Carboxypeptidase A-Catalyzed Hydrolysis of α-(Acylamino)cinnamoyl Derivatives of L-β-Phenyllactate and L-Phenylalaninate: Evidence for Acyl-Enzyme Intermediates

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Abstract: The (CPA) carboxypeptidase A-catalyzed hydrolysis of α-(acetylamino)- (**1**), α-(benzoylamino)- (**2**), or α-[(2-naphthoyl)amino]cinnamoyl ester (**3**) of L-β-phenyllactate manifested small values of both k_{cat} and $K_m$app. On the other hand, the corresponding amides of L-Phe showed enhanced k_{cat} and unaffected $K_m$app values. At −2 °C, accumulation of an intermediate was observed spectrophotometrically immediately after mixing of 6×10^{-5} M CPA with 4×10^{-5} M **3**. This is the most stable (in terms of half-life and $K_m$app) intermediate ever reported for the CPA-catalyzed reactions. For **2** and **3**, k_{cat} was independent of pH over pH 5.5–9.5. Although attempts to trap the intermediate with external or intramolecular trapping reagents were unsuccessful, the very small $K_m$app and the pH independence of k_{cat} for **2** and **3** provide evidence that shows that the accumulating intermediate is the anhydride acyl-CPA intermediate. The temperature dependence and the D_2O effect were measured for the k_{cat} values of **2** and **3**. The different effects of the α-(acylamino)cinnamoyl groups on the kinetic parameters for esters and for peptides were explained in terms of a single mechanism with the intermediacy of an acyl-enzyme.

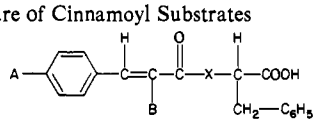
The mechanism of (CPA) carboxypeptidase A-catalyzed reactions has been the subject of extensive studies for the past 2 decades.[1-3] X-ray crystallographic study established that zinc ion, Glu-270, Tyr-248, and Arg-145 are the most important catalytic groups.[1] Although a vast amount of literature has been reported for the chemical modification and the kinetic behavior of the enzyme, the exact catalytic roles of these groups are not understood. In particular, whether the carboxylate of Glu-270 acts as a nucleophile or a general base has been a major controversy in the elucidation of the CPA mechanism.

The spectrophotometric detection of an intermediate has been reported for the CPA-catalyzed hydrolysis of an ester substrate at subzero temperatures.[4] This was originally believed to be the first experimental evidence for the acyl-CPA intermediate and, consequently, for the nucleophilic role of Glu-270.[4-6] A later study, however, revealed that if an anhydride intermediate was formed, it did not accumulate to the extent which could be detected by resonance Raman spectroscopy.[7] Spectrofluorometric study of the CPA-catalyzed hydrolysis of dansyl-containing depsiesters and oligopeptides revealed accumulation of two successive intermediates.[8,9] These intermediates were interpreted to be noncovalent enzyme–substrate complexes. The basis of this interpretation, however, has been criticized.[7]

The most direct evidence for the general base role of the Glu-270 carboxylate, at least in peptidase action, came from an O^{18}-exchange experiment with a dipeptide in the presence of added amino acids or hydroxy acids.[10,11] Based on the results of a model study, however, it was later shown that the O^{18}-exchange results are also explained by the nucleophilic role of the Glu-270 carboxylate.[12,13]

Many important kinetic data have been obtained with para-substituted *trans*-cinnamoyl derivatives of L-β-phenyllactic acid (L-PLA).[4,6,7,14-16] Although these esters contain good chromophores for spectrophotometric measurements, they may lack the additional features available to the natural substrates of CPA. Although CPA cleaves the amide bond adjacent to the C terminus of a polypeptide, distant residues of the polypeptide substrate could induce additional catalytic features. In an attempt to obtain better insight into the action of CPA, we prepared several α-(acylamino)cinnamoyl derivatives of L-PLA or L-Phe. α-(Acylamino)cinnamic acids are *N*-acylamino acids, and the hydrogenation of the olefinic double bond of the cinnamoyl moiety leads to *N*-acyl-Phe. Therefore, the α-(acylamino)cinnamoyl derivatives of L-PLA or L-Phe are *N*-acyldepsiesters or *N*-acyldipeptides with good chromophores. As we expected, α-(acylamino)cinnamoyl substrates produced significant mechanistic data which were not seen with simple cinnamoyl substrates. In this paper, we report kinetic data obtained with the α-(acylamino)cinnamoyl substrates which strongly support accumulation of the acyl-CPA intermediate in ester hydrolysis.

Table I. Structure of Cinnamoyl Substrates



compd	A	B	X
1	H	$-NHCOCH_3$	O
2	H	$-NHCOC_6H_5$	O
3	H	$-NHCO$-2-$C_{10}H_7$	O
4	H	H	O
5	CN	H	O
6	Cl	H	O
7	CH_3CONH-	H	O
8	H	$-NHCOCH_3$	NH
9	H	$-NHCOC_6H_5$	NH
10	H	$-NHCO$-2-$C_{10}H_7$	NH
11	H	H	NH

Experimental Section

Substrates. Structures of a part of the substrates which will be mentioned in this paper are summarized in Table I.

***O*-[*trans*-α-(Acetylamino)cinnamoyl]-L-β-phenyllactic Acid (1).** A pulverized and dried mixture of (*Z*)-2-methyl-4-benzylideneoxazolin-5-one[17] (0.2 g) and L-PLA (0.2 g) was fused for 3 h at 110–120 °C. The resulting product was recrystallized from 1,2-dichloroethane, mp 154–156 °C.

