BIOORGANIC CHEMISTRY 18, 207–220 (1990)

Bifunctional Catalysis by a Metal Ion and a Carboxylate Anion in Hydrolysis of an Alkyl Ester and an Alkyl Amide in Dimethyl Sulfoxide: A Carboxypeptidase A Model

JUNGHUN SUH,[1] BYUNG KEUN HWANG, AND YUNG HYO KOH

Department of Chemistry, Seoul National University, Seoul 151-742, Korea

Received October 10, 1989

Kinetics of the hydrolysis of methyl 2-carboxy-6-(2-imidazoleazo)benzoate (**1**) and *N,N*-dimethyl-2-carboxy-6-(2-imidazoleazo)benzamide (**2**) were investigated in the presence or absence of the Cu(II) or Ni(II) ion in water or in dimethyl sulfoxide (DMSO) containing 5% (v/v) water. In 95% (v/v) DMSO, potassium chloroacetate and chloroacetic acid were used as the buffer species. Potentiometric and spectral titration of chloroacetic acid, **1**, M(II)**1** (metal-complexed **1**), **2**, and M(II)**2** in 95% (v/v) DMSO indicated that the carboxyl groups of M(II)**1** and M(II)**2** ionize almost fully over the pH range examined and that the imidazolyl N–H groups of M(II)**1** and M(II)**2** are nearly as acidic as chloroacetic acid. The kinetic data obtained for the hydrolysis of Ni(II)**1** and Cu(II)**2** in 95% (v/v) DMSO revealed that the ester and the amide bonds are readily hydrolyzed through the catalytic action of the bivalent metal ion and the anionic form, instead of the acid form, of the carboxyl group. The cooperative catalytic action of the carboxylate anion and the metal ion in the hydrolysis of alkyl ester and alkyl amide bonds, which is characteristic of carboxypeptidase A action, is achieved with **1** and **2** by using 95% (v/v) DMSO as the reaction medium. Mechanisms of the model reactions are analyzed, and implications of the present results on the mechanism of carboxypeptidase A are discussed.

INTRODUCTION

Carboxypeptidase A (CPA),[2] a Zn(II) metalloexopeptidase, has been subjected to extensive mechanistic studies by using X-ray crystallographic (*1*), kinetic (*2*), spectroscopic (*3*), and mutagenetic (*4*) methods in addition to other (*5*) chemical methods. The active-site Zn(II) ion and the Glu-270 carboxylate of CPA are believed to be the essential catalytic groups in the hydrolysis of both ester and amide substrates. The most widely proposed role of the Zn(II) ion is the polarization of the carbonyl group of the scissile ester or amide linkage by binding at the carbonyl oxygen. The carboxylate group of Glu-270 is proposed to act as either a nucleophile or a general base, and the exact catalytic role has been the most controversial issue in the mechanistic study of CPA.

Several compounds have been designed as models of CPA (*6*). Although most

[1] To whom correspondence should be addressed.

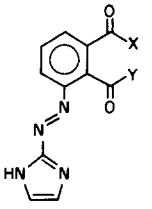
[2] Abbreviations used: DMSO, dimethyl sulfoxide; CPA, carboxypeptidase A; THF, tetrahydrofuran; CA, chloroacetic acid; CAK, potassium chloroacetate.

0045-2068/90 $3.00

of the models tested only one or two catalytic features of the enzyme, some produced informative results for interpretation of the kinetic data obtained with the enzyme (*6g*). In addition, many catalytic systems for the hydrolysis of amides have been investigated as models of proteases in general (*7*). In these systems, hydrolysis of alkyl amides requires proton donors to neutralize the negative charge developed on the leaving nitrogen atoms during the expulsion of the amine moieties. Consequently, the model catalytic systems for amide hydrolysis usually become inactive at neutral pHs, in marked contrast with most of the proteases.

A model of an enzyme would be regarded as a successful one when many of the complicated catalytic features of the enzyme are reproduced. In addition, the model would be still more meaningful if it can also provide clues for interpretation of mechanistic data obtained directly with the enzyme. We have made various attempts to design CPA models which manifest the following catalytic features of the enzyme: (i) a metal ion and a carboxyl group are involved as catalytic groups, (ii) both alkyl ester and alkyl amide linkages are hydrolyzed as the result of the catalysis, and (iii) optimum reactivity is attained when the catalytic carboxyl group is in the anionic form.

In this article, we report kinetic data obtained with the metal-catalyzed hydrolysis of the methyl ester (**1**) and the *N,N*-dimethyl amide (**2**) of a phthalic acid derivative in 95% (v/v) dimethyl sulfoxide (DMSO), which manifest all of the catalytic features of CPA listed above. In addition, implications of the model study on the catalytic roles of various active-site elements of CPA will be discussed.



1: X = OH, Y = OCH_3
1a: X = OCH_3, Y = OCH_3
2: X = OH Y = $N(CH_3)_2$
2a: X = OCH_3, Y = $N(CH_3)_2$
3: X = OH, Y = OH
3a: X = OCH_3, Y = OH

EXPERIMENTAL PROCEDURES

Materials

Methyl 2-carboxy-6-(2-imidazoleazo)benzoate (**1**), dimethyl 3-(2-imidazoleazo) phthalate (**1a**), 3-(2-imidazoleazo)phthalic acid (**3**), and methyl 2-carboxy-3-(2-imidazoleazo)benzoate (**3a**) were prepared according to the literature (*6i*).

