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Intramolecular Nucleophilicity of Amide Groups: Implications on Catalytic Roles of Amide Groups in Enzymatic Reactions

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The rates for the cyclization reactions of phenyl or 2,2,2-trifluoroethyl esters of (Z)-α-(acetylamino)cinnamic acid or (Z)-α-(benzoylamino)cinnamic acid leading to the formation of the corresponding oxazolinones, as well as those of the reverse addition reactions of phenol or 2,2,2-trifluoroethanol to the oxazolinones, were measured. The cyclization reactions involve intramolecular nucleophilic attack of amide oxygen atoms at the alkyl or aryl ester linkages. The efficiency of this intramolecular process was compared with that reported for the carboxyl groups of mono-aryl or mono-alkyl esters of phthalic acid derivatives. The comparison indicated that the amide groups of the present study are as effective as or more effective than the carboxyl groups of the phthalate compounds. The efficient intramolecular nucleophilic attack of amide groups suggests that amide groups can play important catalytic roles in enzyme mechanisms. © 1988 Academic Press, Inc.

Enzymatic catalysis can be viewed as a very efficient intramolecular reaction between the active-site catalytic groups of the enzyme and the reaction sites of the substrate within an enzyme–substrate complex (*1–6*). The catalytic groups of the active sites include polar side chains of amino acids such as carboxyl, hydroxyl (alcoholic or phenolic), imidazole, amino, guanidinium, and thiol groups. Intramolecular reactions of these functional groups have been intensively investigated in small organic compounds in relation to their catalytic roles in enzymatic reactions (*4–6*). In the model studies, efficient intramolecular catalysis has been achieved by these functional groups.

The polar functional group most abundantly available in the active sites of enzymes is the amide group, being present either in the form of a peptide linkage or as the side chain of glutamine or asparagine. The possibility of participation of amide groups in the catalytic processes of enzymes, however, has been ignored in the proposed mechanisms of most of the enzymatic reactions. This is mainly because highly effective intramolecular catalysis by amide groups has not been observed with model organic compounds, although a few reports (*7–10*) have been made previously for the intramolecular participation of amide groups in the hydrolysis of activated esters. If very efficient intramolecular reactivity of amide groups is demonstrated with small model compounds, therefore, participation of

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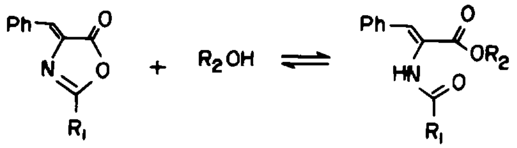
amide groups as catalytic groups must be duly considered in the discussion of mechanisms of enzymatic reactions in general.

In the present study, the interconversion between oxazolinone **1a** or **2a** and the corresponding aryl or alkyl ester (**1b** or **1c**/**2b** or **2c**) was investigated. Thus, kinetics for the addition reactions of phenol (PhOH)[2] or 2,2,2-trifluoroethanol (TFE) to **1a** and **2a** and for the cyclization reactions of **1b**, **1c**, and **2c** were measured. In esters **1b**, **1c**, **2b**, and **2c**, the oxygen atoms, instead of the nitrogen atoms, of the amide groups are geometrically allowed to attack the carbonyl carbon of the ester linkages. It was hoped that close proximity could be achieved between the amide oxygen and the ester carbon atoms in these compounds. Kinetic data revealed very high intramolecular nucleophilicity of amide groups of the ester substrates, which in turn indicated that the catalytic roles of amide groups should not be ignored in the proposed mechanisms of enzymatic reactions (Eq. [1]).



[1]

1a: R_1 = Me
2a: R_1 = Ph

1b: R_1 = Me, R_2 = Ph
1c: R_1 = Me, R_2 = CF_3CH_2
1d: R_1 = Me, R_2 = H
2b: R_1 = Ph, R_2 = Ph
2c: R_1 = Ph, R_2 = CF_3CH_2
2d: R_1 = Ph, R_2 = H

EXPERIMENTAL PROCEDURES

Materials

(Z)-4-Benzylidene-2-methyloxazolin-5-one (***1a***), *(Z)-4-benzylidene-2-phenyloxazolin-5-one* (***2a***), *(Z)-α-(acetylamino)cinnamic acid* (***1d***), and *(Z)-α-(benzoylamino)cinnamic acid* (***2d***). These compounds were prepared as described previously (*11*).

Phenyl (Z)-α-(acetylamino)cinnamate (***1b***). To an *N*-(2-hydroxyethyl)piperazinyl-*N*′-2-ethanesulfonate buffer (0.01 M, 50 ml) at pH 7.0 and 25°C, 1.0 g (11 mmol) PhOH was added. An acetone solution (50 ml) of 0.10 g (0.54 mmol) **1a** was added to the mixture, and the resulting solution was stirred for 3 h at 25°C. The residues obtained after evaporation of acetone under a reduced pressure was recrystallized from ethanol; mp 153–154°C; ^1H NMR ($CDCl_3$) δ 2.10 (s, 3H, CH_3), 7.26–7.43 (m, 11H, aromatic and vinylic H); ir (KBr-disc) 3440 (NH), 1730 (C=O), 1650 (amide C=O); MS (EI), calcd for $C_{17}H_{15}O_3N$: *m/e*, 281, found 281 (M); Anal. C, H, N.

