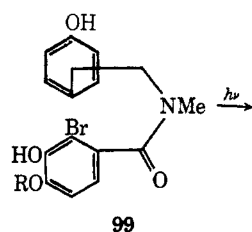

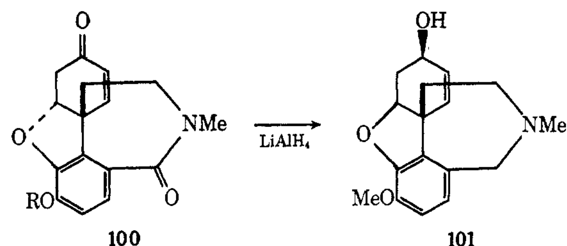

bromo amide **99** was cyclized to the narwedine-type compound **100**, which was a key intermediate to galanthamine (**101**).[35]

In this Account, we have shown simple syntheses of several types of isoquinoline alkaloids. Photolysis provides novel methods for the total synthesis of natural products. The improvements of yield and the availability of stereospecificity or stereoselectivity by appropriate modifications of the reaction provide methods of synthesis which are simpler and more elegant than classical methods which require many steps and much time.

It is a great pleasure to acknowledge the aid of my able coworkers, especially Dr. S. Shibuya and Dr. M. Koizumi. We are indebted to the Department of Education, Japanese Government, and the Japan Society for the Promotion of Science under the Japan–U. S. Cooperative Science Program, for their financial support.

Carboxypeptidase A: a Mechanistic Analysis

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The research to be discussed in this Account was directed toward the mechanistic elucidation of the esterase and peptidase action of the proteolytic enzyme, carboxypeptidase A. This enzyme was chosen for study for a number of reasons, among which are its moderate molecular weight (approximately 34,000), its ready availability in a highly pure crystalline form, and the inherently interesting fact that it is a metalloenzyme requiring the presence of one Zn^{2+} ion per molecule at its active site for catalytic activity. Carboxypeptidase A catalyzes the hydrolysis of the peptide or ester bonds of *N*-acyl α-amino acids and *O*-acyl α-hydroxy acids adjacent to the terminal free carboxyl groups.[3–5]

Our investigation has concentrated on the kinetic analysis of the hydrolysis of selected synthetic substrates because we feel that only by kinetic studies can one measure directly the dynamics of reaction, the principal aspect of enzyme action which any postulated mechanism must explain. When we began our studies, in 1962, the prospects of interpreting the results of kinetic investigations in terms of the structure of carboxypeptidase A were rather limited. No definite information was available concerning the nature of the Zn^{2+} binding ligands in the enzyme and only partial peptide sequence data were in hand. Before the completion of our kinetic studies not only were electron density maps at 2.0-Å resolution obtained for carboxypeptidase A and a complex of the enzyme with the dipeptide glycyl-L-tyrosine[6–8] but also the determination of the entire primary amino acid sequence was reported.[9] Carboxypeptidase A became thus the first metalloenzyme for which the high-resolution structure and sequence were known.

From the X-ray and chemical sequence studies, it is known that there are three amino acid ligands from

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(1) Fellow of the Alfred P. Sloan Foundation, 1968–1970.
(2) Predoctoral Trainee of the National Institutes of Health, 1965–1970.

(3) E. Waldschmidt-Leitz, *Physiol. Rev.*, **11**, 358 (1934).
(4) K. Hoffman and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940).
(5) J. E. Snoke, G. W. Schwert, and H. Neurath, *ibid.*, **175**, 7 (1948).
(6) W. N. Lipscomb, J. A. Hartsuck, G. N. Reeke, Jr., F. A. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead, and J. C. Coppola, *Brookhaven Symp. Biol.*, **21**, 24 (1968).
(7) W. N. Lipscomb, G. N. Reeke, Jr., F. A. Quiocho, and P. H. Bethge, *Phil. Trans. Roy. Soc. London, Ser. B*, **251**, 177 (1970).
(8) W. N. Lipscomb, *Accounts Chem. Res.*, **3**, 81 (1970).
(9) R. A. Bradshaw, L. H. Ericsson, K. A. Walsh, and H. Neurath, *Proc. Nat. Acad. Sci. U. S.*, **63**, 1389 (1969).

the enzyme which bind the metal ion: His-69, Glu-72, and His-196, as well as a water ligand.[10] A cysteine residue[11,12] which at one time had been thought to be a zinc ligand was found from the X-ray study[8] to be far from the zinc ion. It is joined to the other cysteine residue present in the enzyme by a disulfide bridge.[8,13] The most important structural information from the mechanistic point of view was obtained from the 2.0-Å resolution X-ray investigation of the carboxypeptidase A–glycyl-L-tyrosine complex.[7] This study showed that the only parts of carboxypeptidase A which are near enough to the peptide bond of the substrate to be involved directly in catalysis are the Zn^{2+} ion, Glu-270, and Tyr-248. The carbonyl group of the peptide bond has displaced the water ligand from the zinc ion in the complex. The only other group of the protein within 3 Å of a functional group of the substrate is Arg-145, which binds the terminal carboxyl group present in the peptide.

