Proc. Natl. Acad. Sci. USA
Vol. 73, No. 11, pp. 3882–3886, November 1976
Biochemistry

Mechanism of action of carboxypeptidase A in ester hydrolysis

(acyl-enzyme intermediate/cryoenzymology/enzyme kinetics/enzyme mechanisms)

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Communicated by Donald F. Steiner, August 16, 1976

ABSTRACT **The reaction of carboxypeptidase A (peptidyl-L-amino-acid hydrolase; EC 3.4.12.2) with the specific ester substrate *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate has been investigated in the temperature range 25° to −40° with use of organic-aqueous cosolvent mixtures. In the subzero temperature range the hydrolysis reaction is characterized by a biphasic decrease in absorbance specific for the substrate. The kinetic data can be unambiguously analyzed as two consecutive first-order reactions with formation of a covalent acyl-enzyme intermediate. Deacylation of the covalent intermediate is shown to be rate-limiting in the subzero temperature range, and near −60° it is sufficiently stable for spectral characterization. Consideration of the structure of the active site and of the catalytically functional residues of the enzyme leads to the conclusion that the intermediate is a mixed anhydride in which the γ-carboxylate of glutamate-270 is acylated by the substrate. The temperature dependence of the rate constants of the acylation and deacylation steps explains why the intermediate of this enzyme-catalyzed reaction is observed only at low temperatures.**

The ultimate objective in the study of any enzyme reaction is to elucidate the mechanism of action. For this goal, dynamic information must be obtained by detection and characterization of discrete intermediates in the catalytic conversion of substrates to products. The application of rapid mixing devices and relaxation methods (1, 2) to detect enzyme-substrate intermediates and to characterize their lifetimes has been successful for a variety of enzymes. Despite these advances in rapid kinetic methods, studies to detect formation or accumulation of intermediates in the hydrolysis of substrates by pancreatic carboxypeptidase A (peptidyl-L-amino-acid hydrolase; EC 3.4.12.2) have been thus far unsuccessful (3). Furthermore, the mechansism of action of carboxypeptidase A has remained conjectural despite a wide variety of thorough kinetic, structural, and chemical studies (3–5).

One method to overcome problems in detecting catalytically productive enzyme-substrate complexes is to lengthen their lifetime under conditions of very low temperature. To this end the properties of a wide variety of binary and ternary organic-aqueous cosolvent mixtures have been characterized for use in the subzero temperature range (6–8). By application of subzero temperature conditions generally over a range of 0° to −100°, reaction intermediates preceding the rate-limiting step can be accumulated and stabilized for physical characterization with relatively greater ease. The general applicability of these methods has been demonstrated for a variety of enzyme reactions (8).

We report in this communication the application of mixed cosolvent methods at subzero temperatures to determine the mechanism of action of carboxypeptidase A in ester hydrolysis. We present evidence demonstrating the mechanism of the reaction and formation of a covalent enzyme-substrate intermediate. Since mixed cosolvent methods at subzero temperatures have been applied, thus far, only to enzymes for which physical or chemical studies have provided prior evidence of the mechanism and of reaction intermediates (8), these studies consequently illustrate an example of application of subzero cosolvent techniques to determine the mechanism of an enzyme-catalyzed reaction.

EXPERIMENTAL

The α-isozyme of carboxypeptidase A was obtained in crystalline form from Sigma Chemical Co. (lot no. 65C-8410 and 16C-8205). The crystalline preparation was thrice washed in cold deionized water followed by centrifugation and finally dissolved in 0.02 M cacodylate buffer at pH 7.5 containing 1.3 M NaCl. The specific ester substrate *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (ClCPL) was prepared as described earlier (9). Organic-aqueous cosolvent mixtures containing ethylene glycol, methanol (MeOH), and H_2O were prepared according to Douzou *et al.* (7). Carboxypeptidase A was added to buffered ethylene glycol-H_2O near 0° and cooled to at least −20° for the addition of MeOH for kinetic studies with enzyme in excess. This procedure avoided precipitation of the enzyme at high concentrations upon addition of MeOH. This procedure was not necessary when dilute concentrations of enzyme were used with substrate in excess.

