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A Mechanism of Action for Carboxypeptidase A

(x-ray crystallography/esters/peptides/enzyme kinetics/random-pathway model)

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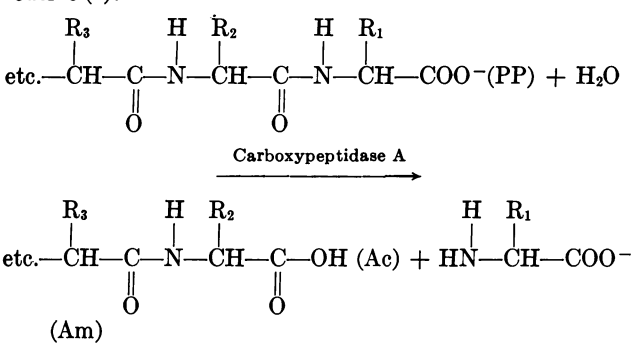
ABSTRACT **In an attempt to gain a better understanding of the mechanism of action of carboxypeptidase A (EC 3.4.2.1), many kinetic studies have been undertaken using numerous substrates—both esters and peptides—that have exhibited substrate linearity, inhibition, activation, and sigmoid-shaped rate plots. Numerous interpretations of the kinetic data have been proposed, none of which are fully in accord both with kinetic data and x-ray crystallographic studies. Much of the kinetic data has been interpreted using multisite binding while the x-ray information seems to severely restrict these possibilities.**

We have examined the feasibility of a simple model with a single active site, without modifier sites, that allows only one substrate molecule to bind the enzyme at a time. A random-pathway model was identified that simultaneously accounts for the nonlinear kinetic data and meets the restrictions imposed by the x-ray crystallographic studies.

INTRODUCTION

Carboxypeptidase A (EC 3.4.2.1) is an exopeptidase that hydrolyzes peptide bonds in peptides and proteins in biological systems, and also esters under experimental conditions, at the carboxy terminal end. It cleaves only L-amino acids with free carboxyl groups adjacent to the peptide or ester bond and is specific for amino acids that have aromatic or hydrophobic side chains, such as phenylalanine, tryptophan, or leucine (4).



If we use the symbols indicated

$$PP + H_2O \xrightarrow{\text{Carboxypeptidase A}} Ac + Am$$

Carboxypeptidase A isolated from the pancreas has a zinc to carboxypeptidase A ratio of 0.96, indicating that the naturally occurring enzyme contains one atom of zinc per protein molecule (5). The zinc atom plays an integral role in the cleavage of the substrate; it polarizes the carbonyl group of the substrate, $Zn^{+}\ldots O^{\delta-} - C^{\delta+}$, in order to render the carbon atom of this carbonyl group more susceptible to nucleophilic attack (3).

Before electron-density maps of carboxypeptidase A were available, chemical studies were undertaken in search of functionally active amino-acid residues at the active site. Among the numerous studies undertaken, acetylation of tyrosine residues (6, 7) was shown to increase esterase activity and decrease peptidase activity. This, coupled with the striking differences in pH–activity profiles between esters and peptides (7), led to the hypothesis that peptides and esters bind at different, but perhaps overlapping, sites on the enzyme. There are exceptions, however, to the effect of acetylation (3) and pH behavior (8).

Kinetic studies—peptides

Kinetic data from the study by Lumry *et al.* (9) show that both carbobenzoxyglycyl-L-tryptophan and carbobenzoxyglycyl-L-phenylalanine exhibit substrate inhibition. It was noted that the extent of inhibition by these substrates is proportional to the fourth power of the substrate concentration. From this relationship, it was concluded that four molecules of substrate are required to inhibit an enzymic center. The model used for interpretation included both a catalytically active and inactive binding site.

Upon investigation of the kinetics of benzoylglycylglycyl-L-phenylalanine and benzoylglycyl-L-phenylalanine, Auld and Vallee (10) found that the latter exhibits substrate activation, while the former is linear. A model proposing the binding of substrates at multiple loci was found to be in accord with the data.