Clearly, one only gets a static picture of an enzyme or enzyme–substrate complex from structure determinations of the sort just described. It is dangerous to draw mechanistic conclusions involving reaction dynamics from such a picture alone. In particular, glycyl-L-tyrosine is a very poor substrate for carboxypeptidase A, and the enzyme–substrate complex observed by X-ray diffraction is likely to be a nonproductive one. There are, however, reasons to believe that the arrangement of the zinc ion, Glu-270, Tyr-248, and Arg-145 in the complexes of carboxypeptidase with reactive peptide substrates is essentially the same as in the case of glycyl-L-tyrosine.[6]

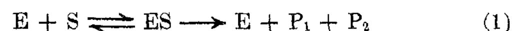
In spite of these reservations, it must be acknowledged that the mechanistic analysis of the kinetic data rests heavily upon the X-ray identification of the groups comprising the active site of the enzyme. The necessity of the zinc ion for enzymatic activity has been firmly established from chemical studies,[14] and there is evidence that chemical modification of the Tyr-248 residue strongly affects the peptidase action of carboxypeptidase A.[6,15] On the other hand, chemical modification data supporting the involvement of Glu-270 as a catalytically essential residue are fragmentary,[16,17] as are results of this type for Arg-145.[18] Certainly, there is need for further chemical modification investigations on carboxypeptidase A.

Although kinetic data for the carboxypeptidase-catalyzed hydrolysis of well over 50 synthetic substrates have been described in the literature, only a few substrates have been examined in sufficient detail to provide reliable results on which mechanistic arguments might be based. Of these, most were found to exhibit complex kinetic behavior in their hydrolyses catalyzed by carboxypeptidase A, such as substrate activation or inhibition and product activation or inhibition. Thus, the hydrolysis of the most thoroughly studied peptide *N*-carbobenzoxyglycyl-L-phenylalanine involves substrate activation, product activation by *N*-carbobenzoxyglycine, and product inhibition by L-phenylalanine.[19] In the hydrolysis of the ester *O*-(*N*-benzoylglycyl)-L-β-phenyllactate, there is substrate inhibition as well as product inhibition by L-β-phenyllactate.[20,21] Fortunately, the kinetic situation for carboxypeptidase substrates is not always so complex. The only significant complicating factor in the hydrolysis of the peptide *N*-(*N*-benzoylglycyl)-L-phenylalanine is product inhibition by L-phenylalanine.[21] For the esters *O*-acetyl-L-mandelate[22] and *O*-(*trans*-cinnamoyl)-L-β-phenyllactate,[23] product inhibition by L-mandelate and L-β-phenyllactate, respectively, was observed, but there was no evidence for either substrate activation or inhibition.

In the following sections, kinetic observations are described which, together with structural data and chemical modification results for carboxypeptidase A, have led to the formulation of a mechanism for the action of this enzyme on esters and peptides.

Kinetic Schemes

Those substrates which show neither substrate activation nor substrate inhibition in their hydrolytic behavior have been most useful in the mechanistic investigations. Typically, the simple Michaelis–Menten scheme represented by eq 1, complicated only by product inhibition as illustrated by eq 2, is sufficient to account for the kinetic data measured. Under conditions where $[S] \gg [E]$, the steady-state rate expression which applies is given by eq 3 where $K_{m\,app} = [E][S]/[ES]$, $K_i = [E][P_1]/[EP_1]$, and $([S]_0 - [S])$ is the concentration of the inhibitory product P_1, at any time t.[22]



$$E + S \rightleftharpoons ES \longrightarrow E + P_1 + P_2 \quad (1)$$

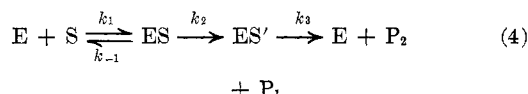
$$E + P_1 \rightleftharpoons EP_1 \quad (2)$$

$$v = \frac{-d[S]}{dt} = \frac{k_{cat}[E]_0[S]}{K_{m\,app} + [S] + K_{m\,app}([S]_0 - [S])/K_i} \quad (3)$$