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[2] Abbreviations used: PhOH, phenol; TFE, 2,2,2-trifluoroethanol.

*2,2,2-Trifluoroethyl (Z)-α-(acetylamino)cinnamate (**1c**).* This compound was prepared according to the procedure described above for **1b** except that a borate buffer at pH 8.5 and 0.78 ml (11 mmol) TFE were used; mp 139–140°C; ^{1}H NMR ($CDCl_3$), δ 2.10 (s, 3H, CH_3), 4.61 (q, J_{HF} = 8 Hz, 2H, CH_2), 7.26–7.45 (m, 6H, aromatic and vinylic H); ir (KBr-disc) 3440 (NH), 1730 (ester C=O), 1670 (amide C=O); MS (EI), calcd for $C_{13}H_{12}O_3NF_3$, *m/e*, 287, found 287 (M); Anal. C, H, N.

*2,2,2-Trifluoroethyl (Z)-α-(benzoylamino)cinnamate (**2c**).* This compound was prepared according to the procedure described above for **1c** except that a 0.59 ml (7.0 mmol) TFE and 0.1 g (0.40 mmol) **2a** were used; mp 141–142°C; ^{1}H NMR ($CDCl_3$), δ 4.65 (q, J_{HF} = 8.8 Hz, 2H, CH_2), 7.26–7.55 (m, 11H, aromatic and vinylic H); ir (KBr-disc) 3440 (NH), 1740 (ester C=O), 1660 (amide C=O); MS (EI), calcd for $C_{18}H_{14}O_3NF_3$, *m/e*, 349, found 349 (M); Anal. C, H, N.

Acetone, acetonitrile, PhOH, and TFE were purified according to the literature (*12*) before being used in kinetic studies. Water was distilled, deionized, and then used in kinetic measurements.

Kinetic Measurements

Reaction rates were measured with a Beckman Model 5260 uv/vis spectrophotometer by observing absorbance changes in the 330- to 370-nm region. Temperature was maintained at 25 ± 0.1°C with a Lauda Brinkman T-2 circulator. pH measurements were performed with a Dongwoo DF-215 pH meter. Buffers (0.01 M unless noted otherwise) used were 4-morpholineethanesulfonate, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonate, borate, and sodium bicarbonate. Ionic strength was maintained at 1.0 M with sodium chloride. Substrate concentrations used in the rate measurements were $0.5–2 \times 10^{-4}$ M. Kinetic measurements were performed in the presence of either 9.1% (v/v) acetonitrile or 40% (v/v) acetone. The pH readings reported with kinetic results are those measured in the presence of the organic solvents.[3]

Product Identification

The uv spectra of the final products for the addition reactions of PhOH or TFE to **1a** or **2a** and for the cyclization reactions of **1b**, **1c**, and **2c** were identical to those of the corresponding cinnamic acid (**1d** or **2d**). For this comparison of spectra, it was necessary to remove acetone from the product solutions in order to eliminate the large absorbance of acetone. The uv spectrum of the intermediate formed by the addition of 0.2 M PhOH to **1a** at pH 9 was identical to that of **1b**. The spectrum was measured only at ≥310 nm, due to the absorbance of added PhOH. Under these conditions, **1b** would be formed quantitatively, because of the equilibration between **1a**, PhOH, and **1b**, as indicated by kinetic results. The uv spectra of the intermediates formed during the addition reactions of PhOH or TFE to **1a** or **2a** in the presence of 40% (v/v) were not measured due to the large absorbance of acetone.

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[3] The pH of buffer solutions increases by up to 1 pH unit upon the addition of 40% (v/v) acetone. The ion product (K_w) of water would decrease as the polarity of the medium is lowered by the addition of organic solvents (*11*).

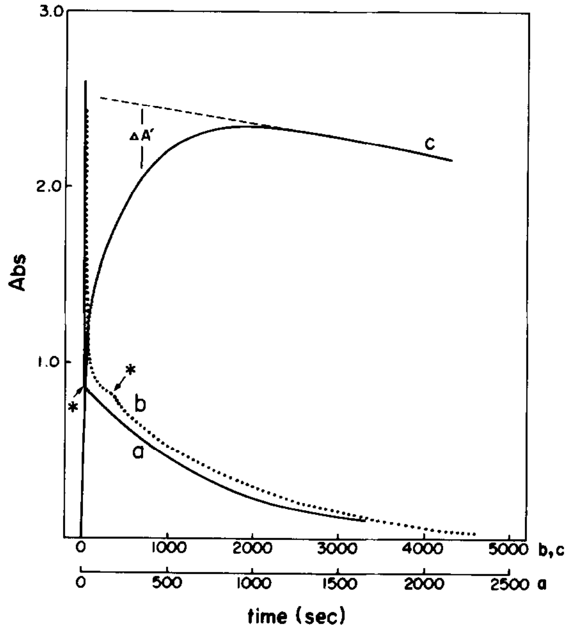

FIG. 1. Typical absorbance changes observed for the addition (curves a and b) of PhOH or TFE to **1a** or **2a** or the cyclization (curve c) of **1b**, **1c**, or **2c**. Curve a is observed at 330 nm in the reaction of 1×10^{-4} M **1a** and 0.02 M PhOH at pH 9.25 in the presence of 9.1% (v/v) acetonitrile, curve b at 360 nm in the reaction of 1×10^{-4} M **2a** and 0.02 M TFE at pH 10.0 in the presence of 40% (v/v) acetone, and curve c at 360 nm in the reaction of 1×10^{-4} M **1b** at pH 9.00 in the presence of 40% (v/v) acetone.

