Kisliuk, R. L., Gaumont, Y., & Baugh, C. M. (1974) *J. Biol. Chem. 249*, 4100–4103.
Koch, A. L., & Levy, H. R. (1955) *J. Biol. Chem. 217*, 947–957.
Kochi, H., & Kikuchi, G. (1974) *J. Biochem. (Tokyo) 75*, 1113–1127.
Krebs, H. A., & Veech, R. L. (1969) *FEBS-Symp. No. 17*, 101–109.
Kung, H., Spears, C., Greene, R. C., & Weissbach, H. (1972) *Arch. Biochem. Biophys. 150*, 23–31.
Leimer, K. R., Rice, R. H., & Gehrke, C. W. (1977) *J. Chromatogr. 141*, 121–144.
Lomax, M. S., & Greenberg, G. R. (1967) *J. Biol. Chem. 242*, 1302–1306.
Marshak, A., & Vogel, H. J. (1951) *J. Biol. Chem. 189*, 597–605.
Meedel, T. H., & Pizer, L. I. (1974) *J. Bacteriol. 118*, 905–910.
Milner, L., Whitfield, C., & Weissbach, H. (1969) *Arch. Biochem. Biophys. 133*, 413–419.
Newman, E. B., & Magasanik, B. (1963) *Biochim. Biophys. Acta 78*, 437–448.
Pastore, E. J., & Friedkin, M. (1962) *J. Biol. Chem. 237*, 3802–3810.
Pizer, L. I. (1965) *J. Bacteriol. 89*, 1145–1150.
Powers, S. G., & Snell, E. E. (1976) *J. Biol. Chem. 251*, 3786–3793.
Ramasastri, B. V., & Blakley, R. L. (1964) *J. Biol. Chem. 239*, 112–114.
Rotman, B., & Spiegelman, S. (1954) *J. Bacteriol. 68*, 419–429.
Salem, A. R., Pattison, J. R., & Foster, M. A. (1972) *Biochem. J. 126*, 993–1004.
Tatum, C. M., Benkovic, P. A., Benkovic, S. J., Potts, R., Schleicher, E., & Floss, H. G. (1977) *Biochemistry 16*, 1093–1102.
Taylor, R. T., Dickerman, H., & Weissbach, H. (1966) *Arch. Biochem. Biophys. 117*, 405–412.
Uyeda, K., & Rabinowitz, J. C. (1967) *J. Biol. Chem. 242*, 4378–4385.
Wall, J. S. (1953) *Anal. Chem. 25*, 950–953.
White, R. H. (1981) *Anal. Biochem. 114*, 349–354.

Cryokinetic Studies of the Intermediates in the Mechanism of Carboxypeptidase A†

Alphonse Galdes, David S. Auld, and Bert L. Vallee*

ABSTRACT: The detection and definition of intermediates in reaction pathways, a problem central to delineation of structure–function relationships in enzymology, have in general resisted solution. We have developed an approach capable of wide application to such studies employing a combination of cryokinetics and cryospectroscopy as exemplified here by using carboxypeptidase A. These studies are performed with a low-temperature stopped-flow instrument which also serves as a cryospectrometer [Auld, D. S. (1979) *Methods Enzymol. 61*, 318]. The intermediates are monitored directly through fluorescence generated by radiationless energy transfer (RET) between enzyme tryptophans and the dansyl group of enzyme-bound substrate. N-Dansylated oligopeptides and their ester analogues exhibit Michaelis–Menten kinetics over the temperature range −20 to +20 °C with k_{cat}/K_m values of (0.3–3) × 10^7 M^{-1} s^{-1} at +20 °C, pH 7.5, and the cryosolvent, aqueous 4.5 M NaCl, does not alter catalysis. There is no chemical evidence of a covalent acyl enzyme intermediate for any of the peptide or ester substrates studied. However, concurrent cryospectroscopy shows that peptides and esters form metallo intermediates whose spectra differ strikingly from one another and from that of the enzyme alone [Geoghegan, K. F., Galdes, A., Martinelli, R. A., Holmquist, B., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry* (in press)]. The cryokinetic data demonstrate for the first time the existence of *two* intermediates during the hydrolysis of both peptides and esters. At −20 °C, the formation, interconversion and breakdown of these intermediates results in three distinct fluorescence steps during substrate hydrolysis: (1) a rapid increase in signal intensity reflects the formation of the Michaelis complex, ES_1, in <15 ms; (2) a slower exponential increase in signal intensity signifies formation of a second hitherto unknown intermediate, ES_2; (3) a slow decrease in signal intensity reflects separation of the dansyl product from the enzyme. All rate and equilibrium constants for the reaction scheme $E + S \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightarrow E + P$ have been determined. The reversible interconversion of ES_1 and ES_2 shows that the C-terminal product is not liberated prior to the rate-limiting step and, hence, deacylation cannot be rate limiting. The cryokinetic studies in conjunction with the chemical evidence demonstrate that there is no acyl intermediate in either ester or peptide hydrolysis. The present cryokinetics and the concurrent cryospectroscopy show that peptides and esters form different metallo intermediates and that these two types of substrates are hydrolyzed through different mechanisms.

