# A Model for Substrate Binding and Kinetics of Carboxypeptidase A*

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ABSTRACT: A schematic model based on multiple modes of substrate binding is proposed which accommodates the kinetic characteristics of carboxypeptidase A. The model postulates that nonidentical but overlapping binding of dipeptides and analogous esters could be one basis for much of the existent kinetic data, characterized by substrate and product inhibition and activation. Up to 38 complexes involving one, two, or three substrate molecules can be incorporated in the minimal model described. Certain of these complexes involve molecules bound in a catalytically productive mode, others in an activating mode, and still others in an inhibitory mode. The model has correctly predicted a reciprocal effect of products and certain analogous compounds on the esterase and peptidase activities of the enzyme. Thus, compounds presumably binding to the nonoverlap loci, which constitute the elements of the site, inhibit one activity and activate the other. Chemical modifications of the active-center tyrosyl residues of carboxypeptidase are postulated to decrease peptidase activity, in part, by interfering with binding to the peptide site and concomitantly to increase esterase activity, in part, by relieving at least one mode of ester substrate inhibition. The model also predicted that certain peptides should be competitive inhibitors of the esterase activity of the modified enzymes. Such inhibition has been observed with a number of peptides. A general mathematical formulation is presented which expresses quantitatively what the model depicts qualitatively. The limitations of the model are pointed out and its relationship to recent studies of oligopeptide- and protein–carboxypeptidase complexes is discussed.

Kinetic studies on carboxypeptidase A over the past 27 years have revealed remarkable complexities in its behavior toward dipeptide and ester substrates (Bergmann and Fruton, 1941; Elkins-Kaufman and Neurath, 1948, 1949; Neurath and Schwert, 1950; Lumry *et al.*, 1951; Smith, 1951; Lumry and Smith, 1955; Hommes, 1962; Kaiser and Carson, 1964, 1965; McClure *et al.*, 1964; Bender *et al.*, 1965; Kaiser *et al.*, 1965; Riordan *et al.*, 1965; Carson and Kaiser, 1966; McClure and Neurath, 1966; Quiocho and Richards, 1966; Whitaker, 1966; Whitaker *et al.*, 1966; Awazu *et al.*, 1967; Auld, 1968; Davies *et al.*, 1968a,b). The enzyme is known to hydrolyze a large number of synthetic substrates, but N-substituted dipeptides, their ester analogs, and a limited number of other esters have served for most studies. The determination of meaningful kinetic constants for the native enzyme with these substrates has proven difficult due to the varying degrees of activation and inhibition by both substrate and products. Moreover, the marked dependence on ionic strength of native carboxypeptidase activity and its inhibition by various buffer ions have added to the difficulties of interpretation and integration of the available data (Neurath, 1960). The kinetic anomalies could arise either from intrinsic features of the enzyme, the properties of the substrates employed, or both (Vallee, 1967a,b), and an analysis of the system to define the contribution of these different parameters has been initiated (Davies *et al.*, 1968a).

The enzymatic properties of carboxypeptidase can be altered either by substitution of other metals for the zinc atom or by chemical modification of the protein side chains, changing the apparent $V_{max}$, the apparent $K_m$, or both (Vallee, 1964a,b). Further, dependent upon the modification, experimental manifestation of the interactions leading to substrate or product inhibition or activation may be shifted to quite different concentration ranges (Davies *et al.*, 1968a).

We have already examined a number of inorganically and organically modified carboxypeptidases to identify derivatives of the enzyme which might be most suitable for future studies. CGP,[1] BGP, and HPLA have served us as substrates so far (Davies *et al.*, 1968a) but additional ones are now being examined (Auld, 1968). Further delineation of the problem may be expected

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CGP, carbobenzoxyglycyl-L-phenylalanine; BGP, benzoylglycyl-L-phenylalanine; HPLA, hippuryl-DL-phenyllactate; BG, benzoylglycine; CG, carbobenzoxyglycine; CGG, carbobenzoxyglycylglycine; CGGG, carbobenzoxyglycylglycylglycine; CGT, carbobenzoxyglycyl-L-tyrosine.