RESULTS AND DISCUSSION

Kinetics of the Addition Reactions of PhOH or TFE to 1a

Oxazolinones **1a** and **2a** absorb strongly at ≥330 nm owing to the heterocyclic five-membered rings. The absorbance in this wavelength region disappears when the heterocyclic rings are opened. The absorbance values decreased when **1a** or **2a** was mixed with PhOH or TFE. The absorbance decreases were biphasic as illustrated by curves a and b of Fig. 1. In the first process, the initial absorbance of **1a** or **2a** approached that (A_{int}) of the intermediate mixture.[4] In the second process, the absorbance further decreased until that (= 0) of hydrolysis product **1d** or **2d** is attained. When the first process was sufficiently faster than the second process, pseudo-first-order constants for the first (k_0^1) and second (k_0^2) processes were calculated without difficulties.

In the reaction of **1a** in the presence of PhOH (>0.01 M) at pH > 9.2, the first process was complete within the manual mixing period of the reaction mixtures as exemplified by curve a of Fig. 1 and, thus, only k_0^2 was measured. The dependence of k_0^2 on [PhOH] was identical to that of A_{int} on [PhOH] as illustrated in Fig. 2. The

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[4] When k_0^1 is sufficiently greater than k_0^2, A_{int} can be taken as those indicated by * in Fig. 1.

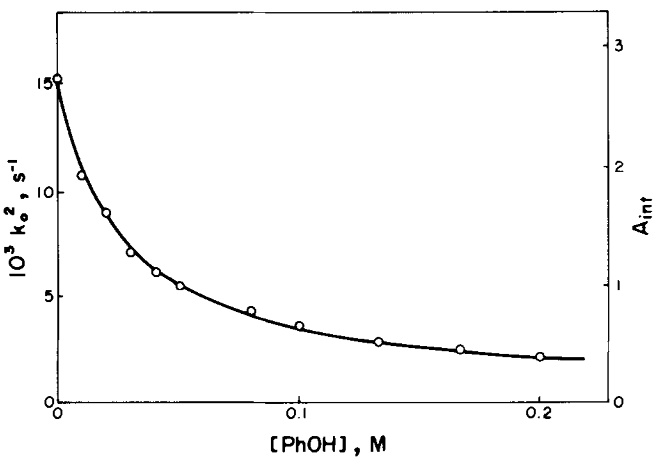

FIG. 2. The dependence of k_0^2 and A_{int} (at 330 nm) on [PhOH] in the reaction of 1×10^{-4} M **1a** with PhOH at pH 10.25 in the presence of 9.1% (v/v) acetonitrile. The circles represent the data points for both k_0^2 and A_{int}. The theoretical curve approaches 0 at infinite [PhOH].

dependence of k_0^2 and A_{int} on [PhOH] was analyzed in terms of Eq. [2]. Here, OX stands for **1a** or **2a**, ROH for PhOH or TFE, ES for the adduct formed as the side-equilibrium product, and P for the hydrolysis product (**1d** or **2d**).

$$\begin{tikzcd} \mathrm{OX} + \mathrm{ROH} \arrow[r, shift left, "k_{ad}"] \arrow[d, "k_h^{OX}"] & \mathrm{ES} \arrow[l, shift left, "k_{cy}"] \arrow[d, "k_h^{ES}"] \\ P & P \end{tikzcd} \qquad [2]$$

The expressions of A_{int}, k_0^1, and k_0^2 for Eq. [2] derived under the conditions of $[\mathrm{ROH}]_0 \gg [\mathrm{OX}]_0$ ($[\mathrm{ROH}]_0$ and $[\mathrm{OX}]_0$ stand for the initial added concentrations of ROH and OX, respectively) and $k_0^1 \gg k_h^{OX} \gg k_h^{ES}$[5,6] are as follows:

$$A_{int} = (A_{OX}^0 + A_{ES}^0 K_{app}[\mathrm{ROH}]_0)/(1 + K_{app}[\mathrm{ROH}]_0) \qquad [3]$$

$$\begin{aligned} k_0^1 &= k_{ad}[\mathrm{ROH}]_0 + k_{cy} \\ &= (K_{app}[\mathrm{ROH}]_0 + 1)k_{cy} \end{aligned} \qquad [4]$$

$$k_0^2 = (k_h^{OX} + k_h^{ES} K_{app}[\mathrm{ROH}]_0)/(1 + K_{app}[\mathrm{ROH}]_0) \qquad [5]$$