Kinetic studies—esters

Data obtained by Awazu *et al.* (11) indicate that the hydrolysis of *O*-(*N*-benzoylglycyl)-L-mandelate exhibits substrate inhibition. The following theoretical equation was obtained by fitting the experimental points.

$$\frac{v_0}{[E_0]} = a[PP]/\{1 + b[PP] + c[PP]^2\}$$

A model with two sites to which the substrate can bind—one catalytic and the other inhibitory—was proposed to account for this equation. The data thus interpreted supported the hypothesis that there are multiple sites to which substrates and modifiers can bind on the carboxypeptidase A molecule.

Using hippuryl-DL-β-phenyllactate, McClure *et al.* (12) noted marked inhibition with excess substrate. It was suggested that these results could be accounted for by the formation of several different inactive complexes or by the coexistence of several forms of the enzyme. An interesting parallel between hippuryl-DL-β-phenyllactate and its peptide

analog carbobenzoxyglycyl-L-phenylalanine was obtained (12) by a generalization of the scheme proposed by Lumry *et al.* (9). Assuming that the enzyme molecules exist in two species, deriving steady-state equations, and fitting them to experimental data, they found that the velocity is inversely proportional to the fifth power of substrate concentration. From this relationship, it was deduced that five molecules of substrate are required to inhibit one enzymic center.

With the investigation of the hydrolysis of *O*-hippuryl-glycolate by Kaiser *et al.* (13), another type of curve—sigmoid—was observed that is typically interpreted as being allosteric. These authors considered the possibility that the hydrolysis of *O*-hippurylglucolate by carboxypeptidase A does exhibit allosteric character and this idea was strengthened by the observation that the sigmoid character observed with the hydrolysis of *O*-hippurylglycolate is no longer apparent after the addition of an activating modifier, 0.05 M *N*-carbobenzyloxyglycine.

The hydrolysis of acetyl-L-mandelate studied by Kaiser and Carson (14) and of cinnamoyl-DL-β-phenyllactate investigated by Awazu *et al.* (11) give linear Lineweaver–Burk plots, which can be interpreted using the Michaelis–Menten model.

General models for interpretation

Two more general models have been propounded in an attempt to account for all of the kinetic data available. Regarding the existence of substrate inhibition for peptidase, and especially esterase activity, Quiocho and Richards (1) stated that there appears to be no mechanism for this phenomenon that requires the assumption of less than two binding sites, and in most cases from three to five binding sites for the substrates. This conclusion was reached from the study of a general mechanism involving a monomeric form of the enzyme with various binding sites. This model can also account for the sigmoid curve obtained by Kaiser *et al.* (13) with *O*-hippurylglycolate. This finding gave further credence to the proposed mechanism involving multiple binding sites for substrates and similar modifiers on a single macromolecule with only one catalytic site.

A second model endeavoring to encompass the observed kinetic properties as a unified whole was proposed by Vallee *et al.* (2). This model, based on multiple modes of substrate binding, assumes discrete, but overlapping, productive binding sites for esters and peptides. Inhibition is conceived of resulting from (*a*) "incorrect" binding of the peptide at the ester site (or of the ester at the peptide site), or (*b*) inversion of the peptide or ester within its respective binding site to yield an "unproductive" complex. Activation results when the mode of substrate inhibition is prevented. Thus, this model is based upon the assumptions that, (*i*) carboxypeptidase A can distinguish between peptides and esters, (*ii*) peptides and esters bind at different sites, and (*iii*) there are multiple modes of substrate binding that can account for activation, sigmoidness, and inhibition.

Structural studies

Electron density maps to 0.20-nm resolution of carboxypeptidase A and its substrate (Gly–Tyr) complex were prepared by Lipscomb *et al.* (3). Upon making a Gly–Tyr difference map, they found that the native enzyme undergoes several conformational changes when Gly–Tyr is bound, re-