For thermostating a spectrophotometer cuvet, a specially constructed chamber (A. K. Churg, M. W. Makinen, and G. Gibson, manuscript in preparation) equipped with a mechanical stirrer having variable chopping frequency and stirring amplitude was adopted for use in a Cary 15 recording spectrophotometer. The mixing time of addition of substrate to enzyme under the highest viscosity conditions of our kinetic studies (about 66 centipoise; 0.066 Pa·sec) was no greater than 15–20 sec and generally was less than 5 sec for a solvent viscosity of less than 30 centipoise (0.030 Pa·sec). Under conditions used to identify the spectrum of the covalent intermediate [40% ethylene glycol, 20% MeOH, 40% H_2O with solvent viscosities of approximately 400 centipoise (~0.4 Pa·sec) near −60° (ref. 7)], the mixing time was approximately 30–50 sec. Temperature control of reaction mixtures to ±0.3° was maintained over the entire temperature range investigated.

Kinetic data were evaluated with use of a nonlinear regression computer program written by Dr. B. A. Blumenstein (Department of Statistics and Biometry, Emory University, Atlanta, Ga.).

RESULTS

The carboxypeptidase A-catalyzed hydrolysis of ClCPL was investigated in the subzero temperature range in efforts to detect directly formation of an intermediate enzyme-substrate

Abbrevations: ClCPL, *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate; MeOH, methanol.

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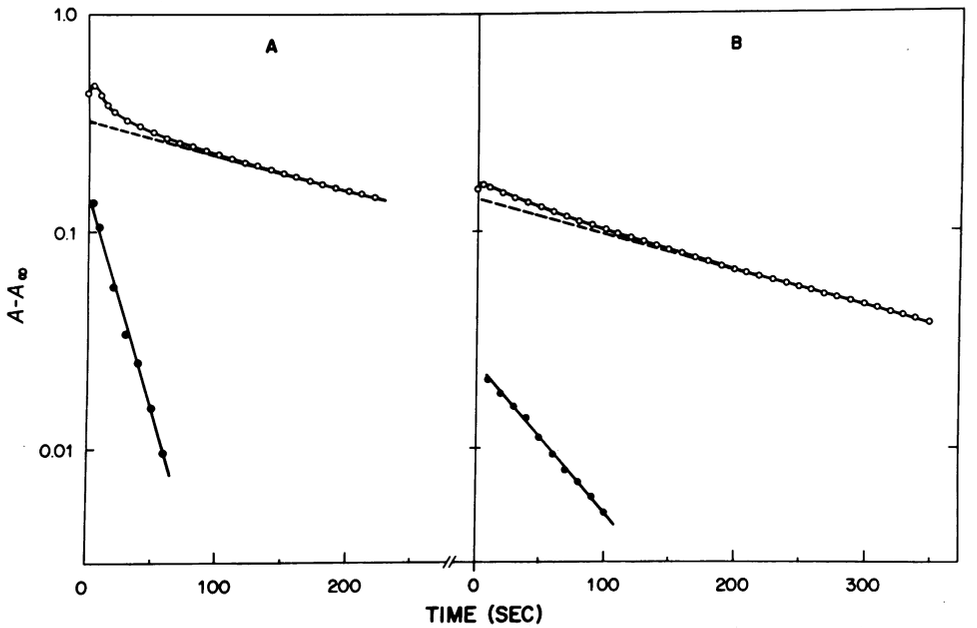


