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The Use of Cycloamylose to Probe the "Charge-Relay" System[1]

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The effect of the combination of imidazolyl and carboxyl groups on the cleavage of *m-t*-butylphenyl acetate in the presence of α-cyclodextrin was examined to shed light on the role of the "charge-relay" system in serine esterases. 2-Benzimidazoleacetic acid, which has both the imidazolyl and carboxyl groups in the same molecule, accelerates the cleavage of *m-t*-butylphenyl acetate in the presence of α-cyclodextrin. On the other hand, neither benzimidazole (which has only an imidazolyl group) nor 2-naphthaleneacetic acid (which has only a carboxyl group) exhibited measurable acceleration. The cleavage of *m-t*-butylphenyl acetate by the α-cyclodextrin-2-benzimidazoleacetic acid system takes place through inclusion complex formation between *m-t*-butylphenyl acetate and α-cyclodextrin, followed by catalysis associated with the combination of the carboxyl anion, the neutral imidazolyl group, and the alkoxide anion. The most probable explanation for the combination of the three groups in the catalysis involves nucleophilic attack by the imidazolyl group, assisted by the carboxyl and alkoxide anions. The mechanism of the combination of the imidazolyl, carboxyl, and hydroxyl groups is apparently different from those shown by the "charge-relay" system in enzymatic reactions.

INTRODUCTION

The enzymatic mechanism in serine esterases has been widely studied for the past two decades. The roles of the hydroxyl, the carboxyl, and the imidazolyl groups in enzymatic reations have been shown by pH–rate constant profiles (*1*, *2*), selective chemical modifications of the enzyme (*3–9*), thermodynamic studies (*10–12*), etc. The "charge-relay" system involving these three groups has been proposed ever since X-ray crystallography (*13*, *14*) uncovered the special arrangement of these groups at the catalytic site of serine esterases. In the "charge-relay" system, the nucleophilicity of the hydroxyl oxygen of the enzyme toward substrates is enhanced by proton relay from the hydroxyl group to the carboxyl group via the imidazolyl group. Acceleration of the imidazole-catalyzed cleavage of phenyl esters by carboxylic acids supported this hypothesis (*15*). Furthermore, cooperativity of hydroxyl, imidazolyl, and carboxyl groups was shown in the general base-catalyzed hydrolysis of ethyl chloroacetate by 2-benzimidazoleacetic acid (II) (*16*). Wright (*17*), however, could not provide support for the "charge-relay" system by X-ray crystallography, since the activation of chymotrypsinogen, which is inactive, to α-chymotrypsin showed only a very minor structural change in the "charge-relay" system. Furthermore, Rogers and Bruice (*18*) found only a 2.8-fold rate enhancement due to cooperativity of the hydroxyl, imidazolyl, and carboxyl groups for

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the intramolecular general base-catalysis in their model compound and concluded that this was insufficient evidence for this mechanism.

In the present paper, the effect of the combination of imidazolyl and carboxyl groups on the cleavage of an ester in the presence of cycloamylose is examined. Cycloamyloses have served as good models of enzymes (*19*, *20*). The acceleration by 2-benzimidazoleacetic acid (II) on the cleavage of *m-t*-butylphenyl acetate (I) included in the cavity of α-cyclodextrin (α-CD) is reported.

The pH–rate constant profile and the deuterium oxide solvent isotope effect for the cleavage catalyzed by the α-CD-II system are also described. Furthermore, to clarify the roles of the carboxyl and imidazolyl groups in the catalysis, the effect of benzimidazole (III) (containing no carboxyl group) on the cleavage and its deuterium oxide solvent isotope effect and the effect of 2-naphthaleneacetic acid (IV) (which has a carboxyl, but no imidazolyl, group) on the cleavage are also examined.