TABLE 1. *Empirical equations for 10 substrates for carboxypeptidase A*

Substrate	Equation	% Standard error	Source of data
Peptides			
1. Carbobenzoxyglycyl-L-tryptophan	$1/\hat{V} = a + b[\text{PP}] + \frac{c}{[\text{PP}]}$	4.24	9
2. Carbobenzoxyglycyl-L-phenylalanine	$1/\hat{V} = a + b[\text{PP}] + \frac{c}{d + [\text{PP}]}$	3.40	9
3. Benzoylglycyl-L-phenylalanine	$1/\hat{V} = a + \frac{b}{c + [\text{PP}]}$	4.83	10
4. Benzoylglycylglycyl-L-phenylalanine	$1/\hat{V} = a + \frac{b}{[\text{PP}]}$	4.10	10
5. Carbobenzoxyglycyl-L-leucine	$1/\hat{V} = a + \frac{b}{[\text{PP}]}$	6.4	9
Esters			
6. *O*-(*N*-Benzoylglycyl)-L-mandelate	$1/\hat{V} = a + b[\text{PP}] + \frac{c}{[\text{PP}]}$	6.4	11†
7. Hippuryl-DL-β-phenyllacetate	$1/\hat{V} = a + \frac{b[\text{PP}]}{c + [\text{PP}]} + \frac{d}{[\text{PP}]}$	5.03	12
8. *O*-Hippuryl-glycolate	$1/\hat{V} = a + \frac{b}{c + d[\text{PP}] + [\text{PP}]^2}$	2.02	13
9. Cinnamoyl-DL-β-phenyllactate	$1/\hat{V} = a + \frac{b}{[\text{PP}]}$	—	11†
10. Acetyl-L-mandelate	$1/\hat{V} = a + \frac{b}{[\text{PP}]}$	2.98	14

* PP = polypeptide.

† Computed by original workers.

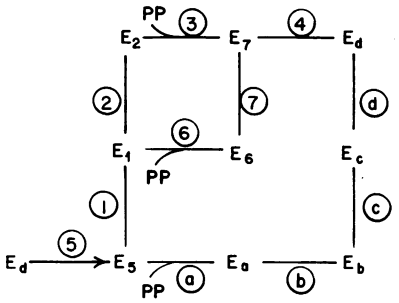


FIG. 1. A plausible random pathway model for the following reaction catalyzed by carboxypeptidase A

polypeptide [PP] + H_2O → cleaved amino acid [Am] + Remaining (poly)peptide [Ac]

Steps 1 and *c*; 2, 7, and *d*; and *b* and 4 may be either rearrangement, water addition, or product-release steps. Step 5 may be product release; rearrangement and product release; water addition and product release; or water addition, rearrangement, and product release, depending upon the assignments made for the other steps.

sulting in several complementary rearrangements in the enzyme structure.

Mechanisms for the cleavage of a peptide substrate were considered. The attack on the susceptible carbonyl group is either (*i*) nucleophilic catalysis by Glu-270, or (*ii*) general base catalysis by water. It was impossible to choose between these two mechanisms.

To account for the inhibition and activation exhibited by kinetic studies, Lipscomb *et al.* (3), through model-building experiments, have elicited two abortive modes of binding for tripeptide substrates—displaced binding and reversed binding—that may result in substrate inhibition, the binding of two tripeptide substrate molecules to give inhibition that is not competitive, and the binding of a tripeptide substrate and a dipeptide product molecule to yield an activation process. Crystallographic experiments devised to demonstrate the abortive modes of binding were unsuccessful. In disagreement with some kinetic analyses, which suggest that from three to five molecules are bound, it was found that the binding region is probably limited to two substrate molecules.

Attempts to prepare crystals of carboxypeptidase with ester substrates have been unsuccessful; however, since (*a*) the very nature of the active site restricts and severely limits the number of productive binding modes, (*b*) the only obvious difference between analogous peptides and esters is the steric and electronic configuration about the ester oxygen, (*c*) peptide nitrogen can be replaced by oxygen in corresponding

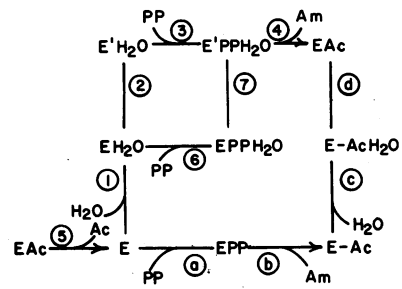


FIG. 2. A specific example of the model in Fig. 1.