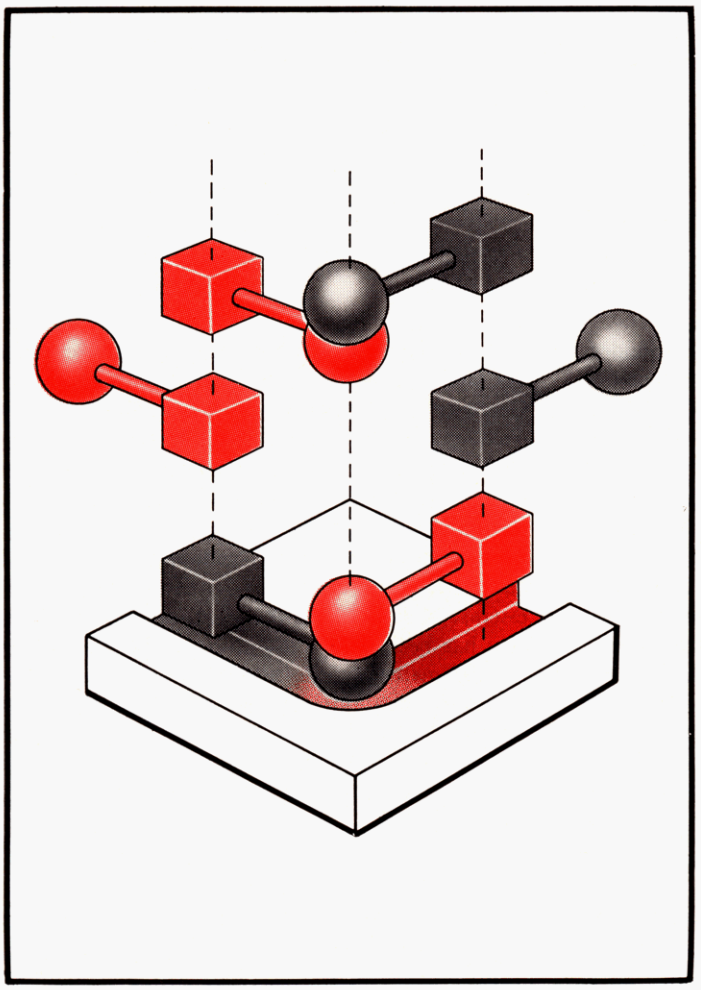

FIGURE 1: Model portraying dual or multiple substrate binding sites. The rectangular area and groove schematically represent the enzyme surface and sites for substrate binding. Peptides, the black symbols, and esters, the red symbols, are thought to interact with the enzyme surface in regions characteristic of each, as indicated by the black and red areas of the groove; an area of overlap, representing one of the possibilities, is indicated by the superpositioning of the symbols and the shading of the groove in the center. Some of the many alternate modes of possible substrate interactions are exemplified in projection and described in the text. The representation of substrates is symbolic only and does not attempt to define the identity and number of substrate or protein residues (or groups) interacting, or the forces involved or any conformational changes brought about either by inhibitors or substrates. The slight displacement of the ester relative to the peptide symbols is only intended to facilitate the simultaneous pictorial representation of both substrates in the same figure and has no significance to the kinetic implications of the model.

as the large numbers of enzyme derivatives and substrates available are explored. The choice and combination of specific enzyme derivatives and substrates should reflect characteristically the various parameters and their combinations.

A model for the mechanism of action of carboxypeptidase, based on the enzymatic consequences of chemical modifications and metal substitutions, was proposed at a time when information on the protein side chains involved in substrate binding was unavailable (Vallee *et al.*, 1963). The effect of chemical modifications on substrate binding and its relationship to activity, therefore, could not be assessed and was deferred (Vallee, 1964a). This model was delineated solely in terms of effects on the catalytic step.

Subsequently, information on substrate and inhibitor binding was obtained through kinetic and equilibrium measurements on the native and on some of the chemically modified enzymes (Vallee, 1964b; Coleman *et al.*, 1966).

This has led to a complementary model, primarily concerned with substrate binding, which has been designed to account for substrate inhibition and activation, for the varied effects of chemical modification on esterase and peptidase activities, and for the apparent differences between esters and peptides in binding to apocarboxypeptidase. The essential features of the model have been presented on several occasions (Vallee *et al.*, 1965; Vallee, 1966, 1967a,b; Vallee and Riordan, 1968). This model has correctly predicted that certain products will reciprocally alter the two activities and that peptide substrates of the native enzyme should inhibit the esterase activity of the acetylated and iodinated enzymes. The present communication explicates the model, its characteristics, and its pertinence to the available data, and expresses it in mathematical terms consistent with the observed kinetics.

## Experimental Section

The relevant experimental techniques have been detailed in previous communications (Davies *et al.*, 1968a, and references cited therein). Each figure or table includes in its caption or heading references to the article delineating the particular methods employed.

## Results and Discussion

The present model assumes that any given enzyme molecule can interact with any substrate or product, that differences in the complexes formed are statistical only in that their concentrations are the result of competing equilibria. However, depending upon the particular substrates employed, the resultant complexes may

differ. Catalysis, inhibition, and activation are all assumed to be the consequence of differing populations of these complexes, accounting for different rates of product formation. These assumptions underlie the specific kinetic scheme to be developed. The pictorial representation is a specific example of the general case (*vide infra*) and should be viewed in that light.

*Pictorial Model.* The model, shown schematically in Figure 1, assumes discrete, productive binding sites for esters, the red symbols, and for peptides, the black symbols.[2] It is thought that the enzyme responds to the over-all structural differences of esters and peptides and not solely to the COO or CONH bonds. In the simplest case, hydrolysis of the peptide will occur only at the peptide site, the black groove, and that of the ester at the ester site, the red groove. However, *each* type of substrate could bind at the site for the *other* without necessarily being hydrolyzed. The model can, of course, incorporate any degree of hydrolysis at either site dependent, *inter alia*, upon the structure of a given substrate. Solely for the sake of simplicity, however, we shall confine our considerations to one productive binding mode for each.

No effort is made to simulate the topology of the enzyme or the chemical details of the protein binding sites of the substrates or the substrate binding sites of the protein (Coleman and Vallee, 1962a,b). The arrangement shown does not represent or imply a unique choice among numerous alternatives. "Sites" are defined to include all those features of the enzyme which interact with a *given* substrate molecule and determine both specificity and catalysis. "Loci" refer to more detailed aspects of the site without specifying the number or identity of specific binding groups (*vide infra*). Hence, both *site* and *locus* are collective designations, the latter constituting elements of the former.

The spheres and cubes, representing the terminals of the substrates, are employed to permit designation of a given orientation. The exact orientation required for hydrolysis cannot be indicated, of course, and hence, these symbols do not represent *specific* structural features of the substrate such as size, shape, number, and nature of residues. The omission of the metal atom (zinc, in the native enzyme), known to be required for binding of ester substrates and certain inhibitors as well as for activity toward both ester and peptide substrates, is in keeping with our intent to remain schematic.

"Correct" or "productive" binding, *i.e.*, that which results in hydrolysis, could depend on complementarity between substrate groups and specific enzyme side chains as well as substrate-induced changes of enzyme conformation, in accord with current concepts of the formation of enzyme–substrate complexes in general (Koshland, 1958). The peptide and ester symbols, directly adjacent to the groove of Figure 1, indicate catalytically productive binding at sites specific for each. An

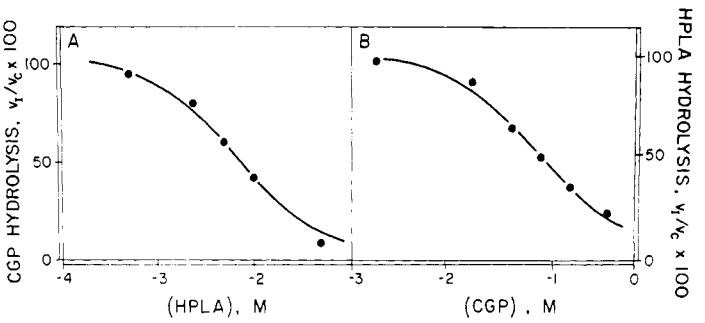


FIGURE 2: Inhibition of peptidase activity by the ester, HPLA, and of esterase activity of carboxypeptidase by the peptide, CGP. (A) The hydrolysis of CGP was measured in the presence of different concentrations of HPLA at 25°, pH 7.5, in 0.05 M Tris–1 M NaCl buffer as described (Davies *et al.*, 1968a). (B) The hydrolysis of HPLA was measured in the presence of different concentrations of CGP, at 25°, pH 7.5, in 0.005 M Tris–0.2 M NaCl buffer as described (Davies *et al.*, 1968a). Inhibition is expressed as per cent of the control. The units of the abscissae are log concentrations.