Here, K_{app} is k_{ad}/k_{cy} representing the equilibrium constant for the addition of ROH to OX ($K_{app} = [\mathrm{ES}]_{eq}/[\mathrm{ROH}]_0[\mathrm{OX}]_{eq}$; $[\mathrm{ROH}]_0$ is $[\mathrm{ROH}]_{eq} + [\mathrm{RO^-}]_{eq}$ under the

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[5] The kinetics for the hydrolysis of oxazolinones **1a** and **2a** have been previously measured and the mechanisms have been elucidated (*11*). Both **1a** and **2a** are hydrolyzed through the nucleophilic attack at the carbonyl carbon under alkaline conditions. Under acidic conditions, only **1a** was readily hydrolyzed. The acidic hydrolysis of **1a** involves the attack of water molecule at the carbon atom of the protonated imine group.

[6] The alkaline hydrolysis of phenyl cinnamate was about 100 times slower than that of **1a** when measured in the presence of 9.1% (v/v) acetonitrile. Phenyl cinnamate was not affected by 1 N HCl for several days.

experimental conditions), A^0_{OX} the absorbance of OX at [OX] = $[OX]_0$, and A^0_{ES} the absorbance of ES at [ES] = $[OX]_0$. Parameter k_h^{ES} is much smaller[6] than k_h^{OX} and $A^0_{ES} \cong 0$ at the wavelengths for the kinetic measurements. When $k_h^{OX} \gg k_h^{ES}K_{app}[ROH]_0$ and $A^0_{OX} \gg A^0_{ES}K_{app}[ROH]_0$, Eqs. [3] and [5] are converted into Eq. [6]. Then, the dependence of A_{int}/A^0_{OX} on $[ROH]_0$ and that of k_0^2/k_h^{OX} on $[ROH]_0$ are identical, as illustrated in Fig. 2.

$$A_{int}/A^0_{OX} = k_0^2/k_h^{OX} = 1/(1 + K_{app}[ROH]_0) \qquad [6]$$

The plots of A_{int} (or k_0^2) against $[ROH]_0$ were analyzed either by nonlinear regression according to Eq. [6] or by linear regression according to Eq. [7], a linear transform of Eq. [6].

$$1/A_{int} = 1/A^0_{OX} + K_{app}[ROH]/A^0_{OX} \qquad [7]$$

Analysis of the dependence of k_0^2 and A_{int} on $[PhOH]_0$ led to K_{app} values. When K_{app} was measured at several pHs, the pH dependence reflects the pK_a of PhOH[7] (Fig. 3), indicating that K_{app} reaches a maximum value (K^0_{app}) when PhOH is protonated (Eq. [8]), in agreement with the stoichiometric relationship indicated by Eq. [2].

$$K_{app} = K^0_{app}/(1 + K_a/[H^+]) \qquad [8]$$

In the addition reaction of PhOH to **1a**, rates of the first processes of the biphasic kinetics were reduced as pH was lowered. Consequently, it was possible to measure k_0^1 at pH 6.1–8.3 in the presence of 9.1% (v/v) acetonitrile or pH 7.2–8.7 in the presence of 40% (v/v) acetone. Parameter k_{cy} can be estimated from the intercept of the plot of k_0^1 against $[ROH]_0$ (Eq. [4]). In order to calculate correct values of k_{cy}, reliable intercept values are needed, which in turn require data points with $K_{app}[ROH]_0$ values considerably smaller than 1. Under the experimental conditions, however, k_0^1 values close to the intercept values were not obtained because of the large values of K_{app}. Therefore, k_{cy} was not correctly estimated from the plot of k_0^1 against $[ROH]_0$. Instead, k_{cy} was estimated from the plot of k_0^1 against ($K_{app}[ROH]_0$ + 1), as indicated in Eq. [4], by using the separately estimated values of K_{app}. In Fig. 4, the proportionality between k_0^1 and ($K_{app}[PhOH]_0$ + 1) is demonstrated. The dependence of log k_{cy} thus obtained on pH is illustrated in Fig. 3. The straight lines of Fig. 3 are drawn with slopes of 1.0. Thus, k_{cy} and k_{ad} illustrated in this figure are proportional to $[OH^-]$. The values of K^0_{app}, k_{cy}^{OH} (= $k_{cy}/[OH^-]$), and k_{ad}^{OH} (= $k_{ad}/[OH^-]$) are summarized in Table 1.[8]

In the addition reactions of TFE to **1a**, k_0^1 became much greater than k_0^2 and k_h^{OX} when [TFE] > 0.1 M. Under these conditions, however, A_{int} was less than 2% of

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[7] pK_a of TFE is 12.43 (Jencks, W. P., and Regenstein, J. (1976) *in* Handbook of Biochemistry and Molecular Biology (Fasman, G. D., Ed.), 3rd ed., Vol. I, pp. 305–351, CRC Press, Cleveland) in water and would be greater in the presence of the organic cosolvents. Thus, TFE is unionized at the pHs employed in the kinetic measurements and the K_{app} measured for the addition of TFE to **1a** or **2a** represents K^0_{app}.

