

Comparable Rates for Cleavage of Amide and Ester Bonds through Nucleophilic Attack by Carboxylate Anion and General Acid Catalysis by Metal-Bound Water in a Carboxypeptidase A Model

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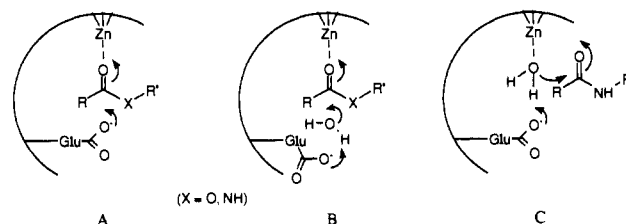
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Abstract: The kinetics of the Cu(II)- or Ni(II)-catalyzed deacylation of the methyl ester (**3**) and the *N,N*-dimethyl amide (**4**) of 2-carboxy-6-[[2-(4-carboxymethyl)imidazolyl]azo]benzoate was measured in dimethyl sulfoxide containing 5% (v/v) water. Efficient catalysis was observed as the result of the cooperation between the metal ion and the benzoyl carboxyl group. In addition, the rate for the metal-catalyzed deacylation of amide **4** was greater or comparable to that of ester **3**. The metal-ion-catalyzed deacylation of **3** and **4** involves the nucleophilic attack of the carboxylate anion as evidenced by the accumulation and the trapping of the anhydride intermediate. Moreover, the general acid catalysis by the metal-bound water in the expulsion of the amine leaving group is discovered as a novel catalytic role for the Lewis acid catalysis by metal ions in organic reactions. Several important catalytic features of carboxypeptidase A (CPA) are reproduced by the metal-ion-catalyzed hydrolysis of **3** and **4**. The most important implication made by the model study on the mechanism of CPA is that, if ester substrates are hydrolyzed by CPA through the anhydride mechanism in which the Glu-270 carboxylate acts as a nucleophile, peptide substrates might be equally well hydrolyzed through the same mechanism.

In the studies of enzyme models, it is attempted to reproduce catalytic features of enzymes with small molecules. In the area of molecular recognition, for example, the ability of enzymes to recognize the structure of substrates is mimicked with small molecules. Although fast catalytic turnover within the resultant supramolecular complexes has been rarely achieved, one of the ultimate goals of this area is to design efficient artificial enzymes. Another major objective of the model studies is to obtain information useful for studies on specific target enzymes. Since it is difficult to investigate enzymatic systems in detail with currently available physical means, studies with small model compounds can provide valuable clues.

Carboxypeptidase A (CPA), a zinc(II) metalloexopeptidase, is one of the enzymes that have been subject to the most intensive mechanistic studies.¹⁻⁶ The most crucial catalytic groups of CPA are the active-site Zn(II) ion and the Glu-270 carboxylate. A large number of papers in the literature have been published on the mechanism of CPA, employing various methods developed in several fields of mechanistic chemistry.⁷⁻²⁵ Controversies

concerning the CPA mechanism, however, have not been resolved, with the major issue being the catalytic role of the Glu-270 carboxylate. In one mechanism, the Glu-270 carboxylate makes nucleophilic attack at the scissile amide or ester linkages (A) leading to the formation of anhydride intermediates.¹⁷⁻²² In the other mechanism, the Glu-270 carboxylate acts as a general base, assisting the attack of an external water (B) of the Zn(II)-bound water (C) at the substrate without the formation of the anhydride intermediate.^{4,5,16}



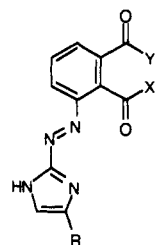
Several catalytic systems have been designed as models of CPA. Catalysis of the hydrolysis of many esters or amides has been investigated using metal ions as the sole catalyst.⁶ Efficient catalysis was observed through the intramolecular nucleophilic attack of metal-bound hydroxide ions at the carbonyl carbons of amides.²⁶⁻²⁹ This may be considered to support the mechanism of C.

Attempts to achieve catalytic cooperation between the metal ion and the carboxyl group in ester or amide hydrolysis have not

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been very fruitful.²⁹⁻³² Recently, we have reported the Cu(II)- or Ni(II)-catalyzed hydrolysis of alkyl ester **1** and alkyl amide **2** as a CPA model.³³⁻³⁵ When dimethyl sulfoxide (DMSO)



- | | | | |
|-----|--|------------------------|--------------------------|
| 1: | X = OCH ₃ , | Y = OH, | R = H |
| 2: | X = N(CH ₃) ₂ , | Y = OH, | R = H |
| 3: | X = OCH ₃ , | Y = OH, | R = CH ₂ COOH |
| 3a: | X = OCH ₃ , | Y = OCH ₃ , | R = CH ₂ COOH |
| 4: | X = N(CH ₃) ₂ , | Y = OH, | R = CH ₂ COOH |
| 4a: | X = N(CH ₃) ₂ , | Y = OCH ₃ , | R = CH ₂ COOH |
| 5: | X = OH, | Y = OH, | R = CH ₂ COOH |
| 5a: | X = OH, | Y = OCH ₃ , | R = CH ₂ COOH |

containing 5% (v/v) water was employed as the reaction medium, the model manifested the catalytic features of CPA such as (1) facile cleavage of alkyl ester and alkyl amide bonds, (2) cooperation among the metal ion, the carboxyl group, and the medium in catalysis, (3) catalytic roles of the metal ion and the carboxyl group similar to those proposed for the action of CPA, and (4) optimum reactivity attained when the catalytic carboxyl group is in the anionic state.^{6,34}

Although several catalytic features of CPA were reproduced by the metal-ion-catalyzed hydrolysis of **1** and **2**, the catalytic role of the carboxylate group in these models has not been elucidated. In the metal-ion-catalyzed hydrolysis of **1** and **2**, the carboxylate anion may act as a nucleophile or as a general base, just like the Glu-270 carboxylate of CPA. This mechanistic ambiguity, however, can be resolved if existence of the anhydride intermediate is demonstrated.