***O*-[*trans*-α-(Benzoylamino)cinnamoyl]-L-β-phenyllactic Acid (2).** (*Z*)-2-Phenyl-4-benzylideneoxazolin-5-one[18] (65 mg) and L-PLA (43 mg) were pulverized together and dried thoroughly and then were fused at 150–160 °C for 3 h. The resulting product was recrystallized from 1,2-dichloroethane–hexane. Alternatively, to a solution of (*Z*)-2-phenyl-4-benzylideneoxazolin-5-one (0.3 g) and L-PLA (0.2 g) in dry THF, sodium (56 mg) dissolved in *tert*-butyl alcohol (5 mL) was added at room temperature. Twelve hours later, the mixture was acidified with

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1:1 HCl and evaporated in vacuo. The residue was washed with water and recrystallized, mp 170–171 °C.

***O*-(*trans*-α-[(2-Naphthoyl)amino]cinnamoyl)-L-β-phenyllactic Acid (3).** A solution of *N*-(2-naphthoyl)glycine (2 g), benzaldehyde (2 mL), and sodium acetate (1 g) in acetic anhydride (30 mL) was refluxed for 3 h and left overnight at room temperature. Greenish-yellow crystals of (*Z*)-2-(2-naphthyl)-4-benzylideneoxazolin-5-one thus obtained were collected by filtration, washed with water and with ethanol, and used in the next step without further purification (mp 171–172 °C). The reaction of the oxazolin-5-one (0.4 g) with L-PLA (0.2 g) in THF in the presence of sodium *tert*-butoxide was carried out as described in the preparation of **2**. The product was recrystallized from 1,2-dichloroethane–hexane, mp 172–174 °C.

***O*-(*trans*-*p*-Cyanocinnamoyl)-L-β-phenyllactic Acid (5).** To a stirred solution of *trans*-*p*-cyanocinnamoyl chloride (1.55 g) in 10 mL of pyridine which was cooled in an ice–salt bath, L-PLA (1.34 g) dissolved in 10 mL of pyridine was added over a period of 30 min. The mixture was stirred for an additional 30 min in the ice–salt bath and for a further 90 min at room tempeature. One hundred milliliters of 1,2-dichloroethane was added to the mixture, and the resulting solution was washed with 80 mL of 1:1 HCl and then with three portions of 50 mL of water. After evaporation of the solvent, the product was recrystallized from benzene–hexane, mp 129.5–131 °C.

***O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenyllactic Acid (6).** This compound was prepared according to the literature.[16]

***O*-[*trans*-*p*-(Acetylamino)cinnamoyl]-L-β-phenyllactic Acid (7).** *trans*-*p*-(Acetylamino)cinnamic acid was coupled with *N*-hydroxysuccinimide in the presence of *N*,*N*′-dicyclohexylcarbodiimide in dry diglyme at 4 °C. After removal of *N*,*N*′-dicyclohexylurea by filtration, the filtrate was evaporated to obtain the crude succinimide ester (mp 250–256 °C). A pyridine solution (10 mL) of the crude succinimide ester (0.3 g) and L-PLA (0.25 g) was refluxed for 3 h. The resultant mixture was washed with 1 N HCl and extracted with ethyl acetate. After decolorization with Norit A and evaporation of the solvent, the crude product was recrystallized from ethyl acetate–hexane, mp 199–200 °C.

***N*-[*trans*-α-(Acetylamino)cinnamoyl]-L-phenylalanine (8).** A solution of (*Z*)-2-methyl-4-benzylideneoxazolin-5-one (0.5 g) and L-Phe ethyl ester (0.57 g) in 10 mL of dioxane was stirred for 24 h. The product was precipitated by adding water to the solution and then recrystallized from ethyl acetate–hexane, mp 148–149 °C. The ethyl ester thus obtained was hydrolyzed at room temperature with 1.5 mL of 1 N NaOH in acetone–water for 1.5 h. The resulting solution was made acidic with Congo Red and extracted with ethyl acetate. The crude product obtained after evaporation of ethyl acetate was recrystallized from ethyl acetate–hexane, mp 206–207 °C.

***N*-[*trans*-α-(Benzoylamino)cinnamoyl]-L-phenylalanine (9).** (*Z*)-2-Phenyl-4-benzylideneoxazolin-5-one (2.5 g) and L-Phe (1.65 g) were added to 10 mL of 1 N NaOH solution, and the resulting mixture was refluxed until a clear solution was obtained. The solution was cooled and treated with 10 mL of 1 N HCl. The crude product separated by filtration was decolorized with Norit A and recrystallized from ethyl acetate–hexane, mp 180.5–181.5 °C.

***N*-(*trans*-α-[(2-Naphthoyl)amino]cinnamoyl)-L-phenylalanine (10).** A solution of (*Z*)-2-(2-naphthyl)-4-benzylideneoxazolin-5-one (0.4 g) and the potassium salt of L-Phe (0.3 g) in 30 mL of Me_2SO was kept for 1 h at 80–90 °C and then evaporated in vacuo. The residue was washed with dilute HCl and then with water. The product was recrystallized from 1,2-dichloroethane–methylene chloride–hexane, mp 100–102 °C.

The *Z* configuration of 2-methyl-(or phenyl)-4-benzylideneoxazolin-5-one was established previously.[19,20] Therefore, the substrates obtained from these oxazolin-5-ones are the derivatives of *trans*-cinnamic acid. It is assumed that the 2-naphthyl-containing compounds have the same configuration as that of the phenyl or methyl compounds.

Satisfactory results were obtained for the elemental analysis (C, H, N) of the new compounds prepared in the present study.

Enzyme. CPA_γ was purchased as a suspension in toluene–water from Worthington Diagnostic. The enzyme stock solutions were prepared by successive dialysis against pH 7.5 buffer (0.5 M NaCl, 0.05 M Tris) or according to the literature.[4] Activity of CPA was determined with either **6** or **2**. At pH 7.5, 25 °C, and ionic strength 0.55, hydrolysis of 0.8×10^{-4} M **6** with CPA shows first-order kinetics, with the average value of k_{obsd}/E_o of $(6.1 \pm 0.2) \times 10^5$ s^{-1} M^{-1}. Under identical conditions, hydrolysis of 2×10^{-4} M **2** with CPA shows zero-order kinetics, with the average value of k_{obsd}/E_o of 0.46 ± 0.02 s^{-1}.

