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Elucidation of the Chemical Nature of the Steady-State Intermediates in the Mechanism of Carboxypeptidase A†

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ABSTRACT: Cryospectrokinetic studies of zinc and cobalt carboxypeptidase A disclosed two intermediates in the hydrolysis of both peptides and depsipeptides and furnished all the rate and equilibrium constants for the reaction scheme $E + S \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightarrow E + P$ [Auld, D. S., Galdes, A., Geoghegan, K. F., Holmquist, B., Martinelli, R. A., & Vallee, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5041-5045]. Since the ES_2 intermediate is the predominate enzyme species present at steady state, its chemical nature is deducible from subzero chemical quench studies done after steady state is established. Extrapolation of the product concentration to zero time, $[P_0]$, measures the concentration of the enzyme species in which bond cleavage has occurred. For peptides, the $[P_0]$ values are zero, indicating that no product is generated prior to turnover and therefore the ES_2 intermediate involves a complex between enzyme and intact peptide substrate. For depsipeptides, $[P_0]$ values are 1 mol of product per mole of enzyme over the entire temperature range -20 to -50 °C, indicating cleavage of the ester bond occurs prior to the rate-limiting step so that ES_2 is more properly denoted by EP_1P_2, where P_1 and P_2 are the substrates for the reverse reaction. The rate-limiting step for depsipeptides thus involves release of the products which may occur directly or through a mandatory conformational change followed by rapid product release.

Carboxypeptidase A is one of the best characterized metalloenzymes and is often considered a prototype of the large family of zinc proteases (Vallee & Galdes, 1984). Until recently, however, its mechanism was largely unknown, although it was the subject of numerous speculations [reviewed in Vallee et al. (1983)]. This uncertainty was a result of its high catalytic efficiency which precluded identification of intermediates formed during substrate hydrolysis. We have recently described a multifaceted strategy designed to obviate the problem and have applied it successfully to establish the pre-steady-state kinetics of the enzyme, as well as to spectrally characterize the intermediates that accumulate at steady state. We have used subzero temperatures to extend the lifetime of the intermediates in nonperturbing cryosolvents and rapid-scanning stopped-flow procedures to monitor their rapid formation and interconversion. The intermediates can be visualized directly through the absorption spectra of the cobalt-substituted enzyme or the fluorescence generated by radiationless energy transfer between enzyme tryptophans and dansylated substrates. The cryokinetic studies disclose two intermediates in the hydrolysis of both peptide and depsipeptide substrates and furnish all the rate and equilibrium constants for the reaction scheme $E + S \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightarrow E + P$. The results show that, contrary to popularly held views (Cleland, 1977; Rees & Lipscomb, 1981), peptide and depsipeptide substrates give rise to different steady-state metallointermediates.

The present work extends our studies by identifying the chemical nature of the steady-state peptide and ester intermediates. This identification was achieved by rapidly quenching reaction mixtures in which the intermediates had accumulated at subzero temperatures and then analyzing the mixtures by high-performance liquid chromatography (HPLC) techniques. The results show that hydrolysis of the scissile amide bond in peptides occurs during, or after, the rate-limiting step, while hydrolysis of the ester bond in depsipeptides precedes the rate-limiting step.

Materials and Methods

Bovine carboxypeptidase A (Cox) was purchased as a crystalline suspension from Sigma Chemical Co. and purified further by recrystallization and affinity chromatography (Galdes et al., 1983).

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Dansylated substrates were synthesized and characterized as previously described (Galdes et al., 1983). For most experiments, 4.5 M NaCl was used as the cryosolvent, and the pH values of solutions at subzero temperatures were corrected for the temperature coefficient of the buffer (Good et al., 1966). The cryosolvents 50% methanol–50% water and 40% ethylene glycol–40% water–20% methanol, both containing 10–20 mM cacodylate and 0.25 M NaCl, were prepared according to the methods of Douzou (1977). All aqueous solutions were rendered metal free by extraction with dithizone in carbon tetrachloride (Thiers, 1957), and precautions were taken to avoid contamination by adventitious metal ions (Vallee & Galdes, 1984).

