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# Model for "charge-relay": Acceleration by carboxylate anion in intramolecular general base-catalyzed ester hydrolysis by the imidazolyl group

(enzyme-like rate/benzoate anion/*endo-endo* structure/dioxane–$H_2O$ mixture)

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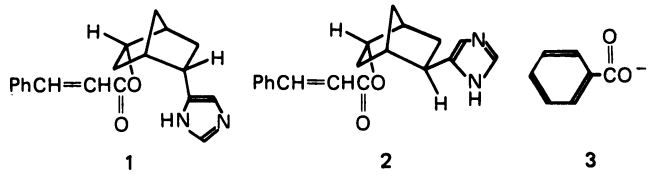
**ABSTRACT** **The effect of benzoate anion on intramolecular general base-catalyzed ester hydrolysis by the imidazolyl group in *endo*-5-[4′(5′)-imidazolyl]bicyclo[2.2.1]hept-*endo*-2-yl *trans*-cinnamate was examined in dioxane/$H_2O$ solutions. Benzoate anion exhibited a remarkable acceleration of the intramolecular general base-catalyzed hydrolysis of *endo*-5-[4′(5′)-imidazolyl]-bicyclo[2.2.1]hept-*endo*-2-yl *trans*-cinnamate by the imidazolyl group. The rate of hydrolysis in the presence of the benzoate anion increased with the dioxane mole fraction and was proportional to the concentration of benzoate anion. On the other hand, the rate of hydrolysis of *endo*-5-[4′(5′)-imidazolyl]bicyclo[2.2.1]hept-*endo*-2-yl *trans*-cinnamate in the absence of benzoate anion decreased with the dioxane mole fraction. Thus, the ratio of the rate in the presence of benzoate anion to that in the absence of benzoate anion drastically increased with the dioxane mole fraction and attained a 2500-fold rate acceleration at a dioxane mole fraction of 0.42 (the highest experimentally attainable) when the concentration of benzoate anion was 0.5 M. The proposed mechanism involves proton abstraction by the benzoate anion from the imidazolyl group, followed by proton abstraction by the imidazolyl group from $H_2O$, resulting in effective general base-catalysis of hydrolysis. The results of the present paper provide support for the "charge-relay" system in serine proteases.**

Recently, much attention has been focused on the roles of "charge-relay" systems in serine proteases. The proposal of the charge-relay system originated in the finding of a "buried" carboxylate anion at the catalytic sites in α-chymotrypsin (1) and subtilisin (2) by x-ray crystallography. Data from the acceleration of the reaction of imidazole with phenyl acetate by carboxylic acid in nearly anhydrous acetonitrile (3) or toluene (4) supported this hypothesis. Furthermore, we found an 8-fold acceleration due to cooperation of the hydroxyl, imidazolyl, and carboxyl groups in the general base-catalyzed hydrolysis of ethyl chloroacetate by 2-benzimidazoleacetic acid, which has both the imidazolyl and carboxyl groups in the same molecule (5). However, Rogers and Bruice found only a 3-fold rate enhancement due to cooperation of the hydroxyl, imidazolyl, and carboxyl groups in the hydrolysis of their model compound and concluded that this was insufficient evidence for the charge-relay system (6).

In a previous paper (7), we found that the imidazolyl group in *endo*-5-[4′(5′)-imidazolyl]bicyclo[2.2.1]hept-*endo*-2-yl *trans*-cinnamate (**1**) functions as an intramolecular *general base* catalyst, as the imidazolyl group of histidine-57 in α-chymotrypsin does.

In the present paper, the acceleration of intramolecular general base-catalyzed hydrolysis of **1** in dioxane/$H_2O$ mixtures by benzoic acid is reported. Benzoate anion (**3**) in dioxane/$H_2O$ mixtures can be used as a model of a buried carboxylate anion in enzymes, because the increase in the dioxane mole fraction makes the surrounding atmosphere of benzoate anion more aprotic (3, 8, 9).