Other Materials. *trans*-α-(Acetylamino)cinnamic acid and *trans*-α-(benzoylamino)cinnamic acid were prepared according to the literature.[21] *trans*-α-[(2-Naphthoyl)amino]cinnamic acid was obtained by the alkaline hydrolysis of the corresponding oxazolin-5-one in 30 mL of 1:2 2 N NaOH–tetrahydrofuran at room temperature for 1 h, followed by acidification and recrystallization from methanol–dichloroethane, mp >200 °C dec. Water was distilled and deionized before being used in kinetic studies. Deuterium oxide (Aldrich) was 99.8 atom % enriched.

Kinetic Measurements. Reaction rates were measured with a Beckman 5260 or 25 UV/vis spectrophotometer. Temperature was controlled to within ± 0.1 °C with a Haake E52 or a Lauda Brinkmann T-2 circulator. pH measurements were carried out with a Fisher Accumet Model 525 pH meter. Buffers used were 2-(*N*-morpholino)ethanesulfonic acid at pH 5.5–7.0, *N*-(2-hydroxyethyl)piperazinyl-*N*′-2-ethanesulfonic acid or tris(hydroxymethyl)aminomethane at pH 7.5–8.0, and boric acid at pH 8.5–9.5. Buffer solutions contained 0.5 M sodium chloride and 0.05 M buffer. The substrates, expect for **3**, were converted to the corresponding sodium salts prior to the preparation of their stock solutions. The solubility of **3** in water was so small, even in the form of the sodium salt, that the stock solution of **3** was made in dimethyl sulfoxide (Me_2SO). The UV spectra of the product solutions of the CPA-catalyzed reactions were identical with those of the solutions containing the corresponding cinnamic acids and L-PLA or L-Phe. In the measurement of the initial rates for the hydrolysis of **5**, a sufficiently low concentration of CPA was initially added so that the absorbance decrease was linear during the initial 1-min period. Then, an additional aliquot of CPA was added to obtain the infinity absorbance value. This together with the initial slope of the reaction was used in calculation of the initial rate.

Results

Kinetic Analysis. The kinetic data of the CPA-catalyzed reactions were analyzed in terms of the simple Michaelis–Menten scheme complicated only by competitive product inhibition (eq 1).[16] The steady-state rate expression for this scheme is derived

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P_1 + P_2$$

$$E + P_1 \underset{K_p}{\rightleftharpoons} EP_1 \qquad (1)$$

as eq 2 under the condition of S_0 (initially added substrate concentration) $\gg E_0$ (total enzyme concentration). Here, k_{cat} is the

$$v = -d[S]/dt = k_{cat}E_0[S]/(K_m app + [S] + K_m app(S_0 - [S])/K_p) \qquad (2)$$

observed catalytic constant ($k_{cat} = k_2$ for eq 1) and $K_m app$ the observed Michaelis constant ($K_m app = K_m = (k_{-1} + k_2)/k_1$ for eq 1).

When $K_m app \simeq K_p$, $v/[S]$ becomes a constant value of $k_{cat}E_0/(K_m app + S_0)$, resulting in pseudo-first-order kinetics. As illustrated in Figure 1, such behavior was observed in the CPA-catalyzed hydrolysis of amides **8** and **9**. For these substrates, the pseudo-first-order rate constant (k_0) obtained with $(1 - 5) \times 10^{-4}$ M S_0 were analyzed according to eq 3. The values of k_{cat} and

$$E_0/k_0 = (1/k_{cat})S_0 + K_m app/k_{cat} \qquad (3)$$

$K_m app$ obtained from the linear plot of $1/k_0$ against S_0 for **8** and **9** are summarized in Table II. The values of $K_m app$ for these substrates are very similar to the K_i value of 5×10^{-4} M of phenylalanine reported in the literature.[23]

When $K_m app$ is not similar to K_p, the reaction should deviate from pseudo-first-order kinetics as predicted by eq 2. For the hydrolysis of **1**, **7**, and **10**, the plot of log [S] against time deviates from a linear line as illustrated in Figure 1. The direction of the deviation indicates that $K_m app < K_p$ for **1** and **10** and $K_m app > K_p$ for **7**.[23] For these substrates, the whole time course of the reaction was analyzed according to the method reported in the literature.[24] This method is based on the integrated form (eq 4) of eq 2. From the values of *A* and *B* obtained with various

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Table II. Values of Kinetic Parameters for the CPA-Catalyzed Hydrolysis of Various Cinnamoyl Esters and Amides[a]

compd	k_{cat}, s^{-1}	$K_m$app, M	10^4K_p, M	k_{cat}/K_mapp, s^{-1} M^{-1}
1	0.725 ± 0.010	(1.24 ± 0.06) × 10^{-5}	0.847	(5.85 ± 0.26) × 10^4
2	0.460 ± 0.010	(2.23 ± 0.08) × 10^{-7}		(2.06 ± 0.09) × 10^6
3	0.276 ± 0.008	(1.24 ± 0.03) × 10^{-7}		(2.26 ± 0.09) × 10^6
4[b]	67.2 ± 1.7	(1.87 ± 0.07) × 10^{-4}	0.578	(3.60 ± 0.16) × 10^5
5[c]	226 ± 2	(1.67 ± 0.02) × 10^{-4}		(1.35 ± 0.02) × 10^6
6[d]	144	1.36 × 10^{-4}		1.01 × 10^6
7	71.4 ± 4.7	(2.91 ± 0.22) × 10^{-4}	0.970	(2.46 ± 0.08) × 10^5
8	0.193 ± 0.006	(6.11 ± 0.21) × 10^{-4}	ca. 6.1	(3.16 ± 0.04) × 10^2
9	5.60 ± 0.47	(4.93 ± 0.47) × 10^{-4}	ca. 4.9	(1.14 ± 0.05) × 10^4
10	2.23 ± 0.11	(1.10 ± 0.07) × 10^{-4}	3.44	(2.03 ± 0.08) × 10^4
11[e]	ca. 0.02	ca. 6 × 10^{-4}		ca. 3 × 10^1

[a] At 25 °C, pH 7.5, ionic strength 0.55. For **3**, 0.8% (v/v) Me_2SO was added in the measurement of k_{cat} and less than 1 × 10^{-3}% (v/v) Me_2SO in the measurement of $K_m$app. [b] Reference 14. [c] Measured by the initial rate method. The k_{cat} of 228 s^{-1} and $K_m$app of 1.14 × 10^{-4} M were reported in ref 21. [d] Reference 21. [e] Estimated from the data reported in ref 22.