FIG. 1. First-order plots of the decrease in absorbance at 310 nm during hydrolysis of ClCPL by carboxypeptidase A at −40° illustrating graphically the characteristic biphasic decrease in substrate absorbance. Enzyme solutions were prepared in the ternary cosolvent mixture containing 25% ethylene glycol, 25% MeOH, and 50% H_2O buffered to pH 7.5 at −40° with 0.01 M sodium cacodylate at an ionic strength of 0.26 M NaCl. (A) The total enzyme concentration is 1.03×10^{-4} M and the initial substrate concentration is 4.83×10^{-5} M. (B) The enzyme concentration is 5.92×10^{-5} M and the initial substrate concentration is 1.0×10^{-5} M. The observed absorbance decrease is indicated by the open circles (○); the separated fast step with rate constant k_f, by the closed circles (●). The dashed line indicates the separated slow step with rate constant k_s.

complex. In these studies enzyme concentrations were maintained in excess of the substrate to avoid complication from product inhibition by L-β-phenyllactate (9). In the range −25° to −40° a biphasic decrease in absorbance specific for the substrate was observed. As illustrated in Fig. 1 for two different enzyme concentrations, a fast first-order reaction was observed as a deviation from the linear portion of a slower step in pseudo-first-order plots of the kinetic data.

The temperature dependence of the two observed rate constants, k_f for the fast reaction and k_s for the slow reaction, is given in Fig. 2 as Arrhenius plots. The plots of log k against $1/T$ for each set of data fall on a straight line, indicating that two separate reactions are monitored spectrophotometrically. The kinetic data thus clearly indicate that the reaction of the enzyme with added substrate is associated with formation of at least one intermediate complex. From Fig. 2 it is apparent that the ratio of k_f/k_s should be approximately 40:1 near −60° and that at this low temperature accumulation of the intermediate, if formed prior to the slow reaction, should be possible.

To demonstrate directly a stable intermediate at this temperature, the ternary mixture of 40% ethylene glycol–20% MeOH–40% H_2O was used because of its lower freezing point (7). In Fig. 3 the solid line shows the difference absorption spectrum in the ultraviolet region of the low temperature stabilized intermediate obtained at −58° for the carboxypeptidase A catalyzed reaction. The difference spectrum was clearly different from that of an equimolar mixture of enzyme and product and at this temperature remained unchanged for periods of time of at least 90 min.

To demonstrate that the species identified in Fig. 3 must be a covalent complex of the substrate bound to the enzyme, we added the tightly bound inhibitor L-benzylsuccinate (10) to 10^{-4} M concentration to the reaction mixture at −58° after formation of the spectrally identified species. The spectrum of the complex formed between enzyme and substrate remained unchanged upon addition of the inhibitor. Furthermore, the difference spectrum of the enzyme-substrate complex near −60° was not observed if substrate was added after addition of the inhibitor to the enzyme solution.

The stability of the covalent intermediate was also investigated under conditions of partial denaturation of the enzyme-substrate complex. Upon formation of the covalent complex at −58°, the solution was adjusted to approximately 3.8 M urea, the maximum concentration that could be achieved at −58°. The difference spectrum of the covalent species under these conditions, illustrated in Fig. 3 by the broken line, remained unchanged for at least 60 min after addition of urea. Breakdown of the covalent intermediate in 3.8 M urea at pH 7.5 in the temperature range −40° to −30° was associated with a rate constant of approximately 10^{-3} sec^{-1}.

The reaction of carboxypeptidase A with ClCPL under conditions of substrate in excess (11–13) was also investigated in the temperature range 25° to −10°. The temperature dependence of log k_{cat} (defined in ref. 3) is indicated in Fig. 2.