N,N-Dimethyl-2-carbomethoxy-6-(2-imidazoleazo)benzamide (**2a**). 2-Carbomethoxy-3-nitrobenzoic acid (*8*) (1.3 g) was stirred with excess thionyl chloride for 1 h at room temperature. The oily residue obtained after evaporation of thionyl chloride under a reduced pressure was dissolved in 30 ml tetrahydrofuran (THF). To the solution kept at 0–5°C, *N,N*-dimethylamine (1 ml) was added and the resulting mixture was stirred for 30 min. The residue (*N,N*-dimethyl-2-carbomethoxy-6-nitrobenzamide) obtained after evaporation of THF and treatment of the resulting mixture with dilute aqueous HCl was recrystallized from ethyl acetate–

hexane, mp 120–122°C. A methanolic solution (20 ml) of this compound (2.3 g) was subjected to catalytic hydrogenation with the Pd–C catalyst. After removal of the catalyst and methanol, the resulting aniline was dissolved in dilute aqueous HCl (15 ml) and the pH of the mixture was adjusted to 2. Diazotization of the aniline was performed by adding an aqueous solution (4 ml) of $NaNO_2$ (1.0 g) at 0–5°C. To an aqueous solution (20 ml) of imidazole (2.4 g) whose pH was adjusted to 9–10 with Na_2CO_3, the solution of the diazonium ion prepared above was added over a period of 1 h at 0–5°C with pH maintained at 8–9 with Na_2CO_3. Thirty minutes later, the mixture was neutralized with 2 N HCl, and the resulting precipitate was recrystallized from chloroform–hexane, mp 173–174°C. Diazo coupling to imidazole is known to occur at the 2 position of imidazole (*6i*). ^{13}C NMR spectrum of **2a** was also consistent with coupling at the 2 position.

N,N-Dimethyl-2-carboxy-6-(2-imidazoleazo)benzamide (**2**). Compound **2a** (0.5 g) was hydrolyzed in 0.1 M NaOH for 2 h at 70–80°C. After the mixture was cooled to room temperature and acidified to pH 3 with HCl, the resulting residue was recrystallized from acetone–hexane, mp 222–223°C.

Other chemicals. DMSO and chloroacetic acid (CA) were purified according to the literature (*9*). Hydrates of cupric nitrate and nickel nitrate were purchased from Aldrich (Gold Label), and concentrations of the metal ions in the aqueous stock solutions of the nitrates were quantitated according to the literature (*10*). Potassium chloroacetate (CAK) was prepared by partial neutralization (ca. 80%) of CA with 2 N aqueous KOH in THF. The precipitates of CAK thus obtained were recrystallized from water–ethanol. Distilled water was used in the kinetic measurements after demineralization.

Kinetic Measurements

Reaction rates were measured with a Beckman Model 5260 uv/vis spectrophotometer. Temperature was adjusted to within ±0.1°C with a Lauda/Brinkman Model RC3 circulator. Kinetic studies in DMSO were performed in the presence of 5% (v/v) water, which was required as a reactant for the hydrolysis reactions. In 95% (v/v) DMSO, ionic strength was maintained at 0.6 M with KNO_3, and pH was adjusted with CA and CAK. Initially added concentrations of the substrates were $(0.5–1) \times 10^{-4}$ M. In 95% (v/v) DMSO, electronic spectra were recorded only in the vis region due to the large absorbance of DMSO in the uv region. Absorbance changes accompanying the hydrolysis in the presence of the Cu(II) or Ni(II) ion were sufficiently large for the spectrophotometric measurement of reaction rates. In the absence of the bivalent metal ions, however, the absorbance changes during the reaction were small. In this case, aliquots (3 ml) of the reaction mixture were taken at various intervals and absorbance values were measured after mixing with 0.3 M $Cu(NO_3)_2$ (0.2 ml). When the kinetic measurements were performed in water, the medium contained 0.8% (v/v) DMSO which was added as the solvent for the stock solutions of the substrates. In water, ionic strength was maintained at 1.0 M with NaCl and the pH was adjusted with 0.02 M chloroacetate (pH 2–3.5) or acetate (pH 3.6–4). Pseudo-first-order rate constants (k_o) were calculated with the absorbance values measured after completion of the reaction. Products of the hydrolysis reactions were identified with their uv–vis spectra.

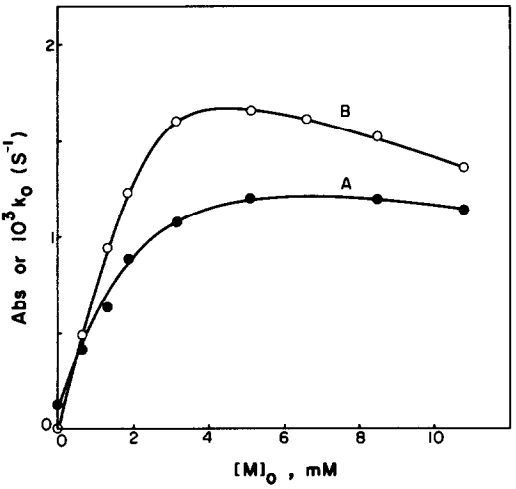


FIG. 1. Typical plots for the dependence of absorbance (curve A) or k_o (curve B) on $[M]_o$. The data were measured with [CAK]/[CA] = 10, 0.03 M $[CA]_o$, and 1×10^{-4} S_o and at 480 nm for the Cu(II)-catalyzed hydrolysis of **2**.

Potentiometric Measurements

Potentiometric titration of CA, **1**, or **2** in 95% (v/v) DMSO was performed at 25°C according to the literature (*11*). The inside of the glass electrode used in the titration was filled with a 95% (v/v) DMSO solution of 0.01 M silver nitrate and 5.2 mM *p*-toluenesulfonic acid.