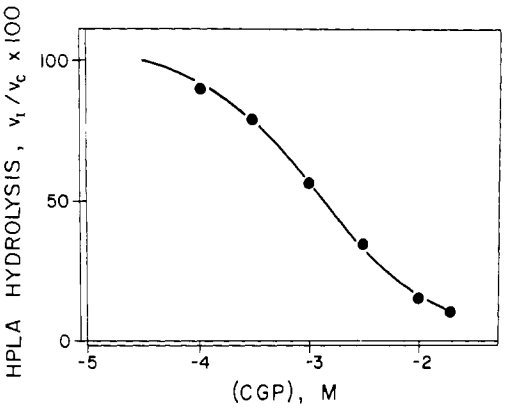


FIGURE 3: Cadmium carboxypeptidase: inhibition of esterase activity by CGP. Hydrolysis of HPLA was measured in the presence of different concentrations of CGP, at 25°, pH 7.5, in 0.005 M Tris–0.2 M NaCl buffer as described (Davies *et al.*, 1968a). Inhibition is expressed as per cent of the control. Cadmium carboxypeptidase is an esterase only. It does not hydrolyze peptides such as CGP. The units of the abscissae are log concentrations.

overlap of the sites is symbolized by the superpositioning of the red and black spheres adjacent to the curved, shaded area of the groove. The extent of overlap could vary for different ester and peptide substrate pairs. The existence of such overlap is consistent with the inhibition of esterase activity by peptide substrates and of peptidase activity by ester substrates of the native enzyme (Figure 2) and with the inhibition by peptide substrates of the cadmium enzyme which exhibits only esterase activity (Figure 3). However, the *literal concept* of physical overlap is not prerequisite to the formal achievement of such results.[3]

The activity *vs.* substrate concentration profiles for many of the peptide and ester substrates of carboxypeptidase examined thus far are indicative of substrate inhibition and/or activation (Lumry *et al.*, 1951; Kaiser

[2] The model is based on data which have been obtained largely with CGP or BGP as the peptide and HPLA as the ester substrate.

[3] Analogous consequences could be expected, *e.g.*, owing to conformational changes of the protein on interaction with substrates.

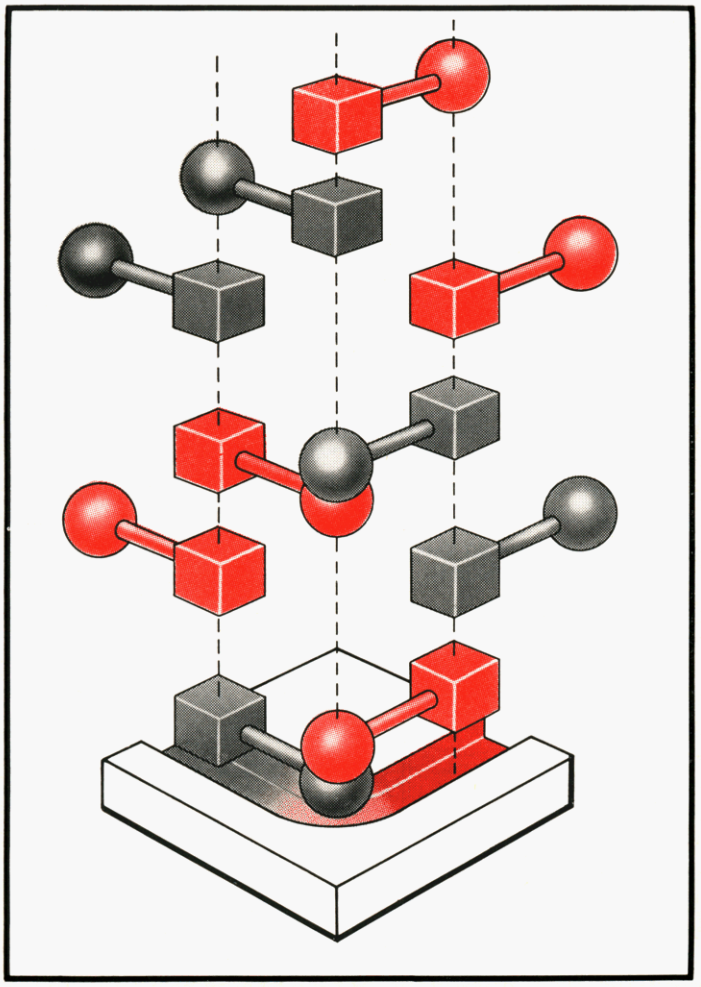

FIGURE 4: Extension of the model to include additional alternate possible modes of binding of esters and peptides to either substrate binding site (see Figure 1).

*et al.*, 1965; McClure *et al.*, 1966; Whitaker *et al.*, 1966; Davies *et al.*, 1968a). The model can account for both phenomena. Thus, in the simplest case, substrate inhibition could result from "incorrect" binding of the peptide at the ester site (or of the ester at the peptide site) to yield an "unproductive" complex. This mode of binding can be visualized by substituting the peptide symbol at the top for that of the ester at the bottom of Figure 1 (or the ester symbol for that of the peptide). It is implicit that this mode of binding could cause substrate inhibition for either substrate owing to the "overlap" of the sites even though the kinetic consequences of such inhibition would not be apparent in a *V vs.* (S) profile. Similarly, substrate activation could result from a mode of binding which can be visualized by substituting the peptide symbol in the middle for that of the ester at the bottom of Figure 1 (or the ester symbol in the middle for that of the peptide). As a result, that mode of substrate inhibition, depicted in Figure 1 (top), would be prevented. The net effect would be apparent activation. Dependent upon the number of loci interacting with a given substrate, a proportionate number of unproductive but activating modes of binding could occur. In the past, substrate inhibition has been accounted for by multiple protein–substrate interactions (*vide infra*). Even in its simplest form the model allows for multiple inhibitory modes of binding (Figure 4). The symbols fourth from the bottom show both peptides and esters interacting with loci of their enzymatically productive site, preventing productive binding, thereby causing inhibition.

