Bioorganic Chemistry **28,** 226–241 (2000)
doi:10.1006/bioo.2000.1161, available online at http://www.idealibrary.com on IDEAL®

Inhibition Effects in the Hydrolysis Reactions of Esters and Peptides Catalyzed by Carboxypeptidase A: An Example of Cooperative Binding Effects with a Monomeric Enzyme

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Received September 1, 1999

N-benzoyl-L-phenylalanyl-L-phenylalanine is an excellent peptide substrate for carboxypeptidase A; at 30°C and pH 7.5, K_m is 2.6×10^{-5} M while k_{cat} is 177 s^{-1} ($k_{cat}/K_m = 6.8 \times 10^6$ M^{-1} s^{-1}). Indole-3-acetic acid is a noncompetitive or mixed inhibitor towards the peptide and toward hippuryl-L-phenylalanine; plots of E/V vs [Inhibitor] are linear. *N*-Benzoyl-L-phenylalanine is a competitive inhibitor of peptide hydrolysis, and plots of E/V vs [Inhibitor] are again linear. One molecule of inhibitor binds per active site, and these inhibitors bind in different sites. At constant peptide substrate concentration and a series of constant concentrations of indole-3-acetic acid, plots of E/V vs the concentration of *N*-benzoyl-L-phenylalanine are linear and intersect behind the E/V axis and above the [Inhibitor] axis. This shows that both inhibitors can bind simultaneously and that binding of one facilitates the binding of the other ($\beta = 0.18$). Employing the ester substrate hippuryl-DL,β-phenyllactate, the same type of behavior is observed in the reverse sense; *N*-benzoyl-L-phenylalanine is a linear noncompetitive inhibitor and indole-3-acetic acid is a linear competitive inhibitor. Again the two inhibitor plot is linear and intersects above the [Inhibitor] axis ($\beta = 0.12$). Previous X-ray crystallographic studies have indicated that indole-3-acetic acid binds in the hydrophobic pocket of the S_1' site, while *N*-benzoyl-L-phenylalanine binds in the S_1–S_2 site. The product complex for hydrolysis of *N*-benzoyl-L-phenylalanyl-L-phenylalanine (phenylalanine + *N*-benzoyl-L-phenylalanine) occupies both of these sites. However, the present work shows that the peptide substrate does not bind to the enzyme at pH 7.5 so as to be competitive with indole-3-acetic acid. The binding sites may be formed via conformational changes induced or stabilized by substrate and product binding.

INTRODUCTION

Carboxypeptidase A (peptidyl L-amino acid hydrolase (EC 3.4.17.1) (CPA)[1] is a Zn(II) metalloenzyme that catalyzes the hydrolysis of C-terminal peptides and *O*-acyl derivatives of β-phenyllactic acid and mandelic acid (1–3). The enzyme is composed of a single chain of 307 amino acids (3). X-ray crystallographic analysis at 2 Å resolution showed that the poor peptide substrate glycyltyrosine is complexed to Zn(II) through the

[1] Abbreviations used: CPA, bovine pancreatic carboxypeptidase A; BPP, *N*-benzoyl-L-phenylalanyl-L-phenylalanine; BP, *N*-benzoyl-L-phenylalanine; IAA, indole-3-acetic acid; HPL, hippuryl-DL, β-phenyllactic acid; HPA, hippuryl-L-phenylalanine; PPA, *N*-(phenoxycarbonyl)-L-phenylalanine; Tris, tris-hydroxymethylaminomethane.

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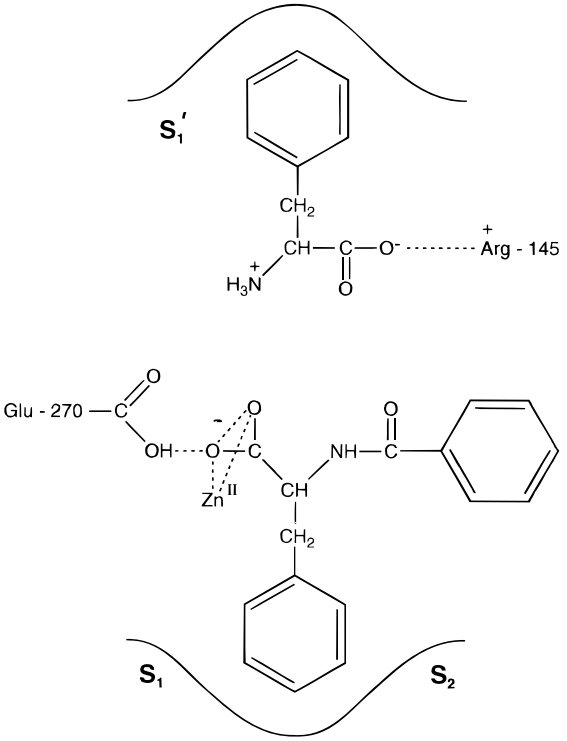
carbonyl oxygen (*3*,*4*). The γ-carboxyl group of Glu-270 is also located in close proximity. On this basis, nucleophilic and general base mechanisms involving Glu-270 were postulated for peptide hydrolysis, and it was assumed that similar or identical mechanisms would occur with ester substrates. Christianson and Lipscomb (5) have suggested that the carbonyl complexation of glycyltyrosine may be anomalous and that the peptide carbonyl is normally polarized by hydrogen bonding from Arg-127.

Christianson and Lipscomb (*5*,*6*) determined that *N*-benzoyl-L-phenylalanine (BP) binds in the S_1–S_2 subsite of CPA with the carboxyl group complexed with the metal ion. In the same complex L-phenylalanine binds in the hydrophobic region of the S_1' subsite with the carboxylate anion electrostatically linked to Arg-145. They considered that I represented the productive complex for the synthesis of *N*-benzoyl-L-phenylalanyl-L-phenylalanine (BPP). If that is the case, then by the principle of microscopic reversibility I should also resemble the productive complex for the hydrolysis of BPP since the transition states for the two reactions must be identical.