RESULTS

Acidity Data

The degree of complexation of **1**, **1a**, **2**, and **2a** to the Cu(II) or Ni(II) ion and the ionization of functional groups present in the consequently produced complexes were analyzed by measuring vis spectra in the presence of various concentrations of the metal ion. In 95% (v/v) DMSO, CA and CAK were used as the buffer agents and log[CAK]/[CA](= pH + log K_a^{CA}, where K_a^{CA} is the acidity constant of CA) was employed as the measure of pH of the reaction medium. An increase in the initially added concentration ($[M]_o$) of the bivalent metal ion was accompanied by an absorbance increase at >440 nm, as exemplified by a typical absorbance change illustrated in Fig. 1. Although the absorbance value usually decreased at high $[M]_o$ concentrations after reaching maximum,[3] the maximum absorbance

[3] The decrease in absorbance or k_o may be taken to reflect the formation of 2:1 ($M(II)_2S$ type) complexes at high $[M]_o$ concentrations. The metal ion may coordinate to the diazo nitrogen and imidazolyl nitrogen atoms in 1:1 (M(II)S type) complexes and the second metal ion may coordinate to the extra nitrogen anion of the deprotonated imidazolyl ring in the 2:1 complexes.

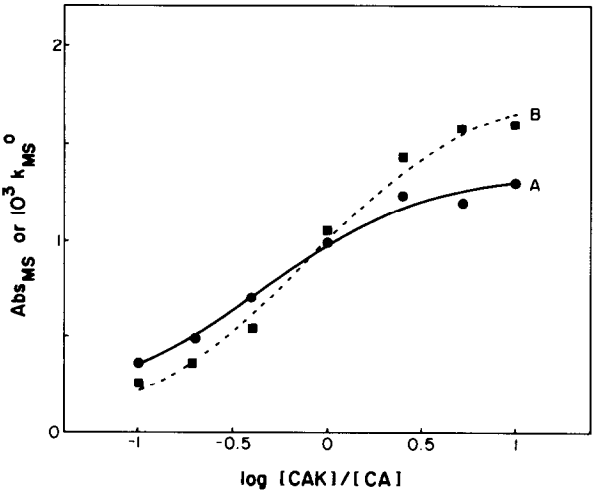


FIG. 2. Typical plots for the dependence of Abs_{MS} (curve A) or k^{o}_{MS} (curve B) on log [CAK]/[CA]. The data were collected for the Cu(II)-catalyzed hydrolysis of **2** under the conditions indicated for Fig. 1.

value obtained over a relatively wide range of $[M]_o$ was taken as the absorbance (Abs_{MS}) of the substrate coordinated to the bivalent metal ion.

A typical plot for the dependence of Abs_{MS} on log [CAK]/[CA] is illustrated in Fig. 2. The curves of spectral titration were analyzed according to Eqs. [1] and [2]. Here, K_a^{app} for S is K_a^S/K_a^{CA} (i.e., $pK_a^{app} = pK_a^S - pK_a^{CA}$) where K_a^S is the acidity constant of S. The values of pK_a^{app} estimated for the metal complexes of the substrates are summarized in Table 1:

$$SH + CA^- \overset{K_a^{app}}{\rightleftharpoons} S^- + CA \qquad [1]$$

$$K_a^{app} = ([S^-]/[SH])([CA]/[CA^-]). \qquad [2]$$

In the absence of the bivalent metal ions, however, the vis spectrum of **1** or **2** in 95% (v/v) DMSO was not affected appreciably by the change in log [CAK]/[CA], and spectral titration of **1** and **2** was not possible at this pH range. Instead, the acidity constants of **1** (K_a^1), **2** (K_a^2), and CA in the absence of the Cu(II) or Ni(II) ion were measured by using a potentiometric method. The pK_a^1 of 7.55, the pK_a^2 of 7.69, and the pK_a^{CA} of 7.31 were thus obtained at 25°C. The pK_a^{app} for **1** and **2** are, therefore, 0.24 and 0.38, respectively.[4] Potentiometric titration of **1** and **2** was not performed in the presence of the Cu(II) or Ni(II) ion in 95% (v/v) DMSO since the compounds were hydrolyzed over the period of time needed for the stabilization of the electrode.

[4] The pK_a^{app} values measured for **1** and **2** are accurate, although pK_a^1, pK_a^2, or pK_a^{CA} might not be very accurate as the electrode was standardized at very acidic pHs.

Kinetic Data Measured in Water

Kinetic data for the hydrolysis of **1** and **1a** in the presence and absence of the Cu(II) or Ni(II) ion in water were reported previously (*6i*).

Rates of the hydrolysis of **2** in the absence of the bivalent metal ion in water were measured in the present study at 50°C and pH 2.3–4. The pH profile of k_o was a descending sigmoid curve, indicating that the acid form of the carboxyl group is catalytic. The pK_a of the carboxyl group and the maximum value of k_o estimated from the pH profile were 3.50 and 4.7×10^{-4} s^{-1}, respectively.

Kinetics of the hydrolysis of M(II)**2** were measured in water in the present study. The spectral changes caused by the addition of the Cu(II) or Ni(II) ion to **2** at 50°C were consistent with formation of 1 : 1 metal–substrate complexes and analysis of the spectral data led to the formation constants (K_f) of 50–90 M^{-1} for Cu(II)**2** at pH 2.3–4.0 and 20–50 M^{-1} for Ni(II)**2** at pH 3.2–4.5. The K_f values decreased as pH was lowered. Over this pH range, a major portion of **2** is bound to the bivalent metal ion when $[M]_o > 0.05$ M. Addition of the Cu(II) or Ni(II) ion up to 0.05 M, however, did not affect the hydrolysis rate appreciably at 50°C.

Addition of up to 0.05 M Cu(II) ion at pH 2.0–3.1 and 50°C to the aqueous solution of **2a** did not change the vis spectrum, indicating that K_f for Cu(II)**2a** in water is much smaller than that for Cu(II)**1a** (*6i*) or Cu(II)**2** in water. In addition, hydrolysis of **2a** was not observed at 50°C and pH 2.0–3.1 for 1 day in the presence or absence of the bivalent ions.