The symbols at the top of Figure 4 present yet another variation which could result in inhibition. The substrate is inverted compared with the one adjoining the groove. Similar inversions could be depicted for each of the other binding modes illustrated, all of which could be unproductive, but which could activate or inhibit depending upon the locus occupied. Of course, for substrate inhibition to be observed kinetically, more than one molecule of substrate must be bound, *i.e.*, more than one of the above modes must be realized simultaneously in a given complex.

The simplest version of the model as depicted, presenting but three loci, permits a total of up to 38 productive and nonproductive complexes. A much greater number could occur if additional loci were to be included.

*Implications of the Pictorial Model.* While the symbols in both Figures 1 and 4 represent binding of *substrate* molecules to carboxypeptidase, the effect of *product* binding can be visualized readily by considering the cubes or spheres alone. Thus, *e.g.*, in Figure 1 the black cube interacting with the end of the red groove could inhibit ester but activate peptide hydrolysis. In fact,

TABLE I: Inhibition of Esterase Activity of Acetylcarboxypeptidase by Peptide Substrates and Analogs.[a]

| Inhibitor | $K_I'$ (M × $10^2$) |
|---|---|
| Glycyl-L-phenylalanine | 0.5 |
| Glycyl-L-tyrosine | 0.7 |
| L-Phenylalanine | 0.75 |
| β-Phenyllactate | 0.5 |
| β-Phenylpropionate | 0.8 |
| Benzoylglycyl-L-phenylalanine | 3 |
| Carbobenzoxyglycyl-L-phenylalanine | 6 |
| Carbobenzoxyglycine | 15 |
| Carbobenzoxyglycyl-L-leucine | 20 |

[a] Concentration of inhibitor required to produce 50% inhibition in the presence of substrate, 0.01 M hippuryl-DL-phenyllactate. Esterase activities were measured on a pH-Stat at 25° in 0.005 M Tris–0.2 M NaCl (pH 7.5) as described (Davies *et al.*, 1968a).

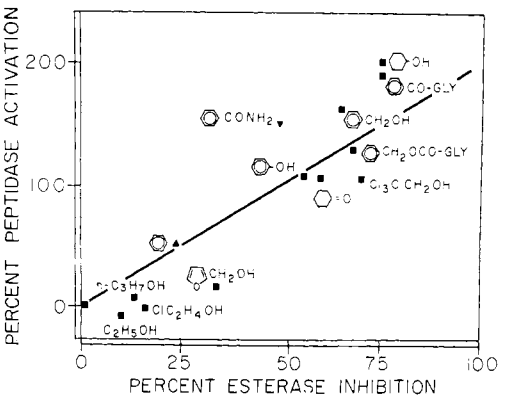


FIGURE 5: Carboxypeptidase. Activation of peptidase and inhibition of esterase activities by products of substrate hydrolysis and by other modifiers. All activities were measured with CGP and HPLA as substrates, both 0.001 M (Davies *et al.*, 1968a). (■) 0.05 M cyclohexanol, benzoylglycine, benzyl alcohol, cyclohexanone, phenol, furfuryl alcohol, 1-propanol, 2-chloroethanol, or ethanol. (▼) 0.025 M benzamide and (▲) 0.01 M benzene (saturated solution). CGG and CGGG, not included in the figure, also accelerate the hydrolysis of CGP. Activation of peptidase and inhibition of esterase activity are expressed as per cent of the control.

this model predicts that any product which could activate the hydrolysis of a peptide substrate bound correctly to its site, should *inhibit* the hydrolysis of an ester substrate bound correctly to its site. Moreover, a product common to the hydrolysis of a peptide and ester pair, and binding at other than the overlap locus, should affect the rates of their hydrolyses in opposite directions, *i.e.*, raising one while lowering the other. This hypothesis has been verified using the N-substituted products of synthetic dipeptide and ester substrates (Davies *et al.*, 1968a,b). BG, a product both of BGP and HPLA, markedly accelerates the hydrolysis of BGP but decelerates that of HPLA (Davies *et al.*, 1968b), while CG, CGG, and CGGG all accelerate the hydrolysis of CGP (Figure 5).

A number of compounds, simulating the hydrophobic character of products, also activate the hydrolysis of CGP and of BGP but inhibit that of HPLA (Figure 5). These effects vary as functions both of modifier (*i.e.*, activator or inhibitor) and of substrate concentration and can be quite marked: at $10^{-3}$ M substrate, cinnamic acid activates CGP hydrolysis fivefold and decreases that of HPLA eightfold (Davies *et al.*, 1968b).

The capacity of the enzyme to differentiate between esters and peptides has also been apparent from its response to chemical modifications. Acetylation of two of its tyrosyl residues markedly diminishes peptidase while increasing esterase activity (Simpson *et al.*, 1963; Riordan and Vallee, 1963). Gel filtration, isotope exchange, and kinetic studies have shown that the decreased peptidase activity of acetylcarboxypeptidase is due, at least in part, to a weakening of peptide binding (Vallee, 1964b; Coleman *et al.*, 1966; Whitaker *et al.*, 1966).