Considering that the metal-ion-catalyzed hydrolysis of **1** and **2** is the only CPA model in which metal ions efficiently cooperate with the carboxylate anion, elucidation of its reaction mechanism would produce important information on the catalytic roles of the Glu-270 carboxylate and the Zn(II) ion of CPA. The metal binding atoms are two imine nitrogens and a carboxylate oxygen in CPA,¹ whereas they are two imine nitrogens in **1** and **2**. In this regard, we modified the model system to include a carboxylate ligand as in **3** and **4**. Catalytic properties of the metal ions may be considerably affected by changing the nature of the ligands. Moreover, we hoped that introduction of an extra coordinating site to the substrate would change the kinetic behavior, producing clues for elucidation of the reaction mechanism.³⁶ In this article, mechanistic information derived from the study on **3** and **4** will be presented, together with its implications on the mechanism of CPA.

Experimental Section

Materials. Methyl 2-Carboxy-6-[[2-(4-carboxymethyl)imidazolyl]azo]benzoate (**3**). Methyl 2-carboxy-6-nitrobenzoate (1.2 g)³⁷ was reduced by catalytic hydrogenation with the Pd/C catalyst in methanol (5 mL). The resultant aniline obtained by separation of the catalyst and evaporation of methanol in vacuo was dissolved in water (10 mL) by adjusting the pH to 2 with 2 N HCl, and then mixed with an aqueous solution (5 mL) of NaNO₂ (0.4 g). The resulting mixture was added dropwise over a period of 30 min to an aqueous solution (20 mL) of 4-imidazoleacetic acid (1.2 g) at 4 °C. The pH of the solution of 4-imidazoleacetic acid was kept at 10–11 with Na₂CO₃ during the diazotization reaction. After stirring for a further 30 min, the mixture was acidified with HCl to pH 4. The precipitates formed were separated and recrystallized from methanol–acetone, mp 210–212 °C. ¹H NMR (DMSO-*d*₆) δ: 3.43 (s, 2 H), 3.83 (s, 3 H), 7.41–7.94 (m, 4 H). Anal. C, H, N. Diazotization to imidazole is known to occur at the 2 position of imidazole.³⁴

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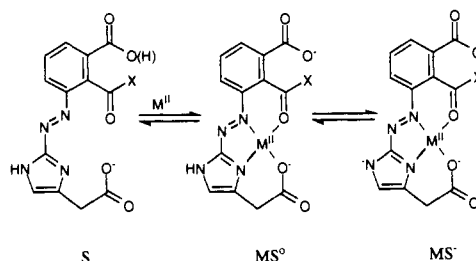
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(36) In a previous study, we were able to alter the kinetic pattern of a metal-catalyzed ester hydrolysis by introducing the carboxyl group as an extra chelating site and, consequently, resolve mechanistic ambiguities (Suh, J.; Han, O.; Chang, B. *J. Am. Chem. Soc.* **1986**, *108*, 1839).

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Scheme 1



Dimethyl 3-[[2-(4-Carboxymethyl)imidazolyl]azo]phthalate (3a). This compound was prepared according to the method described for **3** by using dimethyl 3-nitrophthalate (1.6 g)³⁸ and 4-imidazoleacetic acid (1.5 g) and was recrystallized from ethyl acetate–hexane, mp 180–182 °C. ¹H NMR (DMSO-*d*₆) δ: 3.38 (s, 2 H), 3.87 (s, 6 H), 7.41–8.13 (m, 4 H). Anal. C, H, N.

N,N-Dimethyl-2-carbomethoxy-6-[[2-(4-carboxymethyl)imidazolyl]azo]benzamide (**4a**). This compound was prepared according to the method described for **3** by using *N,N*-dimethyl-2-carbomethoxy-6-nitrobenzamide (1.0 g)³⁴ and 4-imidazoleacetic acid (1.5 g) and was recrystallized from methylene chloride–hexane, mp 174–176 °C. ¹H NMR (DMSO-*d*₆) δ: 2.74 (s, 3 H), 2.98 (s, 3 H), 3.41 (s, 2 H), 3.83 (s, 3 H), 7.73–7.90 (m, 4 H). Anal. C, H, N.

N,N-Dimethyl-2-carboxy-6-[[2-(4-carboxymethyl)imidazolyl]azo]benzamide (**4**). Compound **4a** (0.5 g) was hydrolyzed by stirring in 0.1 N NaOH (10 mL) for 2 h at 70–80 °C. The precipitates obtained by acidification of the cooled reaction mixture to pH 1 with HCl were recrystallized from methanol–acetone, mp 239–241 °C. ¹H NMR (DMSO-*d*₆) δ: 2.72 (s, 3 H), 2.98 (s, 3 H), 3.31 (s, 2 H), 7.43–8.00 (m, 4 H). Anal. C, H, N.

