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General Base-Catalyzed Ester Hydrolysis as a Model of the "Charge-Relay" System[1]

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The validity of the "charge-relay" system in serine esterases was examined by use of the general base-catalyzed hydrolysis of ethyl chloroacetate (I) as a model system. The general base catalytic rate for 2-benzimidazoleacetic acid (II) exhibited an eightfold positive deviation from the Brønsted plot including benzimidazole, imidazole, *N*-methylimidazole (V), and acetate ion, though in nucleophilic catalysis of the hydrolysis of *p*-nitrophenyl acetate, the point for II conformed to the Brønsted relationship together with imidazole and benzimidazole derivatives. The positive deviation of II from the Brønsted plot for the general base-catalysis was attributed to the cooperativity of the carboxyl group of II, the imidazolyl group of II, and the hydroxyl group of water. The present result provides support for the "charge-relay" system. Furthermore, the (essentially) total loss of the enzymatic activity due to N-3 methylation of histidine-57 in α-chymotrypsin is discussed in comparison to the general base-catalysis of V in the hydrolysis of I, which is also favorable for the "charge-relay" system.

INTRODUCTION

Recently, much attention has been given to the catalytic mechanism in serine esterases. The "charge-relay" system, where proton relay from the hydroxyl group to the carboxyl group, via the imidazolyl group in the enzyme, enhances the nucleophilicity of the hydroxyl group toward the substrate, is widely accepted. In fact, X-ray crystallography discovered the triad of these three functional groups, arranged in a geometrical relationship favorable for proton relay, at the catalytic sites of α-chymotrypsin (*1*) and subtilisin (*2*). The "charge-relay" system is consistent with much previous research (*3*), which showed the importance of the hydroxyl, imidazolyl, and carboxyl groups in the enzymatic reaction. The hypothesis, however, has not been firmly established yet, since there is scanty information on the cooperativity of the hydroxyl, imidazolyl, and carboxyl groups in ester hydrolysis (*4*, *5*). Wright (*6*) could not find evidence for the "charge-relay" system by X-ray crystallography (*7*), since no important structural difference of the "charge-relay" system was observed between chymotrypsinogen, which is inactive, and α-chymotrypsin, which is active. Rogers and Bruice (*8*) found only a 2.8-fold acceleration, due to the cooperativity of the three functional groups in their model compound of the enzyme, and concluded that this

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is not sufficient evidence for the "charge-relay" system. Recently, however, the present authors found a 12-fold acceleration due to the cooperativity of the hydroxyl, imidazolyl, and carboxyl groups in the cycloamylose-catalyzed cleavage of an ester (*9*).

In the present paper, the general base-catalyzed hydrolysis (*10*) of ethyl chloroacetate (**I**) by 2-benzimidazoleacetic acid (**II**) is used as a model for the "charge-relay" system. For comparison, the hydrolysis of **I**, either by benzimidazole (**III**) or by 2-naphthaleneacetic acid (**IV**), and that of *p*-nitrophenyl acetate (**VI**) by **II** are also examined. Furthermore, the hydrolysis of **I** by the model (**V**) is compared to methylated α-chymotrypsin (*23*). This comparison also supports the validity of the "charge-relay" system in serine esterases.

EXPERIMENTAL

Materials

I, bp 145°C (lit. *10*, 143.5–144°C), and **V**, bp 192°C (lit. *11*, 191°C), were distilled from commercial products. **II** was obtained by hydrolysis of 2-benzimidazoleacetonitrile and was purified by recrystallization from water; mp 117–118°C (lit. *12*, 116°C). *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. **III** and **IV** were used after recrystallization of commercial products; mp 171°C (lit. *11*, 171–173°C) and mp 143–144°C (lit. *13*, 142°C), respectively. **VI** was synthesized from *p*-nitrophenol and acetic anhydride (*14*) and was purified by recrystallization from ethanol; mp 80–81°C (lit. *15*, 79.5–80°C).