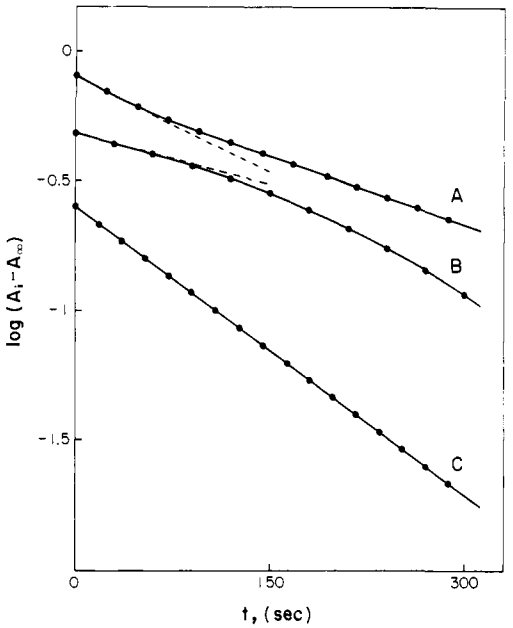


Figure 1. Typical absorbance changes in the CPA-catalyzed hydrolysis of **7** (A), **1** or **10** (B), and **8** or **9** (C).

$$t = A \ln S_0/[S] + B(S_0 - [S]) \quad (4)$$

$$A = K_m\text{app}(S_0 + K_p)/k_{cat}K_pE_0 \quad (5)$$

$$B = (1 - K_m\text{app}/K_p)/k_{cat}E_0 \quad (6)$$

S_0 concentrations, the kinetic parameters (k_{cat}, $K_m$app, and K_p) were estimated. These are summarized in Table II.

In the CPA-catalyzed hydrolysis of esters **2** and **3**, zero-order kinetics were observed whose rate constants were independent of S_0 (1 × 10^{-5} to 3 × 10^{-4} M) within ±5%. The zero-order behavior is expected from eq 2 if $K_m$app ≪ S_0 and $K_m$app ≪ K_p. The zero-order rate constant ($k_{cat}E_0$) directly gives k_{cat} for **2** or **3**, which is summarized in Table II.

Since zero-order kinetics were observed even at 1 × 10^{-5} M S_0, $K_m$app for **2** or **3** was initially estimated as being smaller than 10^{-6} M. For accurate measurement of $K_m$app, however, the spectral changes during the hydrolysis reactions with $S_0 \ll 1 \times 10^{-5}$ M were too small for kinetic measurements. Instead, $K_m$app for **2** or **3** was estimated from the CPA-catalyzed hydrolysis of **5** in the presence of **2** (1–10 × 10^{-7} M) or **3** (0.5–2 × 10^{-7} M). The absorbance changes in the hydrolysis of **5** (0.5–2 × 10^{-4} M) in the presence of **2** or **3** at such low concentrations, reflect only the reaction of **5**. Although **2** and **3** as well as **5** are hydrolyzed according to eq 1, **2** and **3** can be regarded as inhibitors for the hydrolysis of **5**, as far as only the initial rate data are concerned.

The expression of the initial velocity (v_o) for the scheme of eq 1 and 7 is derived as eq 8, assuming that the inhibition is competitive. The plot of E_0/v_o against $1/S_0$ at a given I_0 (total inhibitor concentration) produces a straight line, whose intercept is independent of I_0. The rate data obtained for the CPA-catalyzed hydrolysis of **5** in the presence of **2** are illustrated in Figure 2. The K_i value was calculated from the plot illustrated in Figure 3, which represents the dependence of the slopes of the straight lines in Figure 2 on I_0. The good fit of kinetic data to eq 8 indicates that the inhibition by **2** and **3** is competitive.

$$E + I \underset{K_i}{\rightleftharpoons} EI \quad (7)$$

$$E_0/v_o = K_m\text{app}(1 + I_0/K_i)/k_{cat}S_0 + 1/k_{cat} \quad (8)$$

Table III. Activation Thermodynamic Parameters for the k_{cat} of the CPA-Catalyzed Hydrolysis of **2** and **3**[a]

	2	**3**
E_a, kcal/mol	16.3 ± 0.2	18.8 ± 0.2
$\Delta G^{\ddagger}$, kcal/mol	17.9 ± 0.1	18.2 ± 0.1
$\Delta H^{\ddagger}$, kcal/mol	15.7 ± 0.2	18.3 ± 0.2
$\Delta S^{\ddagger}$, cal/mol K	−7.4 ± 0.8	0.3 ± 0.6

[a] Measured at pH 7.5 and ionic strength 0.55. For **3**, 0.8% (v/v) Me_2SO was added.