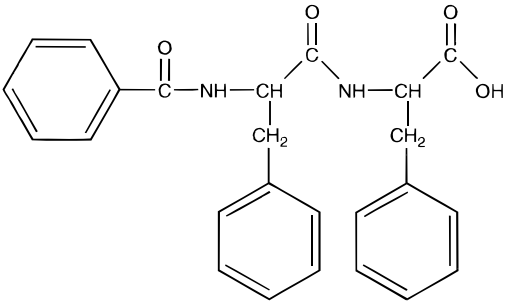


I

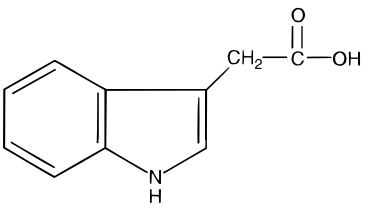
There is strong evidence that peptides and esters bind initially to CPA in different sites (*7*,*8*). Indole-3-acetic acid (IAA) is a competitive inhibitor toward ester substrates but is noncompetitive toward peptides (*7*). The carbamate ester *N*-(phenoxycarbonyl)-L-phenylalanine is a linear competitive inhibitor toward the peptide hippuryl-L-phenylalanine but is a linear noncompetitive inhibitor toward the ester hippuryl-L,β-phenyllactate (*8*). The carbamate ester is also an observable substrate for the enzyme at high enzyme concentration (10^{-6} M), and $K_m = K_i$. The carbamate is therefore binding in the catalytic site for peptides.

The above findings do not rule out similar mechanisms for peptides and esters (*9*). Furthermore, the catalytic site could be formed by a conformational change induced or stabilized by substrate binding. Galdes *et al.* (*10*) and Geoghagen *et al.* (*11*) have found that at least two intermediates occur before the rate-determining step ($ES_1 \rightleftharpoons ES_2$) in the CPA-catalyzed reactions of both peptides and esters. The rate-limiting step in peptide hydrolysis occurs before the formation of an enzyme-product complex (*12*).

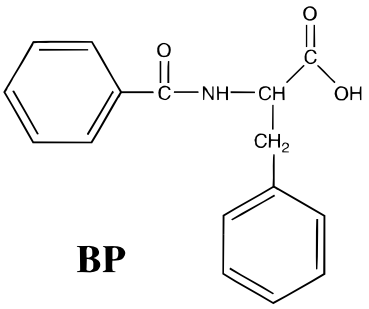
It is clear that insight into the enzyme mechanism is dependent on a deeper understanding of the sites in which peptides and esters bind and of the interrelationships between these sites. We have in the present work studied the CPA catalyzed hydrolysis of the large peptide substrate *N*-benzoyl-L-phenylalanyl-L-phenylalanine (BPP) in the presence and absence of the inhibitors IAA and BP. X-ray crystallographic studies have indicated that these inhibitors bind in the S_1' and S_1–S_2 sites, respectively (*6*,*13*).



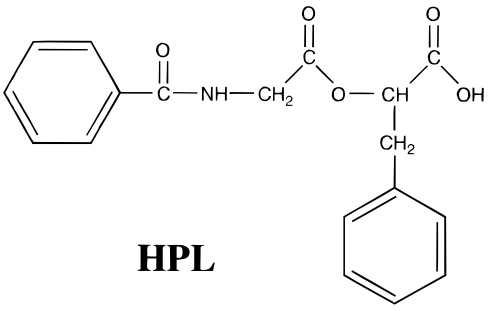
BPP



IAA



BP



HPL

We have also employed these inhibitors together in the reactions of hippuryl-DL,β-phenyllactic acid (HPL), a typical ester substrate for CPA that has been extensively studied in previous investigations (*3,7,8*).

Multiple inhibition analysis can allow the determination of whether binding of the inhibitors to the enzyme is mutually exclusive or whether both can bind simultaneously (*14*). If both can bind simultaneously, then it can be determined whether the binding of one hinders or facilitates the binding of the other. As a consequence, considerable information can be obtained pertaining to the nature of the binding sites and their possible interactions.[2] We have found that IAA and BP can bind simultaneously and that their binding is synergistic, even though CPA is a monomeric enzyme.

EXPERIMENTAL SECTION

N-Benzoyl-L-phenylalanyl-L-phenylalanine (BPP) was prepared by benzoylation of the dipeptide L-Phe-L-Phe (Sigma) by employing the method of Auld and Vallee (16). L-Phe-L-Phe (1 g) was added to 10 ml of water and 1 ml of 10 N NaOH. To this solution was added at 0°C an equivalent amount of benzoyl chloride (Aldrich) in small portions with vigorous stirring over a period of 10 min. The solution was acidified with 6 N HCl. The white precipitate was washed with water. After recrystallization from water-ethanol and vacuum dessication, the crystalline compound melted at 185–186°C. *Anal.* Calcd for $C_{25}H_{24}N_2O_4$: C, 72.10; H, 5.81; N, 6.73. Found: C, 72.19; H, 5.79; N, 6.56.

Indole-3-acetic acid was purchased from Aldrich. After recrystallization from ethyl acetate-hexane it melted at 166–167°C. *N*-Benzoyl-L-phenylalanine, hippuryl-L-phenylalanine, and hippuryl-DL,*β*-phenyllactate sodium salt were purchased from Sigma. Ninhydrin reagent solution (ninhydrin, 20 g/l; hydrindantin, 3 g/l; DMSO, 75%; lithium acetate, 1 M) was purchased from Sigma and stored at 4°C under nitrogen. This assay solution must be stirred gently before its application to the amino acid solutions. All buffer components were reagent grade materials, and deionized water was used throughout.

Carboxypeptidase A from Sigma was dialyzed in 0.05 M Tris-HCl buffer (μ = 0.5 M with NaCl, $[Zn^{2+}] = 10^{-4}$ M), pH 7.5, at 5°C for 36 h. The buffer solution was changed after 18 h. After dialysis the clear enzyme solution was centrifuged at 15,000 rpm for 15 min at 5°C. The supernatant was filtered through Millipore "Millex" filters and stored at 5°C. The protein concentration was determined by employing the extinction coefficient at 222.5 nm ($\epsilon = 5.27 \times 10^5$ M^{-1} cm^{-1}) and 278 nm ($\epsilon = 6.42 \times 10^4$ M^{-1} cm^{-1}) (17).