It is important to consider the extension of eq 1 represented by eq 4 because one of the mechanisms for

(10) The standard abbreviations for the amino acids are discussed in *Biochemistry*, **5**, 1445 (1966).
(11) K. S. V. Sampath Kumar, K. A. Walsh, J.-P. Bargetzi, and H. Neurath, *Biochemistry*, **2**, 1475 (1963).
(12) H. Neurath, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **23**, 1 (1964).
(13) K. A. Walsh, L. H. Ericsson, R. A. Bradshaw, and H. Neurath, *Biochemistry*, **9**, 219 (1970).
(14) J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, **237**, 3430 (1962), and earlier articles cited therein.
(15) O. A. Roholt and D. Pressmann, *Proc. Nat. Acad. Sci. U. S.*, **58**, 280 (1967).
(16) H. Neurath, R. A. Bradshaw, L. H. Ericsson, D. R. Babin, P. H. Petra, and K. A. Walsh, *Brookhaven Symp. Biol.*, **21**, 1 (1968).
(17) P. H. Petra and H. Neurath, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **29**, 666 Abs. (1970).
(18) J. F. Riordan, *ibid.*, **29**, 462 Abs. (1970).
(19) J. R. Whitaker, *Biochem. Biophys. Res. Commun.*, **22**, 6 (1966).
(20) M. L. Bender, J. R. Whitaker, and F. Menger, *Proc. Nat. Acad. Sci. U. S.*, **53**, 711 (1965).
(21) J. R. Whitaker, F. Menger, and M. L. Bender, *Biochemistry*, **5**, 386 (1966).
(22) E. T. Kaiser and F. W. Carson, *J. Amer. Chem. Soc.*, **86**, 2922 (1964).
(23) P. L. Hall, B. L. Kaiser, and E. T. Kaiser, *ibid.*, **91**, 485 (1969).

carboxypeptidase action which was first suggested by Lipscomb,[8,24] on the basis of his comparative studies on the structures of the carboxypeptidase–Gly-L-Tyr complex and of the enzyme itself, involves the attack of the carboxylate group of Glu-270 on the carbonyl group of the substrate. This results in the eventual formation of a covalent anhydride intermediate, which can be represented by ES′, at the active site of the enzyme. The kinetic parameters of eq 3 are related to those of eq 4 as shown in eq 5 and 6.



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (4)$$
$$+ P_1$$

where $K_m = (k_{-1} + k_2)/k_1$.

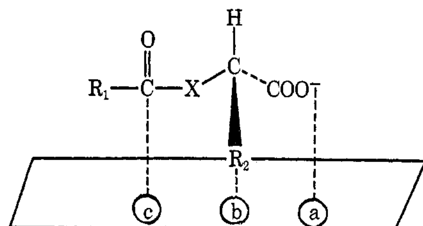
$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad (5)$$

$$K_{m\,app} = K_m\left(\frac{k_3}{k_2 + k_3}\right) \quad (6)$$

Elucidation of Structure–Reactivity Relationships in the Action of Carboxypeptidase on Synthetic Substrates

The analysis of structure–reactivity relationships in carboxypeptidase A catalyzed ester and peptide hydrolysis has been a very useful approach in the development of reaction mechanisms for the action of the enzyme. Since k_{cat} can be a complex quantity, reactivity in enzymatic reactions may be most usefully defined in terms of the values of $k_{cat}/K_{m\,app}$ which for a scheme like that in eq 4 is equal to k_2/K_m.[25] (See equations 5 and 6.)

Following an approach similar to that employed in the case of α-chymotrypsin,[25] the interaction between carboxypeptidase and its substrates can be represented by the following diagram where R_1 is the group attached to the carbonyl moiety of the acyl portion of the substrate, R_2 is the side chain of the α-hydroxy or α-amino acid portion, and X is either O or NH. The



plane represents the surface of carboxypeptidase A where a represents the binding site for the substrate carboxylate group, b, the binding site for R_2, and c, the catalytic site. A form of the Taft[26] equation which can be applied to the carboxypeptidase catalyzed reactions of esters and peptides which have simple kinetic behavior is

$$\log \frac{[k_{cat}/K_{m\,app}][R_1R_2X]}{[k_{cat}/K_{m\,app}][\text{reference compound}]} = \rho_X{}^*\sigma_X{}^* + S_{R_1} + S_{R_2} \quad (7)$$