The dependence of acceleration by **3** on either the dioxane mole fraction, the concentration of **3**, or the difference of $pK_a$ between the carboxyl group of **3** and the imidazolyl group of **1** is shown. Furthermore, the effect of **3** on the hydrolysis of the *exo-endo* isomer of **1** (which is **2**) is also examined as a control. No intramolecular reaction takes place in **2** because of the inadequacy of the stereochemistry of the imidazolyl and *trans*-cinnamoyl groups (7). (See structures **1–3**).




## EXPERIMENTAL

**Materials.** Compounds **1** and **2** were dried under reduced pressure at 80° for 2 hr. The agreement between observed and theoretical values in elemental analyses for these compounds was quite good, as reported by Utaka *et al.* (10). Purities were checked by melting point measurements; **1**, mp 131–133° [mp 130–132° (10)] and **2**, mp 175–177° [mp 174–176° (10)]. Benzoic acid was recrystallized from acetonitrile. All $H_2O$ used in kinetic studies was doubly distilled. Spectrograde dioxane was distilled after refluxing over metallic sodium. Further purification of dioxane by the method of Fieser (11) did not affect the kinetics.

**Kinetics.** The following general procedure was used in the kinetic study. A dioxane/$H_2O$ solution (3 ml) containing **3** was equilibrated at 60 ± 1° for 20 min within the thermostatted cell compartment of a Cary 14 spectrophotometer. The reaction was initiated by the addition of a 20-μl dioxane solution of **1** or **2**, followed by thorough mixing of the solution.

Hydrolyses both of **1** and of **2** were monitored by the decrease in absorbance at 310 nm. All the reactions in the presence of **3** followed first-order kinetics for at least three half-lives. On the other hand, hydrolyses of **1** in the absence of **3** in dioxane/$H_2O$ solutions were so slow that they were only monitored for one or two half-lives, during which time the pH of the solutions was maintained within 0.1 pH unit of the predetermined pH by addition of quite small amounts of aqueous NaOH solution at appropriate intervals. The specific rate constants, $k_{obs}$, were determined by the method of Guggenheim (12). The rate constants were reproducible within ± 5%.

Abbreviations: **1**, *endo*-5-[4′(5′)-imidazolyl]bicyclo[2.2.1]hept-*endo*-2-yl *trans*-cinnamate; **2**, *exo*-5-[4′(5′)-imidazolyl]bicyclo-[2.2.1]hept-*endo*-2-yl *trans*-cinnamate; **3**, benzoate anion.

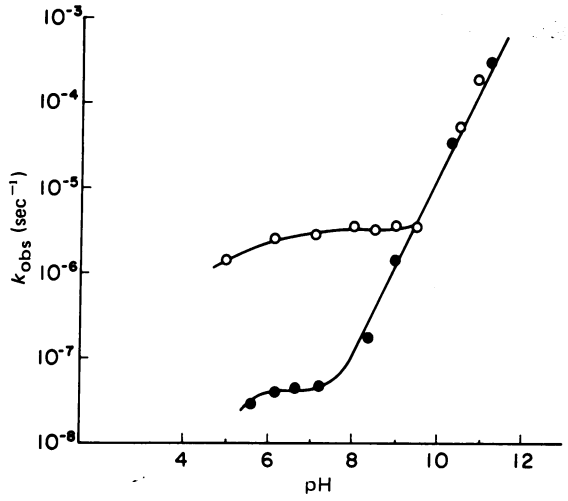



FIG. 1. The pH-rate constant profile for the hydrolysis of 1 in dioxane/$H_2O$ mixture (a dioxane mole fraction 0.17) at 60°, I = 0.2 M (KCl). O, In the presence of 0.1 M of 3; ●, in the absence of 3. In this mixture at 60°, the $pK_a$ of the imidazolyl group of 1 and of the carboxyl group of 3 are 6.7 and 5.7, respectively, as measured by potentiometric titration (data in Table 1). I, ionic strength.