Kinetics

The hydrolysis of **I** was followed by measurement of the concentration of the residual ester by the method of Hestrin (*16*). After a predetermined time, 1 ml of the sample solution was added to 1 ml of a freshly prepared hydroxylamine mixture, to which 4 ml of 10% $FeCl_3 \cdot 6H_2O$ solution in 0.3 *M* HCl was added after a 2-min incubation. After 5 min, the absorbance at 540 nm, which is proportional to the concentration of the residual ester, was measured on a Cary Model 14 spectrophotometer. The hydroxylamine mixture consisted of 5 vol of 3.5 *M* NaOH, 4 vol of 4.0 *M* $NH_2OH \cdot HCl$, and 1 vol of water. For each run at a specific time, the measurement was carried out at least twice; the deviation between measurements was smaller than ±2%. The pseudo-first-order rate constant, k_{obs}, was determined by the method of Guggenheim (*17*).

The hydrolysis of **VI** was followed by absorption at 400 nm due to the release of *p*-nitrophenol. Reactions either for **I** or for **VI** were followed at least three half-lives and were found invariably to be first-order.

The initial concentrations of **I** and **VI** were 6×10^{-3} to 8×10^{-3} *M* and 1×10^{-4} to 5×10^{-4} *M*, respectively. All the reactions were carried out at 25°C. In order to increase the solubility of the substrates, 2% of ethanol and 0.5% of acetonitrile were added to water for the hydrolyses of **I** and **VI**, respectively. The change in pH of the sample solution before and after the reaction did not exceed ±0.02 pH unit. For the deuterium oxide experiments, pD was determined using this equation: pD = meter reading + 0.4 (*18*).

Aliquots of reaction mixture in which hydrolysis of 2×10^{-2} *M* of **I** catalyzed by **II**, **III**, or **V** was almost complete, were analyzed for the presence of free chloride ion

by silver nitrate. However, no indication of chloride ion was found under the conditions where 5×10^{-4} M of chloride ion could easily be detected.

The second-order rate constant, k_2, was obtained from the slope of a plot of the observed first-order rate constant, k_{obs}, versus the concentration of catalyst. The catalytic rate constant, k_c, corresponding to the catalytically active species, was calculated by use of Eq. [1]:

$$k_c = \frac{K_a + [H^+]}{K_a} k_2, \qquad [1]$$

where K_a refers to the imidazolyl moiety of the catalyst and is shown in Table I. Equation [1] is based on the assumption that only the unprotonated imidazolyl residue in the catalyst is the catalytic species, which is confirmed by the pH–k_2 profile in Fig. 2. The concentration of unprotonated imidazolyl component for **II** or **V** in D_2O was determined by potentiometric titration with acid at 0.5 M KCl and 25°C.

RESULTS

Table 1 lists the rate constants for the hydrolyses of **I** by **II**, **III**, **IV**, and **V**. The k_c for **II** was found to be 11 times that for **III**, which shows the important role of the carboxyl group of **II** in catalysis. The deuterium oxide solvent isotope effects for **II** and **V** are 3.8 and 3.1, respectively.

The value of k_c (2.3×10^{-5} M^{-1} sec^{-1}) for **V** is about two-thirds that (3.7×10^{-5} M^{-1} sec^{-1}) for imidazole (*10*). The intercepts, k_i, equal to $k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_{H_2O}$, for **II**, **III**, **IV**, and **V** are identical to each other within experimental error.

Figure 1 shows the Brønsted plot for the general base-catalyzed hydrolysis of **I** at 25°C. The pK_a value (6.1) of **II** was determined from the pH-rate constant profile for the nucleophile-catalyzed hydrolysis of *m-t*-butylphenyl acetate by **II** (*9*). Plots of the logarithm of k_c against pK_a for **III** and **V**, together with those (*10*) for imidazole and acetate ion, exhibited a straight line with a slope of 0.67. The point for **II**, however,

