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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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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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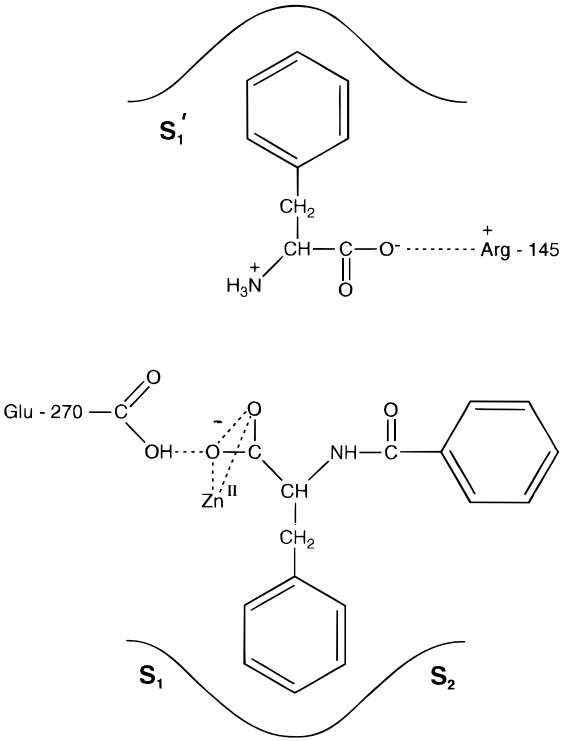
carbonyl oxygen (*3*,*4*). The γ-carboxyl group of Glu-270 is also located in close proximity. On this basis, nucleophilic and general base mechanisms involving Glu-270 were postulated for peptide hydrolysis, and it was assumed that similar or identical mechanisms would occur with ester substrates. Christianson 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.

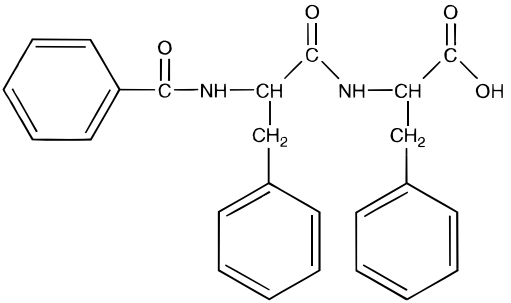


I

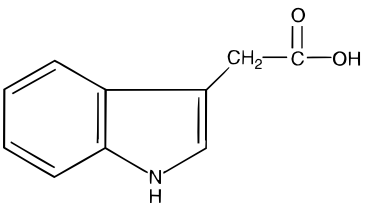
There is strong evidence that peptides and esters bind initially to CPA in different sites (*7*,*8*). Indole-3-acetic acid (IAA) is a competitive inhibitor toward ester substrates but is noncompetitive toward peptides (*7*). The carbamate ester *N*-(phenoxycarbonyl)-L-phenylalanine is a linear competitive 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*).

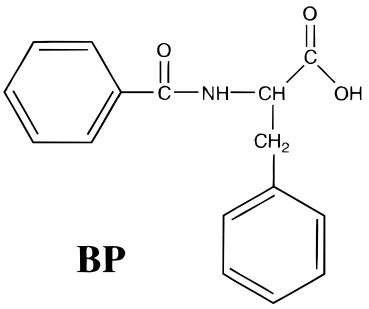
It is clear that insight into the enzyme mechanism is dependent on a deeper understanding of the sites in which peptides and esters bind and of the interrelationships between these sites. We have in the present work studied the CPA catalyzed hydrolysis of the large peptide substrate *N*-benzoyl-L-phenylalanyl-L-phenylalanine (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*).



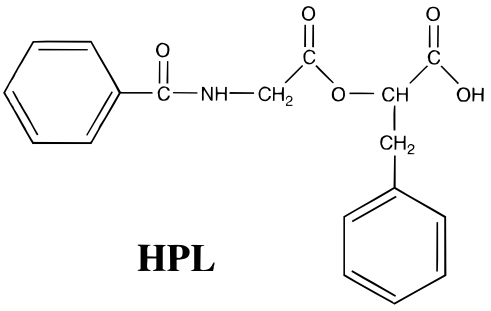
BPP



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 $K_i/K_m = 3.6$.