[8] The K_w value was approximated as 1×10^{-14} M^2 (i.e., $[OH^-] = 10^{-14}/10^{-pH}$). The value of K_w should be smaller than 10^{-14} M^2 and $[OH^-]$ should be smaller than $10^{-14}/10^{-pH}$ in the presence of the organic cosolvents.[3] The actual values of k_{cy}^{OH} and k_{ad}^{OH} should be corrected accordingly.

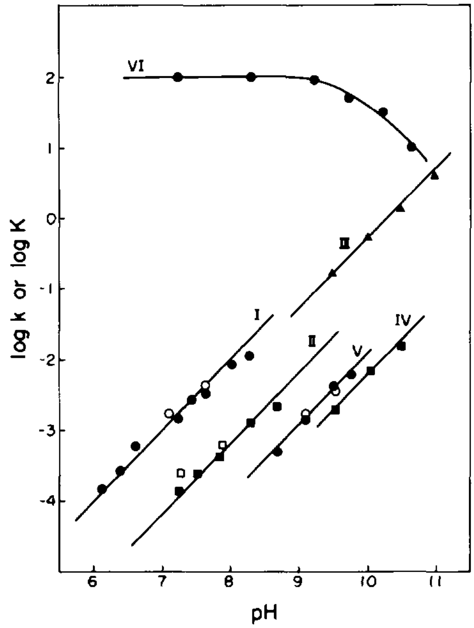

FIG. 3. The pH dependence of log k_{cy} for **1b** (I and II), log k_{ad} for **1c** (III), log k_{cy} for **1c** (IV), log k_{cy} for **2c** (V), and log K_{app} (VI) for **1a**. Lines I and VI were measured in the presence of 9.1% (v/v) acetronitrile and the rest in the presence of 40% (v/v) acetone. For lines I, II, and V, data points ● or ■ were obtained from the kinetic data for the addition of PhOH or TFE to **1a**, whereas data points ○ or □ were from those for the cyclization of **1b** or **1c**. Data points for line III were obtained from the experiments of the addition of TFE to **1a**, while those for line IV were from those of cyclization of **1c**.

A^0_{OX}, and K_{app} was not correctly estimated by using A_{int} values obtained at various [TFE]. Instead, k_{ad} was estimated from the dependence of k^1_0 on [TFE] (Eq. [4]). The value of log k_{ad} is plotted against pH in Fig. 3. The value of k^{OH}_{ad} thus evaluated is summarized in Table 1.

TABLE 1

Values of Kinetic Parameters for the Cyclization and Addition Reactions Involving **1a** and **2a**

Reaction	Solvent[a]	k^{OH}_{cy} (s^{-1} M^{-1})	k^{OH}_{ad} (s^{-1} M^{-2})	K^0_{app} (M^{-1})
1a + PhOH $\rightleftharpoons$ **1b**	A	9400 ± 2600	9.4 ± 2.7 × 10^5[b]	100 ± 6
	B	620 ± 110	6.2 ± 1.3 × 10^3[b]	10 ± 1
1a + TFE $\rightleftharpoons$ **1c**	B	56 ± 5	4.7 ± 0.7 × 10^3	84 ± 15[c]
2a + PhOH $\rightleftharpoons$ **2b**	B	—[d]	—[d]	3.3 ± 0.3
2a + TFE $\rightleftharpoons$ **2c**	B	110 ± 10	1.5 ± 0.2 × 10^3[c]	14 ± 1

[a] A in the presence of 9.1% (v/v) acetonitrile; B in the presence of 40% (v/v) acetone.

[b] Calculated from the values of k^{OH}_{cy} and K^0_{app}.

[c] Calculated from the values of k^{OH}_{cy} and k^{OH}_{ad}.

[d] The rate for the [OH^-]-dependent path was not measured. The pseudo-first-order rate constant for the [OH^-]-independent path was >1 s^{-1}.

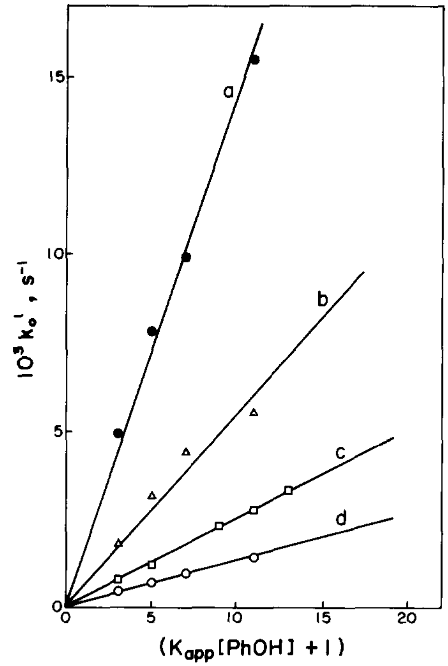

FIG. 4. The plot of k_0^1 against (K_{app}[PhOH] + 1) for the reaction of **1a** with PhOH in the presence of 9.1% (v/v) acetonitrile at pH 7.22 (a), 6.61 (b), 6.38 (c), or 6.11 (d).