Radiationless energy transfer (RET)[1] allows direct detection of transient enzyme–substrate (ES) complexes, thereby providing a powerful approach to the investigation of enzymatic reactions (Auld, 1977; Lobb & Auld, 1979, 1980). RET arises from the transfer of energy between a fluorescent acceptor–donor pair, e.g., enzyme tryptophanyl residues and the blocking

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[1] Abbreviations: RET, radiationless energy transfer; Dns, dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; OPhe, L-β-phenyllactate; SPhe, α-mercapto-β-phenylpropionate; HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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group of bound N-dansylated substrates, and is proportional to the concentration of ES complexes.[2] It directly detects pre-steady-state and steady-state ES complexes and permits determination of the values of k_{cat} and K_m and of the individual rate and equilibrium constants of the reaction mechanism.

Characteristically, the rates of formation and interconversion of pre-steady-state transients are very rapid, so that special means are required to observe them. Thus, stopped-flow techniques which can resolve rates of several hundred reciprocal seconds and RET have been combined successfully to detect enzyme intermediates at ambient temperatures (Lobb & Auld, 1979). The rate of formation and breakdown of intermediates can also be decreased by lowering temperature, facilitating their identification and characterization (Douzou, 1977; Fink, 1977; Auld, 1979).

A stopped-flow instrument operating at temperatures as low as −55 °C (Hanahan & Auld, 1980) combined with RET kinetics provides a potent, novel approach to enzyme mechanisms. A wide variety of substrates and cryosolvents can be screened rapidly, and the kinetic and associated thermodynamic parameters can be determined readily. A unique feature of this instrument is that it is designed to function also as a rapid scanning cryospectrometer, permitting concurrent characterization of intermediates by both kinetic and spectral means (Auld, 1979). The acquisition of such structural information on pre-steady-state intermediates has remained just as difficult as their kinetic characterization.

Studies utilizing this general approach to the structure–function relationship in enzymes have now been performed on carboxypeptidase A, as will be reported in a series of communications beginning here with the kinetic characterization of pre-steady-state intermediates, to be followed in succeeding manuscripts by their structural investigations through synchronous spectroscopic studies.

In spite of detailed kinetic and structural investigations of this enzyme, its mode of action is still uncertain (Vallee et al., 1983, and references cited therein). In particular, two major issues remain unresolved. (i) Are covalent intermediates formed during hydrolysis of substrates? Based on the report of a covalent acyl enzyme intermediate during hydrolysis of the ester *O*-(*trans*-*p*-chlorocinnamoyl)-OPhe by carboxypeptidase A (Makinen et al., 1976), it has been claimed that all substrates form acyl intermediates with this enzyme (Makinen et al., 1979, 1982). This report must be viewed in the light of many others that fail to confirm such findings for other esters and for *any* peptide, including the peptide analogue of this cinnamoyl ester (Breslow & Wernick, 1977). (ii) Are all substrates hydrolyzed through the same mechanism? Among numerous mechanistic speculations, currently the most popular one asserts that both peptide and ester substrates follow the same pathway but have different rate-limiting steps (Cleland, 1977; Rees & Lipscomb, 1981). This hypothesis, however, is at variance with the results of many kinetic and chemical modification studies of carboxypeptidase A, which suggest that these two types of substrate can be hydrolyzed through different mechanisms [for references, see Vallee et al. (1983)].

We have studied the carboxypeptidase A catalyzed hydrolysis of N-dansylated oligopeptides and their depsipeptide analogues by RET cryokinetics and cryospectroscopy to examine these hypotheses. The data demonstrate the presence of two intermediates during the hydrolysis of both peptides and esters. While at room temperature their rates of formation are too fast to be measured, we have succeeded in characterizing them kinetically at −20 °C. No kinetic or chemical evidence for an acyl intermediate in carboxypeptidase A could be found. In addition, the spectral properties of steady-state intermediates formed with cobalt carboxypeptidase have been recorded and found to differ strikingly for peptides and esters, indicating differences in their mechanism of hydrolysis (Geoghegan et al., 1983). A preliminary account of this work has been given (Galdes et al., 1981).

Materials and Methods

Bovine carboxypeptidase A (Cox) was obtained as a crystalline suspension from Sigma Chemical Co. and purified further by affinity chromatography (Bazzone et al., 1979). Yeast carboxypeptidase was a gift from Dr. J. Johansen.

The substrates used were Dns-$(Gly)_n$-$(Ala)_m$-Z where n and m are the number of Gly and L-Ala residues, respectively, and where Z represents L-Phe for peptide substrates or L-OPhe for depsipeptide substrates, synthesized and characterized according to established procedures (Auld & Holmquist, 1974; Lobb & Auld, 1979). Product analysis by HPLC and thin-layer chromatography demonstrates that for all substrates hydrolysis occurs only at the C-terminal –Phe or –OPhe bond.

All aqueous solutions were rendered metal free by extraction with dithizone in carbon tetrachloride, and precautions were taken to avoid contamination by adventitious metal ions (Auld & Vallee, 1971). NaCl (4.5 M) was used as a cryosolvent, and the pH values of solutions at subzero temperatures were corrected for the temperature coefficient of the buffer.