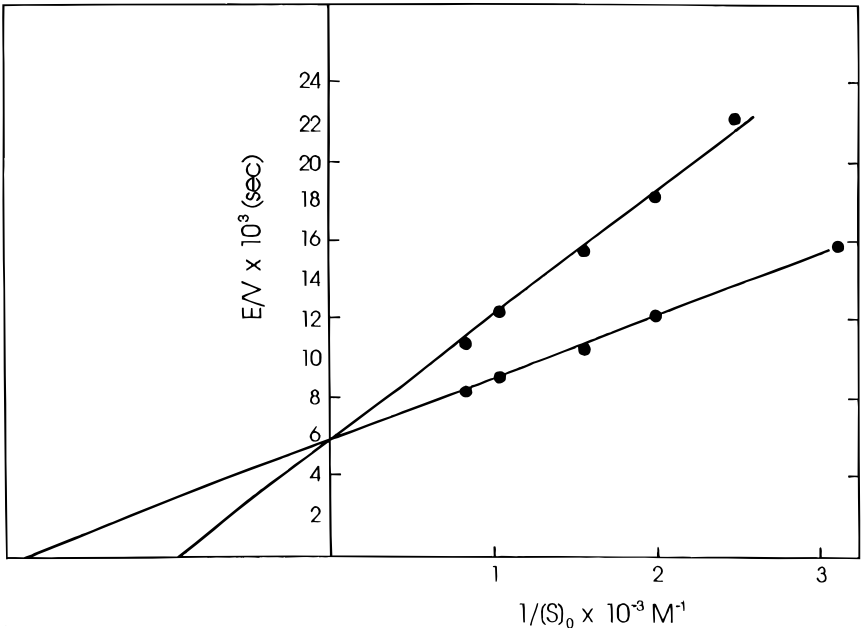


FIG. 1. Plots of E/V vs $1/[S_0]$ for the CPA (1.4×10^{-9} M) catalyzed hydrolysis of BPP in the presence of 0.002 and 0.004 M BP at 30°C, pH 7.5, and μ = 0.5 M NaCl.

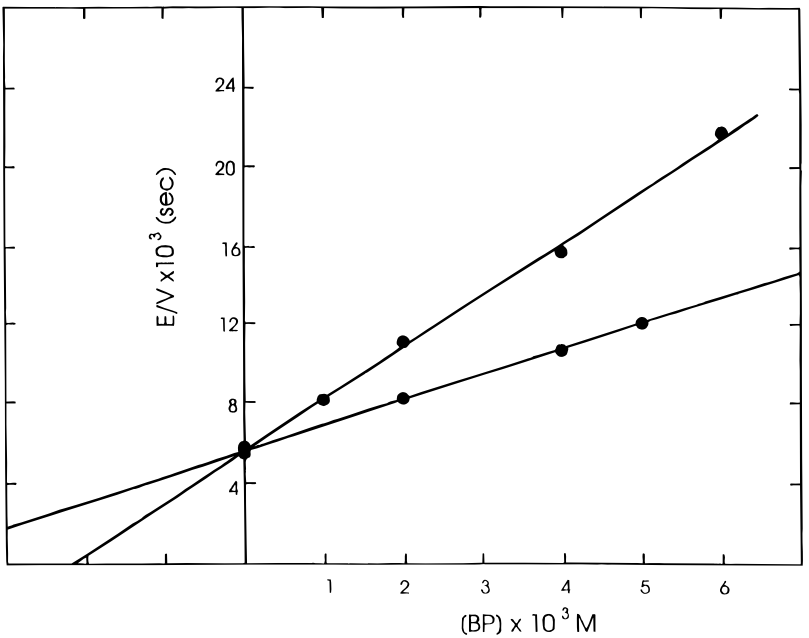
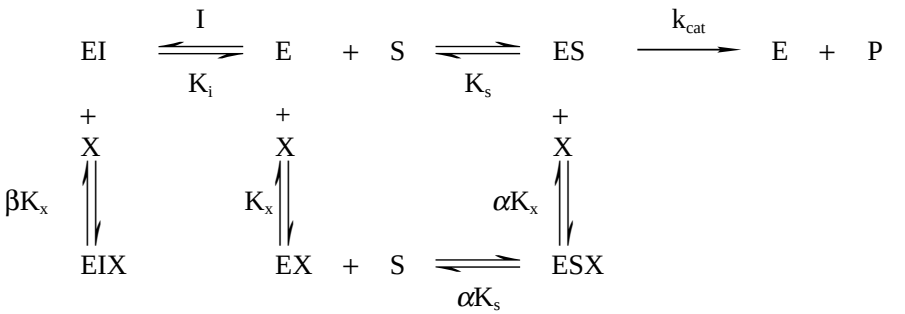


FIG. 2. Plots of E/V vs [BP] for the CPA (1.5×10^{-9} M) catalyzed hydrolysis of 1.3×10^{-3} and 6.4×10^{-4} M BPP at 30°C, pH 7.5, and μ = 0.5 M NaCl.