*Kinetics of the Addition Reactions of PhOH or TFE to **2a***

Because of the limited solubility of **2a** in water, the reactions of **2a** were investigated in the presence of 40% (v/v) acetone. For the addition of PhOH to **2a**, K_{app}^0 (Table 1) was measured from the dependence of A_{int} on [PhOH] at pH 0–7. However, the first process of the biphasic absorbance change was complete within the manual mixing period at pH 7, 2.5, or, even, 1 N HCl in the presence of 0–0.2 M PhOH. As indicated by Eq. [4], k_{cy}/k_0^1 is 0.6–1 at these [PhOH]. Thus, both k_0^1 and k_{cy} are greater than 1 s^{-1} under these conditions. Below pH 7, **2a** was very stable in the absence of added PhOH (*11*).

The k_{cy} value of >1 s^{-1} observed at 1 N HCl is not compatible with mechanisms involving the participation of hydroxide ion.[9] For example, a second-order rate constant exceeding the diffusion control limit (*13*) is needed if the reaction pro-

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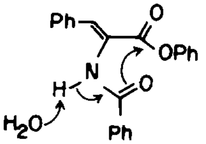
[9] Unlike **1b**, **1c**, and **2c**, **2b** was not prepared. Thus, whether **2b** is the product formed by the addition of PhOH to **2a** is not established with isolated **2b**. In this regard, the very large k_{cy} and k_{ad} values observed for the addition of PhOH to **2a** may be ascribed to the addition of phenol to the imine carbon instead of the carbonyl carbon. This, however, can be excluded on the basis of mechanistic information obtained for the hydrolysis of **1a** and **2a** (*11*). Although **1a** is hydrolyzed through the attack of water molecule at the carbon atom of the protonated imine group under acidic conditions, **2a** resists acid-catalyzed hydrolysis since the phenyl ring of the benzoylamino group sterically hinders the approach of water molecule to the imine carbon. Similarly, the attack of phenol to the imine carbon of **2a** would be severely hampered.

ceeds through the bimolecular reaction between hydroxide ion and **2a.** The pK_a value of the amide group of **2b** would be greater than 15 (*14*). Therefore, a first-order rate constant which is much greater than the upper limit (*13*) for a unimolecular reaction expected for zero free energy of activation is to be assigned if the reaction occurs by the attack of the deprotonated amide group at the ester linkage.[10] The cyclization of **2b** observed at 1 N HCl, therefore, is consistent with nucleophilic reactions of the neutral form of the amide group, such as **A.** The k_{cy}



A

value for this reaction path is >1 s^{-1}, and, thus, it is not clear whether the cyclization rate of **2b** at pH 7 involves contribution from the hydroxide path or is solely due to the path involving neutral species. The much greater reactivity of **2b** compared with **1b** is attributable to the electronic effects exerted by the benzoyl phenyl ring to enhance the acidity of the amide N—H bond or the steric effects to bring the reacting atoms in closer proximity.[11]

In the addition reactions of TFE to **2a,** k_0^1 became much greater than k_0^2 and k_h^{OX} when [TFE] > 0.02 M. The values of K_{app}^0 were estimated from the plot of $1/A_{int}$ against [TFE] according to Eq. [7], by using the data measured with [TFE] > 0.03 M. The value of k_{cy} was estimated from the plot of k_0^1 against ($K_{app}[ROH]_0 + 1$) at each pH (Eq. [4]) and is plotted against pH in Fig. 3. The values of K_{app}^0, k_{cy}^{OH}, and k_{ad}^{OH} thus evaluated are summarized in Table 1.

Kinetics of the Cyclization Reactions of 1b, 1c, or 2c

When **1b, 1c,** or **2c** was dissolved in buffer solutions, the accumulation and the consecutive decomposition of the corresponding oxazolinone were observed spectrophotometrically. The reaction of **2b** was not investigated since it was not isolated due to its very large reactivity (k_{cy}). A typical absorbance change observed during the cyclization of **1b, 1c,** or **2c** is illustrated in Fig. 1 (curve c). When the accumulation of the oxazolinones was sufficiently faster than the subsequent decomposition process and the hydrolysis processes denoted by k_h^{ES} and k_h^{OX}, the rate constant of the accumulation of the oxazolinones, is defined as k_0^1 of Eq. [4]. The values of k_0^1 were calculated from the absorbance changes by using the $\Delta A'$ values which are defined in Fig. 1. When the rate constant (k_0^1) for the accumula-

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[10] If the reaction occurs through the formation of an intermediate adduct (e.g., the tetrahedral intermediate obtained by the attack of hydroxide ion at the amide carbon atom) formed from the reaction of hydroxide ion and **2b,** the formation constant for the adduct should be much greater than 1 M^{-1}, although this is highly unreasonable. Otherwise, the first-order rate constant for the reaction of the intermediate should exceed the upper limit for the first-order rate constant (*13*).

