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Kinetics of the Carboxypeptidase A-Catalyzed Hydrolysis of α-(Benzoylamino)cinnamoyl Derivatives of Various Amino Acids

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Kinetics of the carboxypeptidase A-catalyzed hydrolysis of α-(benzoylamino)cinnamoyl derivatives of various amino acids were measured. The C-terminal amino acid residue of the amide substrates were phenylalanine (Phe), p-substituted phenylalanines (*p*-OH, *p*-NO_2, *p*-Cl, *p*-I, *p*-CH_3 *p*-C_2H_5, and *p*-$CH(CH_3)_2$), tryptophan (Trp), and phenylglycine (phenylGly). Introduction of the *p*-OH, *p*-Cl, *p*-NO_2, or *p*-CH_3 group to the leaving Phe did not lead to considerable changes in the reactivity, while that of the *p*-I or *p*-C_2H_5 group decreased the reactivity remarkably and that of the *p*-$CH(CH_3)_2$ group abolished the reactivity. Substitution of Trp for the leaving Phe did not alter the reactivity appreciably, whereas that of phenylGly for the leaving Phe reduced the reactivity to a large degree. The seemingly scattered pattern of the structure–reactivity relationship was explained by assuming that the hydrophobic pocket in the active site of the enzyme has limited height, which is comparable to the thickness of the benzene ring.

INTRODUCTION

Carboxypeptidase A (CPA)[2] has been subjected to intensive mechanistic studies by using the kinetic method (*1*) as well as other techniques (*2*). Much less kinetic data have been obtained for the peptidase action of CPA than for the esterase action. This is partly because the peptide substrates with good chromophoric properties were not available. *N*-Acyl-α-amino acids are usually hydrolyzed by CPA too slowly for practical kinetic measurements, while analogous *O*-acyl-α-hydroxyacids are good substrates of CPA. The peptide substrates readily hydrolyzed by CPA are *N*-acyl oligopeptides. Recently, we have prepared substrates containing an α-(acylamino)cinnamoyl group, which is an *N*-acyl-α-amino acid residue with a good chromophore (*1f–1h*).

The Glu-270 carboxylate of CPA is believed to be catalytically essential, acting either as a nucleophile or as a general base. In the hydrolysis of amide substrates, it is likely that the expulsion of the leaving amino acid residue is

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[2] Abbreviations used: Phe, phenylalanine; Trp, tryptophan; Gly, glycine; CPA, carboxypeptidase A.

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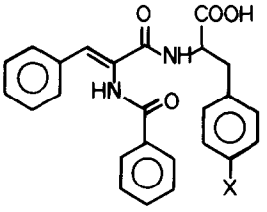
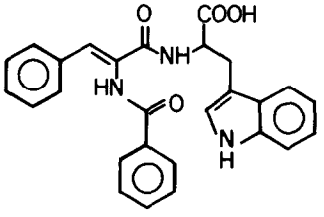
rate-controlling either for the nucleophilic mechanism or for the general base mechanism. Consequently, changes in the side chains of the leaving amino acid residues would affect the enzyme activity considerably and, thus, could provide various lines of information on the mechanism of the catalytic action. In the present study, *trans*-α-(benzoylamino)cinnamoyl-L-phenylalanine (L-**1**) and its analogs (**2–10**) containing various C-terminal amino acids were prepared and kinetics of the hydrolysis of the amide substrates by CPA were measured.



1–8

1: X = H **2:** X = OH
3: X = NO_2 **4:** X = Cl
5: X = I **6:** X = CH_3
7: X = C_2H_5 **8:** $CH(CH_3)_2$



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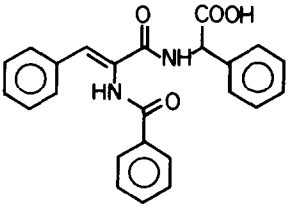

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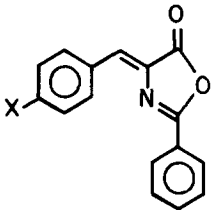
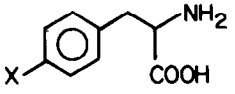
EXPERIMENTAL PROCEDURES

Materials. (*Z*)-4-(*p*-Methylbenzylidene)-2-phenyloxazolin-5-one (**11**), (*Z*)-4-(*p*-ethylbenzylidene)-2-phenyloxazolin-5-one (**12**), and (*Z*)-4-(*p*-i-propylbenzylidene)-2-phenyloxazolin-5-one (**13**): To the solution of hippuric acid (5 g) and triethylamine (5.8 ml) in 80 ml benzene, ethyl chloroformate (2.9 ml) was added, and the resulting mixture was stirred for 1 h at room temperature. After 4-CH_3-, 4-C_2H_5-, or 4-$CH(CH_3)_2$-benzaldehyde (28 mmol) was added, the mixture was refluxed for 10 min. The HCl salt of triethylamine was removed by filtration, and the filtrate was evaporated *in vacuo*. The resulting oily substance was solidified by trituration with ethanol and the precipitates were recrystallized from benzene or ethanol. **11**, mp 139–141°C; **12**, mp 123–124°C; **13**, mp 118–119°C.