DISCUSSION

The data presented in Figs. 1–3 clearly indicate that the two kinetically discrete reactions with rate constants k_f and k_s are correlated with formation and breakdown of a covalent enzyme-substrate complex in the subzero temperature range. The demonstration that two separate processes occur with formation of an intermediate provides an experimental basis for the analysis of the hydrolysis of the substrate ClCPL by carboxypeptidase A in terms of two consecutive first-order reactions represented by the general scheme shown in Eq. 1

$$E + S \xrightarrow{k'} ES' \xrightarrow[+P_1]{k''} E + P_1 + P_2. \qquad [1]$$

Here ES′ represents the covalent enzyme-substrate complex that can be distinguished from a much less tightly bound Mi-

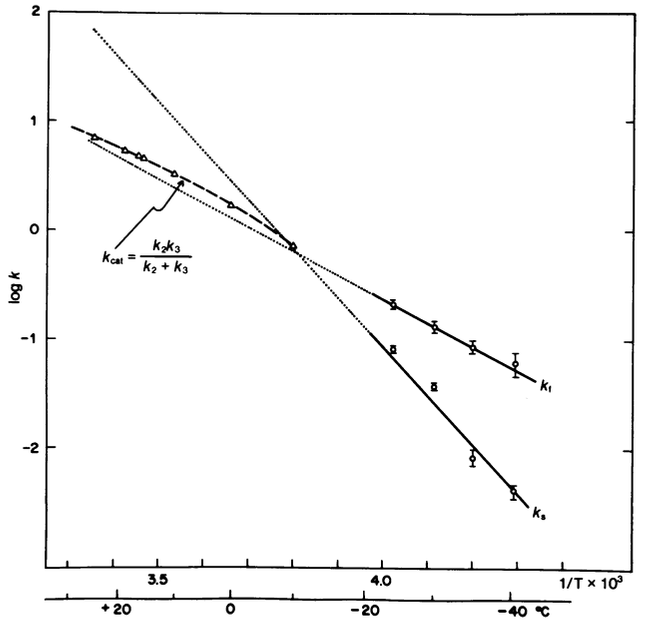


FIG. 2. Arrhenius plots of the temperature dependence of the rate constants of the carboxypeptidase A-catalyzed hydrolysis of ClCPL. The rate constants k_{cat}, k_s, and k_f are defined in the *text*. The k_{cat} values are plotted as open triangles (△). The separate plots for the two rate constants k_f and k_s are accordingly labeled. The dotted lines indicate the extrapolation of k_f and k_s to room temperature. The initial enzyme and substrate concentrations were 1.03×10^{-4} M and 4.83×10^{-5} M, respectively, for determination of the k_s and k_f rate constants. Solvent conditions are otherwise as in the legend of Fig. 1.