Table IV. Solvent Kinetic Isotope Effect of the k_{cat} for the CPA-Catalyzed Hydrolysis of Various Substrates[a]

compd	$(k_{cat})_{H_2O}/(k_{cat})_{D_2O}$
2	1.35 ± 0.05
3	1.53 ± 0.02
4	1.90 ± 0.16[b]
O-(*trans*-*p*-nitrocinnamoyl)-L-PLA	1.51[c]
O-(*trans*-cinnamoyl)-L-mandelate	1.56[c]
O-(*trans*-*p*-nitrocinnamoyl)-L-mandelate	1.09[c]
N-(*N*-benzoylglycyl)-L-Phe	1.33 ± 0.15[b]

[a] Measured at pH 7.50 and pD 8.05[21] (pH reading 7.65), 25 °C, and ionic strength 0.55. For **3**, 0.8% (v/v) Me_2SO was added. [b] Reference 25. [c] Reference 21.

The pertinent rate expressions indicate that the inhibition constant corresponds to the $K_m$app, instead of the dissociation constant of the ES complex, for **2** or **3**. The K_i values, i.e., the $K_m$app values, for **2** and **3** are listed in Table II, together with the kinetic parameters for **5** measured in the presence of **2**.

The pH dependence and the temperature dependence of k_{cat} for the CPA-catalyzed hydrolysis of **2** or **3** were measured, and the results are illustrated in Figures 4 and 5, respectively. From the Arrhenius plot of Figure 5, activation thermodynamic parameters for k_{cat} were calculated and summarized in Table III. The solvent isotope effect was measured for k_{cat} in the hydrolysis of **2** and **3**, and the results are summarized in Table IV.

Because of the limited solubility of **3**, measurement of k_{cat} was performed in the presence of 0.8% (v/v) dimethyl sulfoxide (Me_2SO). Examination of the effect of Me_2SO content on k_{cat} of **3** indicated that 0.8% (v/v) Me_2SO results in ca. 10% underestimation of k_{cat}.

Study of Intermediates. As will be indicated in the Discussion section, the kinetic data of **1–3** are consistent with the rate-determining breakdown of the acyl-CPA intermediate. In order to provide further support for this assignment, the hydrolysis of **3** was examined under the condition of $E_0 > S_0$. Immediately after the addition of **3** to the solution of CPA at −2 °C (S_0 = 0.4 × 10^{-4} M, E_0 = 0.6 × 10^{-4} M), the UV spectrum of the reaction mixture was taken, which is illustrated in Figure 6 after correction for the contribution of the enzyme. The measurement of the spectrum took about 20 s, while the half-life of the intermediate

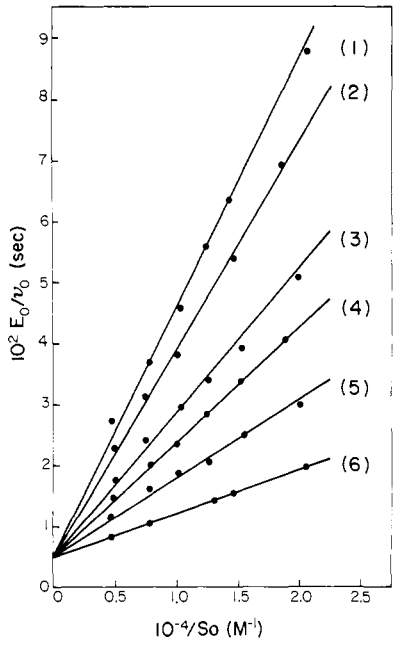


Figure 2. Plot of E_0/v_0 against $1/S_0$ for the initial rate data obtained with the CPA-catalyzed hydrolysis of **5** in the presence of **2**. Compound **2** is added as an inhibitor, whose initial concentration (I_0; μM) is 0 (line 6), 0.154 (line 5), 0.333 (line 4), 0.500 (line 3), 0.700 (line 2), or 1.00 (line 1).

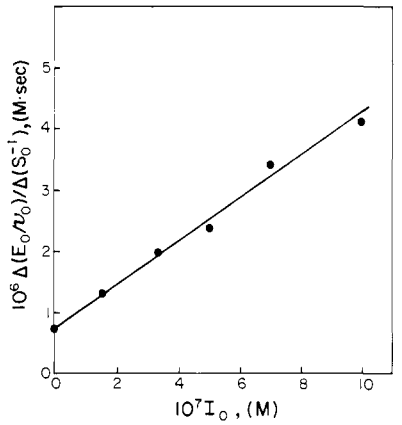


Figure 3. Plot against I_0 of the slopes of the straight lines drawn in Figure 2.

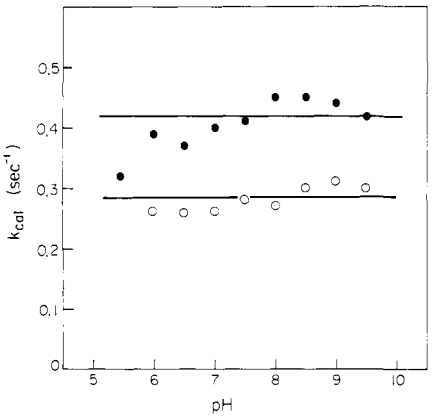


Figure 4. pH dependence of k_{cat} for the CPA-catalyzed hydrolysis of **2** (●) and **3** (○) at 25 °C. For **3**, the data at pH 5.5 were not measured because of limited solubility at low pH.

was about 60 s. The spectral data presented in Figure 6 indicate the accumulation of a CPA–**3** intermediate. The conversion of the intermediate to the product was a pseudo-first-order process, as measured from the absorbance *increase* at 310 nm, with the rate constant of 1.17×10^{-2} s^{-1} at −2 °C. This value is identical

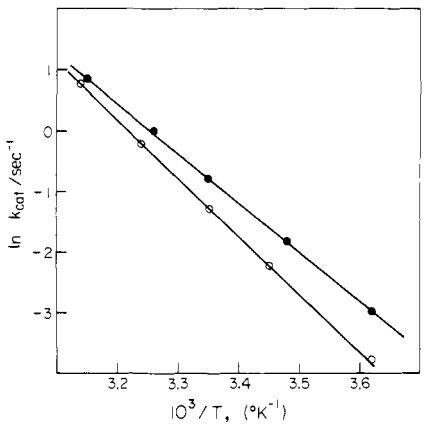


Figure 5. Temperature dependence of k_{cat} for the CPA-catalyzed hydrolysis of **2** (●) and **3** (○).