Kinetic methods. The hydrolysis of BPP cannot be followed spectrophotometrically due to insufficient absorbance changes. Therefore, the hydrolysis reactions were followed using a ninhydrin assay that detects the release of the primary amino group of phenylalanine (*18*). In the initial rate measurements the reaction conditions were chosen so that less than 10% of the substrate was hydrolyzed. Absorbance measurements were carried out using a Zeiss Model PM2DL spectrophotometer. The background absorbance due to the

[2] An example is the multiple inhibitor analysis that was carried out in reactions of D-glyceraldehyde-3-phosphate dehydrogenase (15).

enzyme was negligible at the enzyme concentration employed ($1.4–6 \times 10^{-9}$ M). The reaction measurements were carried out in duplicate. Control duplicates that did not include CPA gave the background absorbance not due to the enzyme. Under the assay conditions the inhibitors BP and IAA do not react with ninhydrin to a significant extent. At pH 7.5, Tris–HCl buffer was employed, which contributes slightly to the background absorbance. An absorbance range of 0.2–1.0 was generally utilized. The reaction temperature was maintained constant at 30 ± 0.1°C. The reaction was stopped using a 1 M citrate, 10^{-4} M EDTA, pH 5.2, solution. The ninhydrin assay has been employed previously to monitor CPA catalyzed peptide hydrolysis in Tris buffer (*16*).

The CPA catalyzed hydrolysis of the ester substrate hippuryl-DL, β-phenyllactic acid (HPL) was monitored spectrophotometrically by following the absorbance changes at 254 nm with a Beckman DU-7500 spectrophotometer. Initial rates were determined. The presence of the D-isomer has been shown not to affect the kinetics of the CPA catalyzed reaction (*17*). The concentrations employed were corrected to reflect that of the L-isomer. The pH measurements were made with a Radiometer PHM-22 pH meter. The peptidase activity of the enzyme stock solution was routinely checked with HPA and the esterase activity with HPL.

The values of the kinetic parameters were obtained from computer analysis of plots of V/E vs $[S_0]$, E/V vs $1/[S_0]$, and E/V vs [Inhibitor]. The solid lines in the plots presented for illustration represent the best fit. The conventional nomenclature (*14*) is employed to describe the inhibition experiments, i.e., competitive, noncompetitive, and mixed. Noncompetitive inhibition is strictly an effect on k_{cat}. If both k_{cat} and the apparent K_m are affected, then the inhibition is mixed. Noncompetitive inhibition has K_i (slope) = K_i (intercept). In the inhibition experiments in which the substrate concentration was varied, at least two constant concentrations of inhibitor were employed.

RESULTS

The CPA catalyzed hydrolysis of *N*-benzoyl-L-phenylalanyl-L-phenylalanine (BPP) at pH 7.5 and 30°C (μ = 0.5 M with NaCl) is characterized by k_{cat} = 177 s^{-1}, and $k_{cat}/K_m = 6.8 \times 10^6$ M^{-1} s^{-1}. The accuracy of rate measurements with the ninhydrin assay was less at very low substrate concentrations (less than K_m) than with higher substrate concentrations. The small apparent K_m of BPP was therefore measured using a suitable competitive inhibitor so that relatively high concentrations of substrate could be conveniently employed. *N*-Benzoyl-L-phenylalanine (BP), a product of the hydrolysis of BPP, was found to be a competitive inhibitor of the CPA catalyzed hydrolysis of BPP. The plots of E/V vs $1/[S_0]$ at constant concentrations of BP shown in Fig. 1 intersect on the E/V axis and give the k_{cat} value of 177 ± 2 s^{-1}. The plots of E/V vs [BP] (Fig. 2) are also linear. Correlation coefficients were greater than 0.99 for the plots of Figs. 1 and 2. Dixon plots of E/V vs $[I]$ intersect at $[I] = -K_i$ when the inhibition is linear competitive. A horizontal line from the point of intersection will then touch the E/V axis at $1/k_{cat}$ (*14*). The intersection of the plots of Fig. 2 for the competitive inhibition by BP at high substrate concentrations occurs close to the E/V axis at $1/k_{cat}$ because of the required scale of the [BP] axis. The average K_m from Figs. 1 and 2 is 2.6×10^{-5} M. A K_i of 9.2×10^{-5} M was found for BP. This K_i value compares well with the value of 8.8×10^{-5} M previously reported in other reactions (19). The slopes of Fig 2. give $K_i/K_m = 3.6$.

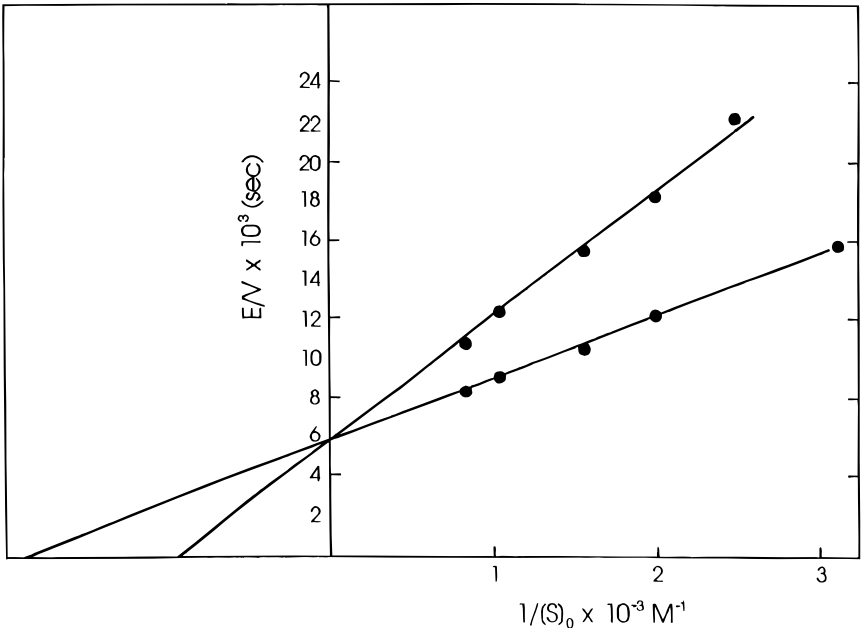


FIG. 1. Plots of E/V vs $1/[S_0]$ for the CPA (1.4×10^{-9} M) catalyzed hydrolysis of BPP in the presence of 0.002 and 0.004 M BP at 30°C, pH 7.5, and μ = 0.5 M NaCl.