EXPERIMENTAL

Materials

α-CD was purified by recrystallization from 2-propanol–water (molar ratio, 2:1), followed by recrystallization from water. I was synthesized from *m-t*-butylphenol and acetyl chloride according to the method of Spasov (*21*), and was fractionally distilled (72°C, 7 mm). The [^{1}H]nmr spectrum showed signals for the nine methyl protons in the *t*-butyl group, for the three protons of the acetyl group, and for the four protons of the phenyl group, respecitively, at 1.1 δ, at 2.0 δ, and at 6.6–7.1 δ. II was obtained by hydrolysis of 2-benzimidazoleacetonitrile, followed by recrystallization from water solution. Anal. Calcd for $C_9H_8N_2O_2$: C, 61.35; H, 4.59; N, 15.90. Found: C, 61.12; H, 4.42; N, 15.86, mp 117–118°C (lit., 116°C, *22*). Other reagents were used after the usual purification of the commercial products.

Determination of Dissociation Constant of the Complex between α-CD and either II or III

Dissociation constants K_d of the complexes (BC) between α-CD (C) and either II or III (B) were determined by use of the equation:

$$\frac{[B]_0[C]_0}{d} = \frac{K_d}{\varepsilon} + \frac{1}{\varepsilon}([B]_0 + [C]_0), \tag{1}$$

where $[B]_0$ and $[C]_0$ are the initial concentrations of B and C, respectively; d is the observed change of absorbance due to complex formation; ε is the difference between the molar absorption coefficient of BC and the sum of the molar absorption coefficients of B and C.

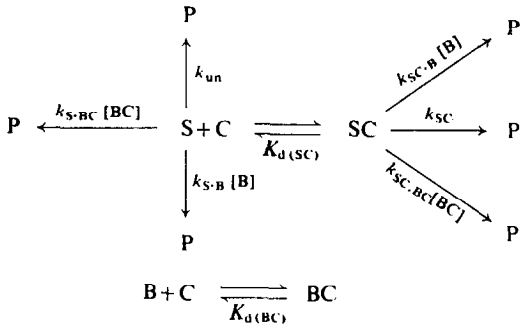
Kinetics

The cleavage of I was carried out in acetonitrile–H_2O solution (30%, v/v),[2] and was followed at 290 nm. All the reactions were pseudo-first-order with respect to the concentration of I for three half-lives. The observed rate constant, k_{obs}, was determined from the spectrophotometric data by the usual first-order equation. At least two measure-

[2] Addition of considerable amount of acetonitrile is necessary because of the scant solubility of I in water.

ments were carried out for every k_{obs} value. Acetate, phosphate, Tris–HCl, and carbonate buffers, respectively, were used in pH ranges of 4.5–6.0, 6.0–8.0, 8.0–9.0, and 9.0–11.5. The ionic strength was kept at 0.2 *M* (KCl) throughout the experiments. For the deuterium oxide experiments, pD was determined using the equation: pD = meter reading +0.4 (*23*).

When both I(S) and B form complexes, SC and BC, with α-CD (C), the scheme for the cleavage of I in the presence of α-CD and B is shown in Scheme I:



where P represents the reaction products, *m-t*-butylphenol and α-cyclodextrin acetate; k_{un} and k_{SC} are the first-order rate constants due to S and SC, respectively. $k_{S\cdot B}$, $k_{S\cdot BC}$, $k_{SC\cdot B}$, and $k_{SC\cdot BC}$ are the second-order rate constants for the reactions, (1) between S and B; (2) between S and BC; (3) between SC and B; and (4) between SC and BC. Note that the first is a reaction of two compounds, the second and third are reactions of a complex and a compound, and the fourth is a reaction of two complexes.

The observed rate constant, k_{obs}, for the cleavage of I can be represented by the following equation:

$$\begin{aligned} k_{obs}[S]_0 = {} & k_{un}([S]_0 - X) + k_{S\cdot B}([S]_0 - X)([B]_0 - Y) \\ & + k_{SC}X \quad + k_{SC\cdot B}X([B]_0 - Y) \\ & + k_{S\cdot BC}([S]_0 - X)Y + k_{SC\cdot BC}XY \end{aligned} \tag{2}$$

where X and Y represent the equilibrium concentrations of SC and BC, respectively, and the subscript zero refers to initial concentrations. The rate constant, $k_{SC\cdot B}$, which is associated with the combination of C and B in the cleavage of S, was determined by subtracting five of the six terms (all except the fourth term, which is the only term involving $k_{SC\cdot B}$) in the right-hand side of Eq. (2) from $k_{obs}[S]_0$. The experimental conditions and procedure for the determination of $k_{SC\cdot B}$ depend on whether B forms a complex with α-CD or not, as will be seen in the following analysis.