Here $\rho_X{}^*$ is the reaction constant, $\sigma_X{}^*$ is the Taft substituent constant[26] for the group X, and S_{R_1} and S_{R_2} are factors which account for the influence of the substituents R_1 and R_2 on substrate reactivity.[27] The results of kinetic measurements on ester and amide derivatives of cinnamate and furylacrylate are compared in Table I. The esters are almost 5000 times more re-

Table I
Influence of X on Carboxypeptidase A Reactions

$$\frac{[k_{cat}/K_{m\,app}]\,[\text{L-}\beta\text{-phenyllactate derivatives}]}{[k_{cat}/K_{m\,app}]\,[\text{L-phenylalaninate derivatives}]}$$

	Cinnamoyl	Furylacryloyl
Ratio ester/amide[a]	4720	4710

[a] These ratios were calculated from the data of W. O. McClure, Ph.D. Thesis, University of Washington (1964).

active than the corresponding peptides to carboxypeptidase A catalyzed hydrolysis. This ratio is comparable with the ratio of the rate constants found for the hydroxide ion catalyzed hydrolysis of methyl acetate *vs.* acetamide which is 5500[25] and indicates that the effect of the variation in the group X on $k_{cat}/K_{m\,app}$ appears only to reflect electronic influences of X and does not involve productive binding of X to the enzyme.[27]

Table II gives data for the influence of R_1 on carboxypeptidase A catalyzed hydrolysis reactions. Ratios found for the difference between the cinnamoyl, furylacryloyl, and p-nitrocinnamoyl derivatives in the L-β-phenyllactate and L-mandelate series are to be com-

Table II
Influence of R_1 on Carboxypeptidase A Reactions[a]

$$\frac{[k_{cat}/K_{m\,app}]\,[R_2R_1X]}{[k_{cat}/K_{m\,app}]\,[R_2R_{10}X]}$$

	Furylacryloyl/ R_{10}[e]	p-Nitro-cinnamoyl/ R_{10}
L-β-Phenyllactate	1.14	5.6
L-Mandelate		4.5
$k_{OH}[R_1CO_2C_2H_5]/k_{OH}[R_{10}CO_2C_2H_5]$[b]	0.8[c]	13[d]

[a] The kinetic constants for the enzymatic reactions are listed in Table 12 of B. L. Kaiser, Ph.D. Thesis, University of Chicago, 1970. [b] k_{OH} for the cinnamoyl ethyl ester is $12 \times 10^{-3}\ M^{-1}\ sec^{-1}$ at 25°, extrapolated from data collected in 60% acetone–water (*Nat. Bur. Stand. Circ.*, **No. 510** (1951)). [c] k_{OH} for furylacryloyl ethyl ester is $9.8 \times 10^{-3}\ M^{-1}\ sec^{-1}$ at 25°, $\mu = 1$, 2.5% ethanol–water (J. F. Kirsch and E. Katchalski, *Biochemistry*, **4,** 884 (1965)). [d] This value is based on the rate constants measured in 60% acetone–water (*Nat. Bur. Stand. Circ.*, **No. 510,** Suppl. I (1956)). [e] R_{10}, cinnamoyl.

(24) G. N. Reeke, J. A. Hartsuck, M. L. Ludwig, F. A. Quiocho, T. A. Steitz, and W. N. Lipscomb, *Proc. Nat. Acad. Sci. U. S.*, **58,** 2220 (1967).
(25) M. L. Bender and F. J. Kézdy, *Annu. Rev. Biochem.*, **34,** 49 (1965).
(26) R. W. Taft, Jr., in "Steric Effects in Organic Chemistry," M. S. Newman, Ed., Wiley, New York, N. Y., 1956, p 556.

(27) We emphasize that our discussion here of substrate reactivity is limited to those substrates in which R_1 contains no amide linkages.

pared with the ratios of the second-order rate constants for hydroxide ion catalyzed hydrolysis of the corresponding ethyl esters. The similarity between the ratios of the rate constants for the enzymatic and nonenzymatic reactions indicates that R_1 exerts only an electronic influence on the rate of the enzymatic reaction.