## RESULTS AND DISCUSSION

Fig. 1 shows the typical pH–$k_{obs}$ profiles for the hydrolysis of 1, both in the presence and in the absence of 3. Here, pH meter readings for dioxane/$H_2O$ mixtures were corrected according to the results of Van Uitert *et al.* (13). The rate constants, $k_{obs}$, varied only slightly when the initial concentration of 1 was varied from 1 to 10 mM. Thus, we conclude that the $k_{obs}$ measured here involves no intermolecular catalysis by the imidazolyl group in one molecule of 1 toward the *trans*-cinnamoyl group in another molecule of 1.

The rate constants for intramolecular catalysis by the imidazolyl group, $k_1$, were determined from the plateau rates in the pH–$k_{obs}$ profiles in the absence of 3. In the previous paper (7), this intramolecular catalysis was definitely assigned to general base catalysis on the basis of deuterium oxide solvent isotope effects. At higher pH, the logarithm of $k_{obs}$ increased with pH (slope 1.0) which is attributable to hydroxide anion catalysis. The rate constants for the alkaline hydrolyses, $k_3$, were not determined because the autoprotolysis constants of $H_2O$ in dioxane/$H_2O$ mixtures at 60° were unknown.

A remarkable increase in the plateau rate was observed when 3 was present in the hydrolysis reaction of 1 in dioxane/$H_2O$ mixtures. The plateau was observed in the pH region above the $pK_a$ of the imidazolyl group of 1, when its $pK_a$ was larger than the $pK_a$ of the carboxyl group of 3, and vice versa. Thus, the plateau regions are associated with the neutral form of the imidazolyl group of 1 and the anionic form of the carboxyl group of 3. A change in the ionic strength from 0.2 M to 0.5 M, by using KCl, scarcely affected the plateau rate in the presence of 3 in dioxane/$H_2O$ mixtures. During hydrolysis at higher pH and in the presence of 3, the pH–$k_{obs}$ profile exhibited a straight line (slope 1.0), which is identical with the straight line effect on the alkaline hydrolysis of 1.

The plateau rate for the hydrolysis of 1 in the presence of 3 in dioxane/$H_2O$ mixtures increased linearly when the concentration of 3 increased from 0.05 M to 0.7 M. This shows the lack of complex formation between 1 and 3 in dioxane/$H_2O$ mixtures. Thus, we can definitely say that 3 functions as an *intermolecular* catalyst for the hydrolysis of 1.

Consequently, the $k_{obs}$ in the presence of 3 in dioxane/$H_2O$ mixtures can be expressed as Eq. 1:

$$k_{obs} = k_1 + k_2[C_6H_5COO^-] + k_3a_{OH^-} \quad [1]$$

in which $k_2$ is the rate constant associated with the combination of the imidazolyl group of 1 and the carboxyl group of 3. The rate constants $k_1$ and $k_3$ have been defined above.

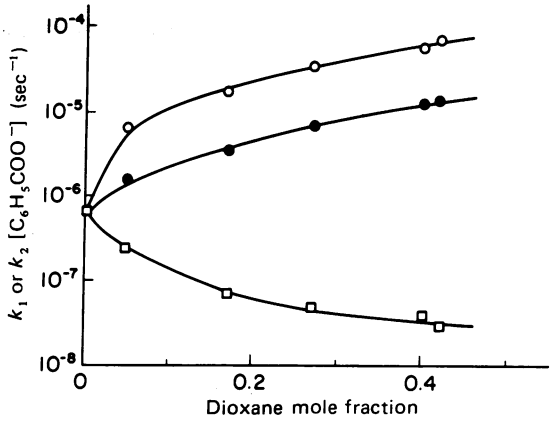



FIG. 2. Dependence of $k_1$ or $k_2[C_6H_5COO^-]$ on the dioxane mole fraction for the hydrolysis of 1 in dioxane/$H_2O$ mixtures at 60°. O, In the presence of 0.5 M 3; ●, in the presence of 0.1 M 3; □, in the absence of 3.

The effect of 3 on the hydrolysis of 2, in which the imidazolyl group is located too far from the *trans*-cinnamoyl group for intramolecular catalysis, was as small as 7–12% of its effect on the hydrolysis of 1. Thus, the second term of Eq. 1 for the hydrolysis of 1 in the presence of 3 almost entirely involves the cooperation of the neutral imidazolyl group of 1 and 3, even though catalysis by 3 without the aid of the imidazolyl group may take place simultaneously and may make a small contribution to the second term of Eq. 1.