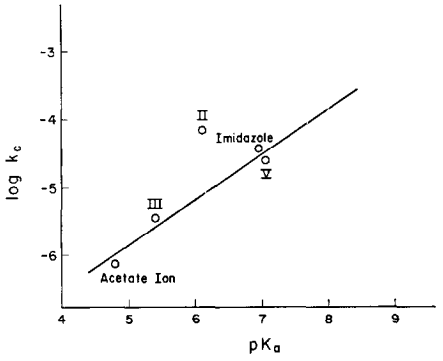


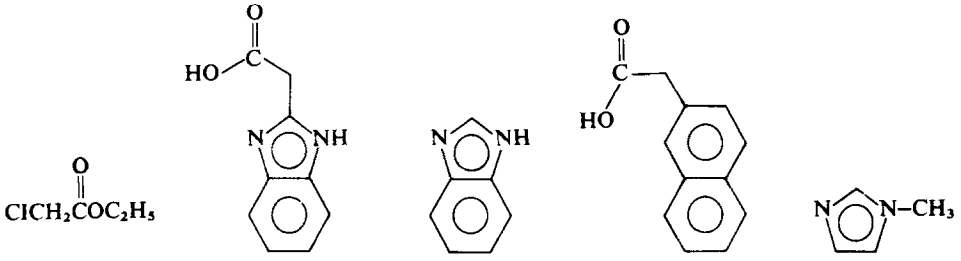
FIG. 1. Brønsted plot for the general base-catalyzed hydrolysis of ethyl chloroacetate (**I**) at 25°C by 2-benzimidazoleacetic acid (**II**), by benzimidazole (**III**), by *N*-methylimidazole (**V**), by imidazole, and by acetate ion; the rate constants are in M^{-1} sec^{-1} units. Data for imidazole and acetate ion from Jencks and Carriuolo (*10*).

TABLE 1

RATE CONSTANTS FOR THE HYDROLYSIS OF I[a]

Catalyst	Solvent	Number of determinations	k_c[b] (10^{-6} M^{-1} sec^{-1})	k_l[c] (10^{-6} sec^{-1})	$\frac{k_c(H_2O)}{k_c(D_2O)}$	pK_a of imidazolyl moiety in H_2O
II[d]						
1.8–9.3 × 10^{-2} M	H_2O	4	60 ± 5	1.5 ± 0.2	3.8 ± 0.9	6.1[e]
2.8–8.2 × 10^{-2} M	D_2O	3	16 ± 3	1.2 ± 0.2		
III[d]						
1.6–8.7 × 10^{-2} M	H_2O	4	5.3 ± 0.8	1.8 ± 0.2	—	5.43[f]
IV[d]						
1.7–28.2 × 10^{-2} M	H_2O	4	0.0 ± 0.1	1.7 ± 0.2	—	—
V[d]						
2.5–26.2 × 10^{-2} M	H_2O	4	23 ± 2	1.6 ± 0.2	3.1 ± 0.6	7.00[g]
2.4–19.3 × 10^{-2} M	D_2O	3	7.5 ± 1.0	1.1 ± 0.2		

[a] 25°C, pH = 7.0 (phosphate buffer), I = 0.5 M (KCl).
[b] k_c = catalytic rate constant.
[c] $k_l = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_{H_2O}$.
[d] See below:



(I) (II) (III) (IV) (V)

[e] Reference (*9*).
[f] V. A. Pavlova, *Zhur. Nauch. i Priklad. Fot. i Kinematog.* **3**, 101 (1958); *Chem. Abstr.* **52**, 20135d (1958).
[g] Reference (*20*).

showed a large positive deviation from a straight line, which corresponds to about an eightfold enhancement of the catalytic activity.

Table 2 shows the rate constants for the hydrolysis of **VI** by **II**. A small deuterium oxide solvent isotope effect ($k_c(H_2O)/k_c(D_2O) = 1.1$) indicates that **II** functions as a nucleophile catalyst in the hydrolysis of **VI**. The nucleophile catalytic site in **II** is the imidazolyl group (*19*), since **IV** barely catalyzes the reaction.

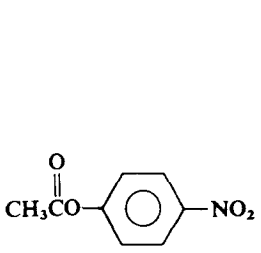
Importantly, the point for **II** fits a straight line of slope 1.12 well in the Brønsted plot for the nucleophile-catalysis of the hydrolysis of **VI**. The plot includes benzimidazole, 4-methylimidazole, 4-hydroxylmethylimidazole (*11*), *N*-methylimidazole, and imidazole (*20*), as well as **II**. This result is much different from the deviation of the

TABLE 2

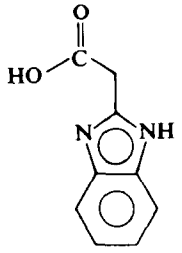
RATE CONSTANTS FOR HYDROLYSIS OF VI BY II[a,b]

Solvent	Number of determinations	k_c[c] (10^{-2} M^{-1} sec^{-1})	k_i[d] (10^{-6} sec^{-1})	$\frac{k_c(H_2O)}{k_c(D_2O)}$
H_2O	4	2.5 ± 0.2	1.7 ± 0.2	1.1 ± 0.3
D_2O	4	2.2 ± 0.2	1.5 ± 0.2	

[a] 25°C, pH = 7.0 (phosphate buffer), I = 0.2 M (KCl), [II] = 1.8–8.8 × 10^{-2} M.
[b] See below:



(VI)



(II)

[c] k_c = catalytic rate constant.
[d] $k_i = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_{H_2O}$.

point for **II** in the Brønsted plot of the general base-catalysis in the hydrolysis of **I** (Fig. 1).