Steady-state and pre-steady-state kinetic parameters were determined from stopped-flow fluorescence measurements as previously described (Lobb & Auld, 1979, 1980, 1984; Galdes et al., 1983).

HPLC analyses were carried out with a Waters Associates high-performance liquid chromatograph equipped with a Model 440 absorbance detector, a Model 510 solvent deliver system, and a 33904 Hewlett Packard integrator. Samples of 10–100 μL were loaded on a 0.78 × 30 cm C_{18} μBondapak column fitted with a Bondapak C_{18}/Corosil guard column and eluted with either 50% acetonitrile–50% water or 65% methanol–35% water containing 0.1% formic acid at a flow rate of 2 mL/min. The dansylated substrates used in this study are well separated from the products of their hydrolysis under both of these elution conditions. The eluting solvents were filtered through 0.6-μm filters (Millipore) and degassed before use. The absorbance of the column effluent was monitored at 254 nm, and the concentrations of the eluted dansylated components were calculated from their peak areas or peak heights, which were normalized to the corresponding areas or heights of an internal standard (see below). The detection limit was 0.1 μM, and analysis of standard solutions of Dns-Ala-Ala-OPhe and Dns-Ala-Ala [where Dns is dansyl [5-(dimethylamino)naphthalene-1-sulfonyl] and OPhe is L-β-phenyllactate] demonstrated that peak areas and heights were linearly proportional to concentration over the 1–100 μM range. Repeated analyses of the same sample showed that reproducibility was better than 5%.

Low-Temperature Quenching Experiments. Solutions of substrate, enzyme, and quenching agent (see below) were equilibrated to the desired subzero temperature in a Cryo Cool CC-60 II Neslab cryobath containing cold methanol and equipped with a cryoflow circulating pump. Temperature was monitored with an Omega Engineering Model 199 copper/constantan digital thermometer controlled to ±0.5 °C with a Neslab Cryo Trol temperature controller. Pipet tips (Rainin Instruments Co.) used to transfer solutions were chilled in a Thermoelectric cold plate (Model TCP-2). Reactions were initiated by addition of 50 μL of the enzyme solution to 200 μL of substrate solution. After the reaction was allowed to proceed for the required time (10 s to 300 min), 250 μL of quenching agent was added and the sample frozen rapidly by immersion in a dry ice–methanol bath. Samples were stored frozen until the HPLC analyses were performed.

The quenching agent, 30 mM HCl in 50% methanol–50% water, contained Dns-Ala, Dns-Val, or Dns-Pro as an internal standard. The inclusion of methanol was essential to solubilize the dansylated compounds at low pH. The quenching agent reduced the pH of the samples to a value of 2, denaturing the enzyme and liberating any enzyme-bound substrate or product. Repeated analyses of quenched samples over a period of several hours showed no further hydrolysis, demonstrating that the enzyme was completely inactivated by the quenching procedure and that the quenching agent itself did not degrade the substrate or products. Control experiments in which substrate was included with quenching agent and both were added to a buffered solution of enzyme at −10 °C showed no detectable substrate hydrolysis, indicating that enzyme denaturation was virtually instantaneous. Furthermore, analysis of quenched enzyme–substrate solutions showed that the amount of product was independent of acid concentration over the range 20–60 mM HCl. Since acid quenching is expected to be a second-order reaction, the constant level of product formed over a 3-fold increase in acid concentration indicates that quenching is much faster than interconversion of enzyme-bound species (Wilkinson & Rose, 1979).

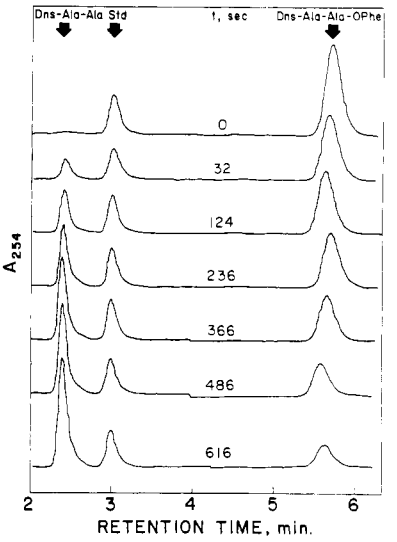

FIGURE 1: HPLC elution profiles for the hydrolysis of 86.9 μM Dns-Ala-Ala-OPhe by 9.5 μM zinc carboxypeptidase A in 10 mM Hepes and 4.5 M NaCl, pH 7.5, at −21 °C. Samples of the reaction mixture were quenched at the indicated times with 30 mM HCl and 50% methanol containing 1% Dns-Ala as an HPLC standard. The HPLC analyses were performed with 50% acetonitrile–50% water as the eluting solvent, as described under Materials and Methods.