(*A*) *α-CD-II system.* Since II does not form a measurable complex with α-CD as determined spectroscopically, Y in Eq. (2) can be neglected. Therefore, under the condition that $[C]_0 \gg [S]_0$, Eq. (3) is derived from Eq. (2),

$$1/\{k_{obs} - k_{un} - k_{S\cdot B}[B]_0\} = 1/\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})[B]_0\} \times \{1 + K_{d(SC)}/[C]_0\} \tag{3}$$

using Eq. (4),

$$X \cong [C]_0[S]_0/\{K_{d(SC)} + [C]_0\}. \tag{4}$$

The values of k_{un} and $k_{S\cdot B}$ on the left-hand side of Eq. (3) were determined from the results for the hydrolyses without α-CD. Thus, a plot of $1/(k_{obs} - k_{un} - k_{S\cdot B}\,[B]_0)$ against $1/[C]_0$, keeping $[B]_0$ constant, gave both the value of $K_{d(SC)}$ and that of $\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\,[B]_0\}$. The values of $(k_{SC} - k_{un})$ and $(k_{SC\cdot B} - k_{S\cdot B})$, in turn, could be determined from the intercept and the slope, respectively, of a plot of $\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\,[B]_0\}$ against $[B]_0$.

When $K_{d(SC)}$ was determined as described above, the rate constant, $k_{SC\cdot B}$, was then determined by Eq. (5),

$$k_{SC\cdot B} = \{k_{obs}\,[S]_0 - k_{un}([S]_0 - X) - k_{S\cdot B}([S]_0 - X)\,[B]_0 - k_{SC}X\}/[B]_0 X, \qquad (5)$$

where the value of X was calculated by use of $K_{d(SC)}$.

(B) α-CD-III system. All six terms on the right-hand side of Eq. (2) must be considered here, since III forms a complex with α-CD, having the dissociation constant $K_{d(BC)}$ determined in the preceding section. However, preliminary experiments showed that either $k_{S\cdot BC}$ or $k_{SC\cdot BC}$ is much smaller than $k_{SC\cdot B}$.[3] Therefore, the determination of $k_{SC\cdot B}$ was effected by use of Eq. (6) under the condition that $[S]_0$, $[B]_0 \gg [C]_0$, where the fourth term in Eq. (2) is much larger than the fifth and sixth terms.[4]

$$k_{SC\cdot B} = \{k_{obs}\,[S]_0 - k_{un}([S]_0 - X) - k_{S\cdot B}([S]_0 - X)([B]_0 - Y) - k_{SC}X\}/X([B]_0 - Y). \qquad (6)$$

The rate constants, k_{un} and $k_{S\cdot B}$, were determined from reactions in the absence of α-CD, while k_{SC} was determined on the α-CD-II system. X and Y were calculated by use of $K_{d(SC)}$ and $K_{d(BC)}$.

RESULTS AND DISCUSSION

No change was observed, either in shape or in intensity of the absorption band of II in the 250–300-nm region on addition of α-CD to a pH 7.0 buffer solution containing II. Therefore, it was concluded that II does not form a detectable complex with α-CD.

However, an increase of the intensity of the band for III in the 260–290-nm region was observed on addition of α-CD at pH 7.0. From the slope and the intercept of the straight line in the plot of $[B]_0\,[C]_0/d$ at 285 nm against $([B]_0 + [C]_0)$, the dissociation constant and the ε value for the α-CD-III complex were obtained:

$$K_{d(BC)} = 1.7 \times 10^{-2}\,M$$
$$\varepsilon\,(285)\,\text{nm} = 130\,(M^{-1}\,\text{cm}^{-1})$$

The uncatalyzed hydrolysis of I is so slow that the rate constant, k_{un}, can be neglected in comparison with those for catalyzed hydrolyses. The buffer-catalyzed hydrolyses are

[3] $k_{obs}\,[S]_0$ under the condition that $[C]_0 > [S]_0 \gg [B]_0$, where most of B is included in α-CD, is almost equal to $\{k_{un}([S]_0 - X) + k_{S\cdot B}([S]_0 - X)\,([B]_0 - Y) + k_{SC}X\}$, calculated by use of both rate constants and the dissociation constants of SC and BC complexes. If either $k_{S\cdot BC}$ or $k_{SC\cdot BC}$ is large compared to $k_{SC\cdot B}$, the acceleration due to the fifth or sixth term on the right-hand side of Eq. (2) should be observed.