Kinetic Data Measured in DMSO

Kinetics of the hydrolysis of **1**, **1a**, **2**, and **2a** in 95% (v/v) DMSO were measured in the presence or absence of the Cu(II) or Ni(II) ion. A typical plot of k_o against $[M]_o$ measured for the hydrolysis reactions in the presence of the bivalent metal ions is illustrated in Fig. 1. Although k_o was reduced somewhat after reaching maximum when $[M]_o$ was raised to high values,[3] the maximum value of k_o observed over a relatively wide range of $[M]_o$ was taken as the rate constant (k_{MS}) for the reaction of M(II)S. At a constant log [CAK]/[CA], k_{MS} was measured at several $[CA]_o$ (= [CA] + [CAK]) concentrations (0.01–0.05 M).[5] The value obtained by extrapolation of k_{MS} to $[CA]_o = 0$ was taken as k^o_{MS}. The dependence of k^o_{MS} on log [CAK]/[CA] is illustrated in Figs. 2 and 3 for the hydrolysis of Cu(II)**1**, Ni(II)**1**, Cu(II)**1a**, and Cu(II)**2**.

The pH dependence of k_o for the spontaneous hydrolysis of **2** in 95% (v/v) DMSO is also illustrated in Fig. 3. Rates for this reaction were not considerably affected by the addition of the Ni(II) ion. The spontaneous hydrolysis of **1**, the spontaneous or Ni(II)-catalyzed hydrolysis of **1a**, and the Cu(II)- or Ni(II)-catalyzed hydrolysis of **2a** in 95% (v/v) DMSO at 74°C were too slow to obtain reliable

[5] In some reactions, k_{MS} either increased or decreased as $[CA]_o$ was raised, although the degree of the change in k_{MS} was not large. The buffer species may act as a general acid/base or may affect the reactivity of the metal center by coordinating to metal ion. Origin of the buffer effects was not examined further. Unlike k_{MS}, Abs_{MS} was not affected considerably by the change in $[CA]_o$ (0.01–0.05 M).

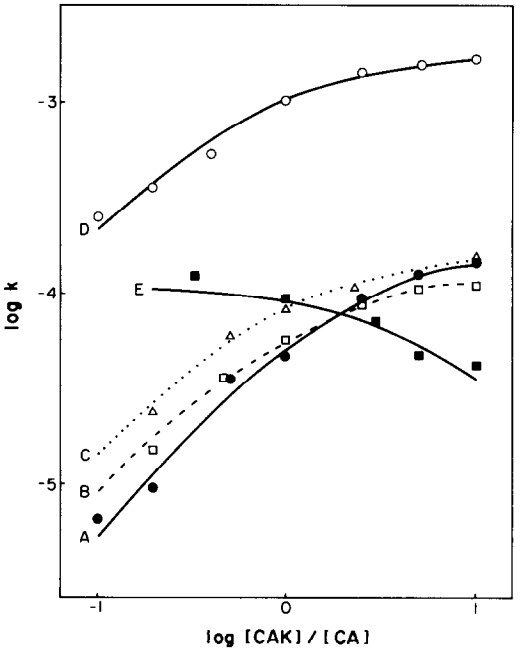


FIG. 3. Plots of log k^{o}_{MS} (for metal-catalyzed hydrolysis) or log k_o (for spontaneous hydrolysis) against log [CAK]/[CA]: curve A, Cu(II)-catalyzed hydrolysis of **1**; curve B, Ni(II)-catalyzed hydrolysis of **1**; curve C, Cu(II)-catalyzed hydrolysis of **1a**; curve D, Cu(II)-catalyzed hydrolysis of **2**; curve E, spontaneous hydrolysis of **2**. Reaction conditions are indicated under Experimental Procedures and Table 1.

kinetic data. The spectral changes observed upon the addition of the Cu(II) or Ni(II) ion indicated that **1a** and **2a** were almost fully complexed to the metal ions under the conditions of the kinetic measurement.

The pH profiles of rate constants illustrated in Figs. 2 and 3 were analyzed in terms of either Eq. [3] or Eq. [4]. Values of the kinetic parameters estimated from the pH profiles are summarized in Table 1:

$$SH \overset{K_a^S = K_a^{app}K_a^{CA}}{\rightleftharpoons} S^- \xrightarrow{k_{MS}^{lim}} \text{products} \qquad \text{(SH: Cu(II)1, Ni(II)1, Cu(II)1a, and Cu(II)2)} \quad [3]$$

$$\text{products} \xleftarrow{k^{lim}} SH \overset{K_a^S = K_a^{app}K_a^{CA}}{\rightleftharpoons} S^- \qquad \text{(SH: 2).} \quad [4]$$

DISCUSSION

Ionization

The dependence of Abs_{MS} on log [CAK]/[CA] for the metal complexes of **1**, **1a**, **2**, and **2a** (Table 1, Fig. 2) indicates that each complex contains a functional group which is almost as acidic as CA.

TABLE 1

Values of pK_a^{app} for Imidazole Moieties and k_{MS}^{lim} Measured for Metal–Substrate Complexes in 95% (v/v) DMSO[a]

Compound	pK_a^{app} estimated by: Spectral titration	pK_a^{app} estimated by: Kinetic data	k_{MS}^{lim} (10^{-4} s^{-1})	Temperature (°C)
Cu(II)**1**	0.03	0.44	1.9	44
			ca. 27[b]	74
Ni(II)**1**	−0.07	0.10	1.3	50
Cu(II)**1a**	−0.19	0.01	1.7	74[c]
Ni(II)**1a**	−0.09	—	≪0.1	74[c]
Cu(II)**2**	−0.33	−0.14	18	50
Ni(II)**2**	−0.06	—	—	50
Cu(II)**2a**	−0.16	—	≪0.1	74[c]