However, chemical modifications affecting the peptide site need not abolish ester substrate inhibition completely, since other inhibitory modes of ester binding to loci of the ester site may be preserved (Figure 6). Indeed, kinetic studies indicate that the observed increase in the rate of hydrolysis of the ester, HPLA, is accompanied by displacement of substrate inhibition to higher substrate concentrations (Davies *et al.*, 1968a; Vallee, 1967a,b). The pictorial representation (Figure 6) refers to what could be an extreme consequence of such chemical modifications. Tyrosyl modification might interfere partially or completely with one or more of the loci of the productive peptide binding site, either directly by blocking a locus, or indirectly through conformational changes accompanying such modifications. Concomitantly, this could interfere with some or all of the unproductive modes of binding possible at this site (Figure 4). In particular, ester binding at some loci of this site would be prevented, thereby eliminating certain modes of ester substrate inhibition. Moreover, the model need not imply any effect of chemical modification on peptide interactions with unmodified loci. Hence, peptide substrates and inhibitors of the native enzyme should inhibit the esterase activity of the acetylated enzyme (Figure 7). Indeed, a wide range of blocked and free dipeptides, products, and product analogs are inhibitors (Table I) and are competitive in the instances investigated thus far (Figure 8).

*Mathematical Model.* This minimal schematic model can account qualitatively for a wide range of kinetic phenomena observed for carboxypeptidase. It can also be defined in quantitative terms.[4] In elaborating the formal kinetic consequences, the possible effect of a number of variables must be considered. Thus, while structural variants of the enzyme are known (Neurath, 1964), possible differences in their kinetic behavior have not been investigated in detail. If $V$ symbolizes the rate

[4] A detailed exposition of the mathematical model and its consistence with experimental results will be published (J. L. Bethune, in preparation).

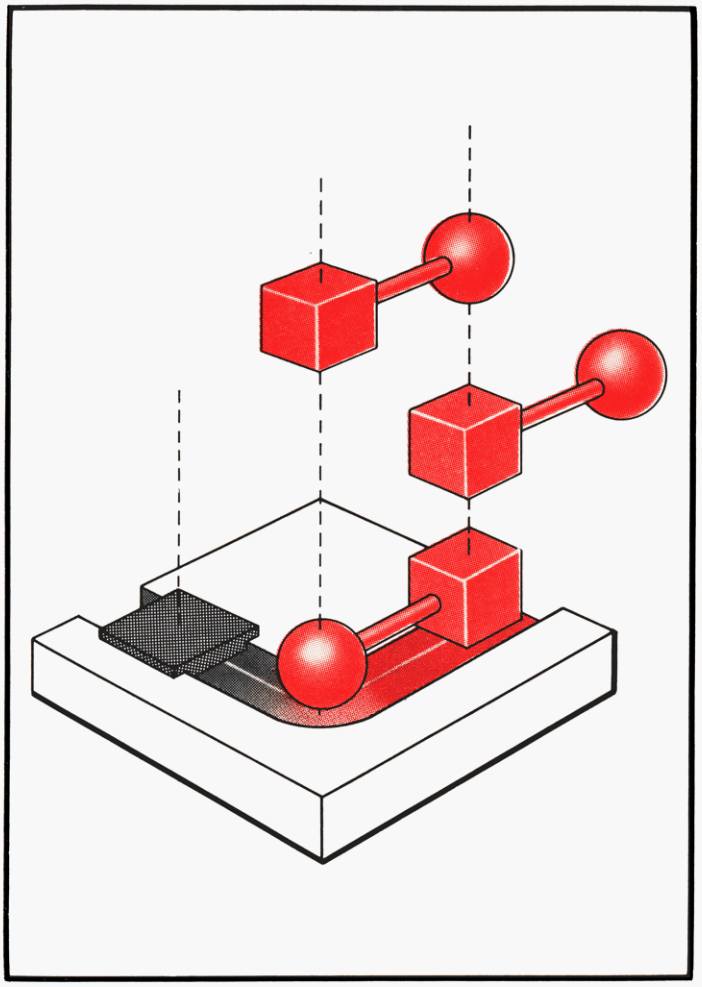

FIGURE 6: Model symbolizing the possible consequence of chemical modification of the productive peptide site. The cross-hatched symbol indicates a weakening or blocking of productive peptide binding as well as nonproductive ester binding to this site. Ester binding can still occur at loci of the productive ester site. Two of the possible permutations are shown. The location of the cross-hatched symbol coincident with that of the peptide cube of Figure 1 has no chemical or physical significance.

of product formation from substrate, then $V = \Sigma V_j$, where $V_j$ is the rate for the $j$th variant. Whatever the distinction between different forms there is no necessary implication that $V_j \neq V_i, i = 1, j$.

The chief difficulty in deriving the relevant rate equations resides in the system of nomenclature devised to designate a given complex of substrate or product with the enzyme. One way of achieving this is to assume a set of coordinates, $\delta$, mapped onto the enzyme, which encompasses all of those features which govern the interactions of the enzyme with substrates and with any molecules, other than substrates, that act as modifiers of enzymatic activity. The identification of any one particular feature with a given coordinate is unique, but the relative location of different features with respect to one another need not be constant.

Then, for each mode of interaction of a given molecule with the enzyme, there is a minimum essential coordinate set identifying the location of that molecule on the enzyme. Each element of this minimum set is a locus. Some or all of the loci for ester and peptide substrates may be identical. In the former case, some regions of the enzyme may be common to the productive interaction of both peptides and esters with the enzyme. Similar considerations apply to the interaction of other molecules (activators or inhibitors) with the enzyme. A second set of coordinates, $\lambda$, is mapped along some axis of the substrate. Minimally, $\lambda$ contains enough information to describe orientation of the substrate among the the loci pertinent to a given mode of interaction. Thus, *e.g.*, it can incorporate effects resulting from the stereochemistry of the substrate.

It must be recognized that in the general formulation, interaction of, *e.g.*, an ester with those loci yielding maximal catalytic rates for blocked dipeptides could result in some degree of ester hydrolysis, albeit at a rate less than that obtained if the interaction occurred at those loci which yield the maximal rate for that ester.

If product can be formed as a result of $p$ different modes of interaction of a given substrate, only one of these can be realized for a single enzyme molecule at a time. Hence, some element of the coordinate subsets describing these modes is common to all of them. These $p$ modes, delineated by $p$ subsets of loci, define the active site for a given substrate.