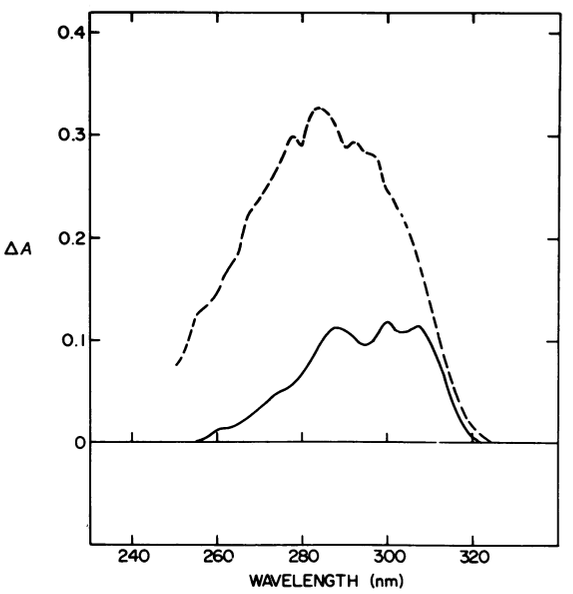


FIG. 3. The ultraviolet difference absorption spectrum of an equimolar mixture of carboxypeptidase A and ClCPL at −58°. The ternary solvent consisting of 40% ethylene glycol, 20% MeOH, and 40% H_2O was buffered with 0.01 M sodium cacodylate at pH 7.5 and contained 0.5 M NaCl in the sample cuvette. The reference consisted of two cuvettes in tandem, one cuvette containing a 1.0×10^{-5} M solution of ClCPL in the same ternary solvent while the second cuvette contained a 1.0×10^{-5} M enzyme solution in a solution consisting of a mixture of 40% ethylene glycol and 60% H_2O buffered otherwise identically with 0.01 M sodium cacodylate at pH 7.5 in 0.5 M NaCl. Both reference cuvettes were maintained at ambient temperature, and MeOH was not added to the reference enzyme solution to prevent precipitation of the protein. The baseline was thus obtained with a 1.0×10^{-5} M solution of the enzyme at −58° compared with an enzyme solution of identical concentration at ambient temperature in the absence of the added substrate. The solid line indicates the difference spectrum of the covalent intermediate. The dashed line indicates the difference spectrum of the covalent intermediate upon addition of 3.8 M urea to the solution of the covalent intermediate and to the reference cuvette containing ClCPL only at ambient temperature. An extinction coefficient of 22,000 M^{-1} cm^{-1} at 310 nm for the intermediate enzyme-substrate complex (ES′) is derived with respect to that of 12,000 M^{-1} cm^{-1} for ClCPL at the same wavelength (13, 19).

chaelis-type complex by addition of the tightly bound inhibitor. We consequently indicate that the product P_1, L-β-phenyllactate, is formed simultaneously with the covalent reaction intermediate. Under conditions of enzyme in excess, we assume that k' can be treated as a first-order rate constant. The validity of this assumption is demonstrated later.

In general, the analysis of two consecutive first-order reactions leads to a straightforward mathematical relationship between the time-dependent change in absorbance and the concentrations of reactant, intermediate, and product (14). For the specific case represented by Eq. 1, we can describe the time-dependent change in absorbance at 310 nm, as illustrated in Fig. 1, by Eq. 2

$$A - A_\infty = S_0[\epsilon_{ST} - \epsilon_{P_1} + \left[\frac{(\epsilon_{ES'} - \epsilon_{P_2})k'}{k'' - k'}\right]e^{-k't} + S_0\left[\frac{(\epsilon_{P_2} - \epsilon_{ES'})k'}{k'' - k'}\right]e^{-k''t} \quad [2]$$

or Eq. 3

$$A - A_\infty = ae^{-k't} + be^{-k''t}. \quad [3]$$

In Eq. 2, S_0 is the initial substrate concentration, $S_0\epsilon_{ST}$ represents the maximum initial absorbance upon mixing, and the various ϵs refer to the molar extinction coefficients of the chemical species of Eq. 1 designated by corresponding subscripts. With respect to the rate data like those presented in Fig. 1, the continuously decreasing absorbance change can be described only by the condition that $b > 0$.

Further analysis of the kinetic data to assign the rate-determining step of Eq. 1 rests on the values of the extinction coefficients of the absorbing species estimated at 310 nm under the reaction conditions. The product P_2, *p*-chlorocinnamate, exhibits an extinction value of approximately 2500 M^{-1} cm^{-1}. In Fig. 3 the difference absorption spectrum of the ES′ intermediate yields an extinction coefficient of 22,000 M^{-1} cm^{-1} under the assumption that all of the added substrate is converted into the covalent intermediate. Computer-aided estimates of $\epsilon_{ES'}$ from kinetic data at −40° yield an absorptivity value of 17,200 M^{-1} cm^{-1}, in good agreement with the difference spectrum. Consequently the condition $b > 0$ holds only if k'' is assigned as k_s for the slow reaction while k' is assigned as k_f of the fast reaction of Fig. 2. These results, thus, provide unambiguous evidence to assign the sequential order of the rate constants associated with the two kinetically discrete processes. We, therefore, conclude that k_f is the observed rate constant for acylation of the enzyme by ClCPL and k_s is the observed rate constant for hydrolysis of the acyl-enzyme intermediate ES′. Consequently, under our low temperature reaction conditions, acylation of the enzyme proceeds kinetically faster than deacylation.