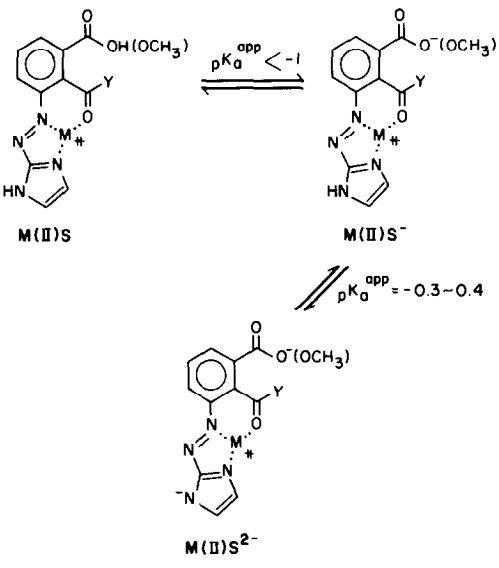
[a] For the spontaneous hydrolysis of **2** in 95% (v/v) DMSO at 50°C, the analysis of rate data (curve E of Fig. 3) according to Eq. [4] leads to $pK_a^{app} = 0.4$ and $k^{lim} = 1.1 \times 10^{-4}$ s^{-1}.

[b] At [CAK]/[CA] = 10, k_{MS}^{o} measured at 70°C was 14 times greater than that at 44°C. The value of k_{MS}^{lim} at 74°C is estimated by multiplication of that at 44°C with 14.

[c] Spectral titration was performed at 50°C.

In **1a** and **2a**, the imidazolyl N–H group is the only ionizable functional group. The acidity of the imidazolyl N–H group of **1** or **1a** in water is considerably enhanced when the imidazole is bound to the Cu(II) or Ni(II) ion (pK_a = 4–5 for M(II)**1** and M(II)**1a**) (*6i*). The spectral titration of M(II)**1a** and M(II)**2a** performed in 95% (v/v) DMSO indicates that the imidazolyl N–H of the complexes ionizes over the pH range examined and, thus, the imidazole group is bound to the metal ion.

In M(II)**1** or M(II)**2**, considerable spectral changes were observed when pH was varied. This is attributable to the ionization of the imidazolyl N–H group in view of the almost identical pK_a^{app} values estimated for M(II)**1** and M(II)**1a** or M(II)**2** and M(II)**2a** by the spectral titration. The pK_a^{app} for **1** and **2** measured potentiometrically in the absence of the Cu(II) or Ni(II) ion in 95% (v/v) DMSO indicates that the carboxyl groups of **1** and **2** ionize partially when log [CAK]/[CA] is varied between −1 and 1. The spectral and potentiometric titration data, however, do not reveal the pK_a of the carboxyl groups of M(II)**1** and M(II)**2**. In M(II)**1** and M(II)**2**, the metal ion would enhance the ionization of the carboxyl group both by exerting an electron-withdrawing effect on the carboxyl group and by the electrostatic stabilization of the resulting carboxylate anion. The electrostatic stabilization would be much more effective in DMSO than in water. Since the acidity of the carboxyl group of **1** or **2** is comparable to that of CA, it is very likely that the ionization of the carboxyl group of M(II)**1** or M(II)**2** is almost complete ($pK_a^{app} < -1$) over the range of log [CAK]/[CA] examined in the present study, whereas the



[M = Cu / Ni , Y = OCH_3 / $N(CH_3)_2$]

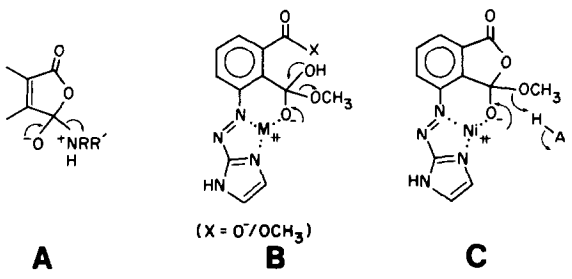
SCHEME 1

imidazolyl groups of the complexes ionize partly over the same pH range. In water, the previous results indicated that pK_a for the carboxyl group of M(II)**1** is smaller by ca. 2 than that for the imidazolyl group of M(II)**1** (*6i*). Ionization of the metal complexes of **1**, **1a**, **2**, and **2a** and the corresponding pK_a^{app} values in 95% (v/v) DMSO are summarized in Scheme 1.

Kinetics and Mechanism

Mechanisms of the hydrolysis of phthalate monoesters including **1**, maleate monoesters, phthalate monoamides, and maleate monoamides in the absence of transition metal ions have been intensively investigated in aqueous media (*6i*, *7*, *8*, *12*). In these reactions, the intramolecular catalysis by the carboxyl group involves the nucleophilic attack by the carboxylate group. Since the expulsion of the leaving alkylamine requires protonation of the amine (e.g., **A**), optimum reactivity for the hydrolysis of phthalate or maleate monoalkyl amides is manifested at low pHs and the reactivity diminishes as the carboxyl group ionizes. In the hydrolysis of phthalate or maleate monoesters, however, the requirement of the protonation of the leaving group becomes less strict as the leaving alkoxide ion becomes less basic (*8*, *12*).

Mechanisms of Cu(II)- or Ni(II)-catalyzed hydrolysis of **1** and **1a** in water have been investigated previously (*6i*). The metal-catalyzed reactions involve rate-determining breakdown illustrated by **B**. The Ni(II)-catalyzed hydrolysis of **1**, however, can also occur through bifunctional catalysis by the metal ion and the



acid form of the carboxyl group. The mechanism of **C** was assigned to this path. Roles of the catalytic groups in the Ni(II)-catalyzed hydrolysis of **1** (**C**) are similar to those of the Zn(II) ion and the Glu-270 of CPA, except that the acid form, instead of the anionic form, of the carboxyl group is the active species in the model system.