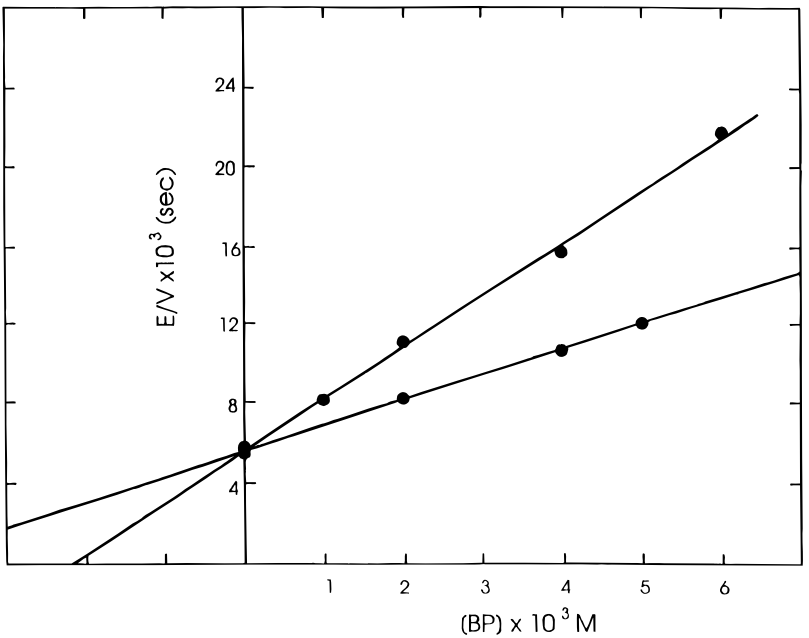
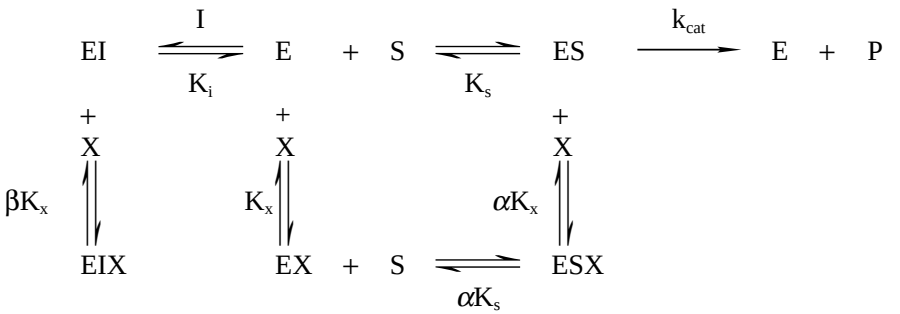


FIG. 2. Plots of E/V vs [BP] for the CPA (1.5×10^{-9} M) catalyzed hydrolysis of 1.3×10^{-3} and 6.4×10^{-4} M BPP at 30°C, pH 7.5, and μ = 0.5 M NaCl.

Indole-3-acetic acid is a noncompetitive or mixed inhibitor of the CPA catalyzed hydrolysis of BPP, and again plots of E/V vs [IAA] are linear as shown in Fig. 3. Varying the constant substrate concentration produced nearly identical lines, as is required for noncompetitive inhibition at high $[S_0]/K_m$ ratios (see equations III-28 and III-29 in (*14*)). The value of K_i is 1.5×10^{-4} M.

Indole-3-acetic acid is a fully noncompetitive inhibitor toward hippuryl-L-phenylalanine (HPA). The plots of E/V vs $1/[S_0]$ at 30°C and pH 7.5 in that case intersect on the $1/[S_0]$ axis, and the values of K_i (slope) and K_i (intercept) are closely similar, 2.1×10^{-4} M and 2.3×10^{-4} M, respectively ($\alpha = 1.1$). The plots of E/V vs [IAA] are linear. From the point of intersection on the [IAA] axis, a K_i of 2.2×10^{-4} M was calculated for IAA inhibition of the CPA catalyzed hydrolysis of HPA.

Figure 4 is a plot of E/V vs the concentration of BP at a constant concentration of BPP and a series of constant concentrations of IAA. Equation [1] is applicable to the case of linear competitive and noncompetitive (or mixed) inhibitors, where I is the competitive inhibitor and X is noncompetitive. The scheme of Eq. [1] leads to Eq. [2].



[1]

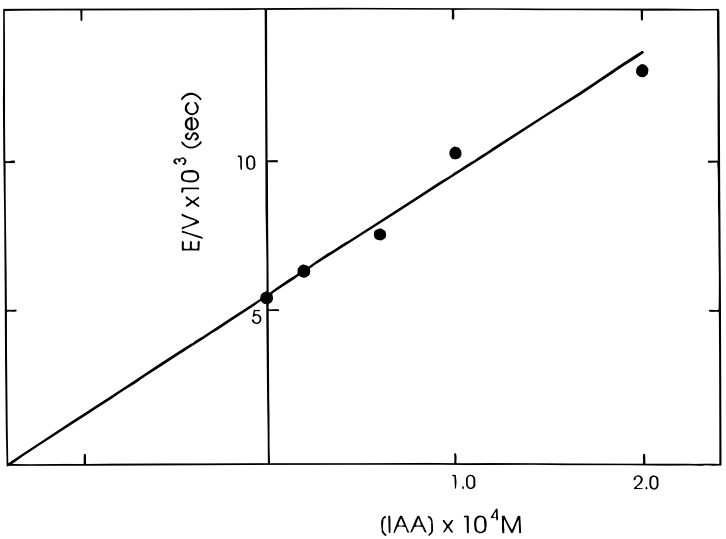


FIG. 3. Plot of E/V vs [IAA] for the CPA (1.5×10^{-9} M) catalyzed hydrolysis of 6.4×10^{-4} M BPP at 30°C, pH 7.5, and $\mu = 0.5$ M NaCl.