11–13



14–16

11: X = CH_3 **14:** X = CH_3
12: X = C_2H_5 **15:** X = C_2H_5
13: X = $CH(CH_3)_2$ **16:** X = $CH(CH_3)_2$

DL-*p*-Methylphenylalanine (**14**), DL-*p*-ethylphenylalanine (**15**), and DL-*p*-i-propylphenylalanine (**16**): To the mixture of **11**, **12**, or **13** (10 mmol), red phosphorous (2 g), and acetic anhydride (13 ml), 57% HI solution (10 ml) was added over a period of 1 h at room temperature, and the resulting mixture was refluxed for 4 h. The reaction mixture was cooled to room temperature and filtered. The filtrate thus obtained was evaporated *in vacuo*, and the residue was dissolved in a 1 : 1 mixture (30 ml) of water and ether. After ether was evaporated by heating, the aqueous solution was neutralized with a 15% ammonia solution. The product which crystallized upon cooling the mixture was washed with cold water. **14**, mp 236–239°C; **15**, mp 233–236°C; **16**, mp 218–220°C.

Substrates **1–10**: The C-terminal amino acids of **1–10** were purchased from Sigma Chemical Co., except for **14–16** which were prepared as described above. The potassium salt of the amino acid was prepared by evaporating the aqueous mixture of each amino acid (3 mmol) and KOH (2.8 mmol). The potassium salt was dissolved in anhydrous dimethyl sulfoxide (25 ml) at 80°C. After (*Z*)-4-benzylidene-2-phenyloxazolin-5-one (2 mmol)(*1f*) was added, the mixture was heated at 70–80°C for 3 h and then cooled to room temperature. White precipitates obtained by the addition of an aqueous 0.5 N HCl solution (150 ml) were quickly separated by filtration, washed with water, dried, and then recrystallized from benzene or ethyl acetate. L-**1**, mp 180.5–181.5°C (lit. (*1f*) 180.5–181.5°C); DL-**1**, mp 180–181°C; L-**2**, mp 106–108°C; L-**3**, mp 104–106°C; DL-**4**, 94–96°C; DL-**5**, mp 97–99°C; DL-**6**, mp 172–173°C; DL-**7**, mp 178–179°C; DL-**8**, mp 186–188°C; L-**9**, mp 127–129°C; L-**10**, mp 114–116°C.

CPA_α (prepared by the Cox *et al.* method (*3*)) was purchased from Sigma Chemical Co. as a suspension in toluene–water. The enzyme was washed twice with deionized water and then dissolved in 2 M NaCl at pH 7.5 (0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate). The enzyme solution was purified by passing through a Sephadex G-25 column at 4°C. Activity of enzyme stock solutions was checked by assay with *trans*-α-(benzoylamino)cinnamoyl-L-β-phenyllactate (*1f*).

Stock solutions of **7** and **8** were prepared in dimethyl sulfoxide, and the rest of the substrates were converted into the sodium salts by neutralization with NaOH

prior to the preparation of the stock solutions in water.

Distilled water was deionized prior to use in the kinetic measurements.

Kinetic measurements. Reaction rates were measured with a Beckman DU-64 spectrophotometer by following absorbance changes at 310–320 nm. Temperature was maintained at 25 ± 0.1°C with a Haake E12 circulator. Buffer solutions for the kinetic measurements contained 0.5 M NaCl and 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (pH 7.50). For **7** and **8**, the reaction mixture contained 0.8% (v/v) dimethyl sulfoxide. When the DL-mixture of a substrate was used, the product spectrum indicated that only half of the substrate is hydrolyzed. Thus, the D-forms of the amide substrates are not hydrolyzed by CPA, in agreement with other substrates. The uv spectra of the hydrolysis products were identical to those of product solutions obtained by mixing authentic samples.

