sents yet another case in which the proton inventory technique has been used to probe hydrolytic reactions in a perturbed aqueous system.[29] It will be interesting to see if this urea effect extends to other hydrolytic reactions.

Experimental Section

Materials. 1-Acetylimidazole, 1-acetyl-3-methylimidazole, and acetonitrile were prepared and purified as previously reported.[4,6] Deuterium oxide (99.75 mol % deuterium, Bio-Rad), deuterium chloride (20% solution in D_2O, Aldrich), urea (Fisher), and urea-d_4 (98+ atom % deuterium, Aldrich) were used as obtained.

Solutions. Stock solutions of urea were prepared by dissolving the appropriate amount of urea in water containing the necessary concentration of HCl to maintain the pH and enough potassium chloride to maintain the ionic strength at 0.2 M. Urea-d_4 was used in the same way with DCl in deuterium oxide to maintain the proper pD. The DCl-D_2O solutions were analyzed for deuterium content. Solvent mixtures were prepared by mixing appropriate volumes of the two stock solutions.

Kinetics. Hydrolysis of **1a** and **1b** was followed at 245 nm as reported earlier.[4-6] Spectral data were collected by using a Micromation computer interfaced to a Cary 118C spectrophotometer. Data points were taken at 1-s intervals for greater than 3 half-lives. The data were then analyzed by using a nonlinear least-squares computer program.

The pH of the solutions used to determine the pH-rate profile was adjusted in two ways with the kinetic results being identical. Different amounts of urea introduced into water previously adjusted to a known pH and the addition of HCl to solutions containing fixed urea concentrations were the methods used. All pH measurements were made by using a Corning 130 pH meter equipped with a combination electrode.

Acknowledgment. We are thankful to Mr. William P. Huskey for technical assistance in the early stages of this work.

Registry No. 1a, 31346-45-9; **1b**, 31399-05-0; urea, 57-13-6; water, 7732-18-5.

Ionization and Intramolecular Reactions of *N,N*-Bis[(2-pyridyl)ethyl]- and *N,N*-Bis[(2-pyridyl)methyl]maleamic Acids. An Enzyme Model

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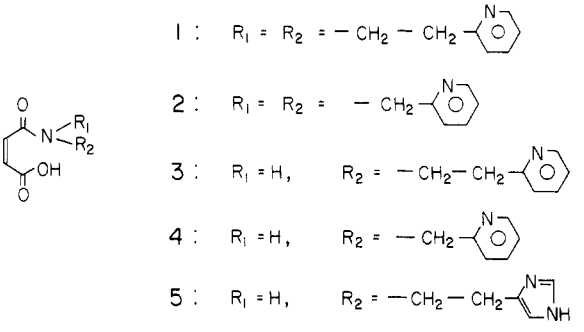
N,N-Bis[(2-pyridyl)ethyl]maleamic acid (1) and *N,N*-bis[(2-pyridyl)methyl]maleamic acid (2) underwent exclusive amide hydrolysis and intramolecular Michael-type addition, respectively. The pH profile of the pseudo-first-order rate constant for the reaction of 1 was a simple descending sigmoid inflecting at the pK_a of the carboxyl group. The pH profile of 2 was a composite of two bell-shaped curves which disclosed the abnormally low pK_a's of the carboxyl group and one of the two pyridinium groups. The change in the reaction path and the abnormal pK_a's observed with the structural variation in maleamic acid derivatives suggest that the change in enzyme specificity and the perturbed pK_a's of the active site functional groups can be achieved with a relatively loose geometry of the enzyme-substrate complex. The failure to observe the metal ion catalysis of the amide hydrolysis of 1 and 2 indicates that the metal complexation of the compounds is inefficient.

The conformation of an enzyme-substrate (ES) complex is extremely important for enzyme catalysis as it determines the relative positions of the functional groups of the enzyme and the substrate.[1,2] Thus, a slight modification of active-site residues or substrate structures often causes remarkable rate changes. However, a completely different reaction can occur upon the alteration of the conformation because both the enzyme active site and the substrate contain more than one reaction site.

One of the most important tools for the elucidation of enzyme mechanisms is the kinetic method.[3,4] Among the information provided by enzyme kinetics, the pH dependence of an enzymatic reaction discloses pK_a values which are utilized in identifying catalytically or conformationally essential functional groups. However, the ionization of an active site functional group can be perturbed by its environment so drastically that the assignment of the pK_a values is often very difficult.