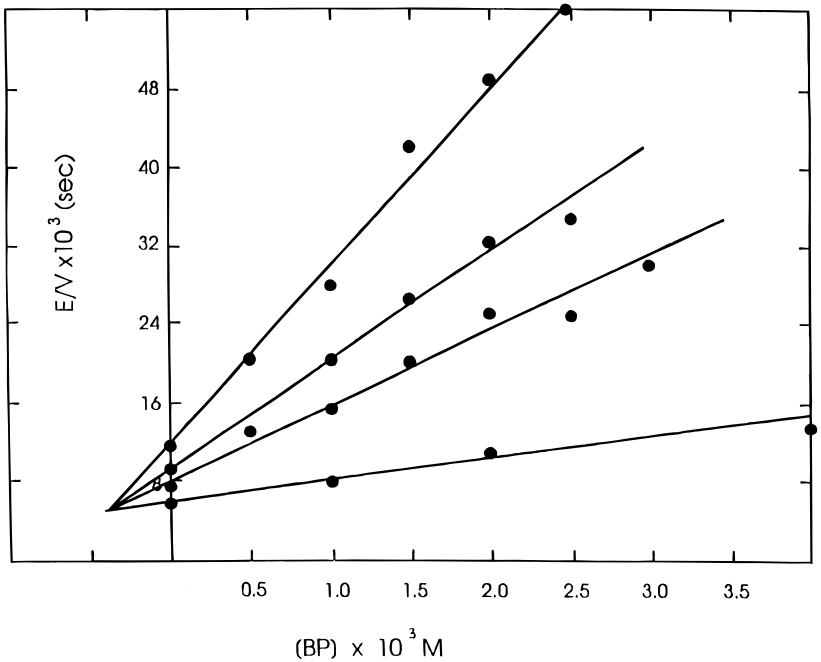


FIG. 4. Plots of *E/V* vs [BP] at a series of constant concentrations of IAA (0, 6×10^{-5}, 1×10^{-4}, and 2×10^{-4} M) in the CPA (1.5×10^{-9} M) catalyzed hydrolysis of 6×10^{-4} M BPP 30°C, pH 7.5, and μ = 0.5 M NaCl.

$$\frac{V}{V_{max}} = \frac{[S]}{K_s\left(1 + \frac{[I]}{K_i} + \frac{[X]}{K_x} + \frac{[I][X]}{\beta K_i K_x}\right) + [S]\left(1 + \frac{[X]}{\alpha K_x}\right.} \qquad [2]$$

The equation for the Dixon plot of $1/V$ vs $[I]$ is given in Eq. [3],

$$\frac{1}{V} = \frac{K_s}{V_{max}K_i[S]}\left(1 + \frac{[X]}{\beta K_x}\right)[I] + \frac{1}{V_{max}}\left[\left(1 + \frac{[X]}{\alpha K_x}\right) + \frac{K_s}{[S]}\left(1 + \frac{[X]}{K_x}\right)\right] \qquad [3]$$

while that of $1/V$ vs $[X]$ is given in Eq. [4] (14).

$$\frac{1}{V} = \frac{1}{\alpha V_{max}K_x}\left[1 + \frac{\alpha K_s}{[S]}\left(1 + \frac{[I]}{\beta K_i}\right)\right][X] + \frac{1}{V_{max}}\left[1 + \frac{K_s}{[S]}\left(1 + \frac{[I]}{K_i}\right)\right] \qquad [4]$$

The plots of Fig. 4 are linear with correlation coefficients of 0.98 or greater, and the slope increases as the concentration of IAA increases. Therefore, both inhibitors bind to the enzyme simultaneously (*14*). The plots intersect behind the *E/V* axis and above the [BP] axis, which shows that the binding of one inhibitor facilitates binding of the other. A linear plot of the slopes of Fig. 4 vs the concentration of IAA gives $-\beta K_X$ as the intercept on the IAA axis. Employing $K_X = 1.5 \times 10^{-4}$ M, β is calculated to be 0.18. A linear plot of the intercepts of Fig. 4 vs [IAA] allows the calculation of α; $\alpha K_x = 1.75 \times 10^{-4}$ M.

The ester substrate hippuryl-DL,β-phenyllactate (HPL), has k_{cat} and K_m values of 750 s^{-1} and 1.0×10^{-4} M, respectively, at 30°C and pH 7.5. A K_m of 9×10^{-5} M was previously reported (*8*). Indole-3-acetic acid is a competitive inhibitor and *N*-benzoyl-L-phenylalanine is noncompetitive. Plots of E/V vs $1/[S_0]$ intersect on the E/V and $1/[S_0]$ axis, respectively, in the presence of constant concentrations of the inhibitor, as illustrated for BP in Fig. 5. In the noncompetitive inhibition by BP the values of K_i (intercept) and K_i (slope) do not differ greatly ($\alpha = 0.9$). The plots of E/V vs [Inhibitor] are linear and intersect on the BP axis. In contrast, the linear plots of E/V vs IAA (not shown) intersect behind the E/V axis and above the IAA axis. A line from the intersection point to the E/V axis is close to $1/k_{cat}$. The linear plots of E/V vs $1/[S_0]$ in the presence of IAA intersect precisely on the E/V axis at $1/k_{cat}$ ($k_{cat} = 752$ s^{-1}). The values of K_i determined from the Dixon plots are 2.2×10^{-4} M for IAA and 1.0×10^{-4} M for BP. Previously reported values of K_i for IAA as a competitive inhibitor of ester hydrolysis and as a noncompetitive inhibitor of peptide hydrolysis are 1.6×10^{-4} M and 1.7×10^{-4} M, respectively (*7*). The linear plots in Fig. 6 of E/V vs the concentration of BP at a constant concentration of HPL and a series of concentrations of IAA, intersect behind the E/V axis and above the [BP] axis ($\beta = 0.12$). Correlation coefficients for the straight lines in Fig. 6 were again greater than 0.99.

DISCUSSION

N-Benzoyl-L-phenylalanyl-L-phenylalanine (BPP) is an excellent peptide substrate for CPA. The K_m value at pH 7.5 of 2.6×10^{-5} M is the smallest that has yet been mea-

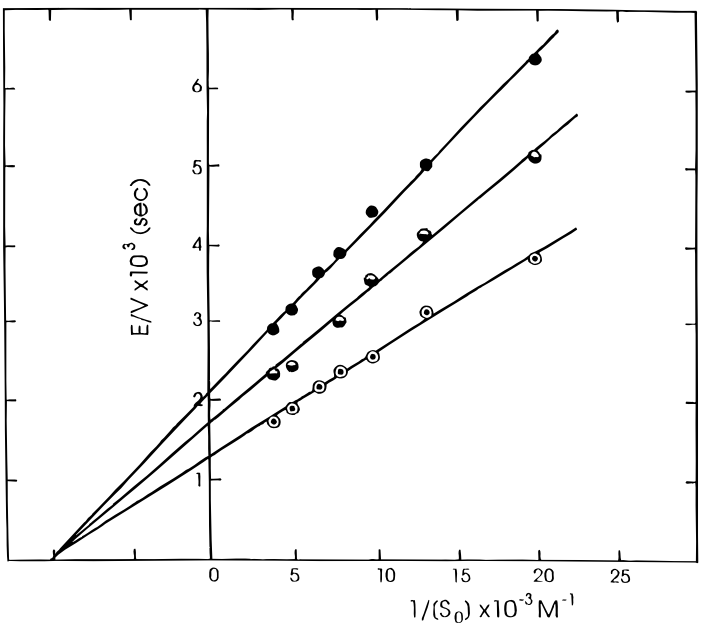


FIG. 5. Plots of E/V vs $1/[S_0]$ for the CPA (2.5×10^{-9} M) catalyzed hydrolysis of HPL in the presence of BP; 1×10^{-4} M (●); 5×10^{-5} M (◒); and in the absence of inhibitor (⊙) at 30°C, pH 7.5, and $\mu = 0.5$ M NaCl.