Stopped-flow fluorescence measurements were performed with a low-temperature stopped-flow instrument interfaced with a Digital Equipment PDP 11/34 computer and VT-55 Descope graphics terminal (Hanahan & Auld, 1980). Steady-state kinetic parameters were determined from RET traces as previously described (Lobb & Auld, 1979, 1980). Turnover numbers, k, were computed from the measured values of maximal fluorescence, F_{max}, and total area described by the stopped-flow trace, A_0, and the known values of initial substrate concentration, $[S_0]$, and total enzyme concentration (Lobb & Auld, 1980). The kinetic parameters k_{cat} and K_m were determined by least-squares analyses of double-reciprocal plots of $1/k$ vs. $1/[S_0]$. Determination of k_{cat} and K_m from the progress curve at a single initial substrate concentration was accomplished by evaluating a series of intensities, F_t, and areas, A_t, at every tenth time interval, generating a series of 10–50 k_t and $[S]_t$ values. First-order rate constants were obtained by linear regression of $-\log (F_\infty - F_t)$ on time. All results were displayed on the VT-55 terminal for visual inspection and automatically recorded on floppy discs.

HPLC product analyses were carried out with a Waters Associates liquid chromatograph; 100-μL samples were applied to a 0.7 × 30 cm μBondapak C_{18} column and eluted with acetonitrile/water at a flow rate of 2 mL/min. The absorbance of the column effluent was monitored at 254 nm.

Results

The N-dansylated peptides and analogous depsipeptides studied are excellent substrates for carboxypeptidase A. The k_{cat} values range from 4 to 190 s^{-1} and those of K_m from 2 to 500 μM when assayed under standard conditions (10 mM Hepes–1 M NaCl, pH 7.5, 20 °C). The cryosolvent, 4.5 M NaCl, does not significantly affect the values of k_{cat} and K_m, which deviate by less than 2-fold from those obtained in 1 M NaCl, a normal constituent of assay mixtures added to render the enzyme soluble. Excellent RET signals are obtained at

[2] Although RET depends on the orientation of, and the distance between, the acceptor–donor pair, for kinetic analyses quantitation of these factors is not necessary.

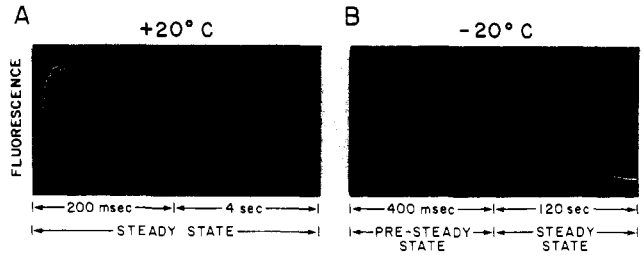

FIGURE 1: Stopped-flow RET measurement of the binding and hydrolysis of 50 μM Dns-$(Ala)_2$-Phe by 1 μM carboxypeptidase A in 10 mM Hepes–4.5 M NaCl, pH 7.5, at (A) +20 °C and (B) −20 °C.

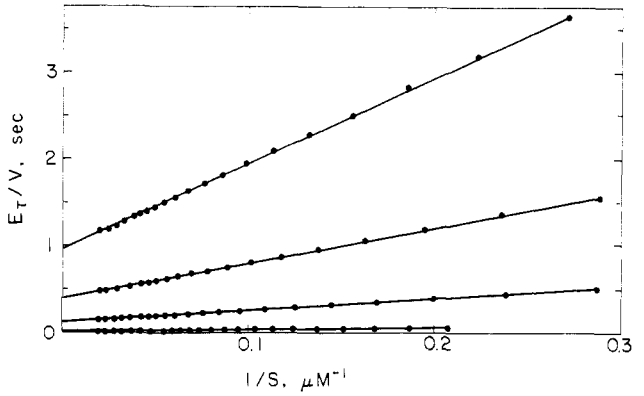

FIGURE 2: Lineweaver–Burk plots for the carboxypeptidase A (1 μM) catalyzed hydrolysis of Dns-$(Ala)_2$-Phe (50 μM) in 10 mM Hepes–4.5 M NaCl, pH 7.5, at temperatures of −19 (top), −11.2, −1.5, and 21.6 °C (bottom). The velocities are determined from the expression $V = F_t\,[S]_t/A_t$ (see Materials and Methods).

substrate concentrations ranging from 0.1 to 100 μM, and assays can be performed coveniently at [S] > [E] < K_m (Lobb & Auld, 1980). Figure 1 shows a typical example using the substrate Dns-$(Ala)_2$-Phe. At +20 °C the initial rapid increase in dansyl fluorescence to its maximum value signals rapid attainment of the steady-state concentration of ES complexes within the dead time of the stopped-flow instrument (Figure 1A). Concomitantly, the enzyme tryptophan fluorescence decreases due to RET (data not shown). The steady-state time interval is characterized by a slow decay in fluorescence which reflects reduction in the concentration of ES accompanying the conversion of substrate to products. In contrast, at −20 °C a pre-steady-state time interval persists for 400 ms (Figure 1B), and two intermediates are observed readily (see below).

All substrates exhibit Michaelis–Menten kinetics over the temperature range from −20 to +20 °C (Figure 2). Their kinetic parameters can be determined from the RET traces both by estimating the turnover number at various initial substrate concentrations and by analyzing the curves at a single substrate concentration (see Materials and Methods).

Arrhenius plots for the substrates are linear over this 40 °C temperature range (Figure 3), indicating that under these conditions the rate-limiting step does not change. For all substrates the dependence of K_m on temperature is slight but that of k_{cat} is strong. Table I lists the thermodynamic parameters for k_{cat} as determined for several peptides and esters.