Maleamic acid derivatives **1-5** can be viewed as a model for an ES complex with multiple functional groups. For example, the nucleophilic groups in **1**, the two pyridyl and



the carboxylate, correspond to the functional groups on the active site, and the electrophilic groups, the carbonyl and the olefinic, to those on the substrate. Thus, we have been interested in the effect of the structural variation in the maleamic acid derivatives on the relative reactivity of the two reaction paths indicated by eq 1 and 2, in relation to the conformational requirements for the changes in enzyme specificity. With respect to the ionization of active-site functional groups, the maleamic acid derivatives can serve as a model since the electrostatic interactions of the charged atoms may alter the ionization behavior of the functional groups. In addition, the amide hydrolysis of **1-5** in the presence of transition-metal ions can be regarded as a model for carboxypeptidase A,[5,6] a carboxyl-

(1) Bruice, T. C. In "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1970; Chapter 4.
(2) Jencks, W. P. "Catalysis in Chemistry and Enzymology"; McGraw-Hill: New York, 1969; Chapter 5.
(3) Segel, I. H. "Enzyme Kinetics"; Wiley: New York, 1975; Chapter 11.
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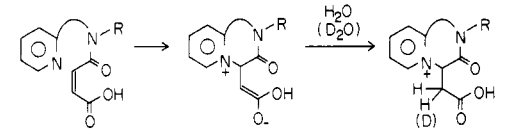
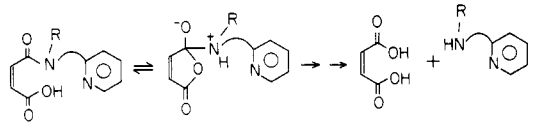
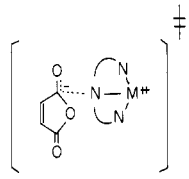


(1)



(2)



A

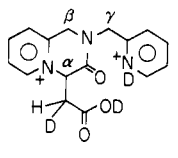
containing metalloprotease. This is because the coordination of a metal ion to the leaving amine portion (A) may accelerate the carboxyl-catalyzed reaction.[7] The results obtained with the intramolecular reactions of 1 and 2 and the implication to enzyme catalysis are reported in this paper.

Results

Reaction Products. The incubation of 1 in buffer solutions at pH 1–6 and 65 °C produced only the hydrolysis products [*N*,*N*-bis[(2-pyridyl)ethyl]amine and maleic acid] as evidenced by the UV spectra of the product solutions. This was supported further by the colorimetric determination of *N*,*N*-bis[(2-pyridyl)ethyl]amine with 2,4-dinitrofluorobenzene.

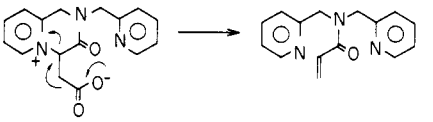
The product solutions obtained after the incubation of 2 in aqueous buffer solutions at pH 0–6 and 65 °C showed UV spectra which were markedly different from the spectra of the hydrolysis products [*N*,*N*-bis[(2-pyridyl)-methyl]amine and maleic acid]. However, when aliquots of the reaction product (0.01 M) obtained in 0.1 N HCl solution were added to buffer solutions at various pH's, the resulting UV spectra were identical to those obtained from direct incubation at the corresponding pH's. Thus, the reaction of 2 at different pH's produced identical products.

The ^{1}H NMR spectrum of the product solution obtained from the incubation of 2 in D_2O with 3 equiv of anhydrous sulfuric acid for 1 h indicated the absence of both [*N*,*N*-bis[(2-pyridyl)methyl]amine[8] and maleic acid]. Instead, the ^{1}H NMR spectrum[9] was consistent with the Michael-type addition product B. The lyophilization of the



B

product solution in D_2O retained the structure as evidenced by the UV spectra. However, when it was neutralized and then lyophilized, a dark red oil was obtained. This can be attributed to the decarboxylation (eq 3) of the Michael-type addition product and the subsequent polymerization of the acryl amide derivative.



(3)

Kinetics. The rates of the reactions of 1 and 2 were measured spectrophotometrically at 65 °C. Changes in the buffer concentration (0.01–0.05 M) did not exert any appreciable effect on the pseudo-first-order rate constants (k_0), indicating the absence of general-acid or -base catalysis by the buffer species.