The influence of R_2 on reactivity may be determined by comparing values of $k_{cat}/K_{m\ app}$ for *O*-acyl esters of L-β-phenyllactate with those for the corresponding derivatives of L-mandelate as shown in Table III. To

Table III
Influence of R_2 on Carboxypeptidase A Reactions

$$\frac{[k_{cat}/K_{m\ app}]\,[R_1R_2X]}{[k_{cat}/K_{m\ app}]\,[R_1R_{2_0}X]}$$

	Cinnamoyl	*p*-Nitrocinnamoyl
L-β-Phenyllactate/L-Mandelate	316	400

separate out the electronic effect which R_2 may exert on the enzymatic reactions, the ratios listed in Table II can be compared with the ratio of rate constants for the hydroxide ion catalyzed hydrolysis of β-phenylethyl acetate and benzyl acetate, where the respective leaving groups are related to the R_2 groups for the L-β-phenyllactate and L-mandelate series. We have calculated that k_{OH}[β-phenylethyl acetate]/k_{OH}[benzyl acetate] = 0.6.[26] Since the $k_{cat}/K_{m\ app}$ ratios recorded in Table III are much higher than this number and since this behavior is similar to that found for groups showing marked hydrophobic interactions in the chymotrypsin case,[25] it is concluded that the R_2 moiety in carboxypeptidase substrates influences reactivity through strong hydrophobic bonding with the enzyme. The X-ray crystallographic results on the carboxypeptidase A–Gly-L-Tyr complex are consistent with this conclusion.

If the choice of hydroxide ion catalyzed reactions as a model is a valid one, then the similarity found for the effects of R_1 and X on the enzymatic and nonenzymatic reactions is consistent with the hypothesis that the mechanism for carboxypeptidase A catalyzed hydrolysis reactions is a nucleophilic one, involving the formation of a tetrahedral intermediate.

pH Dependence of the Reactions of Synthetic Substrates with Carboxypeptidase A

Arguments have been presented[23,28] that the pH dependence of the quantity $k_{cat}/K_{m\ app}$ for the hydrolysis of two ester substrates, *O*-acetyl-L-mandelate and *O*-(*trans*-cinnamoyl)-L-β-phenyllactate, reflects the ionization of groups in the free enzyme which affect its reactivity. The pK_{a_1} values of about 6.5 ± 0.4 obtained for these esters correspond well with those found for dipeptide and tripeptide[29] substrates. If Lipscomb's assignments[8] of the amino acid functions involved in enzymatic peptide hydrolysis are accepted, then these pK_{a_1} values can be attributed to the ionization of the carboxyl group of Glu-270. In other words, in the reactive form of carboxypeptidase A, Glu-270 has its free carboxyl group ionized.

The pK_{a_2} value (~9.4) observed in the hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate is similar to those measured recently for tripeptides.[29] That for the hydrolysis of *O*-acetyl-L-mandelate[28] (~7.5) is considerably lower. The pK_{a_2} values reported in the literature for dipeptide hydrolysis by carboxypeptidase are variable, presumably because of the complex kinetic behavior of these compounds which generally has been incompletely analyzed.

Although acetylation of tyrosine residues in carboxypeptidase A, including Tyr-248, presumably abolishes activity toward *O*-acetyl-L-mandelate,[30] it has no significant effect on $K_{m\ app}$ for *O*-(*trans*-cinnamoyl)-L-β-phenyllactate and only reduces k_{cat} for this reactive ester by a factor of two.[31] It is unlikely, therefore, that the group whose ionization affects the reactivity of the enzyme with the L-β-phenyllactate compound in alkaline solution is Tyr-248. A more likely candidate as the ionizing group the pK_a of which is measured here as pK_{a_2} is the water ligand attached to the Zn^{2+} ion.[32] This assignment is in accord with the X-ray studies which suggest[8] that the water ligand must be displaced from the Zn^{2+} ion by the carbonyl of the substrate when complex formation takes place, a process which would be more difficult if ionization to the Zn^{2+} hydroxide species occurred.

Tentatively, the pK_{a_2} value of ~7.5 seen for *O*-acetyl-L-mandelate is ascribed to the ionization of the phenolic hydroxyl of Tyr-248. While this is consistent with the acetylation results mentioned above, further experiments are needed to test this hypothesis and to establish the mechanistic role of the hydroxyl group.

Inhibition of the Carboxypeptidase A Catalyzed Hydrolysis of a Specific Ester Substrate by a Nonspecific Ester

When the considerable differences in reactivity between *O*-acyl derivatives of L-mandelate and L-β-phenyllactate due to the differences in the structure of the R_2 groups are considered, it seems reasonable to classify the L-mandelate species as nonspecific ester substrates. To investigate the possibility that distinctly different sites on the enzyme are involved in the reactions of the two types of esters, the extent of inhibition of the hydrolysis of a specific ester substrate by a nonspecific one was examined.