TABLE 2. *Some suitable combinations of reaction assignments for the model in Fig. 2*

Steps 1 and *c*	Steps 2, 7, and *d*	Steps 4 and *b*	Step 5
w	*r*	*p*	*p*
w	*p*	*r*	*p*
w	*r*	*r*	*p*, *p*
r	*w*	*p*	*p*
r	*w*	*r*	*p*, *p*
r	*p*	*w*	*p*
r	*r*	*w*	*p*, *p*
r	*r*	*r*	*p*, *w*, *p*
p	*w*	*r*	*p*
p	*r*	*w*	*p*
p	*r*	*r*	*w*, *p*

Symbols: *p* = product-release step, *w* = water-addition step, *r* = rearrangement step.

active substrates, and since (*d*) most of the requisites of substrate and enzyme are the same for peptide and analogous ester substrates, it was hypothesized (3) that the most favorable productive binding mode for ester substrates is, in its essential interactions, the same as that depicted for peptides.

EMPIRICAL EQUATIONS

Empirical equations are a useful way of summarizing experimental data and are valuable in choosing the kinds of models that can account for the data. Most of the kinetic data available for carboxypeptidase A has not been presented in this form; therefore, it was necessary, in most cases, to obtain the data points from published figures and tables and to develop a suitable empirical equation by use of the procedure described by Barton and Fisher (15) for original data. Since only average values could be obtained, each point was arbitrarily assigned a weight of one, whereas with original data the standard deviation for each point was used in weighting the points.

Table 1 shows the results for 10 representative substrates analyzed by this approach. The standard errors are in the range of 2.0–6.4%. It should be noted that the standard error reported by Awazu *et al.* (11) for *O*-(*N*-benzoylglycyl)-L-mandelate is equal to the highest standard error recorded in this paper. Thus, the standard errors defining the experimental data in this paper are within reasonable limits.

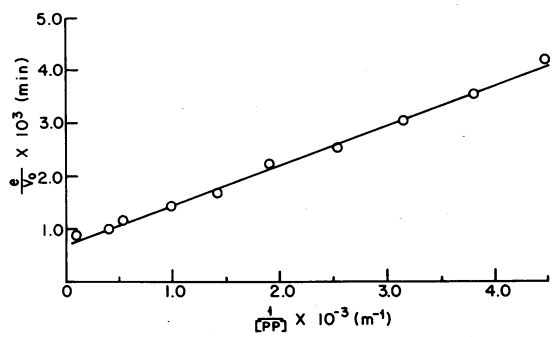


FIG. 3. A Lineweaver–Burk plot of the peptide benzoylglycylglylcyl-L-phenylalanine. The line was calculated from the model proposed in Fig. 1. The data are actual experimental points abstracted from an article by Auld and Vallee (10).

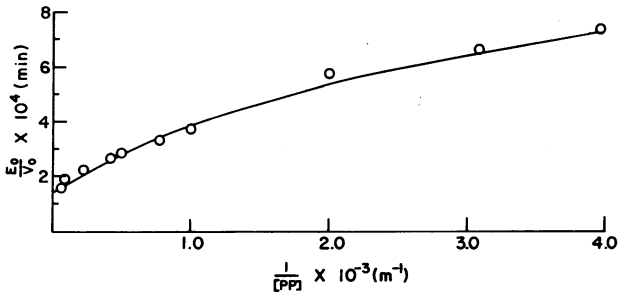


FIG. 4. A Lineweaver–Burk plot of the peptide benzoylglycyl-L-phenylalanine. The line was calculated from the model proposed in Fig. 1. The data are actual experimental points from an article by Auld and Vallee (10).

A PLAUSIBLE MODEL

A reasonable model for reactions catalyzed by carboxypeptidase A should account for the wide variety of reported kinetic properties—substrate linearity, activation, inhibition, and sigmoidness—*without* requiring the binding of more than one substrate molecule to the enzyme at a time. With this in mind, a search for a suitable random pathway model was undertaken.