3-[[2-(4-Carboxymethyl)imidazolyl]azo]phthalic Acid (5). Hydrolysis of **3** (0.5 g) according to the method described for **4** led to **5**, which was recrystallized from methanol–acetone, mp >250 °C. ¹H NMR (DMSO-*d*₆) δ: 3.48 (s, 2 H), 7.31–8.14 (m, 4 H). Anal. C, H, N.

Methyl 2-Carboxy-3-[[2-(4-carboxymethyl)imidazolyl]azo]benzoate (5a). This compound was prepared from methyl 2-carboxy-3-nitrobenzoate (1.0 g)³⁷ and 4-imidazoleacetic acid (1.2 g) according to the method described for **3** and was recrystallized from methanol–acetone, mp 217–219 °C. ¹H NMR (DMSO-*d*₆) δ: 3.43 (s, 2 H), 3.87 (s, 3 H), 7.43–8.03 (m, 4 H). Anal. C, H, H.

DMSO, chloroacetic acid (CA), and hydroxylamine hydrochloride were purified according to the literature.³⁹ Hydrates of cupric nitrate and nickel nitrate were purchased from Aldrich (Gold Label), and the concentrations of the metal ions in the aqueous stock solutions of the nitrates were quantitated according to the literature.⁴⁰ Potassium chloroacetate (CAK) was prepared by partial neutralization (ca. 80%) of CA with 2 N aqueous KOH in tetrahydrofuran. The precipitates of CAK thus obtained were recrystallized from water–ethanol. Distilled water was used in the kinetic measurements after demineralization.

Measurements. Kinetic measurements and spectral titration were performed with a Beckman Model 5260 UV/vis or a Beckman DU-64 spectrophotometer. The HPLC analysis was performed with a Spectra-Physics SP8800/8810 LC pump, a Spectra-Physics SP4290 Integrator, and a Spectroflow 757 absorbance detector. Unless noted otherwise, kinetic and spectral measurements were carried out in DMSO containing 5% (v/v) water at 50 °C. The temperature was adjusted to within ±0.1 °C with a Lauda/Brinkman Model RC3 circulator. The ionic strength was maintained at 0.6 M with KNO₃. The pH was controlled by changing the ratio of [CA] and [CAK]. The total concentration of CA and CAK was kept at 0.02 M, and variation in the total concentration did not affect the kinetic and spectral data appreciably. Initially added concentrations of the substrates were (0.4–1) × 10⁻⁴ M.

Results

Ionization and Rate Data. The kinetic measurements and spectral titration using **3–5** were performed in DMSO containing 5% (v/v) water, as very efficient cooperation between the metal ion and the carboxylate anion in the hydrolysis of **1** and **2** was achieved in this medium.³⁴ Although the medium is totally different from aqueous solutions, it was possible to control the pH by changing [CA] and [CAK], with log [CAK]/[CA] (=pH

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Table I. Parameters Deduced from Kinetic Data for the Metal-Ion-Catalyzed Deacylation of 3 and 4

| metal | substrate | pK_a^{app} | k_{lim}^- , s^{-1} | k_{lim}^0 , s^{-1} | k_{br} , s^{-1} | k_{NH_2OH} , s^{-1} |
|--------|-----------|--------------------|--------------------------------|--------------------------------|----------------------|-------------------------|
| Cu(II) | 3 | 0.55 ± 0.19^d | $(8.4 \pm 1.5) \times 10^{-4}$ | 0 | 6.8×10^{-5} | 1.6×10^{-4} |
| Cu(II) | 4 | -0.33 ± 0.04^d | $(9.9 \pm 0.2) \times 10^{-4}$ | 0 | 6.5×10^{-5} | 1.4×10^{-4} |
| Ni(II) | 3 | 0.06 ± 0.10^d | $(2.8 \pm 0.2) \times 10^{-4}$ | 0 | <i>e</i> | 3.2×10^{-5} |
| Ni(II) | 4 | <i>f</i> | $(1.1 \pm 0.3) \times 10^{-4}$ | $(1.1 \pm 0.3) \times 10^{-4}$ | <i>e</i> | 3.0×10^{-5} |

^aIn data analysis, k_{lim}^0 was assumed to be nil except for Ni(II)-catalyzed deacylation of 4 for which k_{lim}^0 was assumed to be the same as k_{lim}^- . ^bThe rate of breakdown of the accumulating intermediate was measured at $\log [CAK]/[CA] = 1$. ^cAfter the metal (0.6 mM Cu(II) ion or 1.7 mM Ni(II) ion)-catalyzed deacylation was complete at $\log [CAK]/[CA] = 0$, a 0.8 M hydroxylamine solution (0.3 mL) made in methanol containing 10% (v/v) water was added to the reaction mixture (3 mL) and the value of k_{NH_2OH} was measured at 50 °C. The hydroxylamine solution was generated by mixing hydroxylamine hydrochloride with KOH. ^dThe pK_a^{app} values for the Cu(II) or Ni(II) complexes of 1 and 2 were -0.3 to 0.4 .³⁴ ^eToo slow. ^fThe rate was independent of the pH.