An excellent correspondence was found between the

(28) F. W. Carson and E. T. Kaiser, *J. Amer. Chem. Soc.*, **88**, 1212 (1966).
(29) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 4352 (1970), and earlier references cited therein.
(30) Unpublished results of Dr. F. Quiocho, quoted in ref 6.
(31) Unpublished results of Dr. P. L. Hall; see P. L. Hall, Ph.D. Thesis, University of Chicago, 1967.
(32) In agreement with this assignment, nmr dispersion experiments indicate that there is no proton ionization from the hydration shell of manganese carboxypeptidase A in the pH range from 8 to 9. It seems probable that the ionization of a water ligand bound to Mn^{2+} in the enzyme occurs only above pH 9: F. A. Quiocho, J. F. Studebaker, R. D. Brown, S. H. Koenig, and W. N. Lipscomb, to be published (private communication).

inhibition constant, K_i, measured for *O*-(*trans*-cinnamoyl)-L-mandelate as an inhibitor for the hydrolysis of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate, and the $K_{m\ app}$ value measured from the turnover kinetics of the hydrolysis of the L-mandelate ester. This indicates that the binding sites for a nonspecific and a specific ester are similar, if not the same. Proceeding one step further, however, and considering the possibility that eq 4, which was discussed earlier and which postulates that a covalent intermediate ES′ is formed in the catalytic action of carboxypeptidase A, applies to the hydrolysis of esters, these findings can be interpreted to indicate that for *O*-(*trans*-cinnamoyl)-L-mandelate $k_3 \gg k_2$.[33] In agreement with this conclusion, careful spectrophotometric studies on *O*-(*trans*-cinnamoyl)-L-mandelate[34] have not revealed any evidence for the accumulation of an ES′ species under the conditions we used.

Deuterium Oxide Kinetic Solvent Isotope Effects

A significant difference between the deuterium oxide kinetic solvent isotope effects in the carboxypeptidase-catalyzed hydrolysis of the peptide substrate *N*-(*N*-benzoylglycyl)-L-phenylalaninate and the specific ester substrate *O*-(*trans*-cinnamoyl)-L-β-phenyllactate has been observed.[35] A change in solvent from H_2O to D_2O can give rise to several complicating effects, such as conformational changes in the protein, differences in the solvation of the transition state for reaction, or a change in the activity of a nucleophile in the enzyme.[36] Such possible complications must be kept in mind when the kinetic results are interpreted, although some difficulties could be eliminated from consideration by appropriate experiments.

For the carboxypeptidase A catalyzed hydrolysis of the peptide there is no kinetic solvent isotope effect on the value of $k_{cat}/K_{m\ app}$, and the effect on k_{cat} ($k_{cat}^{H_2O}/k_{cat}^{D_2O} = 1.33 \pm 0.15$) is small compared with the effect expected for a reaction in which proton transfer occurs in a rate-determining step.

The values of $K_{m\ app}$ for the ester substrate *O*-(*trans*-cinnamoyl)-L-β-phenyllactate and K_i, the inhibition constant for the inhibitory hydrolysis product L-β-phenyllactate, are nearly unaffected by the change in solvent from H_2O to D_2O. However, the $k_{cat}^{H_2O}/k_{cat}^{D_2O}$ ratio for the reaction of the ester is approximately 2, suggesting that a catalytic step involving proton transfer is important in the carboxypeptidase A catalyzed hydrolysis of this compound.[37]

Proposed Mechanisms for the Carboxypeptidase A Catalyzed Hydrolysis of Synthetic Substrates

In Figure 1 the mechanism proposed for the action of carboxypeptidase A on esters and peptides is illustrated for esters. According to this mechanism, the carboxylate group of Glu-270 acts as a nucleophile, attacking the carbonyl group of the scissile ester (or peptide) bond in the substrate. The zinc ion at the active site of the enzyme serves to orient the carbonyl group of the substrate and perhaps to polarize it, facilitating attack by Glu-270. The tetrahedral intermediate formed breaks down to give an anhydride species with concomitant formation of the α-hydroxy acid product from the C-terminal portion of the substrate. Finally, the anhydride species decomposes, regenerating the free enzyme.