In 95% (v/v) DMSO, the hydrolysis of Ni(II)**1** is faster than that of Ni(II)**1a** or **1** by at least 2–3 orders of magnitude. Thus, both the carboxyl group and the metal ion of Ni(II)**1** appear to act as catalytic groups, just as observed in water. The major difference between the two solvent systems is that the catalytic species is the anionic form of the carboxyl group in 95% (v/v) DMSO and the acid form in water.[6]

In 95% (v/v) DMSO, both Cu(II)**1** and Cu(II)**1a** become reactive when the imidazole groups are deprotonated. In addition, the difference between the rate for the hydrolysis of Cu(II)**1** and that of Cu(II)**1a** is not large enough to assume different mechanisms. In this regard, it is noteworthy that an identical mechanism (**B**) has been assigned to the two reactions in water (*6i*). Thus, it is unlikely that the carboxyl group of Cu(II)**1** acts as a catalytic group, and the mechanism of **B** may be again assigned to the hydrolysis in 95% (v/v) DMSO. Deprotonation of the imidazole group of $M(II)S^-$ (Scheme 1) facilitates the ionization of the hydroxy group of the tetrahedral intermediate **B**, as $M(II)S^{2-}$ and **B** are in the same ionization state.

Both in water and in 95% (v/v) DMSO, the spontaneous hydrolysis of **2** is much faster than that of **2a**, indicating the catalytic participation of the carboxyl group of **2**. In addition, the optimum rate for **2** is observed when the carboxyl group of **2** is in the acid form and the rate decreases as the carboxyl group ionizes at higher pHs. Thus, **2** appears to be hydrolyzed both in water and in 95% (v/v) DMSO through the same mechanism as other phthalate or maleate monoamide derivatives.[7]

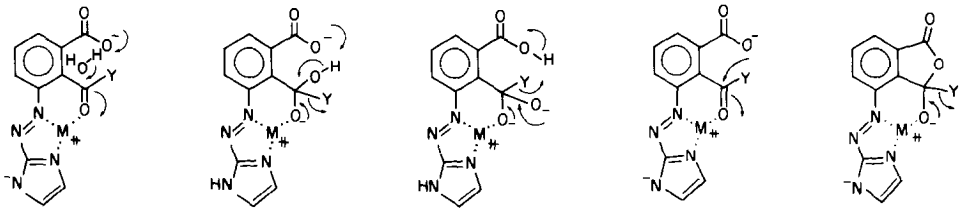
[6] If the acid form of the carboxyl group is assumed to be reactive, it is very difficult to explain the pH profiles illustrated in Fig. 3 for Ni(II)**1** or Cu(II)**2**, even if the carboxyl group is assumed to ionize partially over the pH range examined.

[7] In water, **2** forms the metal complex with Cu(II) or Ni(II) in a 1 : 1 ratio. The hydrolysis rate, however, was not affected by the metal complexation. This may be taken to indicate that the metal complexation does not involve the coordination of the carbonyl oxygen of the amide group to the metal ion. It is known that the introduction of an electron-withdrawing group on the phenyl ring of the phthalate monoalkyl esters, which is mechanistically related to the phthalate monoalkyl amides, does not appreciably alter the hydrolysis rate in water (*8*).

In 95% (v/v) DMSO, the hydrolysis of Cu(II)**2** is much faster than that of **2** or Cu(II)**2a**. Thus, both Cu(II) and the carboxyl group exert catalytic effects. In addition, the anionic form, instead of the acid form, of the carboxyl group of Cu(II)**2** is catalytically active.[6]

The carboxylate anions of Ni(II)**1** and Cu(II)**2** may act either as a general base (**D–F**) or as a nucleophile (**G** and **H**). If the formation (**D** or **G**) of the tetrahedral intermediate is rate-determining for the hydrolysis of Ni(II)**1** or Cu(II)**2**, ionization of the imidazole group would lower the Lewis acidity of Ni(II) and retard the reaction, on the contrary to the observed results.[8,9] At present, it is not easy to rigorously choose the correct mechanisms from the remaining mechanisms which involve the rate-determining processes of **E**, **F**, and **H**. Consideration of the mechanisms of the hydrolysis of Ni(II)**1** in water and those of **1** and **2** in water or in 95% (v/v) DMSO, however, supports the nucleophilic mechanism (**H**). In addition, steric aspects (*7e*) revealed by maleate monoamide derivatives also favor the nucleophilic mechanism.

Regardless of the catalytic role of the carboxylate group of Cu(II)**2**, a proton source is needed for the facile expulsion of the amine moiety from the tetrahedral



D **E** **F** **G** **H**

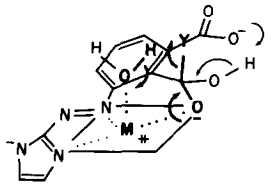
intermediate.[10] This proton may be supplied by the intramolecular carboxyl group (**F**). Here, the proton originates from the nucleophilic water molecule (**D**). On the other hand, the proton source may be the metal-bound water molecule (**I, J**),

[8] For the mechanism of **E** or **F**, deprotonation of the imidazole moiety in $M(II)S^-$ leading to $M(II)S^{2-}$ (Scheme 1) is necessary. If the rate-determining step is **H**, ionization of the imidazole moiety and the consequent decrease in the Lewis acidity of the central metal ion would exert contradictory effects on various processes involved in the overall reaction such as stability of the tetrahedral intermediate, electron push for the cleavage of C–Y bonds, and protonation of the leaving nitrogen and may lead to overall acceleration.