RESULTS

Radiationless energy transfer (RET) cryokinetic studies demonstrate the hydrolysis of Dns-Ala-Ala-OPhe by carboxypeptidase A at −20 °C in 10 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes)–4.5 M NaCl, pH 7.5, is characterized by a k_{cat} value of 0.01 s^{-1} and a K_m of 1.6 μM (Galdes et al., 1983). These studies further establish that under these conditions steady state is attained in less than 0.5 s, at which stage the predominant enzyme-bound species is the intermediate ES_2. We have now determined the time course for the enzyme-catalyzed hydrolysis of this ester by HPLC analyses of reaction mixtures quenched by addition of 30 mM HCl in 50% methanol. Figure 1 shows representative HPLC elution profiles obtained during the hydrolysis of 86.9 μM Dns-Ala-Ala-OPhe by 9.5 μM enzyme at −21 °C. Since the dansylated product, Dns-Ala-Ala, is well separated from the substrate and the internal standard in the elution profile (retention times of 2.39, 5.63, and 3.00 min, respectively), the concentration of each component can be estimated accurately. The enzyme will be virtually saturated with substrate when $[S] \gg K_m$, making it possible to calculate that, under the conditions chosen, the concentration of intermediate ES_2 between 0 and 50% hydrolysis is approximately 9 μM. In addition, under these conditions, the turnover number is essentially equal to k_{cat} so that the time required for the breakdown of ES_2 to an equivalent concentration of products is $1/k_{cat}$

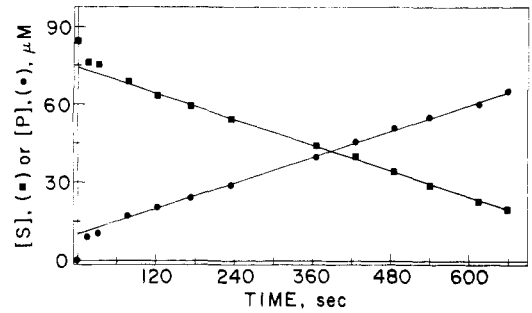

FIGURE 2: Plots of the concentrations of substrate, Dns-Ala-Ala-OPhe (●), and product, Dns-Ala-Ala (■), over time obtained from the HPLC analysis described in Figure 1. Least-squares analysis of the time courses yields a k value of 9.4×10^{-3} s^{-1} for both substrate depletion and product accumulation (determined from the slopes). The y intercepts divided by the total enzyme concentration are 0.96 and 1.01, respectively.

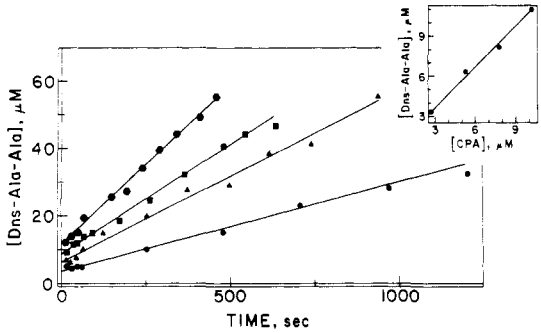

FIGURE 3: Time courses, determined by HPLC analyses, for the hydrolysis of 102.6 μM Dns-Ala-Ala-OPhe by zinc carboxypeptidase A in 10 mM Hepes and 4.5 M NaCl, pH 7.5 at −19.2 °C. The enzyme concentrations were as follows: (●) 10.2 μM; (■) 7.8 μM; (▲) 5.3 μM; (●) 2.7 μM. The k values, determined from the slopes, were 9.4×10^{-3}, 8.3×10^{-3}, 9.5×10^{-3}, and 9.7×10^{-3} s^{-1}, respectively. Insert: Plot of the y intercepts ([Dns-Ala-Ala] at $t = 0$) vs. enzyme [carboxypeptidase A (CPA)] concentration. This plot gives a slope of 1.10 mol of Dns-Ala-Ala/mol of enzyme.