[4] In addition to the smallness of $k_{S\cdot BC}$ and $k_{SC\cdot BC}$ compared with $k_{SC\cdot B}$, Y is considerably smaller than X, since $Y \cong K_{d(SC)}\,[B]_0 X/K_{d(BC)}\,[S]_0$; $K_{d(BC)}$ is much larger than $K_{d(SC)}$, as described under Results and Discussion.

also negligible. Figure 1 depicts the plot of $1/(k_{obs} - k_{un} - k_{S\cdot B}\,[B]_0)$ versus $1/[C]_0$ for the α-CD-II system, with $[B]_0$ constant. As shown by Eq. (3), the slope and the intercept of these plots, respectively, give the values of $K_{d(SC)}/\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\,[B]_0\}$ and $1/\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\,[B]_0\}$.

Both the values of $\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\,[B]_0\}$ and those of $K_{d(SC)}$, obtained from plots such as those in Fig. 1, are listed in Table 1. $K_{d(SC)}$ values were satisfactorily constant either at 25°C or at 50°C, when $[B]_0$ was varied over a wide range.

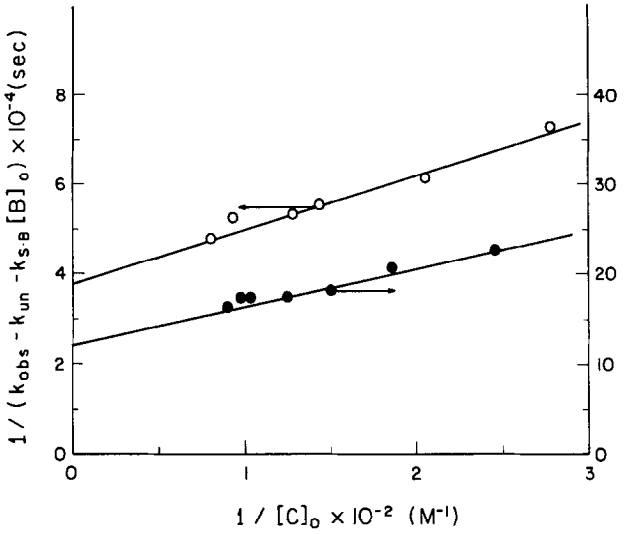


FIG. 1. Plots of $1/(k_{obs} - k_{un} - k_{S\cdot B}\,[B]_0)$ vs $1/[C]_0$ for the cleavage of *m-t*-butylphenyl acetate (I) by the α-cyclodextrin (α-CD)-2-benzimidazoleacetic acid (II) system; 25°C, pH 7.0, $[C]_0 \gg [S]_0$; ○, $[B]_0 = 9.40 \times 10^{-3}\ M$; ●, $[B]_0 = 1.72 \times 10^{-3}\ M$. The slope and the intercept, respectively, correspond to $K_{d(SC)}/\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\,[B]_0\}$ and $1/\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\,[B]_0\}$ (see Eq. (3)). $[S]_0$, $[C]_0$, and $[B]_0$, are the initial concentrations of I, α-CD, and II, respectively.

The values of $(k_{SC\cdot B} - k_{S\cdot B})$ and $(k_{SC} - k_{un})$ were determined from the slopes and intercepts in the plots of $\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\,[B]_0\}$, shown in Table 1, versus $[B]_0$. Table 2 lists the rate constants for the α-CD-II system. It was found that $k_{SC\cdot B}$ for the α-CD-II system is about seven times $k_{S\cdot B}$ either at 25°C or at 50°C.