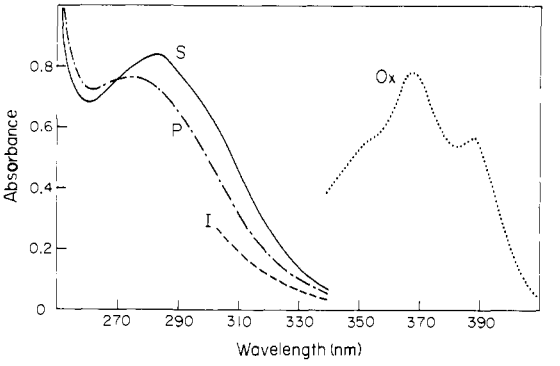
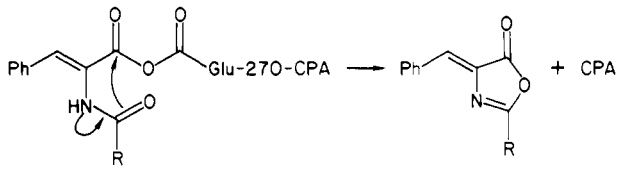


Figure 6. Spectra of **3** (0.4×10^{-4} M; curve S) and its hydrolysis product (curve P; addition of CPA does not affect the spectrum). Curve I is the spectrum obtained immediately after mixing **3** (0.4×10^{-4} M) with CPA (0.6×10^{-4} M) at −2 °C. Curve Ox is the spectrum of 2-(2-naphthyl)-4-benzylideneoxazolin-5-one (0.15×10^{-4} M) in acetonitrile. The spectrum of the 2-phenyloxazolin-5-one derivative shows λ_{max} of 345 nm ($\epsilon° = 35\,500$ M^{-1} cm^{-1}), 359 nm ($\epsilon° = 45\,000$ M^{-1} cm^{-1}), and 378 nm ($\epsilon° = 31\,400$ M^{-1} cm^{-1}).

Scheme I



with that calculated from the Arrhenius plot of Figure 5.

Trapping of the acyl-CPA intermediate was attempted with 0.1 M hydroxylamine in the hydrolysis of **1**–**3** at 25 °C, according to the procedure described previously.[26] Evidence for trapping of the intermediate, however, was not obtained.

Although the oxazolin-5-one derivatives absorb very strongly at 340–400 nm as illustrated in Figure 6, they were not detected after the CPA-catalyzed hydrolysis of **1**–**3**. The intramolecular trapping of the anhydride acyl-CPA intermediate with α-acylamino groups to form the oxazolin-5-one (Scheme I), therefore, does not occur.

Since accumulation of an intermediate was spectrophotometrically observed in the hydrolysis of **3** and the k_{cat} values of **2** and **3** indicated that the half-life of the intermediate was 30–60 s at near 0 °C, denaturation of the intermediate was attempted. When CPA (7×10^{-6} M) at pH 7.5 was incubated with 5.4 M urea or 0.9% sodium dodecyl sulfate for 30 min at 4 and 25 °C, respectively, its activity did not decrease appreciably, as checked by assay with 2×10^{-4} M **2** at 25 °C. Instead, incubation with 5 M guanidinium chloride (pH 7.2) at 4 °C resulted in relatively fast loss of CPA activity (10% loss in 0.5 min, 83% loss in 4 min, >99% loss in 11 min).

Right after mixing **2** or **3** (3×10^{-4} M) with CPA (1×10^{-4}

Table V. Expressions of k_{cat} and $K_m$app for the Scheme of eq 9 under Special Circumstances[a]

case	conditions	k_{cat}	$K_m$app
A	[ES′] ≪ [ES][b]	$k_{cat} = k_2k_3/(k_{-2} + k_3)$	$K_m > K_m\text{app} = (k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2})/k_1(k_3 + k_{-2}) \geq K_s$
B	[ES′] ≪ [ES][b] and acylation is rate-controlling[c]	$k_{cat} = k_2$	$K_m\text{app} = K_m$
C	[ES′] ≫ [ES][d]	$k_{cat} = k_3$	$K_m\text{app} = K_mk_3/k_2 + K_sk_{-2}k_2 \ll K_m$

[a] $K_m = (k_{-1} + k_2)/k_1$, and $K_s = k_{-1}/k_1$. They are sometimes considered to be equal.[8] [b] $k_2 \ll (k_3 + k_{-2})$ since $k_2[ES] = (k_3 + k_{-2})[ES']$ at steady state. ES′ does not accumulate. [c] $k_3 \gg k_{-2}$. [d] $k_2 \gg (k_3 + k_{-2})$. When ES′ accumulates, [ES′]/[ES] ≫ 1.

M) at 0–4 °C, 0.1 mL of 40% trichloroacetic acid or 3 mL of 7.5 M guanidinium chloride precooled at the same temperature was added to 1 mL of the reaction solution. The resulting mixture containing trichloroacetic acid was treated with methyl isobutyl ketone or chloroform, but the oxazolin-5-one derivatives were not detected in the organic layer. The product mixture containing guanidinium chloride also did not contain the oxazolin-5-ones.

Sodium azide (0.1 M) was also added with guanidinium chloride as a part of the denaturing reagent, in an attempt to trap the denatured acyl-CPA with azide ion according to the method[26] described previously. However, no evidence was obtained for the trapping of the intermediate.