The rate constant for the hydrolysis of 1 catalyzed by 3 alone is estimated to be 4–8% or less of that for the hydrolysis catalyzed by the combination of the imidazolyl group of 1 and the carboxyl group of 3. This estimation is based on the result in ref. 7 that the rate constant for intermolecular alkaline hydrolysis of 1 (0.14 $M^{-1}$ $sec^{-1}$ in $H_2O$ at 60°) is 67% that of 2 (0.21 $M^{-1}$ $sec^{-1}$ in $H_2O$ at 60°). Steric hindrance by the adjacent imidazolyl group in intermolecular-catalyzed hydrolysis of 1 by 3 alone should be larger than in intermolecular alkaline hydrolysis of 1.

Fig. 2 shows the dependence of $k_1$ and $k_2[C_6H_5COO^-]$ for the hydrolysis of 1 upon the mole fraction of dioxane in dioxane/$H_2O$ mixtures. The rate constants for the hydrolysis of 1 in the absence of 3, $k_1$, decreased with the increase of the dioxane mole fraction. This is consistent with the marked decrease in the rates of water hydrolyses of esters due to addition of alcohol or acetone (14–16). For example, $k_1$ for a dioxane/$H_2O$ mixture of dioxane mole fraction 0.42 is 25-fold smaller than $k_1$ for a dioxane/$H_2O$ mixture of dioxane mole fraction 0.006. This decrease is mainly due to the preference of the *trans*-cinnamoyl group in 1 in dioxane/$H_2O$ mixtures for solvation by dioxane over solvation by $H_2O$, although other factors may function at the same time. The dioxane molecules solvating the *trans*-cinnamoyl group block the imidazole-catalyzed attack by $H_2O$ on the *trans*-cinnamoyl group.

In contrast to the decrease in $k_1$ with an increase of the dioxane mole fraction, $k_2[C_6H_5COO^-]$, which is associated with the combination of the imidazolyl and carboxyl groups in catalysis, increased with the dioxane mole fraction in the range investigated. In measuring the hydrolysis of 1 by 0.05 M 3 in

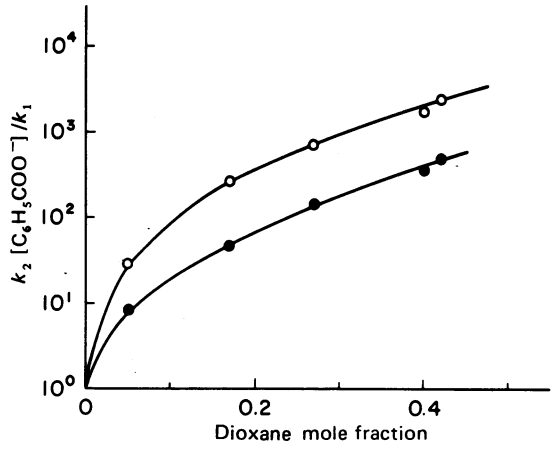



FIG. 3. Plots of $k_2[C_6H_5COO^-]/k_1$ versus the dioxane mole fraction for the hydrolysis of 1 in dioxane/$H_2O$ mixtures at 60°. ○, In the presence of 0.5 M 3; ●, in the presence of 0.1 M 3.

a dioxane/$H_2O$ solution of dioxane mole fraction 0.006, no acceleration at all was observed.

However, $k_2[C_6H_5COO^-]$ in the presence of 0.1 M and 0.5 M 3 at a dioxane mole fraction of 0.42 is 9- and 11-fold larger, respectively, than the rate constants with the same amounts of 3 at a dioxane mole fraction of 0.05.