For the α-CD-III system, $k_{SC\cdot B}$ was determined by Eq. (6), as shown in Table 3. The value of $k_{SC\cdot B}$ remained satisfactorily constant irrespective of the values of $[S]_0$, $[C]_0$, and $[B]_0$, which confirms the validity of Eq. (6) in the present study. In contrast to the α-CD-II system, $k_{SC\cdot B}$ for the α-CD-III system is smaller than $k_{S\cdot B}$ (Table 2). *Furthermore, comparison of $k_{SC\cdot B}$ for the α-CD-II system with that for the α-CD-III system shows that the introduction of the carboxyl group in the neighborhood of the imidazolyl group resulted in a 12-fold acceleration of ester cleavage.*

IV exhibited a retardation effect on the cleavage of I in the presence of α-CD. k_{obs} is 2.8×10^{-6} sec^{-1} under the conditions that $[C]_0 = 1.5 \times 10^{-2}\ M$, $[IV]_0 = 5.2 \times 10^{-3}\ M$, $[C]_0 \gg [S]_0$; pH 7.0, 25°C. On the other hand, $k_{obs} = 3.4 \times 10^{-6}$ sec^{-1} without IV under the same conditions. The retardation by IV, which is attributable to complex formation

TABLE 1

VALUES OF $\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\ [B]_0\}$ AND $K_{d(SC)}$ FOR THE CLEAVAGE OF I BY THE α-CD-II SYSTEM[a]

Temperature (°C)	$[B]_0$ (10^{-3} M)	$\{(k_{SC} - k_{un}) + (k_{SC\cdot B} - k_{S\cdot B})\ [B]_0\}$ (10^{-5} sec^{-1})	$K_{d(SC)}$ (10^{-3} M)
25	9.40	2.7	3.0
	7.03	2.3	3.6
	4.91	1.6	3.7
	1.72	0.83	3.4
Average			3.4
50	8.20	15	14
	5.31	12	10
	3.23	7.1	11
	1.62	5.8	12
Average			12

[a] pH 7.0 phosphate buffer, I = 0.2 M (KCl); k_{SC} = rate constant of the α-CD-I complex; k_{un} = rate constant of uncatalyzed hydrolysis; $k_{SC\cdot B}$ = rate constant of the α-CD-I complex with II; $k_{S\cdot B}$ = rate constant of I with II; $K_{d(SC)}$ = dissociation constant of the α-CD-I complex; α-CD, α-cyclodextrin; I, *m-t*-butylphenyl acetate; II, 2-benzimidazoleacetic acid, $[B]_0$ is the initial concentration of II.

TABLE 2

RATE CONSTANTS FOR THE CLEAVAGE OF THE α-CD-I COMPLEX BY II OR III[a,b]

	II		III
Rate constant	25°C	50°C	25°C
$10^6 k_{SC}$ (sec^{-1})[c]	4.2 ± 0.5	22 ± 3	4.2 ± 0.5
$10^4 k_{S\cdot B}$ (M^{-1} sec^{-1})[d]	4.0 ± 0.3	23 ± 3	2.8 ± 0.3
$10^4 k_{SC\cdot B}$ (M^{-1} sec^{-1})[e]	28 ± 7	150 ± 60	2.4 ± 1.0

[a] pH 7.0 phosphate buffer, I = 0.2 M (KCl).

[b] α-CD, α-cyclodextrin; I, *m-t*-butylphenyl acetate; II, 2-benzimidazoleacetic acid; III: benzimidazole.

[c] k_{SC} = rate constant of the α-CD-I complex.

[d] $k_{S\cdot B}$ = rate constant of I with base (II or III).

[e] $k_{SC\cdot B}$ = rate constant of the α-CD-I complex with base (II or III).

between α-CD and IV, indicates the predominant role of the imidazolyl group of II in the catalysis by the α-CD-II system.