At subzero temperatures the pre-steady-state time interval for all substrates is biphasic,[3] indicating the presence of two

[3] There are three major reasons for a pre-steady-state RET signal change during the interconversion of two ES complexes (Auld, 1977), any or all of which contribute to the observed biphasic trace: (i) the total concentration of the ES complexes changes with time; (ii) the transfer efficiency of the two complexes differs; (iii) the dansyl quantum yield for the two complexes differs.

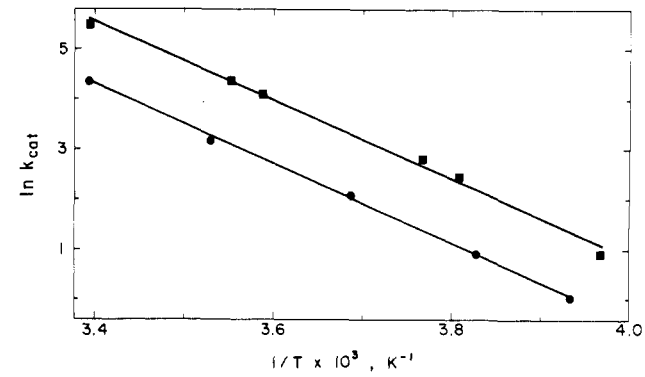

FIGURE 3: Arrhenius plots for the values of k_{cat} of (●) Dns-$(Ala)_2$-Phe in 10 mM Hepes–4.5 M NaCl, pH 7.5 (E_a = 15.8 kcal mol^{-1}), and (■) Dns-$(Gly)_3$-OPhe in 10 mM cacodylate–4.5 M NaCl, pH 7.0 (E_a = 15.6 kcal mol^{-1}).

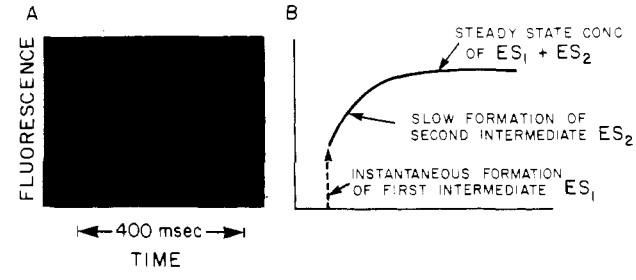

FIGURE 4: (A) Stopped-flow pre-steady-state RET trace for hydrolysis of 100 μM Dns-$(Gly)_3$-OPhe by 0.5 μM carboxypeptidase A in 10 mM Hepes–4.5 M NaCl, pH 7.5, at −20 °C. (B) Schematic representation of this trace, showing the two intermediates observed.

Table I:[a] Thermodynamic Parameters for k_{cat} of the Hydrolysis of Dansylated Substrates by Carboxypeptidase A

substrate	ΔH (kcal mol^{-1})	ΔS (eu)	ΔG (kcal mol^{-1})
peptides			
Dns-$(Ala)_2$-Phe	15.3	2.10	14.7
Dns-Gly-Ala-Phe	11.4	−9.46	14.2
Dns-$(Gly)_3$-Phe	15.2	2.47	14.4
esters			
Dns-$(Ala)_2$-OPhe	18.2	4.78	16.7
Dns-Gly-Ala-OPhe	18.1	8.43	15.5
Dns-$(Gly)_3$-OPhe	15.0	4.45	13.7

[a] Assays were performed in 10 mM Hepes–4.5 NaCl, pH 7.5. The parameters were calculated from Arrhenius plots over the temperature range from 20 to −20 °C by using a standard state of 25 °C and 1 mol L^{-1} for the calculations.

ES complexes, as shown at −20 °C for Dns-$(Ala)_2$-Phe and Dns-$(Gly)_3$-OPhe in Figures 1B and 4A, respectively. When substrate is mixed with enzyme, there is an initial, very rapid (<15 ms) rise in dansyl fluorescence within the dead time of the instrument (Figure 4B), corresponding to formation of the Michaelis complex (ES_1). This is followed by a slower exponential rise, due to formation of a second intermediate (ES_2), with a first-order rate constant, k_{obsd}.

The variation of k_{obsd} with $[S_0]$ is consistent with the following reaction scheme (Auld, 1977)

Scheme I

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \xrightarrow{k_3} E + P$$

It predicts that k_{cat}, K_m, and k_{obsd} are related to the individual rate and equilibrium constants as follows:

$$k_{cat} = k_2k_3/(k_2 + k_{-2} + k_3) \quad (1)$$

$$K_m = K_s(k_{-2} + k_3)/(k_2 + k_{-2} + k_3) \quad (2)$$

$$k_{obsd} = (k_{-2} + k_3) + k_2/(K_s/[S] + 1) \quad (3)$$

or

$$1/(k_{obsd} - k_{-2} - k_3) = K_s/([S]k_2) + 1/k_2 \quad (4)$$

The individual kinetic constants for Scheme I can be determined from the variation of k_{obsd} with [S] in two ways: (a) by nonlinear least-squares fitting to eq 3, which gives $k_{-2} + k_3$ and $k_2 + k_{-2} + k_3$ from the limiting values of k_{obsd} as [S] $\rightarrow 0$ and [S] $\rightarrow \infty$, respectively, and K_s from the value of [S] at $k_{obsd} = k_{-2} + k_3 + k_2/2$; (b) by linear least-squares fitting to eq 4, which gives a y intercept of $1/k_2$ and an x intercept of $-1/K_s$. In this latter instance $k_{-2} + k_3$ is estimated from the limiting value of k_{obsd} at low [S].