Discussion

Accumulation of ES′. When the scheme of eq 1 is modified to include an additional intermediate (eq 9), k_{cat} and $K_m$app become eq 10 and 11.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \xrightarrow{k_3} E + P_i \quad (9)$$

$$k_{cat} = k_2k_3/(k_2 + k_3 + k_{-2}) \quad (10)$$

$$K_m\text{app} = (k_2k_3 + k_{-1}k_3 + k_{-1}k_{-2})/k_1(k_2 + k_3 + k_{-2}) \quad (11)$$

Expressions of k_{cat} and $K_m$app under special circumstances are summarized in Table V.

Spectrum I of Figure 6 indicates accumulation of an intermediate. The rate constant for the first-order breakdown of this intermediate is identical with the k_{cat} obtained from the zero-order kinetics with $S_0 \gg E_0$. This alone, however, does not indicate whether the observed intermediate is ES or ES′ of eq 9.

Observation of two successive intermediates has been achieved with dansyl-containing depsiesters and oligopeptides at −20 °C.[8] The kinteic parameters for the dansyl-containing depsiesters are summarized in Table VI. Parameter values for other ester substrates derived from L-PLA are also listed in this table. The values for **4**–**7** (Table II) and those summarized in Table VI indicate that the $K_m$app values for the L-PLA esters are not much different from 1×10^{-4} M regardless of the structure of the acyl moiety or the magnitude of k_{cat}, except for the dansyl compounds and the α-(acylamino)cinnamoyl compounds.

The small $K_m$app values for the dansyl compounds were due to the accumulation of ES′ (case C of Table V), instead of the formation of a very tight ES complex.[8] The K_s values of the dansyl compounds are indeed about 1×10^{-4} M (Table VI). The $K_m$app values for **2** and **3** are still smaller than those of the dansyl compounds, being the smallest ones ever reported for the CPA-catalyzed reactions. Therefore, the small $K_m$app values for **1**–**3** also should be due to the accumulation of ES′, and spectrum I of Figure 6 represents ES′ instead of ES. Then k_{cat} for **1**–**3** corresponds to k_3. Very small $K_m$app values were also observed for chymotrypsin-catalyzed reactions in which acyl-chymotrypsin intermediates accumulated.[29]

For **6**, formation of an intermediate was observed spectrophotometrically under cryoenzymological conditions.[4] This was originally considered as the first evidence for the acyl-CPA intermediate, although a later study[7] did not reveal the presence of a detectable amount of a cinnamoyl–enzyme covalent intermediate. The $K_m$app for **6** (Table II) indicates that ES′, if it exists, does not accumulate in the hydrolysis of **6** at least at 25 °C.

Table VI. Parameters for the CPA-Catalyzed Hydrolysis of Several *O*-Acyl-L-β-phenyllactate Esters[a]

acyl group	k_{cat}, s^{-1}	$K_m$app, M
3-(2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy)-propen-2-oyl[b]	2.3	0.96×10^{-4}
hippuryl[c]	424	0.53×10^{-4}
hippurylglycyl[d]	520	1.9×10^{-4}
o-hydroxyphenylacetyl[e]	0.16	1.7×10^{-4}
dansyl-$(Ala)_2$[f]	0.062 (−10 °C)	1.6×10^{-6} (−10 °C)[g,h]
dansyl-Gly-Ala[f]	0.14 (−20 °C)	5.6×10^{-6} (−20 °C)[g,i]
dansyl-$(Gly)_3$[f]	3.13 (−20 °C)	22.3×10^{-6} (−20 °C)[g-j]

[a] At 25 °C unless noted otherwise. [b] Reference 6. [c] Reference 27. [d] Reference 28. [e] Reference 26. [f] Reference 8. In this paper, rate equations were derived from eq 9 assuming that $K_m = K_s$ (i.e., $k_{-1} \gg k_2$). [g] Temperature dependence of $K_m$app is reported to be small. [h] $K_s = 1.29 \times 10^{-4}$ M. [i] $K_s = 0.75 \times 10^{-4}$ M. [j] $K_s = 1.64 \times 10^{-4}$ M.

Covalent Nature of ES′. Although the kinetic data are consistent with eq 9, ES′ observed with **3** may be either a noncovalent enzyme–substrate complex or an acyl-enzyme intermediate. The second intermediate observed in the hydrolysis of the dansyl derivatives was proposed to be a noncovalent complex.[8] This proposition was solely based on the fast formation of ES from ES′. This is because the reverse attack of the leaving group at ES′ to form ES would be very slow if the cleaved alcohol or amine diffuses away from ES′. As pointed out in a later study,[7] however, the reverse formation of ES from ES′ should be very efficient even if ES′ is an acyl-CPA intermediate. According to a space-filling model based on the X-ray crystallographic structure, the cleaved amine or alcohol portion of the substrate remains inside the active site until the acyl moiety of the substrate leaves the enzyme.[25] Thus, the leaving group is readily available for attack at ES′ to form ES. Although the reaction of the dansyl compounds was examined by spectrophotometry, spectrofluorometry, or EPR, the spectroscopic data did not establish whether the corresponding ES′ was a covalent intermediate or not.[9]

The covalent nature of ES′ can be determined if the enzymatic reaction is quenched immediately after accumulation of ES′. If ES′ is a noncovalent enzyme–substrate complex, the substrate should be recovered. On the other hand, isolation of the hydrolysis products would be clear evidence for the covalent nature of the accumulating intermediate. In order to obtain unambiguous evidence, however, total and instantaneous denaturation of the accumulating intermediate is necessary, which is very difficult to achieve.