The pH dependence of $k_{SC\cdot B}$ for the cleavage of I catalyzed by α-CD-II system as well as those of k_{SC} catalyzed by α-CD alone and of $k_{S\cdot B}$ catalyzed by II alone were measured at 50°C. The rate constant, k_{SC}, for the α-CD-I complex increased proportionally to pH with a slope of 1.0 in the pH range of 5.0–10.7. Above pH 10.7, the slope becomes gradually smaller, which is consistent with the results in a previous paper (*24*). The pH

TABLE 3

DETERMINATION OF $k_{SC\cdot B}$ FOR THE CLEAVAGE OF I BY THE α-CD-III SYSTEM[a]

$[S]_0$ (10^{-2} M)	$[B]_0$ (10^{-2} M)	$[C]_0$ (10^{-3} M)	k_{obs} (10^{-6} sec^{-1})	$k_{SC\cdot B}$ (10^{-4} M^{-1} sec^{-1})
7.25	6.55	5.01	18.1	2.2
6.48	8.26	4.82	23.1	2.7
3.62	4.26	3.82	11.9	2.3
3.82	2.86	2.46	8.08	2.4
3.05	2.12	1.55	6.04	2.6
2.66	3.02	1.26	8.51	2.3
1.86	2.52	1.82	7.14	2.3
1.60	3.22	1.60	9.04	2.2
1.06	1.02	1.06	3.09	2.6
Average				2.4

[a] pH 7.0 phosphate buffer, 25°, I = 0.2 M (KCl); $k_{SC\cdot B}$ = rate constant of the α-CD-I complex with III; α-CD, α-cyclodextrin; I, *m-t*-butylphenyl acetate; III, benzimidazole; $[S]_0$, $[B]_0$, and $[C]_0$ are the initial concentrations of I, III, and α-CD, respectively.

profile of $k_{S\cdot B}$ corresponds to a functional group of pK_a 6.1. This functional group is definitely assigned to the imidazolyl group in II, since the rate of hydrolysis of I catalyzed by IV is negligibly small in comparison with that catalyzed by II.

Figure 2 shows the pH dependence of $k_{SC\cdot B}$ at 50°C. $k_{SC\cdot B}$ was determined by use of Eq. (5), where $K_{d(SC)}$ was taken as 1.2×10^{-2} M. Below pH 5.8, a plot of logarithm of $k_{SC\cdot B}$ against pH exhibited a straight line of slope 2.0, whereas in the pH range of 6.4–9.7,

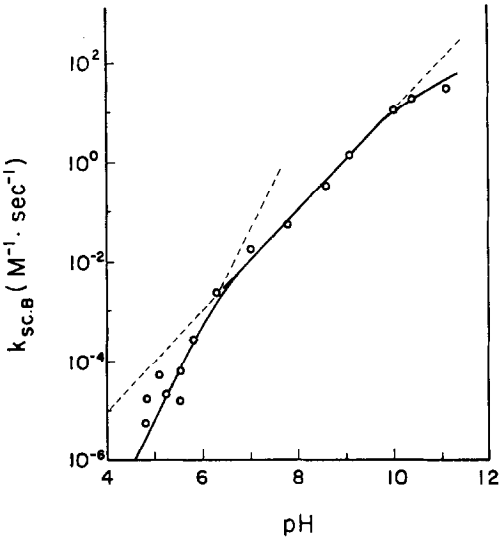


FIG. 2. pH profile of $k_{SC\cdot B}$ for the cleavage of *m-t*-butylphenyl acetate (I) by the α-cyclodextrin (α-CD)-2-benzimidazoleacetic acid (II) system at 50°C; $k_{SC\cdot B}$ = rate constant of the α-CD-I complex with II.

it showed another straight line of slope 1.0. The pH of intersection of these two straight lines is 6.2, which is identical with the pK_a (6.1) of the imidazolyl group in II within experimental error. Above pH 9.7, the slope decreased gradually with pH. The pH–$k_{SC\cdot B}$ profile shows that the $k_{SC\cdot B}$ term depends both on the concentration of alkoxide anion of α-CD and on the concentration of the neutral imidazolyl group of II. The ionization of the carboxyl group was not observed in Fig. 2, since the carboxyl group of II should be entirely ionized above pH 4.5. Comparatively large deviations of points below pH 6.0 in the pH–$k_{SC\cdot B}$ profile are attributable to the subtraction procedure for the determination of $k_{SC\cdot B}$. In this pH region, the observed rate for the α-CD-II system is not overwhelmingly larger than the rate due to the catalysis by II alone.