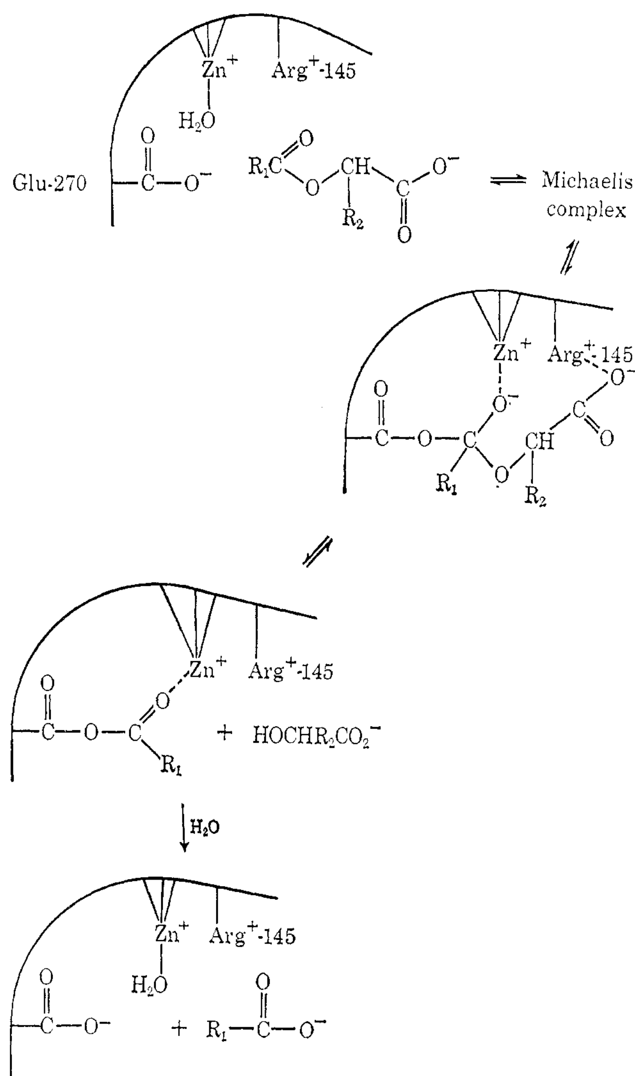


Figure 1. Proposed mechanism of action of carboxypeptidase A as an esterase and a peptidase. The O in an ester is replaced by an NH group in a peptide. A single positive charge is shown for the zinc ion because the other positive charge is neutralized by a carboxylate ligand from the enzyme. For those substrates where modification of tyrosine residues appears to alter carboxypeptidase reactivity significantly, it is still unclear whether the binding or catalytic steps are affected.

We will consider now how this mechanism accommodates the data which have been discussed. First, it is clear that the conclusions drawn from the examination of structure–reactivity relationships for carboxypeptidase A substrates in the hydrolysis of both esters and

(33) B. L. Kaiser, Ph.D. Thesis, University of Chicago, 1970.
(34) G. Tomalin, B. L. Kaiser, and E. T. Kaiser, *J. Amer. Chem. Soc.*, **92**, 6046 (1970).
(35) B. L. Kaiser and E. T. Kaiser, *Proc. Nat. Acad. Sci. U. S.*, **64**, 36 (1969).
(36) W. P. Jencks, *Annu. Rev. Biochem.*, **32**, 657 (1963).
(37) A similar finding was made in the hydrolysis of the very reactive ester *O*-(*N*-benzoylglycyl)-L-β-phenyllactate (unpublished results of Dr. F. A. Quiocho, quoted in ref 8).

peptides are consistent with the mechanism of Figure 1.

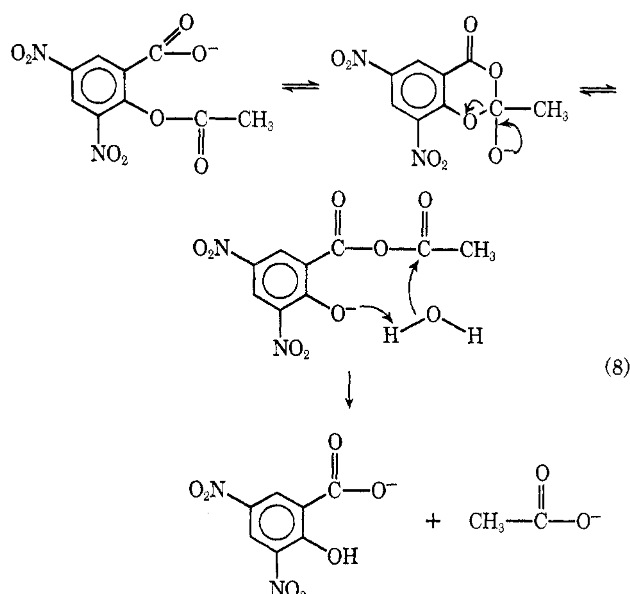
Furthermore, from the pH–rate profile studies described for the carboxypeptidase A catalyzed hydrolysis of esters and peptides, it was suggested earlier that in the reactive state of the enzyme the free carboxyl group of Glu-270 must be in its anionic form and the water ligand bound to the active site Zn^{2+} should be un-ionized. This is in accord with the mechanism of Figure 1.