Activation and/or inhibition may be accounted for by additional modes of interaction of substrate or product with the enzyme. Then for each of the $j$ forms of enzyme with $m$ modes of such modifier interactions a set of $m + p$ associated rate constants, the $k_{cat}$, is postulated.

The $m$ modifier modes of interaction may be divided into two groups. The first, $k$ in number, designate modes of activation and the remainder, $n$ in number, designate

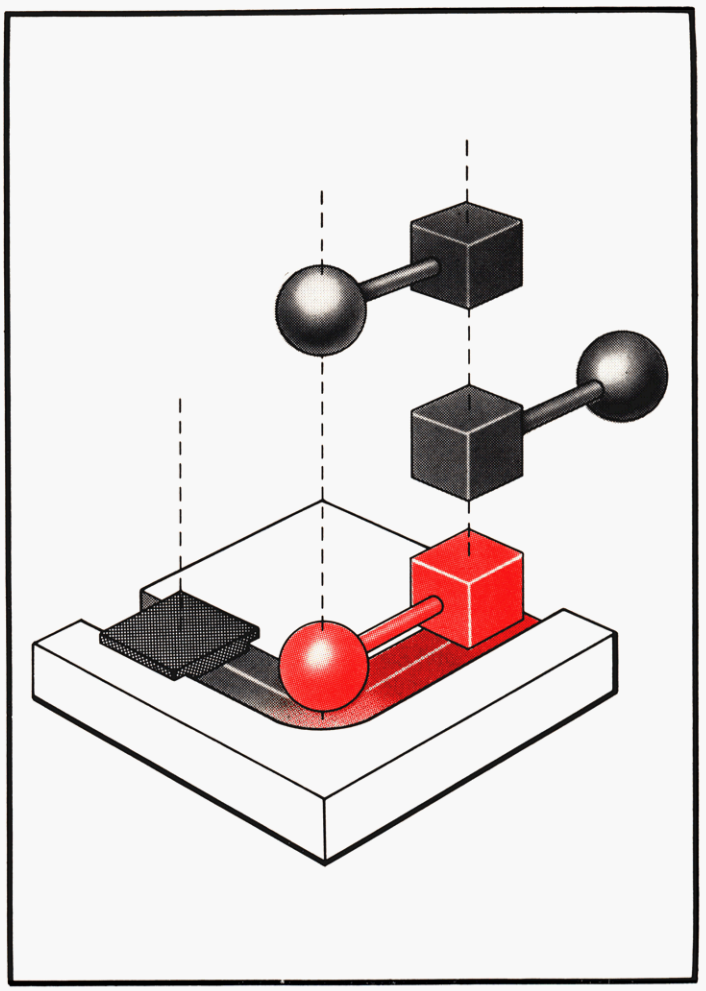

FIGURE 7: Model symbolizing possible consequences of chemical modifications as in Figure 6. Though peptide binding at the productive peptide site is weakened or lost, unproductive peptide binding can still occur at loci of the productive ester site, leading to peptide inhibition of esterase activity.

modes of inhibition. Molecules which interact in these modes may be substrates or products or any other species with analogous effects on activity, all indices being functions of the particular molecular species employed.[5]

Any given complex may be designated by specifying its modes of interaction, which are characterized in turn by coordinate subsets and defining relations, dictating the ordering and union of the $\zeta$ and $\lambda$ subsets, as $(E_tSA_aI_b)_\phi$, where $E_t$ designates enzyme of the $t$ variant ($t = 1, j$), $S$ is substrate, bound in a productive mode, $A_a$ designates $a$ molecules of activator A and $I_b$ designates $b$ molecules of inhibitor I, and $\phi$ is the defined coordinate set. The expression for the velocity is

$$V = \sum_t \sum_\phi k_{\mathrm{cat},t,\phi}(E_tSA_aI_b)_\phi$$

where any of the $k_{\mathrm{cat}}$ may be zero for any or all but one of the complexes.

The total enzyme concentration $(\mathrm{E})^0$ is then

$$(\mathrm{E})^0 = (\mathrm{E}) + \sum_t \sum_\phi (E_tSA_aI_b)_\phi$$

Assuming that all inhibition and activation reactions are in equilibrium, equilibrium constants may be written for each complex and the concentration of each may be expressed, finally, in the form

$$(E_tSA_aI_b)_\phi = (\mathrm{E})_t(\mathrm{S})(\mathrm{A})^a(\mathrm{I})^b/K_{t,\phi}$$

With appropriate values of the parameters, the effects of variations in substrate concentration, for example, can be evaluated from this series of equations.

This highly generalized model can be particularized and restricted to resemble the pictorial representation as follows: (a) three loci at the active center are assumed which can interact simultaneously with three molecules of substrate, (b) the substrate is restricted to two orientations in each interacting mode, *i.e.*, $\lambda$ contains two elements, and (c) one subset of coordinates contains two loci in a single mode, $p$, for each substrate. Thirty-eight complexes can then be described, ten of which are

[5] Of the $k$ activation modes, $q$ may have some loci in common with $p - 1$ productive modes ($q = 0, p - 1$) corresponding to competition with productive modes, characterized by low values of $k_{\mathrm{cat}}$, together with increased productivity of any of the remaining $p - q$ productive modes. Of the remaining $k - q$ modes, $r$ may have loci in common with any of the $n$ inhibition modes ($r = 0, n$), corresponding to release of inhibition by competition for loci common to both. The rest, $k - q - r$ activation modes (which may be zero in number), correspond to activation which is not competitive with productive or inhibitory modes. Of the $n$ inhibition modes, $s$ may have loci in common with the $p$ productive modes ($s = 0, p$) corresponding to competitive inhibition. The remainder, $n - s$, correspond to noncompetitive inhibition. The set $r$ may have loci in common with either set $s$ or $n - s$. In the present highly simplified model, only competitive inhibition and activation by competition for inhibitory loci are postulated; therefore all $k$ are $r$ and all $n$ are $s$.