[11] The accelerating effect of the benzoyl phenyl group is not noticeable for the cyclization of **2c** compared with that of **1c.** Thus, different mechanisms could be operative in the cyclization reactions of the aryl- and the alkyl-ester substrates, at least in the reaction path involving the neutral species.

tion step was not sufficiently greater than that (k_h^{OX}) for the decomposition step, the absorbance change was analyzed as

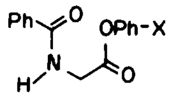
$$[OX] = \frac{S_0 k_0^1}{(k_h^{OX} - k_0^1)} (e^{-k_0^1 t} - e^{-k_h^{OX} t}) \quad [9]$$

The k_0^1 value observed in cyclization of **1b**, **1c**, or **2c** includes (Eq. [4]) the contribution from the reverse attack of the leaving group at the oxazolinone. The k_0^1 value measured with 1×10^{-4} M **1b**, **1c**, or **2c** was, however, unaffected by the addition of 2×10^{-4} M PhOH or TFE, indicating that k_0^1 represents k_{cy} under these conditions. When the buffer concentration was varied as 0.005–0.03 M, the k_{cy} values were not affected significantly. The k_{cy} values thus obtained are illustrated in Fig. 3.

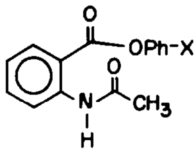
The k_{cy} value measured from the cyclization reactions of **1b** or **2c** agrees (Fig. 3) well with that estimated from the addition reaction of PhOH or TFE to the corresponding oxazolinone. This indicates the validity of both the scheme of Eq. [2] and the analysis of kinetic data based on this scheme.

Intramolecular Nucleophilicity of the Amide Groups

Oxazolinones are involved[12] as intermediates in the hydrolysis of some activated esters such as aryl hippurates (*7–9*) (**B**) and aryl 2-acetylaminobenzoates (*10*) (**C**).[13] Hydrolysis of the alkyl esters of hippuric acid or 2-acetylaminobenzoic acid, however, involves attack of external nucleophiles instead of the intramolecular amide groups at the scissile ester linkage.



B



C

Much greater intramolecular nucleophilicity of amide groups is observed for **1b**, **1c**, **2b**, and **2c** compared with **B**, **C**, or the alkyl esters related to **B** and **C**. The bonds around the methylene carbon atoms of aryl hippurates (**B**) allow free rota-

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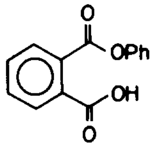
[12] Nucleophilic reactions by amide oxygens leading to the formation of oxazolinones have been involved in the discussion of the mechanism of racemization in peptide-coupling reactions (*15*). The intramolecular nucleophilic reaction of the amide oxygen has been also implicated in the phosphonate ester hydrolysis of dialkyl 2′-phosphonobenzanilide (*16*). The reaction, however, required very high acid concentrations for fast rates. The intramolecular reactions of the amide groups involving the attack of nitrogen atoms instead of oxygen atoms have been observed in ester (*17–21*) and carbamate (*22*) hydrolysis leading to the formation of imides. Intermediate imides, however, may be hydrolyzed at a rate which is slower than the direct hydrolysis of the substrate and the participation of the amide nitrogens in these reactions may not be regarded as catalytic (*17*).

[13] The k_{cy} values for **B** and **C** were proportional to [OH$^-$]. This was explained in terms of the intramolecular nucleophilic attack of the deprotonated amide group or that of the neutral amide group assisted by hydroxide ion acting as a general base to abstract the amide proton (*7–10*). The k_{cy} values for **1b**, **1c**, and **2c** are also proportional to [OH$^-$]. This may be related to similar mechanisms, although more data are needed for reliable mechanistic analysis.

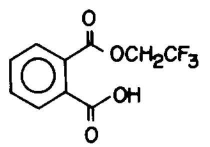
tion. This freedom, however, is substantially reduced in **1b**, **1c**, **2b**, and **2c** due to the presence of the benzylidene group as revealed by space-filling models. Geometries of the transition states for the cyclization reactions are different for **C** and for the ester substrates of the present study. The much greater nucleophilicity of amide groups of **1b**, **1c**, **2b**, and **2c** may be attributed to the enhanced proximity effects (*4–6*) exerted on the amide oxygen atom and the carbonyl carbon atom of the scissile ester bond. Even greater nucleophilicity of amide groups, therefore, can be achieved as the proximity effects are improved in either model systems or enzyme–substrate complexes.

The reactivity of carboxyl groups as intramolecular nucleophiles, general acids, or general bases has been extensively investigated and the results are well-documented (*4–6*). Very efficient intramolecular catalytic effects have been observed for carboxyl groups in several small organic compounds which were designed as enzyme models. Carboxyl groups are regarded as important catalytic groups of enzymes and this is well supported by the results of these model studies.

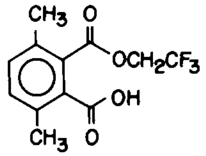
Mono-esters or amides of phthalic acid or maleic acid derivatives have been extensively employed as the models for the estimation of the catalytic efficiency of carboxyl groups in enzymatic systems (*6*). The reactivities of carboxyl groups toward aryl- or alkyl-ester linkages in mono-phenyl phthalate (**D**) (*23*), mono-2,2,2-trifluoroethyl phthalate (**E**) (*23*), and mono-2,2,2-trifluoroethyl 3,6-dimethyl phthalate (**F**) (*24*) reported in the literature are compared in Table 2 with those of amide groups of **1b**, **1c**, **2b**, and **2c**.