D_2O solvent isotope effects were measured at 50°C and pH (pD) 7.5. The rate constant, $k_{SC\cdot B}$, in acetonitrile–H_2O solution was found to be 3.1 ± 0.3 times that in acetonitrile D_2O solution. On the other hand, k_{SC} for α-CD alone and $k_{S\cdot B}$ for II alone, respectively, in acetonitrile–H_2O solution are 3.2 ± 0.3 and 1.2 ± 0.2 times those in acetonitrile–D_2O solution. The 3.1-fold difference in $k_{SC\cdot B}$ is attributable to the difference between the pK_a of α-CD in acetonitrile–H_2O solution and that in acetonitrile–D_2O solution (*25*), since the 3.1-fold difference in $k_{SC\cdot B}$ is equal to the 3.2-fold difference in k_{SC} within experimental error. Thus, a kinetically important D_2O effect was not observed for $k_{SC\cdot B}$. The ionization of the imidazolyl group of II hardly affects the D_2O effect, since pH (pD) 7.5 is far from both the pK_a of II in acetonitrile–H_2O solution (6.1) and that in acetonitrile–D_2O solution (6.5). The small D_2O solvent isotope effect on $k_{S\cdot B}$ indicates nucleophilic attack by the imidazolyl residue of II toward I, which is not included in α-CD. This result is consistent with nucleophile catalysis by imidazole in the hydrolyses of phenyl acetates (*26*).

Since the $k_{SC\cdot B}$ term depends both on the concentration of the alkoxide anion of α-CD and on the concentration of the neutral imidazolyl group of II, both nucelophilic attack by the alkoxide anion toward I and that by the imidazolyl group toward I can be possible pathways. The D_2O experiments could not distinguish these two mechanisms from one another.

However, the following experiment indicates that nucleophilic attack by the imidazolyl group takes place in the catalysis by the α-CD-II system. The rate constant for the cleavage of I by the α-CD-II system remained constant irrespective of conversion up to at least 400% conversion with respect to α-CD, when the initial concentration of I was 10-fold larger than that of α-CD, and the initial concentration of II was 0.1 *M*.

When the catalysis by the α-CD-II system proceeds through nucleophilic attack by the alkoxide anion of α-CD toward I, the rate constant for the ester cleavage should become smaller with conversion up to a limiting value corresponding to the reaction rate between II and I, which is not included in α-CD, since the concentration of α-CD in the solution becomes smaller with conversion because of the small rate constant for the hydrolysis of the resulting α-cyclodextrin acetate (*24*). Furthermore, a preliminary experiment showed that II hardly accelerates the hydrolysis of α-cyclodextrin cinnamate, which is ascribed to the lack of complex formation between α-cyclodextrin cinnamate and II (*27*). However, when the imidazolyl group of II functions as nucleophile in the catalysis by α-CD-II system, the rate constant should remain constant up to high conversion, since II is easily regenerated by fast hydrolysis of acetylated II.

The most plausible explanation for the combination of the imidazolyl group, the

carboxyl anion, and the alkoxide anion, in the cleavage of I is nucleophilic attack by the neutral imidazolyl group, which is assisted by the alkoxide anion. The alkoxide anion can bring the imidazolyl group of II close to I included in the cavity of α-CD through hydrogen bonding. Besides, the hydrogen bonding between a nitrogen atom of the imidazolyl group and the alkoxide anion can increase the nucleophilicity of the other nitrogen atom in the imidazolyl group toward I, as in the hydroxide anion-assisted imidazole catalyses of ester hydrolyses (*28*). Considerable rate enhancement by the introduction of a carboxyl anion is probably attributable to the stabilization of the transition state of nucleophilic attack by the imidazolyl group, which produces a positive charge in the imidazolyl ring, through an electrostatic effect. The comparatively hydrophobic atmosphere at the top of the cavity of α-CD is favorable for this effect.

In summary, the present kinetic study shows the combination of the imidazolyl, carboxyl, and alkoxyl groups in the cleavage of an ester. Catalysis takes place after complex formation between the substrate and α-CD, which is consistent with the formation of the substrate–enzyme complex in enzymatic reactions. This system can be of use to shed light on the "charge-relay" system in serine esterases, though the manner of the combination of the three groups is apparently different from those shown by the enzymes.

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