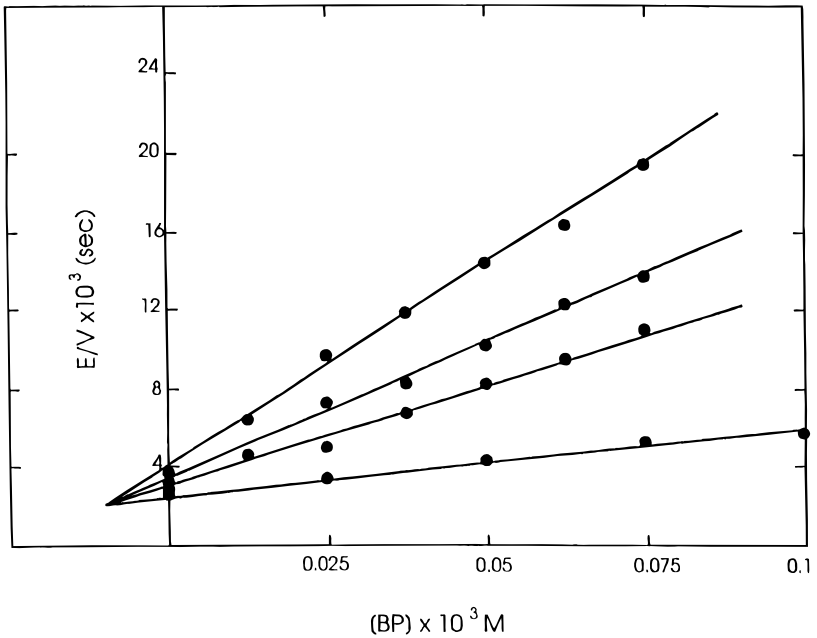


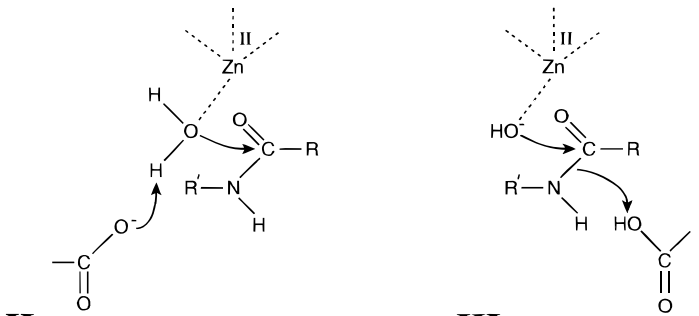
FIG. 6. Plots of E/V vs [BP] at a series of constant concentrations of IAA (0, 1×10^{-4}, 1.5×10^{-4}, and 2×10^{-4} M) in the CPA (2.5×10^{-9} M) catalyzed hydrolysis of 1×10^{-4} M HPL at 30°C, pH 7.5, and μ = 0.5 M NaCl.

sured for benzoylated peptide substrates of the enzyme. It is quite unlikely that the small K_m is influenced by nonproductive binding since k_{cat} (177 s^{-1}), which would be reduced by nonproductive binding, is enhanced over that of usual dipeptide substrates; hippuryl-L-phenylalanine (HPA) has k_{cat} = 100 s^{-1} at pH 7.5 and 30°C (8). The value of k_{cat}/K_m for BPP (6.8×10^6 M^{-1} s^{-1}) is again the largest yet observed for a benzoylated peptide substrate of the enzyme. Only some dansylated peptides provide larger values (*20*). It is probable that these favorable values are produced by the multiplicity of contacts that the substrate can make with the enzyme. In comparison, HPA (*N*-benzoylglycyl-L-phenylalanine) has a K_m of 10^{-3} M and $k_{cat}/K_m = 10^5$ M^{-1} s^{-1} at pH 7.5.

An "edge to face" interaction of a benzyl side chain with an aromatic enzyme residue (Tyr-198) may contribute to the specificity effects for binding in the S_1 subsite (*5*,*21*). Removal of a phenylmethylene group from water to a hydrocarbon environment will proceed with a $\Delta G°$ of −3.6 kcal/mol (*22*). Consequently, the smaller K_m for BPP in comparison with HPA can be explained on the basis of the substitution of a phenylalanyl residue for glycyl. Note that the precision of binding (assuming that $K_m = K_s$, as is the case with other peptide substrates (*16*,*23*,*24*)), manifests itself in a relatively favorable k_{cat}/K_m. The latter constant is the second-order rate constant for reaction of the free enzyme with the substrate and is not affected by any nonproductive binding effects.

The discovery of enzyme substrates that give large rate constants can be mechanistically important. In addition to the general base and nucleophilic mechanisms proposed by Lipscomb (*3*,*4*), more recent suggestions have involved Glu-270 general

base abstraction of a proton from a metal ion bound water molecule (II) (*5*), and the kinetically equivalent attack of metal ion bound hydroxide ion (III) assisted by Glu-270 general acid catalysis (*25*).