The results obtained for Dns-$(Gly)_3$-OPhe from these two procedures are similar (Figure 5 and Table II). In both instances $k_{-2} + k_3$ can be separated into its component parts through relationship 1. The accuracy of the derived kinetic parameters was gauged by estimating K_m from eq 2 and comparing this value with K_m as measured directly (Lobb & Auld, 1979).

Table II shows the rate and equilibrium constants estimated in this manner for several substrates. In every case, K_m calculated from these constants is in close agreement with that determined from Lineweaver–Burk plots (Table II).

Addition of the product OPhe to the reaction for Dns-$(Gly)_3$-OPhe hydrolysis at −20 °C affects the RET parameters as shown in Figure 6. The steady-state time interval of the reaction indicates that OPhe is a competitive inhibitor of ester hydrolysis. Thus, the ratio of maximal fluorescence in the absence and presence of inhibitor, F_c/F_I, increases with increasing inhibitor concentration reflecting a reduction of substrate binding, while the ratio of the corresponding areas, A_I/A_c, remains constant, since the rate-determining step is unaffected (Auld et al., 1972). The slope of the plot of F_c/F_I vs. [OPhe] gives $K_I = 1.4 \times 10^{-4}$ M, which is in good agreement with previous results at +20 °C (Kaiser & Kaiser, 1969).

The slight decrease in k_{obsd} with increased concentration of product (insert of Figure 6) is also consistent with competition between inhibitor and substrate for binding to the enzyme in the initial step of the reaction (eq 5).

$$k_{obsd} = (k_{-2} + k_3) + k_2/[K_s(1 + [I]/K_I)/[S] + 1] \quad (5)$$

Increasing the concentration of I (OPhe) should decrease k_{obsd} as indicated by the theoretical line in the insert of Figure 6.

Hydrolysis reactions were also performed in the presence of a number of nucleophiles, e.g., methanol, ethanol, and hydroxylamine, over a range of temperature from +20 to −40 °C. The products of the reaction were analyzed by HPLC for evidence of any trapped product. As a control, the expected products, viz., Dns-X-OMe, Dns-X-OEt, and Dns-X-NHOH, were incubated with the enzyme and found to be resistant to hydrolysis. Trapped product could not be detected for any of the peptide or ester substrates (Figure 7).

In contrast, when yeast seryl carboxypeptidase was incubated similarly with Dns-$(Gly)_3$-OPhe and these nucleophiles, HPLC analysis readily detected a trapped product even when the reaction was performed at +25 °C (Figure 7).

Discussion

Determination of the mechanism of an enzyme requires a complete kinetic description of the individual steps involved in substrate binding and catalysis, the conformational changes that presumably connect these steps, and the nature of the intermediates involved. It has not been a simple task to detect intermediates directly in protease catalysis since formation of the ES complex and its conversion to products are generally too fast to be observed.

However, the low-temperature stopped-flow RET kinetics described in this study can readily detect intermediates of rapidly hydrolyzed substrates (Figure 1). N-Dansylated tri-

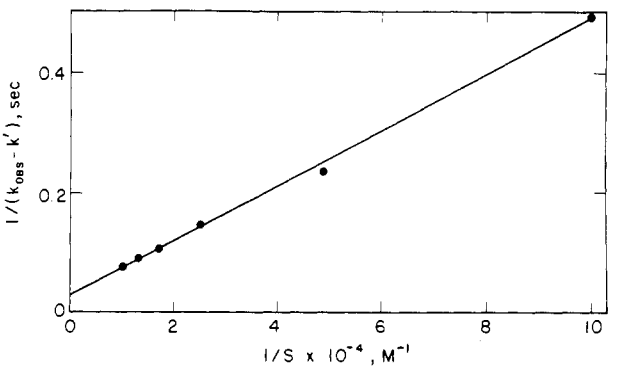

FIGURE 5: Estimation of the individual rate and equilibrium constants for hydrolysis of Dns-$(Gly)_3$-OPhe by carboxypeptidase A in 10 mM Hepes–4.5 M NaCl, pH 7.5, at −20 °C. k' (4.6 s^{-1}) is the limiting value of k_{obsd} at low [S_0]. The line represents the linear least-squares fit to the data, giving K_s = 148 μM and k_2 = 32.6 s^{-1}.

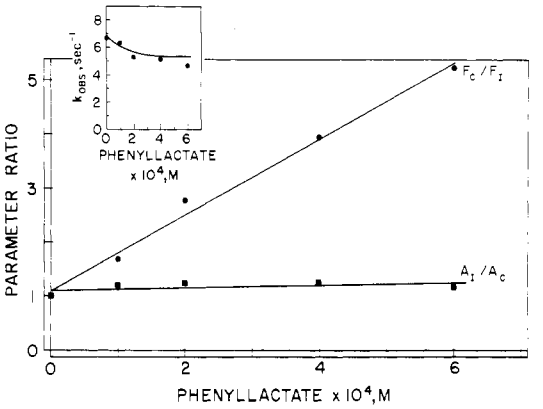

FIGURE 6: Effect of L-phenyllactate on the steady-state (F and A) and pre-steady-state (k_{obsd}) RET kinetic parameters for hydrolysis of Dns-$(Gly)_3$-OPhe at −20 °C. F_c and A_c are the maximal fluorescence and area observed in the absence of inhibitor, and F_I and A_I are those observed in the presence of inhibitor. Conditions of assay are as in Figure 5. The line in the insert is generated from eq 5 by using the values of parameters given in Table II.