Alternatively, trapping of the intermediate can provide direct evidence for its existence. The trapping experiment with external reagents or intramolecular α-acylamino groups was not successful in the presence and absence of denaturing reagents. Similar experiments with **4**, **6**, or *o*-hydroxyphenylacetyl-L-PLA in the absence of denaturing reagents also failed previously.[26] For successful trapping of a native acyl-CPA intermediate,[26] the trapping reagent should have access to the acyl center of the intermediate. For external reagents, its entrance into the active site should not be blocked. For intramolecular groups, the intermediate should have the right conformation. Even when the trapping reagent can attack the acyl center, the rate should be comparable to that of the enzymatic pathway for the breakdown process. When the acyl-enzyme intermediate is denatured, the trapping reagents would have ready access to the acyl center, and the enzymatic deacylation pathway would disappear. However, a model study[30] disclosed that external reagents would attack

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preferentially at the Glu-270 portion of the intermediate instead of the cinnamoyl portion of the denatured intermediate. The intramolecular trapping requires sufficient nucleophilicity of the α-acylamino groups. The efficiency of the intramolecular reaction by the acylamino groups has not been well investigated.[31]

Although the trapping experiments failed to produce positive results, strong support for the assignment of ES′ observed in the hydrolysis of **3** to the anhydride intermediate formed by the acylation of the Glu-270 carboxylate came from the pH profile of k_{cat} for **2** or **3** (Figure 4). The k_{cat} values are essentially pH-independent over pH 5.5–9.5.[32] The breakdown of the anhydride intermediate would occur through the attack of water or hydroxide ion. The k_3 would be independent of pH unless the hydroxide path becomes significant. The pH independence of k_{cat}, therefore, is consistent with the breakdown of the anhydride intermediate mainly through water attack.

Almost all the pH profiles of k_{cat} for the CPA-catalyzed reactions reflect the ionization of a functional group with pK_a of 6–7.[14,16,28,33-37] This group, which should be in the basic form for the enzyme activity, is usually assigned to the Glu-270 carboxylate. If k_{cat} stands for the breakdown of the anhydride intermediate, however, ionization of Glu-270 should not be reflected in the pH profile of k_{cat}, as in the pH profiles for **2** and **3**.

The pH effects have been studied on the breakdown of the intermediate observed for **6** under cryoenzymological conditions. The pK_a value calculated from the observed sigmoid pH profiles was 6.5 when the temperature was extrapolated to 25 °C.[5] Since the observed intermediate was later shown to be a noncovalent complex,[7] the pK_a can be assigned to Glu-270 and the observed reaction is best described as the acylation of Glu-270.

The intermediate observed during the hydrolysis of **3** is the most stable intermediate ever reported for CPA, in terms of both the half-life ($0.693/k_3$) and the magnitude of $K_m$app. In addition, it contains a good chromophore. Thus, the intermediate is well suited for spectroscopic characterization.

Activation Thermodynamic Parameters and Solvent Isotope Effects. Usually, k_{cat} for an enzymatic reaction is contributed to by several rate constants, and, therefore, its physical meaning is not straightforward. For **2** and **3**, k_{cat} represents k_3, and its temperature dependence and D_2O effect are related solely to the deacylation of ES′.

The activation entropy change (Table III) is not large, suggesting that the unimolecular breakdown of ES′ involves little changes in conformational freedom.

Among the substrates listed in Table IV, k_{cat} for *N*-(*N*-benzoylglycyl)-L-Phe (BGP) likely represents the acylation of Glu-270 (k_2). For **2** and **3**, the observed solvent isotope effect is rather small, being similar to that of BGP, for the nucleophilic attack of water at an acyl center. For the rest of the substrates listed in Table IV, k_{cat} is a complex quantity. No obvious trend is seen among the values of the solvent isotope effect. Instead, the data of Table IV demonstrate the limitation of solvent isotope effects in the mechanistic study of enzymes. Even with simple organic reactions, the solvent isotope effect does not necessarily differentiate the nucleophilic and general base mechanisms.[38] In enzymatic reactions, the contribution of the secondary isotope effects intrinsic to the enzyme would be even more significant.[39]

Effects of α-Acylamino Groups on Kinetic Parameters. The values of the rate parameters for **7** (Table II) fit (not shown) the Hammett plot[21] obtained for various para-substituted derivatives of **4**. Thus, the *p*-acetylamino group exerts only electronic effects. On the other hand, the α-acylamino groups of **1–3** lead to remarkably small k_{cat} and $K_m$app. When α-acylamino groups are attached to the cinnamoyl peptide substrates, k_{cat} is enhanced while $K_m$app is affected only slightly.

Several differences in the behavior of CPA toward ester and peptide substrates, such as the effects of metal substitution, inhibitors, or chemical modification, have been reported. This was explained in terms of different mechanisms[34,40,41] for esters and peptides but was also accounted for by a single mechanism[28,42] with different rate-determining steps. Similarly, the effects of the α-acylamino groups on the kinetic parameters for esters and for peptides can be explained in terms of a single mechanism of eq 9.

For peptides **8–11**, the acyl-CPA intermediate (ES′) does not accumulate and the acylation step is likely to be rate-determining. Thus, case B of Table V holds for **8–11**. Cases A and C of Table V apply to esters **4–7** and **1–3**, respectively. Thus, the effects of the α-acylamino groups on the kinetic parameters of these substrates are explained by assuming that k_2 is raised, k_3 is lowered, and K_m or K_s is little affected by the introduction of the α-acylamino groups.

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Studies of Silicon–Phosphorus Bonding

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Abstract: Ab initio calculations are presented for several species containing a silicon–phosphorus bond. The types of bonding studied include "normal" single, double, and triple bonds, as well as an ylide-like structure. The latter is found to be much less strongly bound than the carbon analogue, with a smaller stretching force constant than that in silylphosphine. The insertions of silylene into the phosphine bond and of phosphinosilylene into H_2 are discussed, with the former being illustrated using localized molecular orbitals along the intrinsic reaction coordinate (IRC). Silylene to silene isomerizations in both the closed-shell singlet and the lowest triplet states of $SiPH_3$ are analyzed in the same manner.

I. Introduction

As part of an ongoing program investigating the nature of bonding of silicon in a variety of molecular environments, the present paper presents an analysis of the bonding between silicon and phosphorus in a variety of prototypical environments. These include "normal" triple (I), double (II), and single (III) bonds, as well as the apparently hypervalent ylide (IV).

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