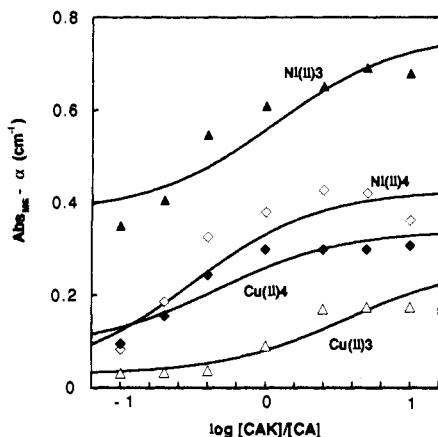


Figure 1. pH dependence of Abs_{MS} (measured at 520 nm) for the Cu(II) or Ni(II) complex of 3 or 4. The value of α is 0.3 for Cu(II)-3, 0 for Ni(II)-3, 0.5 for Cu(II)-4, and 0.35 for Ni(II)-4. The initially added concentrations of 3 or 4 were 7.48×10^{-5} M for Cu(II)-3 and Ni(II)-3 and 7.22×10^{-5} M for Cu(II)-4 and Ni(II)-4. The lines are constructed on the basis of the pK_a^{app} values (Table I) obtained by analysis of the pH dependence (Figure 1) of k_{MS} . The line for Ni(II)-4 is constructed with pK_a^{app} of -0.5 .

+ $\log K_a^{CA}$, where K_a^{CA} is the ionization constant of CA being employed as the measure of the pH.³⁴

As described previously for 1 and 2,³⁴ complexes are formed upon mixing 3 or 4 with Cu(II) or Ni(II) ion and ionization of the imidazolyl N-H group is enhanced by the metal complexation (Scheme I). Previous potentiometric titration indicated that the benzoyl carboxyl group of the metal complexes of 1 is fully ionized over the pH range of $\log [CAK]/[CA] = -1$ to 1 .³⁴ As the initially added concentration ($[M]_0$) of the bivalent metal ion was raised, the absorbance of 3 and 4 at 500–600 nm increased, approaching a maximum value, as was illustrated previously for 1 and 2.³⁴ This maximum absorbance obtained over a wide range of $[M]_0$ at a given pH was taken as the absorbance (Abs_{MS}) of the metal-substrate complex. The dependence of Abs_{MS} on $\log [CAK]/[CA]$ is illustrated in Figure 1.

The $[M]_0$ concentrations at which the Abs_{MS} was attained were 0.4–0.6 mM for the Cu(II) or Ni(II) complexes of 3 or 4. These are about 10 times smaller than those observed for the corresponding complexes of 1 or 2.³⁴ This indicates that the introduction of the carboxymethyl group to 1 or 2 leading to 3 or 4, respectively, results in a ca. 10-fold increase in the formation constants for the corresponding metal complexes.

In the presence of Cu(II) or Ni(II) ion, 3 and 4 produced stable anhydride intermediates, as is shown below. When $[M]_0$ was raised, the pseudo-first-order rate constant (k_0) for the deacylation reaction increased, approaching a limiting value, as was illustrated previously for 1 and 2.³⁴ This limiting value of k_0 was taken as the rate constant (k_{MS}) for the reaction of MS leading to the formation of the anhydride intermediate. The range of $[M]_0$ used for the measurement of k_{MS} was identical with that of Abs_{MS} . The dependence of k_{MS} on $\log [CAK]/[CA]$ is illustrated in Figure 2.

As indicated by the pH dependence illustrated in Figure 2, MS^- is the reactive form and the reactivity of MS^0 is negligible for the

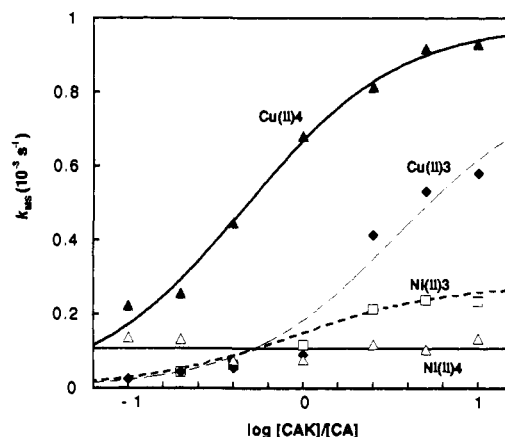
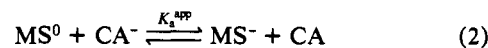


Figure 2. pH dependence of k_{MS} for the Cu(II)- or Ni(II)-catalyzed deacylation of 3 or 4. The curves are drawn with the parameter values summarized in Table I.

Cu(II)-catalyzed deacylation of 3 and 4 and the Ni(II)-catalyzed deacylation of 3. On the other hand, MS^0 is as reactive as MS^- for the Ni(II)-catalyzed deacylation of 4. The pH dependence of k_{MS} was analyzed according to eq 4, and the parameter values thus obtained are summarized in Table I.⁴¹



$$K_a^{app} = ([MS^-]/[MS])([CA]/[CA^-]) \quad (3)$$

$$k_{MS} = \frac{k_{lim}^- + k_{lim}^0([CA]/[CAK])/K_a^{app}}{1 + ([CA]/[CAK])/K_a^{app}} \quad (4)$$

In the absence of Cu(II) or Ni(II) ion, the deacylation of 3 or 4 did not occur appreciably over several hours when followed by spectrophotometric measurement or by HPLC analysis, and precise measurement of the rate constants was not made.⁴² The deacylation of 3a and 4a in the presence of Cu(II) or Ni(II) ion was found to be also very slow. The rate constants for these reactions are estimated as $<3 \times 10^{-6} s^{-1}$ at $\log [CAK]/[CA] = -1$ to 1 .