Table II:[a] Michaelis–Menten Parameters and Rate and Equilibrium Constants for the Hydrolysis of Dansylated Substrates by Carboxypeptidase A

substrate	K_m (μM)	k_{cat} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	k_3 (s^{-1})	K_s (μM)	K_m[b] (μM)
peptides							
Dns-$(Ala)_2$-Phe	13.5	1.18	40.0	3,48	1.32	102	10.9
Dns-Gly-Ala-Phe	12.9	3.72	228.0	6.12	3.88	418	17.6
esters							
Dns-$(Ala)_2$-OPhe[c]	1.6	0.062	53.3	0.50	0.062	129	1.3
Dns-Gly-Ala-OPhe	5.6	0.14	15.6	0.66	0.15	75	3.7
Dns-$(Gly)_3$-OPhe	22.3	3.13	32.6	1.41	3.61	164	21.9

[a] Conditions of assay were −20 °C in 10 mM Hepes–4.5 M NaCl, pH 7.5. The Michaelis–Menten parameters were calculated from plots of $1/k$ vs. $1/[S_0]$ (see Materials and Methods), while the rate and equilibrium constants were calculated by nonlinear least-squares analysis of eq 3 as described in the text. [b] Calculated from eq 2. [c] At −10 °C.

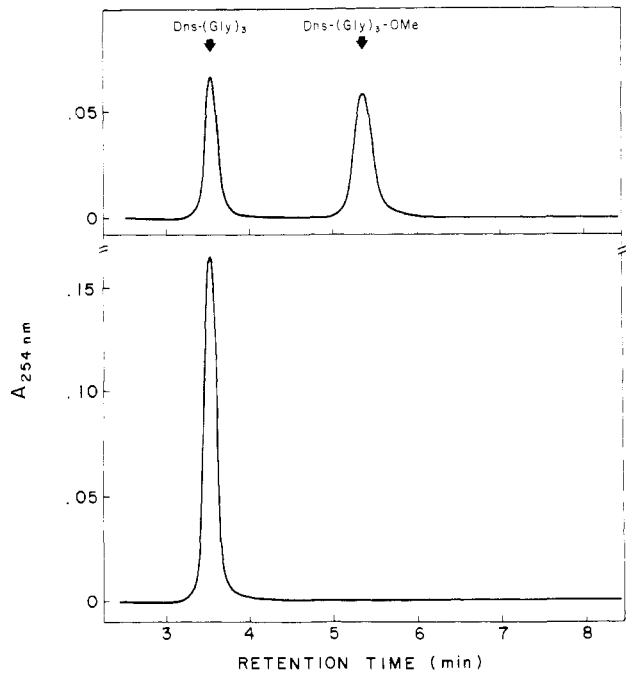

FIGURE 7: HPLC product analysis for hydrolysis of 100 μM Dns-(Gly)$_3$-OPhe in 50% MeOH–0.25 M NaCl–10 mM cacodylate, pH 7.5, by 5 μM of yeast carboxypeptidase (above) and carboxypeptidase A (below). The ester was incubated in methanolic solvent at the desired temperature and reaction initiated by addition of enzyme. Reaction mixtures at 25, 0, −20, and −40 °C yielded identical results. HPLC analysis were performed as described under Materials and Methods.

and tetrapeptide and depsipeptide substrates follow Michaelis–Menten kinetics (Figure 2) with k_{cat}/K_m values of $\sim 10^7$ M^{-1} s^{-1} at room temperature, values which are equal to, or exceed, those of the best substrates of carboxypeptidase. While the intermediates formed with these substrates are too transient to be characterized at room temperature, the cryoenzymological conditions employed stabilize them for a sufficient period to permit their characterization (Figures 1 and 4).

The intrinsic properties of carboxypeptidase A greatly facilitate cryokinetic studies, since this enzyme, in contrast to many others, is readily soluble only in the presence of salt (Putnam & Neurath, 1946); for this reason virtually all physical and kinetic studies over the past two decades have been performed in solutions containing 1 M NaCl. Increasing the NaCl concentration to 4.5 M minimally affects activity (see above) but depresses the freezing point sufficiently to allow kinetic measurements at −20 °C.

The Arrhenius plots (Figure 3) for all substrates examined here are linear over the 40 °C temperature range employed (+20 to −20 °C), indicating that the rate-limiting step is the same at subzero temperatures as at room temperature. This is in accord with previous studies on carbobenzoxy- and benzoyl-blocked tripeptides which exhibit linear Arrhenius plots over the temperature range from +35 to +5 °C (Auld & Vallee, 1971). Hence, the mechanism of the enzyme that can be deduced from cryokinetic RET experiments also pertains at these higher temperatures.