From the equations in Table 1, some more complicated terms such as $c/\{d + [PP]\}$, $b[PP]/\{c + [PP]\}$, and $b/\{c + d[PP] + [PP]^2\}$ can be noted. These terms serve as the principle guide in building a suitable model. It can be noted that a single cycle has the capacity to produce the first two terms, but not the third. One of the simplest ways to generate such a term is to have a cycle within a cycle, as illustrated in Fig. 1. Mathematical procedures for handling such models have been presented (16). For a cycle within a cycle to account for the critical term given above, it is necessary for substrate-addition steps to appear in both the large and small cycles and play an important role in determining the fractional velocities flowing through each cycle. There is no specifically required arrangement of product release, rearrangement, or water addition steps. Some suitable combinations are given in Table. 2.

The presence of rearrangement steps is deemed feasible by the fact the Lipscomb *et al.* (3) noted that the native enzyme undergoes several conformational changes with the binding of Gly–Tyr. With relaxation spectrometry, Hammes (17, 18) found evidence to support the hypothesis that rapid conformational changes or isomerizations involving binary and ternary complexes occur in dehydrogenase systems, and evidence for the existence of at least eight different states of ribonuclease A.

Fig. 2 illustrates one of the specific models given in Table 2 that are inherent in the model presented in Fig. 1. It should be emphasized that this specific model is not being presented as "the" model from the more general model, but merely to exemplify in more concrete terms the types of specific models that are being suggested. The specific model in Fig. 2 illustrates the first set of assignments given in Table 2.

A few comments regarding the specific model in Fig. 2 are in order. One of the pathways in the specific model is a ping pong-type ordered sequence involving: substrate [PP] addition (*a*), release of one product (cleaved amino acid [Am]) (*b*), binding of water (*c*), hydrolysis of an acyl enzyme bond (*d*), and release of the second product [the remaining (poly)peptide [Ac] after cleavage of an amino acid] (5). It should be

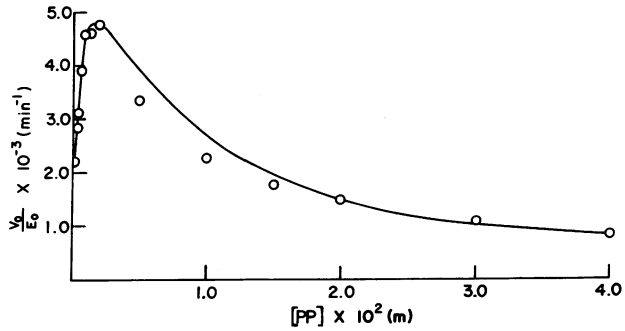


FIG. 5. A plot of velocity against substrate concentration for the ester *O*-(*N*-benzoylglycyl)-L-mandelate. The line was calculated from the model proposed in Fig. 1. The data are actual experimental points from an article by Awazu *et al.* (11).

noted that rearrangement steps (2) and (7) do not represent the same process as step (*d*). The other pathways involve similar processes but include a ternary complex, which is absent in the ping pong sequence described above. Some of the suitable combinations listed in Table 2 do not include both ping pong and ternary-complex pathways; therefore, this combination is not an essential feature.

To determine whether the proposed model can indeed yield the empirical equations for each substrate, rate constants for the model in Fig. 1 were assigned values of small, medium, and large, differing by 10^2, in conformity with the law of microreversibility. These assignments reduced the general rate equation to the desired empirical equations for the particular substrate in question. In this way it was shown that the model can yield empirical equations that define the observed data for all 10 substrates.

Exact values were assigned to the rate constants of four substrates, which manifest linearity, activation, inhibition, and sigmoidness, in order to demonstrate that (*a*) the preliminary assignment of the rate constants yielded valid results, (*b*) when rate constants are assigned exact numerical values, the actual empirical equations can be generated, and when plotted, produce a curve through, or in close proximity to, the observed data points, and (*c*) the necessary assigned values are reasonable.