The pH dependence of k_0 for 1 is a simple descending sigmoid, reflecting the ionization of a single group as illustrated in Figure 1. The pH profile was analyzed according to eq 4[7]. The values of limiting k_0 (k_{lim}) and pK_a

$$k_0 = k_{lim}/(1 + K_a/[H^+]) \quad (4)$$

obtained from the analysis of the pH profile for the hydrolysis of 1 are summarized in Table I, together with those for the hydrolysis of other maleamic acid derivatives.

The pH profile of k_0 for the intramolecular Michael-type addition of 2 is illustrated in Figure 2. Although the curve is bell shaped, the decrease in k_0 in the basic limb occurs over a much wider pH range than expected from the ionization of a single group. The basic limb indicates the presence of at least two groups ionizing over pH 1–6 and

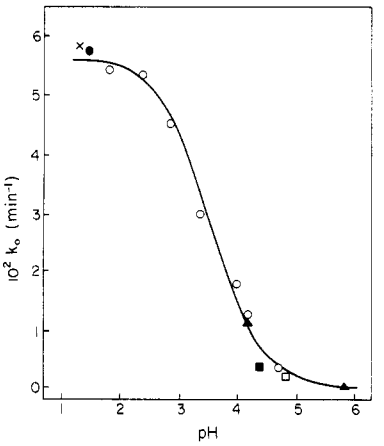


Figure 1. pH dependence of k_0 for the hydrolysis of 1 at 65 °C: ○, at ionic strength 0.05; ●, 0.1 M $HClO_4$ solution; ×, 0.1 M HCl solution at ionic strength 1.0 with added NaCl; ▲, in the presence of 0.01 M Ni^{2+} and at ionic strength 0.05; ■, in the presence of 0.05 M Cu^{2+}; □, in the presence of 0.01 M Cu^{2+} and at ionic strength 0.05.

Table I. Parameter Values for the Hydrolysis of Maleamic Acid Derivatives at 65 °C

compd	pK_a[d]	$10^2 k_{lim}$, min^{-1}
N-methylmaleamic acid[a]	4.0[e]	8.9[f]
1[b]	3.55	5.6
3[c]	3.60	5.6
4[c]	3.55	2.7
5[c]	3.80	7.3[g]

[a] Reference 12. [b] This study. [c] Reference 7. [d] pK_a's for other *N*-alkylmaleamic acid derivatives at 39 °C were 3.2–4.2.[12] [e] At 39 °C. [f] Calculated by using the activation thermodynamic parameters reported in ref 12. The k_{lim} measured at 39 °C was 3.9×10^{-3}. [g] k_{lim} at 39 °C is 4.8×10^{-3} as calculated with the activation thermodynamic parameters.[7]

(5) Kaiser, E. T.; Kaiser, B. L. *Acc. Chem. Res.* **1972**, *5*, 219–224.
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(7) Suh, J.; Baek, D.-J. *Bioorg. Chem.* **1981**, *10*.
(8) The methylene groups of *N*,*N*-bis[(2-pyridyl)methyl]amine trihydrochloride show a sharp singlet at δ 4.47 in D_2O.
(9) The peaks revealed by the compound over δ 0–10 are δ 3.63 (d, 1 H), 5.33 (s, 2 H), and 7.95–9.15 (m, 8 H). The peaks of the methine group (C_α) and one of the methylene groups (C_β or C_γ) appear to overlap (δ 4.8–5.0) with that of HOD.

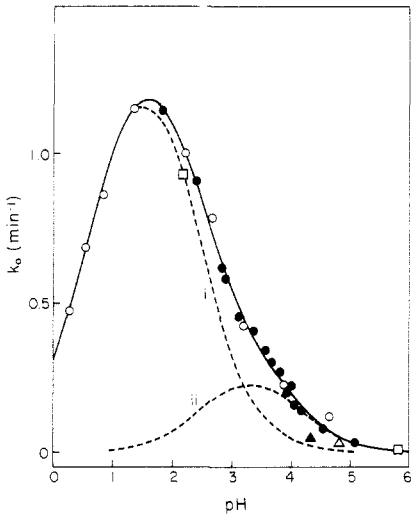
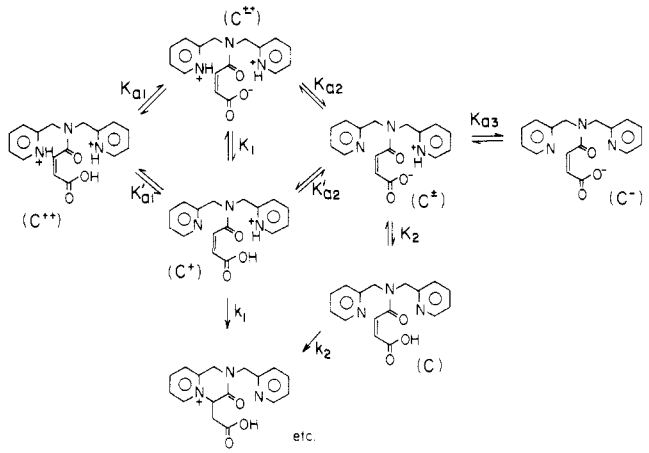