II **III**

The latter mechanism is attractive because it should be capable of generating large rate constants and large rate enhancements (*25*,*26*), whereas mechanism II has not been shown to be chemically feasible. Metal ion promoted OH^- catalysis has been considered to be a general mechanism for hydrolytic metalloenzymes (*27*). Kinetically equivalent mechanisms may be distinguished if one of the mechanisms demands a rate constant that is greater than that for a diffusion controlled reaction (10^8–10^9 M^{-1} s^{-1} in an enzymatic reaction). If the apparent pK_a values of 6.3 and 9 are assigned to the carboxyl group of Glu-270 and a metal ion bound water molecule, as suggested (*1*,*2*,*28*,*29*), then the concentration of active site II is 500-fold larger than that of active site III at any pH.[3] Therefore, from the experimental rate constant for BPP at pH 7.5 (7×10^6 M^{-1} s^{-1}), the true rate constant for mechanism III is calculated to be 3.5×10^9 M^{-1} s^{-1}. This value is slightly greater than that usually considered for a diffusion-controlled enzyme reaction, but does not allow mechanism III to be ruled out. However, it is clear that the pK_a of zinc ion bound water cannot be much greater than 9 for mechanism III to be possible. On the other hand, a water pK_a much less than 9, e.g., 6.3, would preclude the need for general base catalysis by Glu-270 (Mechanism II) because of the high concentration of the ionized species. A mechanism involving attack of metal ion bound OH^- without involvment of Glu-270 in the rate-determining step would be consistent with the pH dependence of k_{cat} in peptide hydrolysis (*23*) if the water pK_a is as low as 6.3.[4]

[3] The ratio of the concentrations of the monoanionic active sites in II and III is given by the ratio of the dissociation constants for ionization of metal ion bound water and ionization of the γ-carboxyl group of Glu-270, assuming that these dissociations do not influence each other. Thus:

$$\frac{K_{COOH}}{K_{M^{II}-H_2O}} = \frac{[COO^-][M^{II}-H_2O]}{[COOH][M^{II}-OH^-]}$$

[4] The value of the dissociation constant of the Zn^{II}–H_2O of CPA has not been determined unambiguously; pK_a values ranging from near 6 to 9 have been suggested. The pK_a of the aquo complex of Zn(II) is 8.8.

Inhibition of CPA. Linear inhibition is observed in the CPA catalyzed hydrolysis of both peptide and ester substrates. The competitive inhibition by the carbamate ester *N*-phenoxycarbonyl-L-phenylalanine (PPA) in the CPA catalyzed hydrolysis of the peptide hippuryl-L-phenylalanine, gives rise to linear E/V vs [PPA] plots (*8*). The same type of behavior is observed with the competitive inhibitor BP when the peptide BPP is the substrate. Indole-3-acetate is a noncompetitive or mixed inhibitor towards peptides, and plots of E/V vs [IAA] are again linear. The reverse is the case when the substrate is the ester HPL, IAA is then a linear competitive inhibitor, whereas PPA and BP are linear noncompetitive. The linear plots of E/V vs [I] for the CPA catalyzed hydrolysis of constant concentrations of BPP, HPA, or HPL, show that one molecule of inhibitor (PPA, BP, or IAA) binds per active site at pH 7.5.

Linear plots of E/V vs $1/[S_0]$ intersecting on the E/V axis in the presence of an inhibitor indicate competitive inhibition but do not establish that the substrate and inhibitor bind in the same site on the enzyme. Partially competitive inhibition, in which an EIS complex can break down to products will also produce such plots. Partially competitive inhibition can be identified by hyperbolic plots of $1/V$ vs [I] at a constant substrate concentration. To establish that substrate and inhibitor are mutually exclusive, it is necessary for plots of $1/V$ vs [I] to be linear. Since the plots of E/V vs [I] are linear in the CPA catalyzed reactions of BPP, HPA, and HPL, an EIS complex is not formed in the competitive cases, and there is direct competition for binding to the active site.

As only one molecule of inhibitor binds per active site at pH 7.5, and the K_i values are nearly constant regardless of whether the inhibition is competitive or noncompetitive (*7,8*), it might be reasonably inferred that the binding of each inhibitor is to a single favored site. If it is considered that the inhibitors are binding in the sites indicated by the X-ray analysis, then the linear competitive inhibition of peptide hydrolysis by BP in conjunction with the observed binding of BP in the S_1–S_2 subsite in the X-ray crystallographic studies of Christianson and Lipscomb (*5,6*), indicates that peptide substrates bind in that site. In view of its structural similarity to BP, PPA probably also binds to the same site (*8*). L-Phenylalanine binds in the S_1' site with the phenyl group in the hydrophobic pocket, as in structure I (*3,5*). Structure I, the suggested complex for the synthesis of BPP, would then resemble the productive complex for the forward hydrolysis reaction. However, the lack of apparent competitive inhibition of IAA toward BPP and HPA at pH 7.5 poses an interpretive problem.

The X-ray structure of the IAA–CPA complex shows that IAA binds in the hydrophobic pocket of the S_1' subsite with the carboxylate group salt-linked to Arg-145 in the same manner as phenylalanine (*13*). No other binding sites for IAA were observed at the concentrations employed (0.01 M). The competitive inhibition shown by IAA toward esters was considered by Auld and Holmquist to indicate the same binding site for those substrates and IAA (*7*). A different binding site was indicated for peptides toward which IAA was noncompetitive. Lipscomb suggested that peptides initially bind in a remote site and that the rate-determining step is migration into the S_1' site, i.e., there are different initial binding sites and rate-determining steps for esters and peptides but the same catalytic site (*9*). The difficulty of binding peptides in S_1' was ascribed to a twisting effect necessary to achieve binding. However, the large k_{cat}/K_m for BPP and dansylated peptides (*10,20*) implies that the prevalent ES species is the one in which the substrate is bound efficiently to the catalytic site. Also, rate-

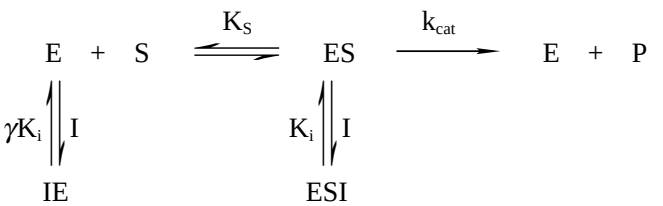
determining binding of peptides in S_1' is not in accord with structure-reactivity considerations (*8*). Thus, the problem of the observed noncompetitive inhibition by IAA in peptide hydrolysis remains in apparent conflict with the X-ray results.