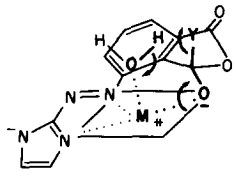
[9] The potassium salt of *N,N*-dimethyl-2-carboxyl-6-[2-(*N*-methyl)imidazoleazo]benzamide, which is analogous to **2** except that one of the imidazolyl nitrogens is methylated, was prepared in the present study in an attempt to block the ionization of the imidazole portion. In 95% (v/v) DMSO, however, this compound did not form a complex with up to 15 mM Cu(II) ion. The *N*-methylation appears to affect the complex formation considerably. As described under Results, **2** does not compex with Cu(II) in water, while it readily coordinates to Cu(II) in 95% (v/v) DMSO. In addition, **1** compexes readily with Cu(II) both in water and in 95% (v/v) DMSO. At present, it is not clear why the minor structural modification involved in these compounds leads to such a large difference in complexation behavior.

[10] The proton source might not be required in the ester hydrolysis of Ni(II)**1** in view of the results obtained for the spontaneous hydrolysis of some activated phthalate monoalkyl esters (*8, 12*). In addition, the reaction path of **H** (Y = OCH_3) without protonation of the leaving group can be accelerated by the dipolar aprotic solvent as discussed in this paper.

which is located in a position suitable for the protonation of the leaving nitrogen atom. In this regard, the acidity of water is considerably enhanced upon coordination to metal ions (*13*).



I



J

The change in the reaction medium from water to 95% (v/v) DMSO greatly enhances the reactivity of the anionic form of the carboxyl groups in Ni(II)**1** and Cu(II)**2**. In the rate-controlling transition states for the hydrolysis of these substrates (**E, F, H, I,** or **J**), charge is considerably more dispersed compared with the corresponding ground states ($M(II)S^{2-}$ of Scheme 1). It is well established that reactions are greatly accelerated in dipolar aprotic solvents such as DMSO when the charge is more dispersed in the transition state than in the ground state (*14*). The high catalytic activity of the anionic form of the carboxyl group in 95% (v/v) DMSO, therefore, is attributable to the solvent effects.

In summary, the ester hydrolysis of Ni(II)**1** and the amide hydrolysis of Cu(II)**2** in 95% (v/v) DMSO involves the collaboration among the metal ion, the carboxyl group, and the medium effects exerted by DMSO. As a consequence of the multiple catalytic factors, facile hydrolysis of an alkyl ester and an alkyl amide[11] is achieved with the anionic form of the carboxyl group.

Implications on the Mechanism of Carboxypeptidase A

The present model system contains some structural elements in common with CPA and reproduces several catalytic features of the enzyme. The carboxyl group and the metal ion of M(II)**1** or M(II)**2** correspond to the Glu-270 and the active-site Zn(II) ion of the enzyme, respectively. Furthermore, DMSO is chosen as a medium mimicking the microenvironment of the active site.[12]

The ready cleavage of the alkyl ester and alkyl amide linkages with the carboxyl group in the anionic form is one of the major catalytic features of CPA reproduced by the present model. This is achieved when the medium was switched from water

[11] Recently, an amide derived from a cyclohexanetricarboxylic acid was reported to be attacked by an adjacent carboxyl group very readily at neutral pHs, although the attack led to the formation of a stable anhydride intermediate instead of overall hydrolysis (*7h*). The fast cleavage of the amide bond was due to the high pK_a of the nucleophilic carboxyl group. Thus, the acid form of the carboxyl group was the reactive unit, in contrast to the present model system.

[12] In a previous report (J. Suh, Y. Kim, E. Lee, and S. H. Chang (1986) *Bioorg. Chem.* **14,** 33), DMSO has been shown to accommodate both hydrophobic and electrostatic interactions, just as the microenvironments of active sites of enzymes. This property is unique in that hydrophobic interaction is favored in polar solvents while the electrostatic interaction is facilitated in nonpolar solvents.

to 95% (v/v) DMSO. Results of the present study, therefore, suggest the importance of the microenvironment of active site of CPA for the catalytic efficiency.

As the source of the proton needed in the expulsion of the amine moiety, the metal-bound water molecule (**I** and **J**) as well as the nucleophile water molecule (**F**) are proposed in the present study. In CPA, the active-site Zn(II) ion has five coordination sites in contrast to the Zn(II) ions present in most of small inorganic complexes (*1*). In addition to the side chain bases of His-69, Glu-72, and His-196, the carbonyl oxygen of the substrate and a water molecule can occupy the coordination sites (*1*). In the CPA-catalyzed hydrolysis of amide substrates, the Zn(II)-bound water molecule may act as a general acid to protonate the leaving amine in analogy with the metal-bound water in the present model system. In a previous model study (*6h*), the nucleophilic role of the Glu-270 carboxylate in the peptidase action of CPA was questioned because this mechanism does not apparently provide the proton needed for the protonation of the leaving amine. For this reason, the general base role of Glu-270 was favored. The present model system, however, suggests that the nucleophilic role of Glu-270 in the hydrolysis of peptide substrates may not be excluded solely based on this reason because the Zn(II)-bound water can serve as the proton source.

ACKNOWLEDGMENT

This work was supported by a grant from the Korea Science and Engineering Foundation.