The active-site metal ion of CPA is Zn(II). Kinetic studies for the deacylation of 3 and 4, however, were not performed in

(41) Analysis of data was performed with a computer program based on the nonlinear regression method reported in the literature (Yamaoka, K.; Tanigawara, Y.; Nakagawa, T.; Uno, T. *J. Pharm. Dyn.* 1981, 4, 879).

(42) In the spontaneous hydrolysis of 3 and 4 and the Cu(II)- or Ni(II)-catalyzed hydrolysis of 3a and 4a, the visible spectra of reactants were distinctly different from those of the expected products. On the other hand, the reactant spectra were almost the same as the product spectra in the spontaneous hydrolysis of 1 and 2.³⁴ The rates for the spontaneous hydrolysis of 1 and 2, however, were measured by the spectrophotometric analysis of the metal complex of the hydrolysis product after adding metal ions to portions of the reaction mixtures. The rate data measured in this way for the spontaneous hydrolysis of 1 and 2 may have been significantly overestimated by partial hydrolysis during the process of quantitation of the metal complex of the hydrolysis product.

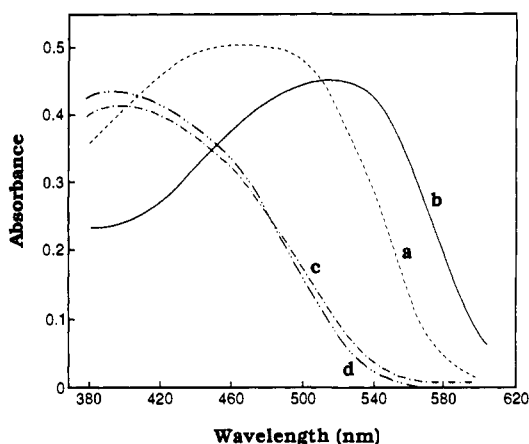


Figure 3. Visible spectra of **3** in the presence of Ni(II) ion taken immediately after manual mixing (a), the intermediate accumulating after the Ni(II)-catalyzed deacylation of **3** (b), **5** in the presence of Ni(II) ion (c), and **3** in the absence of Ni(II) ion (d). The concentrations of the azo compounds are 4.49×10^{-5} M, that of Ni(II) is 1.7 mM, and $\log [\text{CAK}]/[\text{CA}]$ is 1. Under these conditions, **3** is completely bound to Ni(II) as manifested by the dependence of absorbance on $[\text{Ni(II)}]$. The spectrum of the intermediate accumulating after the Ni(II)-catalyzed deacylation of **4** is the same as spectrum b.

the presence of Zn(II) ion due to its limited solubility in DMSO containing 5% (v/v) water.

Trapping of the Intermediate. In the case of **1** and **2**, hydrolysis products were obtained after completion of pseudo-first-order reactions in the presence of Cu(II) or Ni(II) ion.³⁴ In marked contrast to this, the product spectra obtained after completion of the pseudo-first-order reactions of **3** or **4** in the presence of Cu(II) or Ni(II) ion were totally different from those expected to be obtained by the hydrolysis of the substrates, as illustrated by typical spectra presented in Figure 3. The spectrum of the accumulating intermediate for **3** was identical with that for **4** when the same metal ion was added. The product spectra did not change for several hours at low values of $\log [\text{CAK}]/[\text{CA}]$ and were converted slowly into the spectra of hydrolysis products at high values of $\log [\text{CAK}]/[\text{CA}]$. The rate constants (k_{br}) for the breakdown of the intermediates are summarized in Table I. After accumulation of the intermediates was complete, hydroxylamine was added and the rate constants ($k_{\text{NH}_2\text{OH}}$; Table I) for the breakdown of the intermediates were measured. The kinetic data presented in Table I indicate that a common intermediate accumulates in the metal-catalyzed deacylation of **3** and **4** in the presence of the same metal ion.

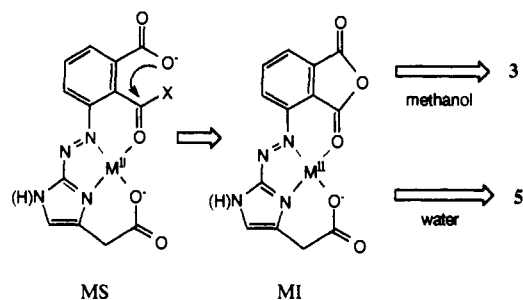
In order to obtain more evidence for the assignment of the structure of the accumulating intermediate, trapping of the intermediate with methanol was carried out. After pseudo-first-order process of **3** or **4** in the presence of Cu(II) or Ni(II) ion was complete ($[\text{M}]_0 = 0.6$ mM, $\log [\text{CAK}]/[\text{CA}] = 1$), methanol (2 mL) was added to the reaction mixture (2 mL) and the resulting solution was incubated for 0.5–1 h at room temperature. A 0.6 N methanolic HCl solution (1.3 mL) was added to the solution cooled in an ice bath, and the mixture was extracted with 5 portions of 5 mL of ethyl acetate. The visible spectra and the HPLC analysis of the ethyl acetate extracts indicated that **3** and **5** were extracted in 90–95% yield, with the molar content of **3** being 70–82%.