The observation that *O*-(*trans*-cinnamoyl)-L-mandelate, a nonspecific ester substrate, binds at a site similar to that occupied by the specific ester *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactate supports the hypothesis that both nonspecific and specific ester substrates hydrolyze *via* the mechanism of Figure 1.

Lastly, the absence of a deuterium oxide solvent isotope effect on $k_{cat}/K_{m\ app}$ in the carboxypeptidase A catalyzed hydrolysis of *N*-(*N*-benzoylglycyl)-L-phenylalanine is also consistent with the mechanism of Figure 1. The observation of a significant effect would not be expected if anhydride formation were rate controlling. However, a deuterium oxide kinetic solvent isotope effect of 2 on the value of the first-order rate constant has been found for the spontaneous hydrolysis of acetic anhydride at 25°.[38] This indicates that in the carboxypeptidase A catalyzed hydrolysis of the specific ester substrate *O*-(*trans*-cinnamoyl)-L-β-phenyllactate the finding of a similar kinetic solvent isotope effect can be rationalized by postulating rate-controlling hydrolytic breakdown of an anhydride intermediate. Failures to detect the buildup of such intermediates[34] in the hydrolysis of ester substrates can be understood in this case in terms of the reversibility of the anhydride formation step of Figure 1. Presumably, the overall equilibrium between the starting materials (enzyme and substrate) and the anhydride intermediate plus α-hydroxy acid lies to the side of the starting materials in this case,[39] and the intermediate does not accumulate in solution.

Model System Studies

An organic model system relevant to the mechanism proposed in Figure 1 for the action of carboxypeptidase is the hydrolysis of 3,5-dinitroaspirin which involves rate-controlling breakdown of an anhydride intermediate, exhibiting a deuterium oxide kinetic solvent isotope effect of 2.[40] The mechanism proposed for this reaction is given in eq 8. The equilibrium between the starting material and the anhydride intermediate lies to the side of the starting material, and the concentration of the anhydride intermediate does not build up to a spectrophotometrically detectable level. The rate of the hydrolysis of the starting ester is controlled by the breakdown of the anhydride intermediate although the latter species is present at a very low concentration. This behavior is similar to what we postulate occurs in the carboxypeptidase-catalyzed hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate.



(8)

Few model system studies on zinc ion catalysis have been reported and, in general, relatively small catalytic effects due to this metal ion have been seen in ester[41] or peptide hydrolysis. Additional studies on model systems designed to simulate the basic features of carboxypeptidase action would obviously be an aid in furthering the understanding of the efficiency of catalysis by this enzyme.

Conclusion

In conclusion, we would stress that the mechanism proposed is the simplest one we can devise to account for the data now available on the carboxypeptidase A catalyzed hydrolysis of synthetic ester and peptide substrates which do not exhibit complex kinetic behavior.[42] We are continuing to test this mechanistic hypothesis and to examine further the complex phenomena of substrate activation and inhibition.[43]

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(38) A. R. Butler and V. Gold, *J. Chem. Soc.*, 2305 (1961).

(39) Although an attempt to detect carboxypeptidase A catalyzed transesterification was not successful in the case of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate this does not rule out the reversibility of the anhydride formation step of Figure 1 (P. L. Hall and E. T. Kaiser, *Biochem. Biophys. Res. Commun.*, **29**, 205 (1967)). It may mean simply that the rate of binding of free L-β-phenyllactate from solution to the anhydride species is slow compared to the hydrolysis of this intermediate. Perhaps only the enzymatically bound L-β-phenyllactate generated by the anhydride formation step reverses this step appreciably.

(40) A. R. Fersht and A. J. Kirby, *J. Amer. Chem. Soc.*, **90**, 5818 (1968).

(41) R. Breslow and D. Chipman, *ibid.*, **87**, 4195 (1965).

(42) At the present time, however, an alternative fairly simple hypothesis which was considered earlier cannot be ruled out.[35] According to that hypothesis, the carboxylate of Glu-270 might act as a nucleophile in the hydrolysis of peptides like *N*-(*N*-Bz-Gly)-L-Phe and as a general base catalyst in the hydrolysis of some esters like *O*-(*trans*-cinnamoyl)-L-β-phenyllactate.

(43) NOTE ADDED IN PROOF. Since the submission of this Account, chemical modification studies have been reported which are in accord with the assignment made here of pK_{a_1} to the ionization of the carboxyl group of Glu-270: G. M. Hass and H. Neurath, *Biochemistry*, **10**, 3541 (1971), and references therein.