REFERENCES

1. (a) LIPSCOMB, W. N. (1970) *Acc. Chem. Res.* **3,** 81. (b) HARDMAN, K. D., AND LIPSCOMB, W. N. (1984) *J. Amer. Chem. Soc.* **106,** 463. (c) CHRISTIANSON, D. W., AND LIPSCOMB, W. N. (1986) *J. Amer. Chem. Soc.* **108,** 4998. (d) CHRISTIANSON, D. W., DAVID, P. R., AND LIPSCOMB, W. N. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1512.
2. (a) KAISER, E. T., AND KAISER, B. L. (1972) *Acc. Chem. Res.* **5,** 219. (b) MAKINEN, M. W., YAMAMURA, K., AND KAISER, E. T. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3882. (c) BRESLOW, R., AND WERNICK, D. L. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1303. (d) GEOGHEGAN, K. F., GALDES, R. A., MARTINELLI, R. A., HOLMQUIST, B., AULD, D. S., AND VALLEE, B. L. (1983) *Biochemistry* **22,** 2255. (e) SUH, J., CHO, W., AND CHUNG, S. (1985) *J. Amer. Chem. Soc.* **107,** 4530. (f) SUH, J., HONG, S. B., AND CHUNG, S. (1986) *J. Biol. Chem.* **261,** 7112. (g) SUH, J., CHUNG, S., AND CHOI, G. B. (1989) *Bioorg. Chem.* **17,** 64.
3. (a) QUIOCHO, F. A., MCMURRAY, C. H., AND LIPSCOMB, W. N. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 2820. (b) JOHANSEN, J. T., AND VALLEE, B. L. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1403. (c) STEPHENS, R. S., JENTOFT, J. E., AND BRYANT, R. C. (1974) *J. Amer. Chem. Soc.* **96,** 8041. (d) HOFFMAN, S. J., CHU, S. S.-T., LEE, H., KAISER, E. T., AND CAREY, P. R. (1983) *J. Amer. Chem. Soc.* **105,** 6971. (e) KUO, L. C., LIPSCOMB, W. N., AND MAKINEN, M. W. (1986) *J. Amer. Chem. Soc.* **108,** 5003.
4. HILVERT, D., CARDELL, S. J., AND KAISER, E. T. (1986) *J. Amer. Chem. Soc.* **108,** 5298.
5. (a) HASS, G. M., AND NEURATH, H. (1971) *Biochemistry* **10,** 3535, 3541. (b) RIORDAN, J. F. (1973) *Biochemistry* **12,** 3915. (c) JOHANSEN, J. T., AND VALLEE, B. L. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2006. (d) SANDER, M. E., AND WITZEL, H. (1985) *Biochem. Biophys. Res. Commun.* **132,** 681. (e) SCHAFFER, A., AND AULD, D. S. (1986) *Biochemistry* **25,** 2476.

6. (a) BRESOLOW, R., AND MCALLISTER, C. (1971) *J. Amer. Chem. Soc.* **93,** 7096. (b) BRESLOW, R., MCCLURE, D. E., BROWN, R. S., AND EISENACH, J. (1975) *J. Amer. Chem. Soc.* **97,** 194. (c) BRESLOW, R., AND MCCLURE, E. E. (1976) *J. Amer. Chem. Soc.* **98,** 258. (d) FIFE, T. H., PRZYSTAS, T. J., AND SQUILLACOTE, V. L. (1979) *J. Amer. Chem. Soc.* **101,** 3017. (e) SUH, J., CHEONG, M., AND SUH, M. P. (1982) *J. Amer. Chem. Soc.* **104,** 1654. (f) FIFE, T. H., AND PRZYSTAS, T. J. (1982) *J. Amer. Chem. Soc.* **104,** 2251. (g) SUH, J., AND HAN, H. (1984) *Bioorg. Chem.* **12,** 177. (h) SCHEPARTZ, A., AND BRESLOW, R. (1987) *J. Amer. Chem. Soc.* **107,** 1814. (i) SUH, J., CHUNG, S., AND LEE, S. H. (1987) *Bioorg. Chem.* **15,** 381.
7. (a) BENDER, M. L., CHOW, Y.-L., AND CHLOUPEK, F. (1958) *J. Amer. Chem. Soc.* **80,** 5380. (b) KIRBY, A. J., AND FERSHT, A. R. (1971) Progress in Bioorganic Chemistry (KAISER, E. T., AND KEZDY, F. J., Eds.), Vol. 1, p. 1, Wiley, New York. (c) KIRBY, A. J., MCDONALD, R. S., AND CLINTON, S. R. (1974) *J. Chem. Soc. Perkin Trans. 2,* 1495. (d) KLUGER, R., CHIN, J., AND CHOY, W.-W. (1979) *J. Amer. Chem. Soc.* **101,** 6976. (e) KIRBY, A. J. (1980) *Adv. Phys. Org. Chem.* **17,** 183. (f) SUH, J., KIM, M. J., AND SEONG, N. J. (1981) *J. Org. Chem.* **46,** 4354. (g) SOMAYAJI, V., KEILLOR, J., AND BROWN, R. S. (1988) *J. Amer. Chem. Soc.* **110,** 2625. (h) MENGER, F. M., AND LADIKA, M. (1988) *J. Amer. Chem. Soc.* **110,** 6794.
8. HURST, G. H., AND BENDER, L. M. (1971) *J. Amer. Chem. Soc.* **93,** 704.
9. PERRIN, D., ARMAREGO, W. L. F., AND PERRIN, D. R. (1966) Purification of Laboratory Chemicals, Pergamon, London.
10. SKOOG, D. A., AND WEST, P. M. (1982) Fundamentals of Analytical Chemistry, 4th ed., Chap. 31, CBS College Publishing, New York.
11. (a) RITCHIE, C. D., AND MEGERIE, G. H. (1967) *J. Amer. Chem. Soc.* **89,** 1447. (b) RITCHIE, D. D., AND USCHOLD, R. E. (1967) *J. Amer. Chem. Soc.* **89,** 1721. (c) RITCHIE, C. D., AND USCHOLD, R. E. (1968) *J. Amer. Chem. Soc.* **90,** 2821.
12. (a) THANASSI, J. W., AND BRUICE, T. C. (1966) *J. Amer. Chem. Soc.* **88,** 747. (b) ALDERSLEY, M. F., KIRBY, A. J., AND LANCASTER, P. W. (1974) *J. Chem. Soc. Perkin Trans. 2,* 1504.
13. BASOLO, F., AND PEARSON, R. G. (1962) Mechanisms of Inorganic Reactions, 2nd ed., p. 32, Wiley, New York.
14. PARKER, A. J. (1969) *Chem. Rev.* **69,** 1.