Fig. 3 shows plots of the ratio $k_2[C_6H_5COO^-]/k_1$ versus the mole fraction of dioxane for the hydrolysis of 1 in dioxane/$H_2O$ mixtures. $k_2[C_6H_5COO^-]$, normalized by $k_1$ at the same dioxane mole fraction, can indicate the net effect of 3 on the general base-catalyzed hydrolysis by the imidazolyl group in 1, if the solvation of *trans*-cinnamoyl and imidazolyl groups of 1 is hardly affected by the addition of 3. Thus, surrounding dioxane molecules blocking the *trans*-cinnamoyl group against imidazole-catalyzed attack by $H_2O$ should cause an effect of the same magnitude on the hydrolysis in the presence of 3.

The ratio of the rate in the presence of 3 to that in the absence of 3, $k_2[C_6H_5COO^-]/k_1$, drastically increased with the dioxane mole fraction and attained a 2500-fold rate acceleration (Fig. 3) at a dioxane mole fraction of 0.42 (the highest experimentally attainable) when the concentration of 3 was 0.5 M. The 2500-fold acceleration by 0.5 M 3 observed here was about 16-fold larger than the 160-fold acceleration of the intermolecular imidazole-accelerated cleavage of *p*-nitrophenyl acetate in an acetonitrile/$H_2O$ solution by 0.5 M 3 (3).

One of the important reasons for the much larger effect of 3 in dioxane/$H_2O$ solutions than in $H_2O$ can be the change in the $pK_a$ of the carboxyl group of 3 as well as that of the imidazolyl group of 1, which is attributable mostly to the fact that the dielectric constant decreases with an increase of the dioxane mole fraction. Dioxane has a much smaller dielectric constant (2.2 at 25°) than $H_2O$ (78.5 at 25°) (17). The values of $pK_a$ were determined by potentiometric titration. As shown in Table 1, the $pK_a$ of 3 increased from 4.1 to 9.4 when the dioxane mole fraction changed from 0.006 to 0.42, whereas the $pK_a$ ($ImH^+$) decreased by only 0.4 pH unit. These results are consistent with previous work; the transfer of acetic acid from $H_2O$ to 82% dioxane/$H_2O$ solution increased the $pK_a$ of its carboxyl group by 5.8 units (18), whereas the transfer of ammonia from $H_2O$ to 80% ethanol/$H_2O$ solution decreased its $pK_a$ by 0.5 unit (19).

In Fig. 4, the ratio $k_2[C_6H_5COO^-]/k_1$ was plotted versus $\Delta pK_a$, the difference between the $pK_a$ of the carboxyl group of 3 and that of the imidazolyl group of 1. When $\Delta pK_a$ was much smaller than zero (i.e., the basicity of the imidazolyl group

Table 1. $pK_a$ of the carboxyl group of 3 ($C_6H_5COOH$) and the imidazolyl cation of 1 ($ImH^+$) in dioxane/$H_2O$ mixtures (at 60°, I = 0.1 M KCl)*

| Dioxane mole fraction | $pK_a$ | | $\Delta pK_a$ |
|---|---|---|---|
| | $C_6H_5COOH$ | $ImH^+$ | |
| 0.006 | 4.1 | 6.8 | −2.7 |
| 0.05 | 4.7 | 6.8 | −2.1 |
| 0.17 | 5.7 | 6.7 | −1.0 |
| 0.27 | 7.0 | 6.6 | +0.4 |
| 0.40 | 8.6 | 6.5 | +2.1 |
| 0.42 | 9.4 | 6.4 | +3.0 |

* pH meter readings were corrected according to the results in ref. 13 by assuming that the pH meter corrections at 60° are equal to those at 30°. These corrections do not affect the values of $\Delta pK_a$, which are the differences of $pK_a(C_6H_5COOH)$ minus $pK_a(ImH^+)$. I, ionic strength.