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]

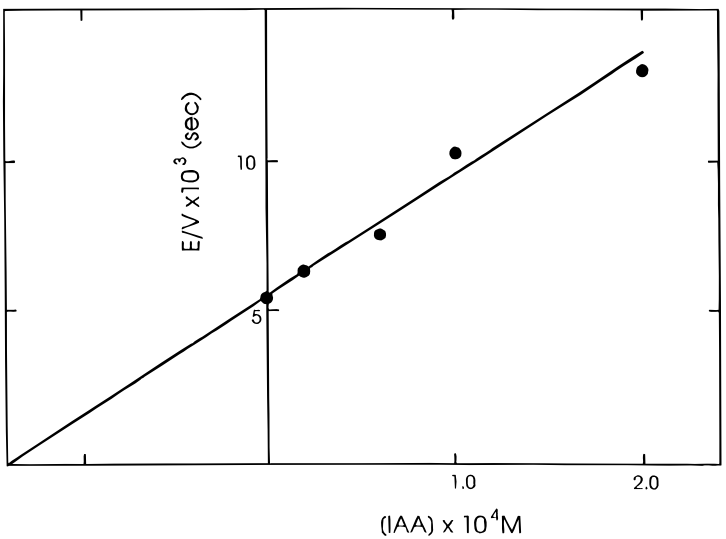


FIG. 3. Plot of E/V vs [IAA] for the CPA (1.5×10^{-9} M) catalyzed hydrolysis of 6.4×10^{-4} M BPP at 30°C, pH 7.5, and $\mu = 0.5$ M NaCl.

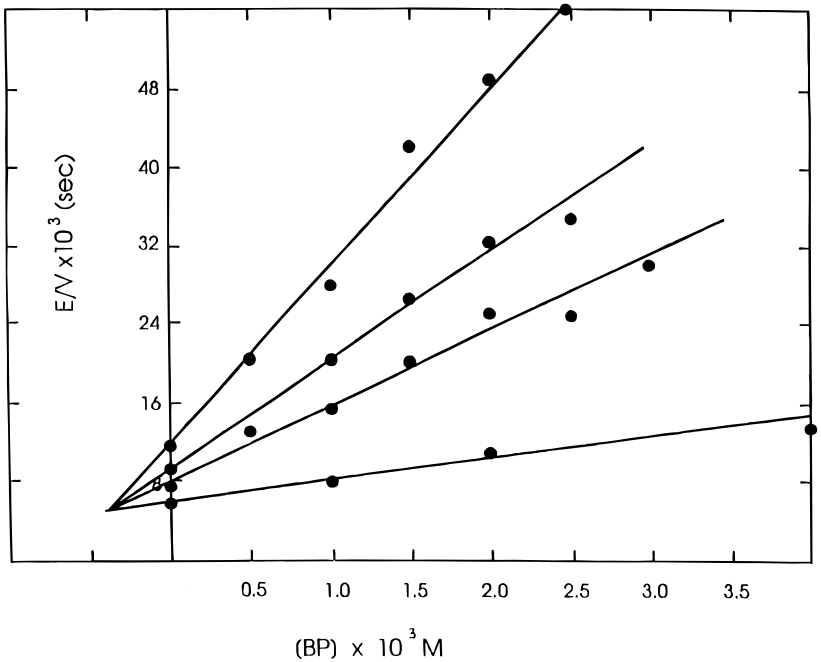


FIG. 4. Plots of E/V vs [BP] at a series of constant concentrations of IAA (0, 6×10^{-5}, 1×10^{-4}, and 2×10^{-4} M) in the CPA (1.5×10^{-9} M) catalyzed hydrolysis of 6×10^{-4} M BPP 30°C, pH 7.5, and $\mu = 0.5$ M NaCl.

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

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.