Prior to formation of the covalent intermediate ES′, the reaction of carboxypeptidase A with ClCPL must involve simple binding of the substrate to the enzyme as a Michaelis complex.

Table 1. Comparison of activation energy parameters for the hydrolysis of esters and anhydrides

	E_a	$\Delta G‡$	$\Delta H‡$	$\Delta S‡$
		(kcal/mole)		(e.u.)
Ester saponification[a]	15	20.3	13.7	−22.13
Anhydride hydrolysis[b]	11	21.0	9.8	−37.6
Deacylation of acetyl-α-chymotrypsin[c]	10.3	20.4	9.7	−35.9
Deacylation of *trans*-(cinnamoyl)-α-chymotrypsin[c]	11.8	20.1	11.2	−29.9
Acylation of carboxypeptidase A by ClCPL[d]	8.9 ± 1.9	16.4 ± 1.9	8.3 ± 1.9	−27.0 ± 6.0
Deacylation of (*trans*-*p*-chlorocinnamoyl)-carboxypeptidase A[d]	20.6 ± 2.3	14.9 ± 2.3	20.0 ± 2.3	17.0 ± 7.7

[a] Average values obtained for a series of alkyl ester derivatives (24, 25).
[b] Results are those of Gold (21) for acetic anhydride in pure aqueous medium. Comparable values are observed for aromatic anhydrides and mixed anhydrides (26).
[c] Ref. 22.
[d] Errors are estimated on the basis of the maximum and minimum slopes that can be drawn within the error bars for the data plotted in Fig. 2.

On this basis the reaction represented by Eq. 1 can be modified to that of Eq. 4

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_1 + P_2 \qquad [4]$$
$$\qquad\qquad +P_1$$
$$E + P_1 \overset{K_p}{\rightleftharpoons} EP_1.$$

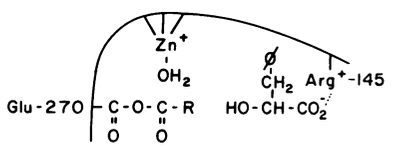
For this mechanistic scheme of the kinetics of hydrolysis of ClCPL by carboxypeptidase A, appropriate equations defining the rate parameters k_{cat} and $K_{m_{app}}$ under conditions of competitive product inhibition have been derived (12, 13).

Under conditions of excess enzyme used for first-order plots, the extrapolation of k_f to 25° in Fig. 2 yields a value of 6 sec^{-1}, in reasonable agreement with the observed k_{cat} value of 6.34 ± 0.02 sec^{-1}. The demonstration in Fig. 2 that the graphically extrapolated value of k_f at 25° is identified with k_{cat} provides the basis for the conclusion that the two processes with observed rate constants k_f and k_s correspond, respectively, to the acylation and deacylation steps of the enzyme reaction in Eq. 4.

We also note in Fig. 2 that the extrapolated values in the high temperature range are consistent with the condition that $k_{cat} \simeq k_2$ when $k_3 \gg k_2$. Under normal room temperature conditions, therefore, ester hydrolysis proceeds with acylation of the enzyme as the rate-limiting step, and this accounts for previous failures to detect or accumulate intermediates of hydrolysis reactions of carboxypeptidase A with esters (3, 9). It is, thus, significant to point out that in the low temperature region the deacylation step has become rate-limiting for hydrolysis of ClCPL, a condition that permits accumulation of the covalent intermediate.