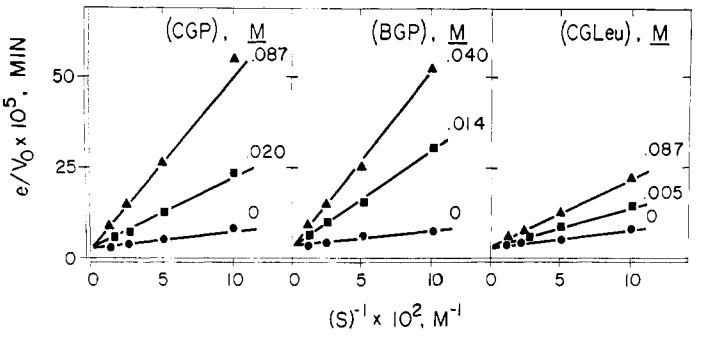


FIGURE 8: Acetylcarboxypeptidase: inhibition of esterase activity by CGP, BGP, and CGLeu. The inhibition constants, $K_I$, for CGP, BGP, and CGLeu are $1.0 \pm 0.5 \times 10^{-2}$ M, $3.7 \pm 0.5 \times 10^{-3}$ M, and $3.7 \pm 0.1 \times 10^{-2}$ M, respectively, and were calculated from the equation $K_I = K_I'K_M/((S) + K_m)$, where $K_I'$ is the apparent inhibition constant and $K_m$ is $7.6 \times 10^{-5}$ M (Davies *et al.*, 1968a) for acetyl carboxypeptidase. Esterase activity was measured at 25°, pH 7.5, in 0.005 M Tris–0.2 M NaCl buffer using HPLA as the substrate as described (Davies *et al.*, 1968a).

with one, twenty with two, and eight with three molecules of substrate. One of the single substrate complexes is productive, *i.e.*, $p = 1$, seven are inhibited completely, and two correspond to activation modes. Of the twenty interactions with two molecules, two are productive and eighteen are inhibitory, while all complexes with three molecules of substrate are nonproductive. If the equilibria involved are noninteracting, then only six equilibrium constants are necessary to describe the system.

Furthermore, if the $j$ forms of the enzyme are all described by the same set of constants and only one catalytic constant applies to any one substrate, the final expression for the rate equation becomes

$$V' = [\theta_1(S) + \theta_2(S)^2]/[1 + \theta_3(S) + \theta_4(S)^2 + \theta_5(S)^3]$$

where the $\theta_i$ are functions of the equilibrium constants and $V'$ is now a reduced rate, $V/V_{max}$.

This equation is written for a single substrate interacting with the enzyme in multiple modes, thereby resembling all previous models. If, however, in the situation for which the present model is designed, both esterase and peptidase activities are simultaneously accommodated and all coordinate subsets for the two substrates are identical, and the product of only one of the substrates is measured, then

$$V'_S = [\theta_1(S) + \theta_2(S)^2 + \theta_6(S)(S')]/[1 + \theta_3(S) + \theta_4(S)^2 + \theta_5(S)^3 + \theta_3'(S') + \theta_4'(S')^2 + \theta_5'(S')^3 + \theta_7(S)(S') + \theta_8(S)^2(S') + \theta_9(S)(S')^2$$

Here the subscript refers to the product obtained from substrate, S, S′ refers to the other substrate, $\theta_1$ to $\theta_5$ are as above; $\theta'_3$ to $\theta'_5$ are analogous expressions for S′ and $\theta_6$ to $\theta_9$ are mixed expressions involving both sets of equilibrium constants. This equation allows for mutual inhibition and activation corresponding to the model visualized in Figure 1.

If chemical modifications lead to changes in certain equilibrium constants, none of the $\theta_i$ are eliminated necessarily. Thus, if the $K'_m$ for substrate S′ is increased substantially, and the interaction of S′ with these loci is, therefore, weakened, all remaining loci are still available.[6] The possibility of modification of $k_{cat}$ is not excluded.

For a given set of the parameters a computer program, written in FORTRAN IV for an IBM 7094 computer, has generated curves of $V$ *vs.* (S) from these equations which suffice to fit experimental data.

*Extensions to Other Substrates.* Various models have been proposed in the past to explain the substrate inhibition of carboxypeptidase by HPLA, CGP, and CGT. Thus, for CGP, Lumry *et al.* (1951) considered two variants of enzyme, occurring in equal amounts. One of them was postulated to be inhibited by four molecules of substrate. Similarly, McClure *et al.* (1964) fitted the kinetics of HPLA hydrolysis by assuming two species of enzyme (in a ratio of 61:39), one binding one substrate molecule, the other interacting with as many as five. Alternatively, the kinetics could be accounted for by one species of enzyme interacting noncompetitively with five substrate molecules, each yielding catalytically inactive complexes, or by four coexistent species of enzyme. To account for the residual activity found at high substrate concentration, it has been proposed that none of the enzyme molecules are inactivated totally by combination with more than one molecule of substrate (Quiocho and Richards, 1966).

Another scheme for the substrate and product activation observed in the hydrolysis of CGP assumes that the binding of a second substrate molecule, or of CG, can increase $V_{max}$ without affecting binding in the catalytically active complex (Whitaker, 1966). Substrate activation with the ester hippuryl glycolate has been considered evidence for allosteric effects (Kaiser *et al.*, 1965). Basically, these and the present model all postulate multiple protein–substrate interactions, but differ primarily in the number of protein or substrate molecules assumed to interact. However, the present model further encompasses the dual specificity of the enzyme toward esters and peptides as critical to its design.