The pH dependence of pK_i for IAA noncompetitive inhibition is a gentle sigmoid (*30*), similar to that of phenylacetate (*31*). L-phenylalanine also gives a sigmoidal log K_i vs pH plot (*32*). In contrast, the log K_i vs pH profile for the inhibition toward peptides of L-benzylsuccinate, which may bind to the metal ion, shows a large pH dependence that is nearly linear from pH 6–10 (*33*). These results are consistent with the view that IAA in its noncompetitive mode does not bind to the metal ion.

Carboxylic acids can exhibit different types of inhibition effects (*34*). The partially competitive inhibition of HPL hydrolysis by cyclohexyl acetate was interpreted in terms of an EI_2 complex (*35*). At high concentrations (greater than 0.01 M), a second binding site can be detected for carboxylate inhibitors of CPA, and the inhibition toward peptides changes from noncompetitive to mixed or competitive at high pH (> 8) (*31*,*36*). In view of the linear inhibition, binding of IAA in the two sites is mutually exclusive at pH 7.5. Furthermore, a competitive component is not detected when the peptide HPA is the substrate at pH 7.5; the IAA inhibition is linear noncompetitive, and $\alpha = 1.1$.

Linear mixed inhibition can result from the binding of an inhibitor at two different but mutually exclusive sites (*37*). A scheme involving mixed competitive and noncompetitive inhibition, in which there are two exclusive sites for binding of inhibitor, can give results characteristic of noncompetitive inhibition if coincidentally K_i (slope) = K_i (intercept) (*37*). However, two preexisting sites for the binding of inhibitor is not consistent with the X-ray results for IAA (*13*) unless binding in the second site is very poor.

A scheme involving mixed competitive and uncompetitive inhibition with exclusive binding of inhibitor in two different sites is shown in Eq. [5].[5] This scheme will also appear to be pure noncompetitive



[5]

inhibition if $\gamma = 1$. In Eq. [5] the second binding site for inhibitor is created by the binding of substrate. A scheme of that type can explain the observed noncompetitive inhibition by IAA in peptide hydrolysis without invoking different binding sites for esters and peptides or conflicting with the X-ray data. The difficulty in the scheme stems from the requirement that $\gamma = 1$.

L-Phenylalanine might, of course, move into the S_1' site *after* the C–N bond breaking step. Such an alternative would imply that the X-ray crystal structure depicted in

[5] Uncompetitive inhibition has been reported in CPA catalyzed ester hydrolysis reactions (34).

I does not represent the initially formed product complex in water. Failure of the C-terminal phenylalanine residue of BPP to bind in S_1' would then imply a rigid active site. Conformationally inflexible ES and EP complexes are not, however, consistent with the present double inhibitor experiments.

The double inhibitor plots of E/V vs the concentration of one inhibitor at a series of fixed concentrations of the other inhibitor (Figs. 4 and 6), are linear and intersect, which shows that both inhibitors can bind simultaneously (*14*). The value of β influences the slope of the plot and determines whether the lines intersect. Intersection indicates the presence of the ternary complex EIX. Moreover, β denotes the effect of the binding of one inhibitor on the binding of the other (see Eqs. [3] and [4]). A β of ∞ indicates that binding of the inhibitors is mutually exclusive, and the lines are parallel. $\beta = 1$ indicates that binding of one inhibitor has no effect on the binding of the other (Dixon plots intersect on the [inhibitor] axis), while $\beta < 1$ indicates that binding of one inhibitor promotes the ease of binding of the other. That the plots intersect above the [inhibitor] axis indicates that binding is synergistic, i.e., binding of one inhibitor favors binding of the other. The value of the inhibitor interaction factor β was found to be ~ 0.2 when either the peptide BPP or the ester HPL is the substrate. Thus, the shape of the plots and the values of β are the same with these structurally different peptide and ester substrates with which different assay methods were employed. There are at least two distinct but not independent sites for the binding of the two inhibitors. A conformational change is occurring in CPA catalyzed reactions that is either induced or stabilized by inhibitor binding, and these conformational effects are communicated between sites.

When HPL is the ester substrate and BP is the noncompetitive inhibitor, the values of K_i (intercept) and K_i (slope) are similar although not identical ($\alpha = 0.9$). If the K_i for IAA acting as a competitive inhibitor vs. HPL is taken as a fixed value, then α in the IAA inhibited reactions of BPP (K_i (noncompetitive)/K_i (competitive)) is 0.68. Thus, synergistic effects occur predominantly within the EIX complex. A large substrate may make sufficient contacts with the enzyme so that the optimum conformation for binding is achieved with minimum mutual binding effects. Clearly, cooperative binding effects are highly dependent on the structure of the ligands.

Conformational changes of CPA have been noted previously (*3*,*5*), in particular, the movement of Tyr-248 and the associated peptide chain, when substrates bind to the enzyme. Such conformational changes may affect peptides and esters differently. Tyrosyl acetylation reduces k_{cat} for peptides to 3–6% of the k_{cat} for the unmodified enzyme (*16*,*38*), but the ester HPL is still hydrolyzed efficiently. Both k_{cat} and K_m for HPL hydrolysis are increased by acetylation. Likewise, conformational changes involving Arg-127 occur that could be mechanistically important with peptide substrates (*5*,*39*). The extended conformation of Arg-127 can bind to the substrate carbonyl oxygen and thereby exert a polarizing effect. Thus, conformational changes can be crucial in the formation of the catalytic site and are very likely dependent on the ligands.

Cooperative binding of ligands has been frequently observed with enzymes composed of subunits but is rare in reactions of monomeric enzymes (see (40) and references therein for an example). The classical equilibrium explanations for cooperativity (*41*,*42*) cannot be easily applied to monomeric enzymes. The positive or negative cooperativity effects that often occur with enzymes composed of subunits can be considered to arise

so that the enzyme can respond to drastic increases or decreases in the substrate concentration. However, with a monomeric enzyme such as CPA, intersubsite cooperativity is very likely a consequence of the large binding site required for the binding of peptides and the necessity of high velocity with different substrates. A conformational change of a flexible active site induced or stabilized by the binding of substrate would allow the formation of the catalytic site in the most advantageous manner with a variety of peptide substrates.[6] With a flexible active site a conformational change need not be energetically prohibitive. Small molecules that can bind simultaneously in different regions of the active site might then bind cooperatively.

ACKNOWLEDGMENT

This work was supported by a grant from the National Science Foundation.

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[6] CPA does, of course, have an absolute specificity requirement in the free C-terminal carboxyl group, but numerous structurally different peptides and esters will serve as substrates.

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