Figure 2. pH dependence of k_0 for the intramolecular Michael-type addition of **2**: ●, at ionic strength 0.05; ○, at ionic strength 1.0; □, in the presence of 0.01 M Ni^{2+} and at ionic strength 0.05; ▲, in the presence of 0.05 M Cu^{2+}; △, in the presence of 0.01 M Cu^{2+} and at ionic strength 0.05.

Scheme I



the acidic limb that of a group ionizing below pH 1.

The pH dependence of k_0 for **2** can be explained in terms of the mechanism summarized in Scheme I.[10] In this scheme, six forms of **2** in various ionization states are illustrated. However, only C^+ and C, in which the carboxyl group is protonated and at least one pyridyl nitrogen is in the basic form, are assumed to undergo the addition reaction. The rate expression for Scheme I is shown in eq 5. The first term of eq 5 represents the reaction of C^+,

$$k_0 = \frac{k_1/\left(1 + \frac{1}{K_1}\right)}{\frac{[H^+]}{K_{app1}} + 1 + \frac{K_{app2}}{[H^+]}} + \frac{k_2/\left(1 + \frac{1}{K_2}\right)}{\frac{[H^+]}{K_{app2}} + 1 + \frac{K_{app3}}{[H^+]}} \quad (5)$$

$$pK_{app1} = pK_{a1}(1 + K_1) \approx pK_{a1} \quad (6)$$

$$pK_{app2} = pK_{a2}(1 + K_2)/(1 + K_1) \approx pK_{a2} \quad (7)$$

$$pK_{app3} = pK_{a3}/(1 + K_2) \approx pK_{a3} \quad (8)$$

and the second that of C. Each of the terms leads to a pH profile in the shape of a symmetrical bell.

(10) The equilibrium constants are defined according to the direction of the arrows. For example, $K_{a1} = [C^{\pm+}][H^+]/[C^{++}]$ and $K_1 = [C^+]/[C^{\pm+}]$.

Table II. Values for the Parameters Listed in Schemes I and II

compd	parameter	value
2	$k_1/(1 + 1/K_1)$	1.4 min^{-1}
	$k_2/(1 + 1/K_2)$	0.30 min^{-1}
	pK_{a1}	0.55
	pK_{a2}	2.50
	pK_{a3}	4.10
4[a]	$k_{21}/(1 + 1/K_{21})$	0.14 min^{-1}
	pK_{a21}	3.55
	pK_{a22}	4.35

[a] Reference 7.

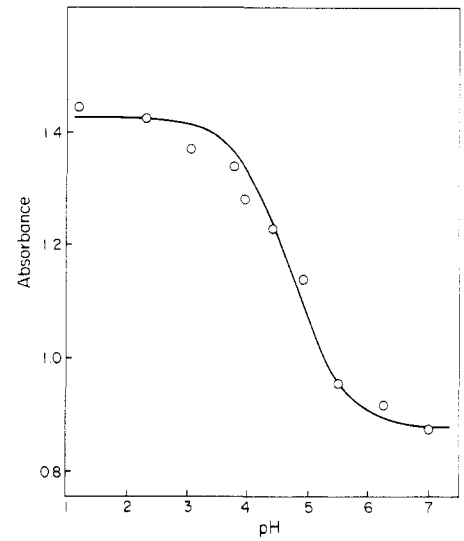


Figure 3. Spectral titration curve for **1** measured at 262 nm with 1.0×10^{-4} M **1**.