Discussion

Mechanism. The rates for the deacylation of **3** and **4** in the absence of Cu(II) or Ni(II) ion are much slower than those of **3** and **4** measured in the presence of Cu(II) or Ni(II) ion, indicating that the metal ions are necessary for the efficient reaction. Furthermore, the rates for the deacylation of **3a** and **4a** in the presence of Cu(II) or Ni(II) ion are much slower than those of **3** and **4** measured in the presence of Cu(II) or Ni(II) ion, indicating that the benzoyl carboxyl group is required for the effective catalysis. Thus, the efficient deacylation of **3** and **4** in the presence

of Cu(II) or Ni(II) ion is due to the bifunctional catalysis by the benzoyl carboxyl group and the metal ion.

The spectra of the metastable intermediates and the kinetic data (k_{br} and $k_{\text{NH}_2\text{OH}}$ in Table I) for the breakdown of the intermediates indicate that an identical intermediate accumulates in the deacylation of ester **3** and amide **4** in the presence of the same metal ion. Only the anhydride (MI) formed by the nucleophilic attack of the benzoyl carboxyl group at the ester or amide linkage can be the common intermediate with considerable stability, and this prediction is confirmed by the trapping experiments. Trapping of anhydride intermediate MI by methanol and water would produce **3** and **5**, respectively.⁴³



The intermediacy of MI indicates that the benzoyl carboxyl group of MS acts as the nucleophile. Although the catalytic role of the benzoyl carboxyl group was not identified in the previous study with **1** and **2**,³⁴ the present study clarifies the mechanistic ambiguities concerning **1–4**.⁴⁴ Under the conditions of kinetic measurements, the benzoyl carboxyl group would be fully ionized, according to the previous potentiometric measurements.³⁴ The sigmoidal pH profiles of Abs_{MS} (Figure 1) and k_{MS} (Figure 2) reflect ionization of the same group. The large spectral changes accompanying the ionization process indicate that the ionizing group is the imidazole ring.³⁴ The shape of the pH dependence of k_{MS} indicates that both the benzoyl carboxyl group and the imidazolyl moiety are to be in the anionic forms in order to manifest full catalytic reactivity for the Cu(II)-catalyzed deacylation of **3** and **4** and the Ni(II)-catalyzed deacylation of **3**. If either the carboxyl or the imidazolyl group is or both groups are to be in the neutral form for full catalytic reactivity, the rate would be either independent of the pH or would rapidly decrease as the pH is raised over the pH range examined.

In the case of amide hydrolysis, the high basicity of amide anion requires that leaving nitrogen atom be protonated either in the tetrahedral intermediate or in the transition state for the expulsion step.^{47–52} In the Cu(II)-catalyzed hydrolysis of **4**, the Cu(II)-bound water molecule is the only source of the acidic proton needed for the fast deacylation reaction. Thus, it is highly likely that the water molecule bound to the Cu(II) ion is involved as a general

(43) Compound **5** might have been formed in part by the spontaneous hydrolysis of **3** in the work-up stage.

(44) In the previous study on the Ni(II)-catalyzed hydrolysis of **1** or the Cu(II)-catalyzed hydrolysis of **2**,³⁴ accumulation of the corresponding anhydride intermediates was not observed. This may be taken to indicate that the introduction of the carboxymethyl group to MI retards the hydrolysis of the anhydride. The extra six-membered chelate ring present in MI apparently leads to geometric changes resulting in the slow anhydride hydrolysis. The slow hydrolysis of the anhydride intermediates formed during the reactions of **3** or **4** in the presence of Cu(II) or Ni(II) ion may be taken to reflect the low nucleophilicity of water in 95% (v/v) DMSO. Then, the fast hydrolysis of the anhydride intermediates formed in the metal-catalyzed reactions of **1** and **2** may be attributed to more efficient metal ion catalysis^{45,46} in anhydride hydrolysis in the case of **1** and **2**.

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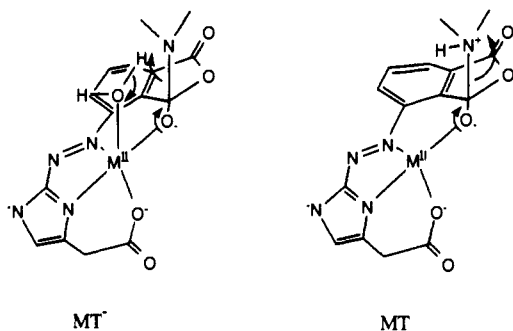
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acid to protonate the leaving nitrogen atom (e.g., MT^-).⁵³



In the Ni(II)-catalyzed hydrolysis of **4**, the rate is independent of the pH over the pH range investigated. At high pH, the anionic forms of both the carboxyl group and the imidazole groups are involved in the Ni(II)-catalyzed deacylation and the mechanism of MT^- would be operative. The pH independence of k_{MS} may be accounted for by the mechanism of MT^- except that the ionization of the imidazolyl N-H group does not affect the reactivity. The kinetic data obtained for the Ni(II)-catalyzed deacylation of **4**, however, are also compatible with the mechanism of MT at low pH. In this case, however, it should be assumed that the rate for the path of MT is the same as that of MT^- .