RESULTS AND DISCUSSION

Steady-state kinetic measurements were performed under the conditions of S_o (initially added concentration of substrate) $\gg E_o$ (total enzyme concentration). The initial rates (v_o) were calculated by the statistical treatment described in the literature (*4*). The values of k_{cat} and K_m^{app} for each substrate were calculated by plotting E_o/v_o against $1/S_o$ according to Eq. [1]. Although the kinetics of CPA-catalyzed reactions are usually complicated by product inhibition, this complication was avoided by using the v_o data:

$$E_o/v_o = 1/k_{cat} + (K_m^{app}/k_{cat})(1/S_o). \quad [1]$$

The parameter values obtained for the substrates are summarized in Table 1. When the DL-compound was used as the substrate, the concentration of the L-form was used as S_o. The close agreement between kinetic parameters for L-**1** and those for DL-**1** indicates that D-**1** does not affect the kinetics of the CPA-catalyzed hydrolysis of L-**1** in the range of S_o ($S_o < 3K_m^{app}$) employed in the kinetic measurements.

The actual process of an enzymatic reaction is much more complicated than the simple Michaelis–Menten scheme, involving multiple forms of enzyme–substrate complexes and various intermediates. In this regard, the meaning of k_{cat}/K_m^{app} is much more straightforward than that of k_{cat} or K_m^{app}, representing the bimolecular reactivity of the enzyme toward the substrate (*1a, 6*).

In terms of k_{cat}/K_m^{app}, L-**1** is 400 times more reactive than cinnamoyl-L-Phe, an *N*-acyl amino acid, and is as reactive as *N*-acyl oligopeptide substrates such as benzoyl-$(Gly)_2$-L-Phe, benzoyl-$(Gly)_3$-L-Phe, or dansyl-$(Gly)_3$-L Phe.[3] In this regard, **1** behaves as an *N*-acyl dipeptide, although **1** contains an unnatural amino acid, α-aminocinnamate.

The reactivity of **10** is 150 times smaller than that of **1**. Replacement of the

[3] In comparison with benzoyl-$(Gly)_2$-L-Phe, benzoyl-$(Gly)_3$-L-Phe, or dansyl-$(Gly)_3$-L-Phe, **1** manifests less effective k_{cat}, more effective K_m^{app}, and comparable k_{cat}/K_m^{app}.

TABLE 1

Kinetic Parameters for the CPA-Catalyzed Hydrolysis of Various Amide Substrates[a]

Compound	k_{cat} (s^{-1})	K_m (10^{-4} M)	k_{cat}/K_m (10^4 s^{-1} M^{-1})
L-**1**[b]	4.4	3.8	1.2
DL-**1**	3.6	3.5	1.0
L-**2**	2.1	1.8	1.1
L-**3**	0.65	1.6	0.41
DL-**4**	1.6	2.7	0.59
DL-**5**	0.42	5.8	0.072
DL-**6**	2.4	6.2	0.39
DL-**7**	0.38	11	0.035
DL-**8**		No reaction	
L-**9**	1.7	2.2	0.78
L-**10**	0.16	23	0.0070
Benzoyl-$(Gly)_2$-LPhe[c]	20	10	2.0
Benzoyl-$(Gly)_3$-L-Phe[c]	43	37	1.2
Dansyl-$(Gly)_3$-L-Phe[c]	70	8.0	8.8
Cinnamoyl-L-Phe[d]	ca. 0.02	ca. 6	0.0030

[a] Standard deviations of the parameter values measured in the present study are less than 10% of the respective parameter values. When DL-forms were used, only the L-form was hydrolyzed as discussed in the text.

[b] Similar values have been obtained (*1f*) with CPA_γ prepared by the Anson (5) method.

[c] Ref. (*1b*).

[d] Ref. (*1f*).

benzyl side chain with the phenyl side chain in analogous ester substrates also leads to large rate decreases. For example, k_{cat}/K_m^{app} for cinnamoyl-L-mandelate is 300 times smaller than that for cinnamoyl-L-β-phenyllactate (*1a*).