The observed rate constant of 10^{-3} sec^{-1} for hydrolysis of the denatured covalent intermediate provides a meaningful indication of the nature of the enzyme residue that has been acylated by the substrate. The corresponding rate constant for hydrolysis of the phenyl ester of *p*-chlorocinnamic acid (15, 16) corrected to pH 7.5 at 25° would be approximately 10^{-10} sec^{-1}. We conclude, therefore, that catalytic participation of active site tyrosine residues (17, 18) in carboxypeptidase A as an attacking nucleophile to form a covalent intermediate of ester linkage is unlikely and remains incompatible with the rate constant for deacylation of the denatured intermediate. Furthermore, acetylation of tyrosine-248 has no influence on the activity of carboxypeptidase A towards ClCPL (19).

The results of high resolution x-ray studies (4, 20) have indicated that the only remaining residues in the active site region that are near enough to the substrate to be catalytically involved are the Zn^{+2} ion and the side-chain of glutamate-270, while arginine-145 binds the terminal carboxylate group on an electrostatic basis. We conclude that the covalent intermediate ES′ of the reaction of carboxypeptidase A with ClCPL in Eq. 4 is a mixed anhydride whereby the carbonyl carbon of the *p*-chlorocinnamoyl portion is bonded to a carboxylic oxygen atom of glutamate-270. In support of this conclusion, we note that the rate constant for the decomposition of the denatured intermediate is not unlike that expected for a mixed anhydride (21) at −30°. The increase in positive absorption over the long wavelength region, moreover, indicates retention of the α,β-conjugated aromatic structure of the cinnamoyl portion, suggesting that the covalent species identified in Fig. 3 is not associated with a change of the substrate moiety to a tetrahedral configuration at the cinnamoyl carbonyl carbon. We suggest that the covalent intermediate identified in Fig. 3 has Structure I



STRUCTURE I.

in which the general structural relationships between the catalytically functional residues and the substrate acyl moiety are schematically illustrated.

As seen in Table 1, comparison of the activation energy parameters obtained here with other data clearly indicates that the mechanism of deacylation of the covalent intermediate of carboxypeptidase A is different from that for the hydrolysis of simple esters and of the corresponding *trans*-cinnamoyl-α-chymotrypsin intermediate studied by Bender *et al.* (22). For no hydrolytic enzyme catalyzed reaction has a comparably large positive entropy of activation been observed. This large positive value indicates that the covalent intermediate is more highly constrained and ordered than the products of the reaction and that its formation is clearly associated with an efficient hydrolytic process resulting in its breakdown. There are several factors that should contribute to the positive entropy of activation observed in deacylation. The more important of these are probably release of the L-β-phenyllactate group and the motional freedom acquired by side chains of the protein upon hydrolysis of the covalent intermediate. Additionally, it has not escaped our attention that a positive entropy of activation frequently is associated with disordering of structured water, as in replacement by a ligand of a metal ion coordinated water molecule (23).

Despite the wide variety of chemical, structural, and kinetic studies of carboxypeptidase A, the mechanism of action for both peptide and ester hydrolysis has remained hitherto conjectural. On the basis of x-ray diffraction studies (4, 20), including a variety of difference Fourier syntheses of bound substrates, the detailed structural basis for the catalytic role of the Zn^{+2} ion and of the side chains of glutamate-270, arginine-145, and tyrosine-248 has been outlined for peptide hydrolysis. Significantly, however, no esterolytic substrate has been sufficiently sluggish in reactivity with the enzyme to permit x-ray data collection at normal temperatures for difference Fourier studies (4). The value of using cryoenzymologic techniques is consequently well demonstrated, particularly since our results have indicated the covalent intermediate to be a mixed anhydride formed by acylation of the γ-carboxylate group of glutamate-270. The demonstration through our studies of not only the mechanism of the ester hydrolysis reaction with use of a specific ester substrate, but also of a covalent acyl-enzyme intermediate serves consequently as an incisive step in better understanding the structural basis of catalytic function of this important enzyme.

We are indebted to Dr. A. K. Churg for assistance in the construction and design of the cuvet chamber and mixing device used in these studies. We acknowledge helpful discussions with Prof. F. J. Kèzdy. This work has been supported by grants of the National Institutes of Health awarded to M.W.M. (GM 21900) and to E.T.K. (AM 07957). M.W.M. is an Established Investigator of the American Heart Association.