Several catalytic roles of metal-bound water and hydroxide ion have been discovered. For example, the metal-bound hydroxide ion can act as a nucleophile²⁶⁻²⁹ or as a general base.⁵⁶ Nucleophilic attack by the metal-bound water molecule has been reported.⁵⁷ Upon coordination to metal ions, water becomes weak acids^{58,59} and may act as a general acid catalyst. The general acid participation of metal-bound water molecules, however, has not been clearly demonstrated yet. On the basis of the mechanistic evidence collected in the present study, the general acid role of the metal-bound water molecule is added as a novel catalytic feature of metal ions acting as Lewis acid catalysts in organic reactions.

Implication to Carboxypeptidase A. Although several lines of evidence have been presented in support of mechanisms A-C, none of the mechanisms have been clearly established as the mechanism of CPA action. Mechanism A would be unambiguously accepted if the existence and the structure of the anhydride acyl-CPA intermediate are proved. Spectral observation of the accumulation of intermediates and trapping of the intermediate with ³H-containing reducing agents have been reported.^{17-23,60} Some criti-

cisms, however, have been raised against these results.^{4,5,31} Several attempts to trap the anhydride intermediate with external trapping agents or intramolecular trapping groups have failed.^{20,61-63} Results of the unsuccessful trapping experiments, however, cannot be used as evidence against⁴ the anhydride mechanism.^{20,63,64}

Most of the kinetic data, including the pre-steady-state kinetic data,^{4,14} which were measured directly with CPA and were taken to support the general base role of the Glu-270, can be equally well accounted for by the nucleophilic role of Glu-270. The most convincing evidence for the general base role of Glu-270 was obtained from the CPA-catalyzed ¹⁸O-exchange experiments.¹⁶ The results were, however, later shown by a model study to be also compatible with the nucleophilic role of Glu-270.⁶⁵ Mechanism C was proposed for the peptidase activity of CPA based on the X-crystallographic structures of static and unproductive complexes of CPA formed with inhibitors or pseudosubstrates.^{5,66}

In the CPA-catalyzed reactions, k_{cat} values for specific amide substrates are greater than or comparable to those of specific ester substrates.^{4,67} The equal or greater catalytic efficiency of CPA toward peptides than toward esters has been taken to suggest that product release is rate-limiting for the esters but not for the amides and that cleavage of the ester is still faster than that of the amide.^{4,68} The k_{MS} values measured for amide **4** is greater than or comparable to that for ester **3** in the presence of Cu(II) or Ni(II) ion.⁶⁹ As the result of the combination of the nucleophilic attack by the carboxylate anion, the Lewis acid catalysis by the metal ion, and the general-acid catalysis by the metal-bound water in 95% (v/v) DMSO, cleavage of the amide bond of **4** becomes faster than or similar to that of the ester bond of **3**.

The carboxylate anion of **3** and **4** acts as a nucleophile as proposed by mechanism A for the CPA action. Cooperation of the carboxylate anion with metal ions is required for both the model and CPA. In addition, 95% (v/v) DMSO may resemble the microenvironment of the active site of CPA.⁷⁰ The results of the present study, therefore, demonstrate that if ester substrates are hydrolyzed through mechanism A in the CPA action, peptide substrates⁷¹ might be equally well hydrolyzed through the same mechanism.

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(53) That both the carboxyl and the imidazolyl groups are to be ionized for full catalytic activity in the Cu(II)- or Ni(II)-catalyzed deacylation of **3** may be taken to suggest that the Cu(II)- or Ni(II)-bound water molecule acts as a general acid also in the deacylation of **3**. The possibility, however, cannot be excluded that methoxide anion is expelled from the tetrahedral intermediate without assistance of protonating agents in the metal-catalyzed deacylation of **3**. Moreover, the rate-controlling step might be the attack by the carboxylate anion. In the intramolecular nucleophilic catalysis by the carboxyl group in the phthalic monoalkyl esters,^{36,34,35} the rate-controlling step is the expulsion of the protonated alkoxy group when an unactivated alcohol such as methanol is the leaving group. When the leaving alcohol is activated, however, protonation of the leaving alcohol is not required. When the leaving alcohol has an intermediate leaving ability (e.g., propargyl alcohol), the rate is independent of the pH.

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(60) We have observed accumulation of intermediates in quantitative amounts in the CPA-catalyzed hydrolysis of ester substrates containing α -acylamino cinnamoyl moieties.^{20,23} Common intermediates were observed with ester substrates containing different alcohol moieties with considerably different reactivities.²¹ The kinetics of the breakdown of the intermediates suggested that the intermediate is an anhydride.^{20,21} Furthermore, we were able to show that the accumulating intermediate is the productive intermediate leading to the formation of the products, instead of being the product of a side equilibrium.²² In order to prove the validity of mechanism A, however, positive identification of the structure of the accumulating intermediate is needed.⁵

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(69) In the previous study on **1** and **2**,³⁴ catalysis by Cu(II) ion was demonstrated with **2** but not with **1**, and that by Ni(II) ion was positively detected with **1** but not with **2**. In the present study, catalysis by Cu(II) or Ni(II) ion is observed with both **3** and **4**, and, therefore, it is now possible to compare catalytic efficiency toward ester and amide substrates for a given metal ion.