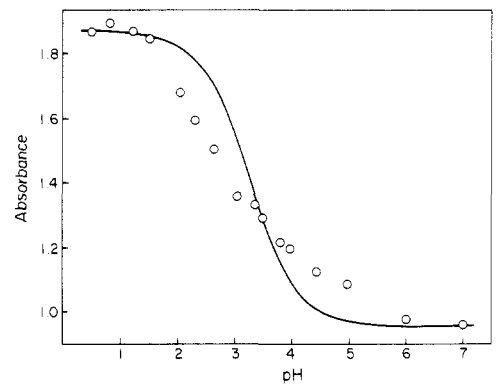


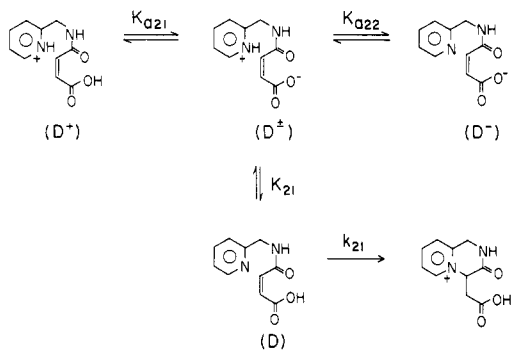
Figure 4. Spectral titration curve for **2** measured at 264 nm with 1.1×10^{-4} M **2**.

The theoretical curve of Figure 2 is constructed by using eq 5 and the parameter values summarized in Table II. The solid line is drawn as the sum of dotted curves i and ii, which represent the reactions of C^+ and C, respectively.

The addition of cupric or nickel ion to the buffer solutions containing **1** or **2** did not affect the product distribution. The reaction rate was not enhanced by the added transition metal ions as indicated in Figures 1 and 2.

Spectral Titration. The spectral titration curves of **1** and **2** measured at near the absorption maxima of the pyridinium portions are illustrated in Figures 3 and 4. Because of the instability of the compounds, the spectral titration was performed at 25 °C. The pH values given in Figures 3 and 4 are, therefore, measured at 25 °C while those for the pH profiles illustrated in Figures 1 and 2 are the values at 65 °C. The solid lines of Figures 3 and 4 are constructed by treating the compounds as monoacidic

Scheme II



bases. The data for 1 do not deviate appreciably from the sigmoid line while the ionization of the pyridyl groups in 2 occurs over a wider pH range.

Discussion

Reaction Paths. The previous study in this laboratory revealed that 4 undergoes the intramolecular Michael-type addition (eq 1) as well as amide hydrolysis (eq 2) while 3 and 5 are subject to exclusive amide hydrolysis.[7] The present study indicates that amide hydrolysis is the only reaction with 1. In this regard, 1, 3, and 5 behave in the same way as *N*-alkylmaleamic acid derivatives.[11-14] The failure to observe the Michael-type addition for 1, 3, and 5 can be ascribed to the unfavorable ring size of the transition state.[7]

On the other hand, 2 undergoes exclusive intramolecular Michael-type addition. Thus, the presence of an additional N substituent in 2 compared with 4 and the difference in the number of methylene units between 1 and 2 or 3 and 4 result in a remarkable change in the reaction path.

The failure of the carboxylate-catalyzed amide hydrolysis of 2 is due to the increased rate of the Michael-type addition instead of the decreased rate of the hydrolysis in comparison with 4, as will be discussed in the following section.

The kinetic scheme and parameter values for the intramolecular Michael-type addition of 4[7] are summarized in Scheme II and Table II. The Michael-type addition of 2 is much faster than that of 4, although this does not necessarily reflect the magnitude of k_1 or k_2 relative to k_{21}. One may expect that the productive conformations for the addition reaction is more probable with 2 than with 4, and this can be related to the faster reaction of 2.

pH Profiles. Several studies have been performed on various types of maleamic acid derivatives.[11-17] Thus, the hydrolysis of simple maleamic acid derivatives includes the attack of the carboxylate group at the carbonyl carbon and the subsequent rate-determining expulsion of the amine moiety to produce maleic anhydride.[7,12,13] As the amine expulsion requires the protonation of the leaving nitrogen, the maximum rates are observed at low pH, and the pH profiles inflect at the pK_a of the carboxyl group. Thus, descending sigmoid pH profiles similar to that of 1 (Figure 1) have been observed for the hydrolysis of simple maleamic acid derivatives including 3 and 5.