1. Gibson, Q. H. (1966) *Annu. Rev. Biochem.* **35,** 435–456.
2. Hammes, G. & Schimmel, P. R. (1970) in *The Enzymes,* ed. Boyer, P. D. (Academic Press, New York), 3rd ed., Vol. II, pp. 67–114.
3. Kaiser, E. T. & Kaiser, B. L. (1972) *Acc. Chem. Res.* **5,** 219–224.
4. Hartsuck, J. A. & Lipscomb, W. N. (1971) in *The Enzymes,* ed. Boyer, P. D. (Academic Press, New York), 3rd ed., Vol. III, pp. 1–56.
5. Makinen, M. W. (1975) in *Techniques and Topics in Bioinorganic Chemistry,* ed. McAuliffe, C. A. (Macmillan, London), pp. 1–106.
6. Hui Bon Hoa, G. & Douzou, P. (1973) *J. Biol. Chem.* **248,** 4649–4654.
7. Douzou, P., Hui Bon Hoa, G., Maurel, P. & Travers, F. (1976) in *Handbook of Biochemistry and Molecular Biology,* ed. Fasman, G. D. (Chemical Rubber Co., Cleveland, Ohio), 3rd ed., in press.
8. Douzou, P. (1973) *Mol. Cell. Biochem.* **1,** 15–27.
9. Tomalin, G., Kaiser, B. L. & Kaiser, E. T. (1970) *J. Am. Chem. Soc.* **92,** 6046–6049.
10. Byers, L. D. & Wolfenden, R. (1973) *J. Biol. Chem.* **247,** 606–608.
11. Carson, F. W. & Kaiser, E. T. (1966) *J. Am. Chem. Soc.* **88,** 1212–1223.
12. Hall, P. L., Kaiser, B. L. & Kaiser, E. T. (1969) *J. Am. Chem. Soc.* **91,** 485–491.
13. Suh, J. (1975) Ph. D. Dissertation, University of Chicago.
14. Benson, S. W. (1960) *The Foundations of Chemical Kinetics* (McGraw-Hill, New York), pp. 33–36.
15. Satpathy, K. K., Nayak, P. L. & Rout M. K. (1971) *Indian J. Chem.* **9,** 680–681.
16. Bernhard, S. A., Lau, S. J. & Noller, H. (1965) *Biochemistry* **4,** 1108–1118.
17. Johansen, J. T. & Vallee, B. L. (1975) *Biochemistry* **14,** 649–660.
18. Johansen, J. T., Klyosov, A. A. & Vallee, B. L. (1976) *Biochemistry* **15,** 296–303.
19. Suh, J. & Kaiser, E. T. (1976) *J. Am. Chem. Soc.* **98,** 1940–1947.
20. Quiocho, F. A. & Lipscomb, W. N. (1971) *Adv. Protein Chem.* **25,** 1–78.
21. Gold, V. (1948) *Trans. Faraday Soc.* **44,** 506–518.
22. Bender, M. L., Kèzdy, F. J. & Gunter, C. R. (1964) *J. Am. Chem. Soc.* **86,** 3714–3721.
23. Basolo, F. & Pearson, R. G. (1967) *Mechanisms of Inorganic Reactions* (Wiley & Sons, Inc. New York), 2nd ed., pp. 193–207.
24. Smith, H. A. & Levenson, H. S. (1939) *J. Am. Chem. Soc.* **61,** 1172–1175.
25. Levenson, H. S. & Smith, H. A. (1940) *J. Am. Chem. Soc.* **62,** 1556–1558.
26. Bunton, C. A., Fuller, N. A., Perry, S. G. & Shiver, V. J. (1963) *J. Chem. Soc.* **1963,** 2918–2926.