of 1 was much larger than that of 3), $k_2[C_6H_5COO^-]$ increased drastically with each increase in $\Delta pK_a$. However, decreases in the slope with $\Delta pK_a$ were gradual. For example, in the presence of 0.5 M 3, the increase in $\Delta pK_a$ from +2.1 to +3.0 produced only a 1.4-fold increase in the ratio $k_2[C_6H_5COO^-]/k_1$, but the increase in $\Delta pK_a$ from −2.1 to −1.0 increased the ratio $k_2[C_6H_5COO^-]/k_1$ by 9.0-fold. This indicates that catalysis by the combination of the imidazolyl and carboxyl groups is associated with proton abstraction from the imidazolyl group of 1 by the carboxyl group of 3. Above a $\Delta pK_a$ of zero, proton abstraction is not governed by the $\Delta pK_a$ but is mainly diffusion controlled (20). Proton abstraction from $H_2O$ by a nitrogen atom of the imidazolyl group of 1, which is an essential process for intramolecular general base catalysis in 1, can be enhanced by proton abstraction by the carboxylate anion from the other nitrogen atom of the imidazolyl group, resulting in effective catalysis. This mechanism is consistent with that shown by the charge-relay systems in enzymes.

Recent nuclear magnetic resonance and infrared spectroscopic studies on charge-relay systems have indicated that it is not the imidazolyl group of histidine-57, but rather the carboxyl group of aspartate-102 that has an apparent $pK_a$ of 7 and that controls both the acylation and deacylation steps in serine protease-catalyzed reactions (21, 22). According to these studies, the imidazolyl group of histidine-57 has a lower $pK_a$ than the

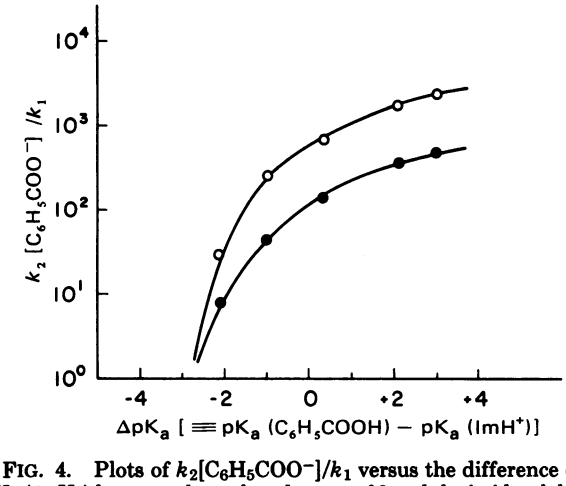



FIG. 4. Plots of $k_2[C_6H_5COO^-]/k_1$ versus the difference of the $pK_a$ ($\Delta pK_a$) between the carboxyl group of 3 and the imidazolyl group of 1 for the hydrolysis of 1 in dioxane/$H_2O$ mixtures at 60°. ○, In the presence of 0.5 M 3; ●, in the presence of 0.1 M 3.

carboxyl group of aspartate-102, and this has also been proposed through quantum chemical calculations (23). The present hydrolysis of 1 in the presence of 3 in dioxane/$H_2O$ mixtures is associated with these kinds of hypotheses, because the $pK_a$ of the carboxyl group of 3 is larger than that of the imidazolyl group of 1 in dioxane/$H_2O$ mixtures of large dioxane mole fraction.

In addition to the increase in the difference between the $pK_a$s of the carboxyl group and the imidazolyl group of dioxane/$H_2O$ mixtures, the carboxylate anion may be more bare in dioxane/$H_2O$ mixtures than in $H_2O$, because of the lack of hydrogen bonding between the carboxylate anion and dioxane. This also should facilitate proton abstraction from the imidazolyl group by the carboxylate anion.

We found neither formation of a complex by benzoate anion with the imidazolyl group of 1 nor aggregation of benzoate anions, because the catalytic rate in the presence of 3 increased linearly with the concentration increase of 3 as described above. Furthermore, this linear dependence rules out a change in the rate-determining step from the formation of the tetrahedral intermediate to its decomposition in the hydrolysis of 1 in dioxane/$H_2O$ mixtures (24).

The electrostatic effect in which a negatively charged benzoate anion enhances proton abstraction from $H_2O$ by the imidazolyl group of 1 through electrostatic stabilization of a resulting (partial) positive charge in the imidazolyl ring in the catalysis by the combination of the imidazolyl group and benzoate anion is too small to be significant.