Figure 2 shows the pH–k_2 profile for the hydrolysis of **I** by **II**. The pH–k_2 profile corresponds to catalysis by a functional group of pK_a 6.1, which is identical with the pK_a of the imidazolyl group of **II** (*9*). Figure 2 indicates that the unprotonated imidazolyl residue is the only catalytic species.

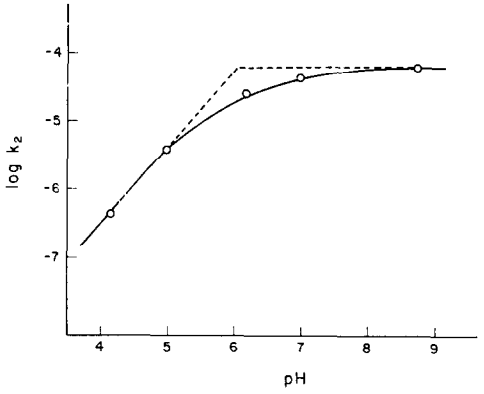
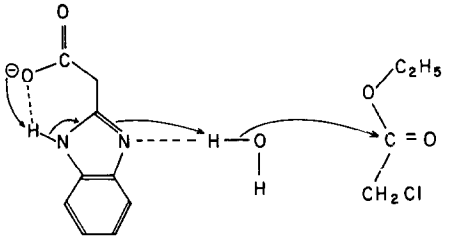


FIG. 2. The pH–k_2 profile in the hydrolysis of ethyl chloroacetate (**I**) by 2-benzimidazoleacetic acid (**II**); 25°C, I = 0.5 M (KCl). Solid line is the theoretical line calculated by $k_c = 6 \times 10^{-5}$ M^{-1} sec^{-1} and pK_a = 6.1, using Eq. [1].

DISCUSSION

The large positive deviation, corresponding to an eightfold acceleration of the point for **II** *from the Brønsted plot in the general base-catalyzed hydrolysis of* **I** *is associated with the carboxyl group of* **II**. However, the carboxyl group itself cannot catalyze the reaction, since **IV** barely catalyzes the reaction. Therefore, the enhancement of **II** in general base-catalyzed hydrolysis can be attributed to the cooperative function of the carboxyl group, both with the imidazolyl group of **II** and with the hydroxyl group of water. Consequently, a mechanism of the hydrolysis of **I** by **II** can be proposed, as in Scheme 1.



SCHEME 1.

In Scheme 1, the nucleophilicity of the hydroxyl oxygen atom of water toward the substrate is enhanced through proton removal by the nitrogen atom of the imidazolyl group, the nucleophilicity of which is in turn enhanced through proton removal by the carboxyl anion from the other nitrogen atom of the imidazolyl group. Obviously, Scheme 1 is substantially identical with the "charge-relay" system in serine proteases. The enhancement of **II** in catalytic activity, due to the cooperativity of the hydroxyl, imidazolyl, and carboxyl groups, indicates, in this instance, the validity of the "charge-relay" system.

The proton abstraction of the carboxyl anion from the imidazolyl group through the hydrogen bonding in Scheme 1 is supported by ir spectroscopy (*21*). In the solid state, the carbonyl oxygen of the carboxyl group of **II** is hydrogen bonded to the nitrogen atom of the imidazolyl group. Since almost all the carboxyl groups are ionized at pH 7.0, **II** can have the configuration depicted in Scheme 1 with very high probability, through stabilization by hydrogen bonding.