The value of k_{lim} for 1 is almost identical with that for 3 and falls within the range of those for simple maleamic acid derivatives (Table I). The pK_a for 1 is also similar to those for simple maleamic acid derivatives. Therefore, the hydrolysis of 1 occurs through the same mechanism as the other maleamic acid derivatives, and the pH profile of 1 reflects the ionization of the carboxyl group. The absence of general-acid or -base catalysis also agrees with the rate-determining expulsion of the amine moiety.[12,13]

The peculiar shape of the pH profile of k_0 for the intramolecular Michael-type addition of 2 is explained as the sum of two bell-shaped curves as illustrated in Figure 2. The values of $k_1/(1 + 1/K_1)$ and $k_2/(1 + 1/K_2)$ (Table II) determine the peak heights of curves i and ii. Thus, the greater peak height of curve i does not necessarily indicate the larger rate constant of C^+ (k_1) than that of C (k_2). Instead, the relative magnitude of K_1 and K_2, which is related to the concentrations of C^+ and C at their respective optimum pH's may be more important.

As the rate of the amide hydrolysis of maleamic acid derivatives is not affected appreciably by the nature of the N substituents (Table I), the rate of the amide hydrolysis of 2 is expected to be similar to that of 4 or 1 and, thus, is much slower than the rate of the Michael-type addition of 2. This accounts for the failure to observe the hydrolysis products for the reaction of 2.

Ionization of Functional Groups. As is seen in Table II, the pyridinium group of $C^{\pm+}$ (pK_{a2}) and the carboxyl group of C^{++} (pK_{a1}) are much more acidic than the pyridinium group of $D^{\pm}$ (pK_{a22}) and the carboxyl group of D^+ (pK_{a21}), respectively. This suggests that the electrostatic interaction between the charged atoms is important for the ionization of 2. In other words, the electrostatic repulsion between the two pyridinium cations of C^{++} is partially relieved upon the ionization of the carboxyl group, resulting in the low value of pK_{a1}. The electrostatic repulsion in $C^{\pm+}$ is then removed completely upon the deprotonation of one of the pyridinium ions, leading to the low value of pK_{a2}.

The pK_a of the carboxyl group of D^+ (pK_{a21}) is similar to those of the carboxyl groups of simple maleamic acid derivatives (Table I), indicating the absence of electrostatic stabilization in $D^{\pm}$. This in combination with the similar values of pK_{a3} and pK_{a22} discloses the absence of electrostatic interaction between the two oppositely charged atoms in $C^{\pm}$. Thus, the electrostatic effect in 2 appears to be important only when both of the two pyridiyl groups are protonated as in C^{++} and $C^{\pm+}$.

Similar electrostatic consideration may be extended to 1. However, the pK_a of the carboxyl group of 1 falls within the range of the corresponding pK_a's of simple maleamic acid derivatives (Table I) and, thus, such electrostatic interaction is not operative in this case. This may be ascribed to the presence of additional methylene units in 1 compared with 2 and the consequent greater conformational freedom and larger separation between the two pyridinium nitrogens.

The spectral titrations of 1 and 2 illustrated in Figures 3 and 4 indicate that the two pyridyl groups of 1 ionize independently while the protonation of one of the two pyridyl groups of 2 retards that of the other. The spectral titration, thus, supports the preceding conclusion regarding the ionization behavior.

Metal Ion Effect. It has been anticipated that the coordination of a metal ion at the leaving nitrogen (eq 9

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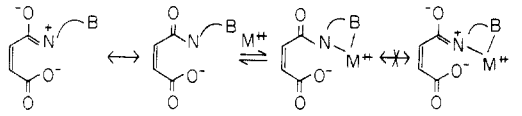
(17) Kluger, R.; Chin, J.; Choy, W.-W. *J. Am. Chem. Soc.* **1979**, *101*, 6976–6980.

and 10) could accelerate the amide hydrolysis.[7] Metal ion



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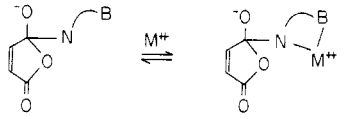

(10)

catalysis has not been observed for the hydrolysis of **3–5** in which the leaving nitrogens form bidentate ligands.[7] Although **1** and **2** contain tridentate amine moieties with a greater tendency for complex formation, metal ion catalysis (A) was also not observed. This indicates that the complex formation of the tetrahedral intermediate or the substrate with cupric or nickel ion is still inefficient due to the short lifetime of the intermediate or weak binding.