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Registry No. 3, 141344-43-6; 3a, 141344-44-7; 4, 141344-45-8; 4a, 141344-46-9; 5, 141344-47-0; 5a, 141344-48-1; CPA, 11075-17-5; Cu, 7440-50-8; Ni, 7440-02-0; methyl 2-carboxy-6-nitrobenzoate, 6744-85-0; methyl 6-amino-2-carboxybenzoate, 113579-20-7; dimethyl 3-nitrophthalate, 13365-26-9; *N,N*-dimethyl-2-carboxymethoxy-6-nitrobenzamide, 129356-57-6; methyl 2-carboxy-3-nitrobenzoate, 21606-04-2;

4-imidazoleacetic acid, 645-65-8.

Supplementary Material Available: Values of Abs_{MS} and k_{MS} for the deacylation of 3 and 4 at various values of $\log [CAK]/[CA]$ (1 page). Ordering information is given on any current masthead page.

Systematic Kinetics of High Nuclearity Metal Carbonyl Clusters. Associative Substitution Reactions of $Ru_6C(CO)_{17}$ with P-Donor Nucleophiles

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Abstract: The high nuclearity carbonyl cluster (HNCC) $Ru_6C(CO)_{17}$ has been shown to react with a wide variety of P-donor nucleophiles exclusively by associative processes. The rate constants, k_2 , can be subdivided into electronic and steric contributions which depend on the σ -donicity (pK'_a values) and size (Tolman cone angles, θ) of the P-donor nucleophiles. The data fit well to the equation $\log k_2 = \alpha + \beta(pK'_a + 4) + \gamma(\theta - \theta_{th})\lambda$. The standard reactivity for a weak and small hypothetical nucleophile of $pK'_a = -4$ is defined by $\log k_2^\circ = \log k_2 - \beta(pK'_a + 4)$ and shows an exceptionally high value of 1.51 ± 0.26 . The electronic sensitivity ($\beta = 0.41 \pm 0.04$) is also very high and indicates a high degree of bond making in the transition state. The steric threshold, θ_{th} , below which no steric effects are apparent (switching function (λ) = 0) is 119° , showing that the transition states are quite congested as expected from the high degree of bond making. These transition states are proposed to contain a well-defined $Ru_6C(CO)_{17}$ moiety that is isomeric with the ground-state cluster and which is opened up to an extent that it can accommodate all nucleophiles of various sizes up to the cone angle 119° , defined by θ_{th} , without any steric repulsions. Above the steric threshold, when $\lambda = 1$, the flexibility of the transition state is found to be exceptionally low as indicated by the value $\gamma = -0.20 \pm 0.01 \text{ deg}^{-1}$ which means that the rate of the reaction will decrease by 37% for each degree increase in the cone angle of the incoming nucleophile when electronic effects are absent or constant. This steric effect leads to values of k_2° that vary by 10^{10} over the θ range 119 – 170° . Values of ΔH_2^\ddagger and ΔS_2^\ddagger are low (7 to 13 kcal mol $^{-1}$) and very negative (-10 to $-36 \text{ cal K}^{-1} \text{ mol}^{-1}$), respectively, again showing the exceptionally high degree of bond making in the transition state. These kinetic parameters are compared with those found for smaller clusters and a mononuclear carbonyl.

Introduction

The importance of electronic and steric properties of P-donor nucleophiles in determining rates of associative reactions of metal carbonyls has been known for many years,¹ and the quantitative separation of their contributions to the rates has more recently been shown to be possible for a growing number of reactions.^{1d,e,2-5} Virtually all published or otherwise available data have been found⁶ to fit extremely well to eq 1, which is essentially the same as that

$$\log k_2 = \alpha + \beta(pK'_a + 4) + \gamma(\theta - \theta_{th})\lambda \quad (1)$$

proposed by Giering et al.⁵ The parameter λ is a switching factor that is zero when θ (the Tolman cone angle for the nucleophiles⁷)

$\leq \theta_{th}$, so that steric effects are not important for nucleophiles smaller than the steric threshold, θ_{th} . When the nucleophile cone angles exceed the steric threshold, λ becomes unity, steric effects come into play, and their importance is quantified by the steric parameter γ . The parameter β defines quantitatively the sensitivity of the rates to the σ basicity, pK'_a ,⁸ of the nucleophiles and can be related to the extent of bond making in the transition states.^{1b,3} For a standard, weakly basic ($pK'_a = -4$), and small ($\theta \leq \theta_{th}$) hypothetical nucleophile the value of $\log k_2^\circ = \log k_2 - \beta(pK'_a + 4)$ provides a measure of the standard reactivity (SR) of the carbonyl, and this can be taken as an approximate estimate of its intrinsic reactivity.^{4,9}

The excellent fit of all available data⁶ to eq 1 is the more fortunate because eq 1 therefore provides an objective way of deriving values for the parameters SR, β , and γ , together with their uncertainties, through a multilinear regression analysis.^{5,10} This requires the choice of an initial value for θ_{th} which can be obtained graphically, the best value being obtained subsequently by variation of θ_{th} until a minimum value of the root-mean-square deviation (RMSD) of the values of $(\log k_2)_{calc}$ and $(\log k_2)_{expt}$ is obtained.⁶

(8) Although experimental pK'_a values² can be used successfully, we have used pK'_a values derived from the χ_d values obtained by Giering et al.⁵ essentially by multiplying them by a constant factor to allow for the change of units and choosing $\theta = 145^\circ$ as a reference for steric correction.⁶ This has the advantage of eliminating the steric effects, evident⁵ on experimental pK'_a values, due to various degrees of hydration of the protonated P-donor ligands, without losing the advantage⁴ of obtaining dimensionless β values.

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