The magnitude of the D_2O effect in the hydrolysis of **I** by **V** (3.1) is quite similar to that (3.0) by imidazole found by Jencks and Carriuolo (*10*). The large values of the D_2O solvent isotope effect indicate that both **II** and **V** function as general base-catalysts in the hydrolysis of **I**. The catalysis of hydrolysis by **IV** is much smaller than by **II**, indicating that catalysis by **II** is not by the carboxyl group, but rather by the imidazolyl group. The general base-catalysis of the imidazolyl group in the hydrolysis of **I** is consistent with the finding by Jencks and Carriuolo (*10*).

The positive deviation for **II** in general base-catalysis from the Brønsted plot in Fig. 1 might be attributed to the stabilization of the hydrogen abstraction from the water molecule by the imidazolyl group through an electrostatic effect by the carboxyl anion. The electrostatic effect could be transferred through space or through bonds. However, this does not seem to be the case. If an electrostatic effect were to explain the deviation in the general base-catalysis, it should have appeared, and been larger, in the nucleophile-catalyzed hydrolysis of **VI** by **II**. This is because charge development

on the imidazolyl residue in nucleophile-catalysis is usually larger than it is in general base-catalysis. But no deviation for **II** was observed in the Brønsted plot for nucleophile-catalysis. Thus, the positive deviation for **II** in the Brønsted plot for general base-catalysis is not attributable to an electrostatic effect.

The positive deviation for **II** in Fig. 1 cannot be ascribed to the "abnormal" pK_a due to the adjacent anion either. Epstein et al. (*22*) proposed that the pK_a of a positively charged phenol is not a correct measure of the basicity of the corresponding phenolate ions toward neutral substrate, since the dissociation of protons from positively charged phenols is facilitated by electrostatic repulsion. In **II**, the adjacent anion suppresses the release of the proton from the protonated imidazole, resulting in a pK_a value larger than expected from its basicity toward neutral substrate alone. Thus, the effect of the "abnormal" pK_a value, if it functions here, should cause a negative deviation for **II** from the Brønsted plot, rather than the positive deviation found in the present study. In other words, the deviation for **II** from the Brønsted plot can be larger than eightfold, if the effect of the "abnormal" pK_a is absent. Thus, it is quite reasonable to propose Scheme 1 for the hydrolysis of **I** by **II**, though all other possibilities have not been definitely ruled out.

The slope of the Brønsted plot (0.67) for the general base-catalyzed hydrolysis of **I** is slightly larger than that (0.47) for the general base-catalyzed hydrolysis of ethyl dichloroacetate (*10*). This higher selectivity of **I** toward catalyst is attributable to the reactivity of **I** being smaller than that of ethyl dichloroacetate. **I** is activated by one chlorine atom, whereas ethyl dichloroacetate is activated by two chlorine atoms.

The result in the present report showed that **V** can function as a general base-catalyst in the hydrolysis of **I** in the same manner as imidazole (*10*); the methylation of the nitrogen atom reduced the k_c value of the imidazolyl group by only about one-third. This result is important, since the methylation of the nitrogen in position 3 of the imidazolyl group of histidine-57 in α-chymotrypsin showed essentially total loss of enzymatic reactivity (*23*). The essentially total loss of enzymatic reactivity is ascribed to a decrease of the rate constants, not to a change in the binding of the substrate to the enzyme; *N*-methylation of α-chymotrypsin reduced the rate constants of acylation and deacylation steps by 2×10^5- and 5×10^4-fold, respectively, for *N*-acetyl-L-tyrosine ethyl ester, while it did not affect the dissociation constant of the enzyme–substrate complex (*24*).

The general base-catalysis of **V** is directly concerned with the enzymatic reaction by methylated α-chymotrypsin, since the *N*-methylimidazolyl group of modified histidine-57 functions as a general base-catalyst (*24*, *25*). For this general base-catalysis in the modified enzyme, however, the movement of the side chain of histidine-57 must locate the N-1, instead of the N-3, of modified histidine-57, close to serine-195, resulting in the formation of the hydrogen bond between histidine-57 and serine-195 and the destruction of that between histidine-57 and aspartate-102 (*24*). Thus, the essentially total loss of enzymatic activity due to N-3 methylation indicates the importance of the "charge-relay" system in native α-chymotrypsin, since k_c for **V** is two-thirds that for imidazole as shown in the present paper. Finally, it should be noted that N-3 methylation of α-chymotrypsin can reduce the enzymatic activity through the lack of the general acid-catalysis by the imidazolyl group of histidine-57 (*26*) as well as through the breakdown of the "charge-relay" system.

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