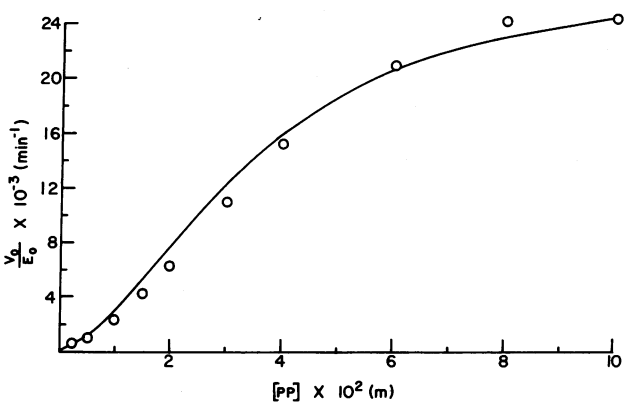


FIG. 6. A plot of velocity against substrate concentration for the ester *O*-hippuryl-glycolate. The line was calculated from the model proposed in Fig. 1. The data are actual experimental points from an article by Kaiser *et al.* (13).

Two peptides—benzoylglycylglycyl-L-phenylalanine, which gives linear plots, and benzoylglycyl-L-phenylalanine, which manifests activation—and two esters—*O*-(*N*-benzoylglycyl)-L-mandelate, which gives inhibition, and *O*-hippurylglycolate, which gives sigmoid plots—were chosen. The actual rate constants were assigned values ranging from 26 to 5×10^6 min^{-1} for unimolecular processes and from 6.0×10^4 to 5.2×10^9 M^{-1} min^{-1} for bimolecular processes. Graphs of the lines calculated with these rate constants and the general rate equation are shown in Figs. 3–6. The observed data points abstracted from the literature are also plotted on these graphs, showing the correspondence.

DISCUSSION

Random pathway models, which require only one active site and no modifier sites, can account for the substrate kinetic data for carboxypeptidase A found in the literature. This has been demonstrated by (*a*) defining by empirical equations the kinetic data from the literature for ten representative substrates, both esters and peptides, which exhibit linearity, activation, inhibition, and sigmoidness; (*b*) proposal of a simple, random pathway model that can account for the data; (*c*) showing semiquantitatively for all 10 substrates that the empirical equations for each substrate can be derived from the model; and (*d*) making actual rate-constant assignments for four substrates, two esters, and two peptides, which manifest substrate linearity, activation, inhibition, and sigmoidness, and plotting the resulting data in the form of curves with the observed data points abstracted from the literature.

The general random pathway model proposed is in harmony with crystallographic studies by Lipscomb *et al.* (3). It is also able to offer a resolution to the disparity between structural data and previous interpretations of, and models for, substrate kinetic data. Some interpretations of nonlinearity have led to the proposal of the binding of up to five substrate molecules at the active center; of two sites, one catalytic and the other inhibitory; and also of allosteric character. Quiocho and Richards (1) proposed a more general model which, assuming a monomeric form of the enzyme, requires more than two, and in most cases the binding of three to five substrate molecules, to account for inhibition. Vallee *et al.* (2) were able to account for all the observed phenomena by a model based on multiple modes of substrate binding, assuming discrete but overlapping binding sites for esters and peptides. Unfortunately, these models are not in accord with crystallographic studies (3). Gly–Tyr difference maps demonstrated the binding of only one molecule of Gly–Tyr at the active site of carboxypeptidase A. Model-building experiments have shown that it is feasible to have abortive substrate binding, binding of two substrate molecules, and binding of one substrate and one product molecule. Under no circumstances does it appear plausible to have the binding of more than two substrate molecules at the active site. Experiments demonstrating binding of more than one substrate molecule or abortive binding, however, are lacking. Upon study of the topography of the active site and of the essential features of esters and peptides, it has been hypothesized that the essential mode of interaction of peptides and esters is probably the same (3). Thus, one can see that there is strong structural evidence for productive binding of esters and peptides at identical sites, and also the binding of, at most, two substrate molecules at the one and only active site.

In conclusion, a feasible randon pathway model to account for the substrate kinetic data for carboxypeptidase A has been proposed. In this model a plausible relationship between substrate-addition steps has been proposed, but no attempt has been made to order rearrangement or product-release steps. In contrast to previous interpretations, this model is in accord with x-ray crystallographic studies.

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