D



E



F

In the reaction of **D–F**, carboxylate anions acts as the intramolecular nucleophiles. Thus, the rates for the intramolecular path remain constant as pH is raised once the carboxyl groups are fully ionized. On the other hand, the rates for the intramolecular paths for **1b** and **2b** increase as pH is raised. The amide group of **2b** is much more efficient than the carboxylate of **D**. When compared at pH 7–9, the amide groups of **1c** and **2c** are as effective as the carboxylate groups of **E** and **F** toward the alkyl-ester linkage.

As mentioned in the introduction section, the catalytic roles of active-site amide groups have been ignored in the discussion of enzyme mechanisms mainly because highly effective intramolecular catalytic effects of amide groups have not been observed with small model compounds. The present results indicate that the intramolecular nucleophilicity of amide groups toward alkyl- and aryl-esters can become as efficient as carboxyl groups. Carboxyl groups are implicated as catalytic groups in the mechanisms of many enzymes. A specific example in which an active-site carboxyl group is involved as a nucleophile in the hydrolysis of ester

TABLE 2

Comparison of the Intramolecular Nucleophilicity of Amide Groups of **1b**, **1c**, **2b**, and **2c** with That of the Carboxyl Groups of **D–F**[a]

Compound	Half-life	pH	Temperature (°C)	pH dependence
Phenyl esters				
1b	12 min	7	25	$k_{cy} \propto [OH^-]$
	7 s	9	25	
2b	<1 s	7[b]	25	Unknown
D	9s	≥5	30	$pH_{opt} \geq 5$
2,2,2-Trifluoroethyl esters				
1c	34 h	7[b]	25	$k_{cy} \propto [OH^-]$
	20 min	9[b]	25	
2c	17 h	7[b]	25	$k_{cy} \propto [OH^-]$
	10 min	9[b]	25	
E	17 min	≥5	65	$pH_{opt} \geq 5$
	16 h	≥5	25[c]	
F	43 s	≥5	40	$pH_{opt} \geq 5$
	4 min	≥5	25[d]	

[a] Solvents: 9.1% (v/v) acetonitrile for **1b**, 40% (v/v) acetone for **2b**, **1c**, and **2c**, and without significant amounts of organic solvents for **D–F**.

[b] Since K_w is considerably smaller in the presence of 40% (v/v) acetone (*11*, *13*), $[OH^-]$ of a buffer solution containing 40% (v/v) acetone would be significantly smaller than that of a pure aqueous buffer solution of the same pH.[7]

[c] Calculated by using the E_a value which was taken as the average of that for mono-methyl phthalate (23.1 kcal/mol) and that for **F** (22.3 kcal/mol) (*21*, *22*).

[d] Calculated by using the E_a value (*22*) reported for **F**.

substrates is the carboxyl group of Glu-270 of carboxypeptidase A (*25*, *26*).[14] Since amide groups are the most abundant polar functional group of active sites and since they can be located very close to the reaction sites of the substrates in

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[14] Previously, we have reported results on the carboxypeptidase A-catalyzed hydrolysis of *O*-[(*Z*)-α-benzoylaminocinnamoly]-L-β-phenyl lactate (**2e**) (similar to **2c** except that $R_2 = CH(CH_2Ph)COO^-$; see Eq. [1]), in which a stable intermediate accumulated (*25*, *27*). On the basis of several lines of kinetic evidence (*25*, *27*), this intermediate was assigned as the anhydride formed between (*Z*)-α-benzoylaminocinnamic acid and Glu-270 of the enzyme. The present results indicate that the cyclization of an anhydride derived from (*Z*)-α-benzoylaminocinnamic acid and another small carboxylic acid to form **2a** should be extremely fast. During the carboxypeptidase A-catalyzed hydrolysis of **2e**, formation of **2a**, i.e., the intramolecular trapping of the anhydride intermediate, was not observed. The failure to trap the intermediate, however, does not exclude the presence of the intermediate. As discussed previously (*25*, *28*), successful intramolecular trapping of the acyl-carboxypeptidase A intermediate requires that the trapping group should be conformationally allowed to attack the anhydride group. Although this is allowed for small compounds such as **1b–1c** and **2b–2c**, it is possible that the anhydride group of acyl-carboxypeptidase A derived from **2e** is sterically protected from the attack of the α-benzoylamino group. For example, the acylamino group may be bound by the additional binding sites disclosed by a recent X-ray crystallographic study (*29*), and its attack at the anhydride linkage could be thus prohibited. (*Z*)-benzoylaminocinnamoyl-Ser-195-chymotrypsin did not produce **2a** during deacylation reaction (*30*), although the leaving ability of Ser-195 could be enhanced considerably. This is also attributable to the conformational restriction imposed on the attack of the acylamino group by the enzyme.

Michaelis complexes, amide groups should be given due consideration in the discussion of enzyme mechanisms.

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