (20 mM: ion-pairing agent) and sodium acetate (20 mM). The wavelengths for UV detection were 257 nm for Phe-containing substrates, 275 nm for Gly-Tyr, and 280 nm for Gly-Trp.

In order to detect the substrates and the products showing no UV absorption, the post-column *o*-phthalaldehyde method was employed (Figure 1a). First, the samples were eluted through a Tosoh TSK-GEL Aminopak column, with 66.7 mM citrate/sodium citrate buffer (pH 3.3) containing *n*-caprylic acid (0.1 mL L^{-1}) and ethanol (80 mL L^{-1}). Then, the compounds were treated in situ with *o*-phthalaldehyde (1.0 g L^{-1}) and 2-mercaptoethanol (2 mL L^{-1}) in the solution containing boric acid (24.7 g L^{-1}), sodium hydroxide (16 g L^{-1}), and Brij 35 (polyoxyethylene(23) lauryl ether (30% solution), 3 mL L^{-1}), and detected by the emitted fluorescence; $\lambda_{ex} = 345$ nm, $\lambda_{em} = 455$ nm.

The assignment of all HPLC peaks was achieved by co-injection with authentic samples (when necessary, several different HPLC conditions were used to confirm the assignment). Furthermore, the products were analyzed by ^{1}H NMR spectroscopy, which was totally consistent with the HPLC analysis. All reactions obeyed pseudo first-order kinetics (see Figure 1 in the Supporting Information). The rate constants presented in the present paper are the averages of the results of duplicate or triplicate runs which coincided with each other within 5%.

^{1}H NMR Spectroscopy: The ^{1}H NMR spectra were recorded in D_2O at room temperature on a JNM-270 FT-NMR spectrometer. As the internal standard, *tert*-butyl alcohol was used. The pD values were adjusted with DCl and NaOD in D_2O. The pH-meter reading was corrected by the equation: pD = pH-meter reading + 0.41.[35]

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[21] The metal ions other than Ce^{IV} were used as their chloride salts. The catalytic activity of the Ce^{IV} is not affected by sodium chloride. Furthermore, nitrate and perchlorate salts of the lanthanide(III) ions

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gave almost the same activities as the corresponding chloride salts. Neither counter anion nor ionic strength shows any significant effects on the results in Figure 2.

[22] In spite of the formation of the hydroxide gel, the concentration of the active species linearly increases with $[Ce(NH_4)_2(NO_3)_6]_0$. Diffusion of the substrates and the products in the gel is not rate-limiting. Thus, the present reactions can be satisfactorily treated by Michaelis–Menten kinetics. Other Ce^{IV} ions in the gel assist this catalysis, as described in the Discussion.

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