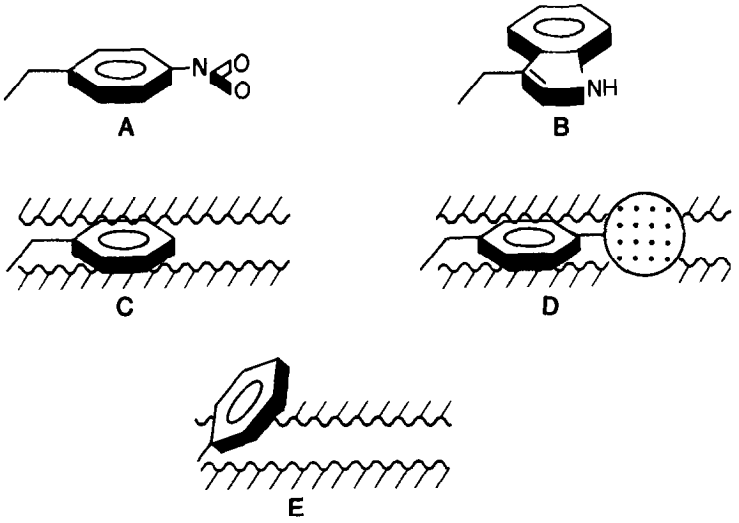
Replacement of Phe of **1** with Trp did not alter the reactivity considerably. Introduction of *p*-OH to **1** did not affect the reactivity, and that of *p*-Cl, *p*-NO_2, or *p*-CH_3 lowered the reactivity only by two to three times. On the other hand, the introduction of *p*-I or *p*-C_2H_5 resulted in a 15- or 30-fold decrease in the reactivity, and that of *p*-$CH(CH_3)_2$ abolished the reactivity.

The reactivity of **1–10** is not simply correlated with the size of the hydrophobicity of the side chain of the leaving amino acid. The reactivity difference between **1** and **10** may be related to the greater size or the higher hydrophobicity of the benzyl side chain compared with that of the phenyl side chain. On the other hand, the reactivity is decreased as the alkyl or halogen p-substituent of **2** becomes bulkier and more hydrophobic, and is totally lost when *p*-$CH(CH_3)_2$ is introduced. The reactivity is similar for **1**, **3**, and **9**, although the side chain of **3** or **9** is considerably bulkier than that of **1**.

The X-ray crystallographic studies indicated (*2a*) that the side chain of the

leaving amino acid residue of an amide substrate of CPA interacts with the hydrophobic pocket of the enzyme in the enzyme–substrate complex. However, the introduction of hydrophilic p-substitutents such as OH and NO_2 to **1** did not lower the reactivity considerably.[4]

The differences in the reactivity of the amide substrates examined in this investigation would originate from several factors concerning binding and catalytic processes. The introduction of various side chains would affect the fitting of the side chain into the hydrophobic pocket of the enzyme. Therefore, the kinetic data of the present study might be related to the structure of the hydrophobic pocket. The dimension of the pocket would not allow accommodation of side chains of substrates beyond a certain size limit. The reactivities of **3** and **9** suggest that the pocket is deep enough to accommodate the bulky side chain of *p*-NO_2Phe or Trp. The very low reactivities of **5** or **8**, therefore, cannot be ascribed to the limited depth of the pocket. In **3** and **9**, the substituted aromatic portions are plate-shaped as illustrated by **A** and **B**, respectively. If the height of the hydrophobic pocket is similar to the thickness of the benzene ring as illustrated by **C**, the thin side chains of substrates **2–4**, **6**, and **9** would be readily accommodated by the pocket, aligning the substrates in catalytically suitable positions. On the other hand, the *p*-substituents of slowly reacting amides such as **5**, **7**, and **8** may not fit snugly into the hydrophobic pocket in the same binding mode since the *p*-substituents are thicker than the benzene plane as illustrated by **D**. The binding of **10** to CPA might be less efficient than that of **1** as illustrated by **E**. The low reactivity of **5**, **7**, **8**, or **10**, therefore, may be ascribed to the lack of precise alignment of the substrate within the active site.



[4] The uncharged polar groups such as OH or NO_2 are apparently well accommodated by the hydrophobic pocket of the enzyme. The X-ray crystallographic study on CPA complexed with Gly-Tyr, a pseudosubstrate, indicated that the OH of Tyr does not form a hydrogen bond to the enzyme (*2a*). Thus, specific interactions may not be present between the hydrophobic pocket of CPA and the *p*-OH or *p*-NO_2 group of **2** or **3**.

Information on the shape of the hydrophobic pocket may be obtained from the results of X-ray crystallographic studies performed on static complexes of CPA formed with pseudosubstrates or inhibitors. However, it is possible that the binding of amide substrates by the enzyme may induce changes in the shape of the hydrophobic pocket. The hypothesis proposed in this study concerning the shape of the hydrophobic pocket is one way to rationalize the seemingly scattered pattern of the structure–reactivity relationship of amide substrates **1–10**.

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