The carboxylate anion of 2-benzimidazoleacetic acid exhibited no measurable electrostatic effect in general base-catalyzed hydrolysis of ethyl chloroacetate by the imidazolyl group of 2-benzimidazoleacetic acid in $H_2O$ solution, although the carboxyl and imidazolyl groups were linked closely in the same molecule by covalent bonds (5). However, an 8-fold positive deviation for 2-benzimidazoleacetic acid from the Brönsted plot for the general base-catalyzed hydrolysis of ethyl chloroacetate has been ascribed to proton abstraction by the carboxylate anion from the imidazolyl group, followed by proton abstraction by the imidazolyl group from $H_2O$, which is consistent with the charge-relay system in enzymatic reactions (5).

Furthermore, no electrostatic effect by carboxylate anion was observed in the intramolecular imidazole-catalyzed hydrolysis of the model compound used by Rogers and Bruice (6). In our study, benzoate anion is an external catalyst and, as such, it cannot cause a considerable electrostatic effect.

It is interesting to compare the rate constant of the hydrolysis of the model compound 1 in the presence of 3 with that of the hydrolysis of *trans*-cinnamoyl-$\alpha$-chymotrypsin. For this purpose, the rate constant of intramolecular general base-catalyzed hydrolysis of 1 in the absence of 3 ($6.4 \times 10^{-7}$ $sec^{-1}$ in $H_2O$ at 60°; ref. 7) can be multiplied by the ratio ($k_2[C_6H_5COO^-]/k_1$), which is 2500 (at 0.5 M 3), the dioxane mole fraction being 0.42 (Fig. 3), resulting in a rate constant of $1.6 \times 10^{-3}$ $sec^{-1}$. This value is still much smaller than that of the rate constant of the hydrolysis of *trans*-cinnamoyl-$\alpha$-chymotrypsin ($1.3 \times 10^{-1}$ $sec^{-1}$ at 60°; ref. 25). However, it should be noted that $1.6 \times 10^{-3}$ $sec^{-1}$ was estimated for intramolecular general base catalysis by the imidazolyl group of 1, assisted by *intermolecular* catalysis by 0.5 M 3 in the dioxane/$H_2O$ mixture. The hydrolyses of acyl enzymes are catalyzed by intramolecular general base catalysis by the imidazolyl group, which is assisted by *intramolecular* catalysis by a buried carboxylate anion. In charge-relay systems the carboxyl and imidazolyl groups, as well as the esterified hydroxyl groups, are oriented appropriately for catalysis. Considering the fact that the concentrations of intramolecular catalysts are in general equivalent to about 10 M intermolecular catalysts (26), rather than to 0.5 M intermolecular catalysts which are experimentally accessible, it is estimated that the rate constant for the hydrolysis of the *trans*-cinnamoyl group of 1, catalyzed by the combination of the imidazolyl and carboxyl groups, is $3.2 \times 10^{-2}$ $sec^{-1}$ at 60° when the benzoate anion in the dioxane/$H_2O$ mixture functions as an intramolecular catalyst, instead of as an intermolecular catalyst, as in the present study. The rate constant, $3.2 \times 10^{-2}$ $sec^{-1}$, is about 25% that for the hydrolysis of *trans*-cinnamoyl-$\alpha$-chymotrypsin ($1.3 \times 10^{-1}$ $sec^{-1}$) (25). The ratio of intramolecular catalysis by carboxylate anion to its intermolecular catalysis may be too conservative. For example, intramolecular catalysis by acetate anion in the hydrolysis of mono-*p*-nitrophenyl glutarate is equivalent to *ca* 600 M external catalyst (27). Thus, it can be said that the effectiveness of intramolecular general base catalysis by the imidazolyl group in the hydrolysis of 1 *in the presence of benzoate anion* is quite close to that in the hydrolysis of acyl enzymes.

In summary, the hydrolysis of 1 in the presence of 3 in dioxane/$H_2O$ mixtures is a good model for the charge-relay system and supports the validity of the charge-relay system in serine proteases.

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