At −20 °C the kinetic profiles of both peptide and ester substrates clearly reveal the existence of *two* intermediates. The first, ES_1, is formed rapidly and represents the Michaelis complex, which then converts into a second enzyme-bound species, denoted above by ES_2. The calculated rate and equilibrium constants (Table II) show that for all substrates examined the rate of formation of ES_2 (k_2) is 10–100-fold greater than its rate of breakdown ($k_{-2} + k_3$) and that $k_{-2} \neq 0$.

The interconversion of ES_1 and ES_2 by two first-order rate constants, k_2 and k_{-2}, indicates that ES_2 does not represent an acyl intermediate. In classical acyl mechanisms, e.g., as established for serine proteases (Fersht, 1977), formation of the acyl–enzyme complex (EA) is accompanied by release of the C-terminal moiety, P_1, of the substrate, as depicted in Scheme II.

Scheme II

$$E + S \overset{K_s}{\rightleftharpoons} ES \underset{k_{-2}[P_1]}{\overset{k_2}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P_2$$

$$k_{obsd} = k_3 + k_{-2}[P_1] + k_2/(K_2/[S] + 1) \qquad (6)$$

P_2 represents the N-terminal moiety of the product, k_2 is the rate constant for acylation, and k_3 is the rate-limiting deacylation rate constant.

In this mechanism, reversal of the acyl complex occurs in a second-order rate process ($k'_{-2} = k_{-2}[P_1]$) and, hence, is essentially irreversible in the absence of added product. Furthermore, product P_1 is a mixed inhibitor and its addition increased k_{obsd} (Fersht, 1977; Lobb & Auld, 1979). Chymotrypsin, which is known to form an acyl intermediate, meets all these expectations when examined by stopped-flow RET kinetics (Lobb & Auld, 1979). These studies have shown that k'_{-2} *is zero* in the absence of added product, that P_1 is a *mixed* inhibitor, and that its addition markedly *increases* k_{obsd} (Lobb & Auld, 1979).

However, carboxypeptidase A fulfills none of these expectations. For all substrates studied k_{-2} is *greater than zero*, in fact usually larger than k_3 (Table II), the P_1 products, Phe and OPhe, are *competitive* inhibitors, in accord with previous studies (Auld et al., 1972), and k_{obsd} *decreases* upon addition of P_1 (Figure 6). Minimally, these results demonstrate that the C-terminal product is not liberated prior to the rate-limiting step and hence, by inference, that deacylation cannot be rate limiting. This is entirely in accord with previous steady-state kinetic studies employing other peptide and ester substrates of carboxypeptidase A, which also led to the conclusion that deacylation cannot be rate limiting (Tomalin et al., 1970; Bunting & Kabir, 1977; Breslow & Wernick, 1977).

Furthermore, in enzyme mechanisms known to operate through acyl intermediates addition of nucleophiles traps the acyl moiety, regardless of whether or not deacylation is rate limiting (Fersht, 1977). In this regard, it is of special relevance that yeast seryl carboxypeptidase behaves in accord with predictions based on the acyl mechanism of chymotrypsin (Figure 7): when hydrolysis is carried out in the presence of methanol the substrate Dns-(Gly)$_3$-OPhe is readily converted to Dns-(Gly)$_3$-OMe. However, when carboxypeptidase A acts on this same substrate, there is no evidence that the product is trapped under any of the conditions investigated, in complete accord with previous studies of other substrates (Breslow & Wernick, 1977; A. Klyosov and B. L. Vallee, unpublished observations). Methanol, in particular, has proven to be a very effective nucleophile in reactions of other proteases (Bender et al., 1964; Lowe & Williams, 1965), since its intrinsic nucleophilicity is much greater than that of water. Yet, methanol concentrations of up to 50% (v/v) fail to trap any intermediate during hydrolysis by carboxypeptidase A.

The only reports of covalent acyl intermediates for this enzyme come from studies on two acylphenylalanine ester analouges, *S*-(*trans*-cinnamoyl)-SPhe (Suh & Kaiser, 1975) and *O*-(*trans*-*p*-chlorocinnamoyl)-OPhe (Makinen et al., 1976); the former is thought to acylate Tyr-248 and the latter

Glu-270. However, such covalent acyl intermediates have not been identified for any peptide or any other ester substrates. Therefore, it remains to be ascertained whether acylation with these two particular esters is related to the mechanism of hydrolysis of other esters and peptides or whether it is coincidental and due to the particular stereochemistry and electronic properties of the *trans*-cinnamoyl group. All the data on oligopeptides and their depsipeptide ester analogues strongly indicate that an acyl intermediate mechanism does not pertain to any of these.

Our results also support our earlier inferences that peptides and esters are hydrolyzed through different mechanisms (Vallee, 1964; Vallee et al., 1983). In particular, previous kinetic and chemical modification studies have indicated that the metal plays a critical but different role in the mechanism of peptide and ester hydrolysis [reviewed in Vallee et al. (1983)]. The metal was envisaged to be unimportant for binding of peptides but to aid their hydrolysis by coordination of the amide carbonyl group, while it was considered critical for the binding of esters through coordination of their carboxyl group. Thus, the steady-state intermediate (ES_2) for both types of substrate was proposed to coordinate to the metal, albeit in a different manner.