Implication on Enzymology. Enzyme specificity can be altered in several ways when the structure of the ES complex is modified. Thus, the reaction site of the substrate can be changed as exemplified by some of the active-site-directed modifications. In some cases, the enzyme uses a different functional group when the substrate structure is modified. An example is the carboxypeptidase A catalyzed hydrolysis of thiol esters. Thus, the Glu_{270} carboxylate attacks at the carbonyl carbon of L-*S*-cinnamoyl α-mercapto-β-phenylpropionate while the Tyr_{248} phenolate attacks at the carbonyl carbon of the corresponding D isomer.[18]

When **1–5** are viewed as a model of an ES complex, the change in the reaction paths caused by the structural variation in **1–5** corresponds to the change in the reaction sites of both the enzyme and the substrate, leading to a totally different reaction. The structural variation involved in **1–5** is not large. Furthermore, the compounds have much greater conformational freedom compared with that for an ES complex. Thus, the alteration of enzyme specificity may be achieved with a rather simple geometric change in the ES complex.

There are many examples of active site functional groups with abnormal pK_a's due to their microenvironments. Thus, the abnormally high pK_a of the Glu_{270} carboxylate of carboxypeptidase A ($pK_a = 6.5$) and the Glu_{35} carboxylate of lysozyme ($pK_a = 6$) and the abnormally low pK_a's of the Tyr_{248} phenolate of carboxypeptidase A ($pK_a = 8$) and the Ile_{16} amine of chymotrypsin ($pK_a = 8.5$) are related to the hydrophobicity or the electrostatic interactions in the active site.[19-21] The pK_a's of these active-site groups differ from the respective normal pK_a's by about 2–3 pK units. A similar degree of perturbation in pK_a is observed with the carboxylate and the pyridyl groups of **2**.

Conformationally, the charged atoms in **2** can affect the ionization of each other more effectively than those in **1** or **4**. The conformation of **2** is, however, much less rigid compared with an ES complex. Thus, the abnormal pK_a's of **2** suggest that the ionization of an active site group can be influenced by another active site group located in a rather remote part.

Experimental Section

Maleamic Acid Derivatives. Compounds **1** and **2** were prepared by adding the solutions of *N,N*-bis[(2-pyridyl)ethyl]-amine[22] or *N,N*-bis[(2-pyridyl)methyl]amine[22] in anhydrous ether dropwise to the well-stirred solutions of maleic anhydride in anhydrous ether at 4 °C over the period of 30 min and stirring the mixtures for further 30 min at 4 °C. The products were separated as white crystals which were filtered and washed with ether and methylene chloride. Compound **1** was recrystallized from acetone–hexane below room temperature; mp 132.5–134.5 °C. Compound **2** was recrystallized from acetone–ether below room temperature; mp 115–117 °C. Elemental (C, H, N) analysis on **1** and **2** gave satisfactory results.

Kinetic Measurements. Reaction rates were measured with a Beckman Model 5260 UV/vis spectrophotometer. Temperature was controlled to within ±0.1 °C with a Haake E52 circulator. The reactions were carried out at 65 °C, and the evaporation of water during incubation for the kinetic measurements at this temperature was prevented by sealing cuvettes tightly with serum caps. Kinetics were performed at an ionic strength of 0.05 or 1.0. The ionic strength was adjusted with sodium perchlorate unless noted otherwise. The buffers used were chloroacetate (pH 2.5–3), formate (pH 3–4.5), acetate (pH 4.5–5.5), and 4-morpholinoethanesulfonate (pH 5.5–7). The pseudo-first-order rate constants were calculated either with the infinity absorbance readings being measured or by the Guggenheim method.

Miscellaneous. Solutions of nickel perchlorate and cupric perchlorate were prepared by dissolving the corresponding metal oxides (Aldrich, "Gold Label") with perchloric acid. Water was redistilled and deionized before being used in the kinetic studies. The colorimetric determination of amines was performed with 2,4-dinitrofluorobenzene according to the literature.[23] The pH measurements were carried out with a Chemtrix Type 60A or a Fisher Accumet Model 525 pH meter. ^1H NMR spectra were obtained with a Varian EM 360 NMR spectrometer at 60 MHz. Chemical shifts are quoted as parts per million downfield relative to tetramethylsilane.

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Registry No. **1**, 78763-63-0; **2**, 78763-64-1; *N,N*-bis[(2-pyridyl)-ethyl]amine, 15496-36-3; *N,N*-bis[(2-pyridyl)methyl]amine, 1539-42-0; maleic anhydride, 108-31-6.

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