Kinetic anomalies of carboxypeptidase catalysis have been encountered in the study of virtually all the dipeptides and esters which have served as substrates in the past. It should be recalled that the synthesis of these compounds initially provided the technical opportunity to study the kinetics of this enzyme (Bergmann and Fruton, 1941). The anomalies, observed on hydrolysis of these substrates, were inferred *pari passu* to pertain also to other potential substrates. It has become apparent, however, that the kinetics of hydrolysis of acylamino acids, dipeptides, polypeptides, and protein substrates

[6] Any of these complex models result in rate equations involving the quotient of two polynomials (Hearon *et al.*, 1959). Those in which the degree of the polynomial constituting the numerator is less than that of the denominator yield, in the limit of infinite substrate concentration, zero velocity. If the degree of both polynomials is the same, a constant nonzero level of activity may be reached. However, with a given set of parameters, one may closely resemble the other. Indeed, investigation of the derivatives of the above equation reveals that while only one extremuum can exist, up to four inflection points are possible.

do differ significantly (Coombs and Vallee, 1966, and pertinent references therein). These variations may, of course, be but expressions of the enzyme's physiological capacity to accommodate diverse substrates. Among these, dipeptides and depsipeptides might not complement all the substrate's binding loci available on the enzyme uniquely, but other substrates might progressively approximate this condition as a function of their composition, sequence, and stereochemistry. This conjecture can be tested readily as the kinetics of larger substrates are studied in greater depth.

It is already known that certain polypeptides and proteins bind more firmly to apocarboxypeptidase than do dipeptides. Acetylation of the apoenzyme with *N*-acetylimidazole drastically reduces dipeptide binding but does not prevent the formation of polypeptide or protein complexes, at least in those instances which have been studied (Coombs and Vallee, 1966). Apparently, these larger molecules bind to groups on the surface of the apoenzyme in addition to those concerned with productive dipeptide binding. The systematic kinetic study of alanyloligopeptides of varying chain length, blocking groups, and C-terminal residues has aided greatly in the understanding of these problems. The turnover numbers and $K_m$'s observed with such peptides containing strategically placed L and D positional isomers have led to the conclusion that the active center of the enzyme may contain as many as five residues (Schechter and Berger, 1966, 1967; Abramowitz *et al.*, 1967). Perhaps these comprise loci such as discussed. If these larger substrates occupy all of the loci uniquely, normal kinetics could be expected and indeed have been already observed when tripeptides are employed (Vallee, 1967a; Auld, 1968). Alternatively, the size of such substrates might preclude the manifestation of inhibition by preventing the formation of complexes involving more than one substrate molecule.

Examination of enzyme–substrate complexes by X-ray diffraction would be one direct approach to examine such hypotheses. The rapid turnover of active enzyme–substrate complexes would, of course, deplete the substrate, and hence the active complex, a circumstance which may well account for the difficulties encountered in the structural studies of most enzyme–substrate complexes examined thus far. The time periods for X-ray diffraction studies and for kinetic studies differ by orders of magnitude. In contrast, unproductive modes of binding could be discernible readily. The definitive structural identification of either mode of binding will require the development of suitable methods.

Structural studies at 6-Å resolution have been reported already for complexes of crystalline carboxypeptidase with one substrate, glycyl-L-tyrosine, with inactive peptide substrate analogs, *e.g.*, peptide amides, and with inhibitors (Lipscomb *et al.*, 1967; Steitz *et al.*, 1967). At least two different but competitive binding sites in the vicinity of the zinc atom can be discerned for the inhibitor, $\beta$-(*p*-iodophenyl)propionate. Two regions of increased electron density, labeled A and B, are also detected on binding of both glycyl-L-tyrosine and L-lysyl-L-tyrosinamide. These results are consistent with a model such as proposed here. In view of the above considerations it cannot as yet be judged unequivocally which of the modes of binding the data reflect. While such agreement between the model and structural data might be inferred to support the model, much broader experimental evidence with substrates of different structure will be required to seek answers to the hypotheses here advanced.

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# The Action of Dipeptidyl Transferase as a Polymerase*

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ABSTRACT: The electrophoretic separation of the components of reaction mixtures in the action of dipeptidyl transferase on Gly-Tyr-$NH_2$ or Ala-Phe-$NH_2$ has permitted the analytical determination of the products that are formed. When $^{14}C$-labeled dipeptide amides are used as substrates at pH 7.5, appreciable quantities of labeled tetrapeptide amide (Gly-Tyr-Gly-Tyr-$NH_2$ or Ala-Phe-Ala-Phe-$NH_2$) or hexapeptide amide (Gly-Tyr-Gly-Tyr-Gly-Tyr-$NH_2$) appear, in addition to the insoluble octapeptide amide derived from Gly-Tyr-$NH_2$ or the insoluble hexapeptide amide derived from Ala-Phe-$NH_2$. The data indicate that, with increasing substrate concentration and at constant enzyme concentration, the proportion of dipeptidyl units going to free dipeptide (by hydrolysis) decreases to a small value (*ca.* 10%), but the total fraction of dipeptidyl units going to free dipeptide and tetrapeptide amide remains relatively constant. This finding suggests the formation of an intermediate dipeptidyl–enzyme that reacts either with water or with dipeptide amide. With increasing enzyme concentration and at constant substrate concentration, the partition of dipeptidyl units shifts in favor of higher oligopeptides (hexapeptide amide and octapeptide amide), suggesting the possibility that the chain-propagation reaction involves the cooperative interaction of separate enzyme molecules bearing activated dipeptidyl units. The available data are inconsistent with chain elongation by the addition of dipeptidyl units to the $\alpha$-amino group of oligopeptide amides, but favor rather a mechanism in which oligopeptidyl–enzyme intermediates are formed.

Dipeptidyl transferase, an enzyme purified from beef spleen (Metrione *et al.*, 1966), catalyzes the transfer of dipeptidyl units from suitable peptides, dipeptide amides, and dipeptide esters to acceptor nucleophiles ($H_2O$, $NH_2OH$, and $NH_2R$). The specificity of the enzyme with respect to the structure of the dipeptidyl unit has been defined in previous studies (Voynick and Fruton, 1968, and earlier papers cited therein). The present communication deals with the ability of dipeptidyl transferase to catalyze the transfer of one dipeptidyl unit acting as an acyl donor to another dipeptidyl unit acting as an acceptor amine. As reported in earlier papers from this laboratory (Jones *et al.*, 1952; Fruton *et al.*, 1953; Würz *et al.*, 1962; Nilsson and Fruton, 1964), such transfer reactions are involved in the polymerization of suitable dipeptide amides to yield sparingly soluble products, shown to be higher oligopeptides. The average chain length of the insoluble product derived from Gly-Tyr-$NH_2$ (as well as from

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