This view has been challenged on the basis of a kinetic hypothesis (Cleland, 1977). This now popular hypothesis (Rees & Lipscomb, 1981) proposes that peptides and esters follow the same mechanism but that for peptides the rate-limiting step occurs earlier along the pathway than for esters. According to this proposal, the rate-limiting step in peptide hydrolysis is thought to be rotation of the scissile amide bond toward the metal atom, while for depsipeptides the rate-limiting step is assumed to occur after rotation of the corresponding ester bond and its ligation to the metal. However, as has been discussed (Vallee et al., 1983), this hypothesis cannot account for the specificity of the enzyme toward hydrophobic peptides or the differential effects of both chemical modification and carboxylic inhibitors on peptide and ester hydrolysis. Our present results provide further evidence against this hypothesis, since they contradict three of its key predictions, namely, that (i) peptides form one less pre-steady-state intermediate than esters, (ii) the rate-limiting step for peptides is always slower than that of their depsipeptide analogues, and (iii) only esters form steady-state metallo intermediates. Thus, we find that hydrolysis of both peptides and depsipeptides involves formation of the same number of pre-steady-state intermediates, i.e., two intermediates for each type of substrate (Figures 1 and 4), and k_3, the rate-limiting rate constant, can be larger for peptides than for their exact depsipeptide analogues (Table II). Cryospectroscopic investigations of the hydrolysis of these same substrates by cobalt carboxypeptidase A, performed concurrently with RET studies, clearly demonstrate that peptides and esters form steady-state metallo intermediates with distinctly different spectroscopic characteristics (Geoghegan et al., 1983), in accord with our long-standing inferences (see above).

In conclusion, the present RET kinetic results, and the concurrent cryospectroscopic studies, show that peptides and esters form metallo intermediates, rather than covalent acyl ones, during catalysis and that peptide and depsipeptide substrates are hydrolyzed through different mechanisms.

Registry No. Dns-$(Ala)_2$-Phe, 84623-92-7; Dns-Gly-Ala-Phe, 84623-93-8; Dns-$(Gly)_3$-Phe, 37923-00-5; Dns-$(Ala)_2$-OPhe, 84623-94-9; Dns-Gly-Ala-OPhe, 84623-95-0; Dns-$(Gly)_3$-OPhe, 71136-36-2; carboxypeptidase A, 11075-17-5.

References

Auld, D. S. (1977) in *Bioorganic Chemistry* (Van Tamelin, E. E., Ed.) Vol. 1, pp 1–17, Academic Press, New York.

Auld, D. S. (1979) *Methods Enzymol. 61*, 318–335.

Auld, D. S., & Valee, B. L. (1971) *Biochemistry 10*, 2892–2897.

Auld, D. S., & Holmquist, B. (1974) *Biochemistry 13*, 4355–4361.

Auld, D. S., Latt, S. A., & Vallee, B. L. (1972) *Biochemistry 11*, 4994–4999.

Bazzone, T. J., Sokolovsky, M., Cueni, L. B., & Vallee, B. L. (1979) *Biochemistry 18*, 4362–4366.

Bender, M. L., Clement, G. E., Gunter, C. R., & Kezdy, F. J. (1964) *J. Am. Chem. Soc. 86*, 3697–3703.

Breslow, R., & Wernick, D. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1303–1307.

Bunting, J. W., & Kabir, S. H. (1977) *J. Am. Chem. Soc. 99*, 2775–2778.

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273–387.

Douzou, P. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 157–272.

Fersht, A. (1977) in *Enzyme Structure and Mechanism*, pp 180–185, W. H. Freeman, San Francisco.

Fink, A. L. (1977) *Acc. Chem. Res. 10*, 233–239.

Galdes, A., Vallee, B. L., & Auld, D. S. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1655 (Abstract 666).

Geoghegan, K. F., Galdes, A., Martinelli, R. A., Holmquist, B., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry* (in press).

Hanahan, D., & Auld, D. S. (1980) *Anal. Biochem. 108*, 86–95.

Kaiser, B. L., & Kaiser, E. T. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 36–41.

Lobb, R. R., & Auld, D. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2684–2688.

Lobb, R. R., & Auld, D. S. (1980) *Biochemistry 19*, 5297–5302.

Lowe, G., & Williams, A. (1965) *Biochem. J. 96*, 199–204.

Makinen, M. W., Yamamura, K., & Kaiser, E. T. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3882–3886.

Makinen, M. W., Kuo, L. C., Dymowski, J. J., & Jeffers, S. (1979) *J. Biol. Chem. 254*, 356–366.

Makinen, M. W., Fukuyama, J. M., & Kuo, L. C. (1982) *J. Am. Chem. Soc. 104*, 2667–2669.

Putnam, F. W., & Neurath, H. (1946) *J. Biol. Chem. 166*, 603–619.

Rees, D. C., & Lipscomb, W. N. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5455–5459.

Suh, J., & Kaiser, E. T. (1975) *Biochem. Biophys. Res. Commun. 64*, 863–869.

Tomalin, G., Kaiser, B. L., & Kaiser, E. T. (1970) *J. Am. Chem. Soc. 92*, 6046–6049.

Vallee, B. L. (1964) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 23*, 8–17.

Vallee, B. L., Galdes, A., Auld, D. S., & Riordan, J. F. (1983) in *Metal Ions in Biology—Zinc Proteins*, Vol. 5, Wiley, New York (in press).