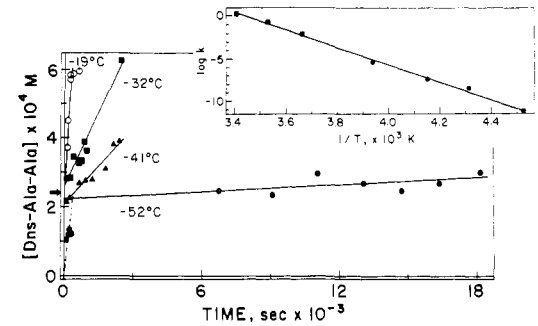

FIGURE 4: Time courses, determined from HPLC analyses, for the hyrolysis of 930 μM Dns-Ala-Ala-OPhe by 24 μM cobalt carboxypeptidase A in the ternary cryosolvent 40% ethylene glycol–20% methanol–40% water containing 20 mM cacodylate and 0.25 M NaCl, pH 7.5, at the following temperatures: (○) −19 °C; (■) −32 °C; (▲) −41 °C; (●) −52 °C. The enzyme concentration is denoted by the arrow on the y axis. The intercepts on the y axis divided by the enzyme concentration are 1.05, 1.05, 1.08, and 0.92, respectively. Insert: Arrhenius plot for the k values determined in the ternary cryosolvent over the temperature range +20 to −52 °C. The data were obtained from RET cryokinetics (■) and by HPLC analysis (●). The slope of this plot yields an activation energy of 20.5 kcal/mol.

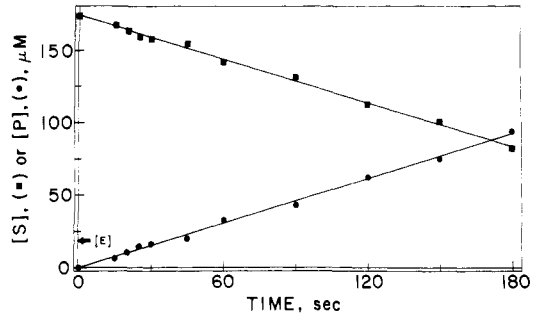

FIGURE 5: Time courses, determined by HPLC analyses, for the depletion of Dns-Gly-Phe (■) and the formation of Dns-Gly (●) during the hydrolysis of 174 μM Dns-Gly-Phe by 19 μM cobalt carboxypeptidase A in 10 mM Hepes and 4.5 M NaCl, pH 7.5 at −20 °C. The slopes yield a k value of 0.028 s^{-1}.

seconds (Segel, 1975). For the case under consideration, since k_{cat} is 0.01 s^{-1}, the appearance of 9 μM Dns-Ala-Ala resulting from a single catalytic cycle should take approximately 100 s. The HPLC analyses, however, clearly demonstrate that the initial appearance of product, and the concomitant disappearance of substrate, occurs much faster than indicated by the turnover number (Figure 2). Thus, one full equivalent of Dns-Ala-Ala is formed within 16 s of the mixing of substrate with enzyme, the shortest quenching time feasible for this quenching procedure. Once product formation becomes linear with time ($t > 100$ s), the slope of [Dns-Ala-Ala] vs. time yields a turnover number of 0.0094 s^{-1} (Figure 2) which is in excellent agreement with the k_{cat} value determined by RET analysis. Furthermore, the substrate and product time course plots extrapolate to 74.6 and 9.7 μM, respectively, at zero time, demonstrating the rapid hydrolysis of 1 mol of substrate/mol of enzyme prior to catalytic turnover (Figure 2).

The relationship between the concentration of Dns-Ala-Ala produced prior to enzyme turnover and the enzyme concentration is shown in Figure 3. In this experiment, the hydrolysis of 102.6 μM Dns-Ala-Ala-OPhe at −20 °C is followed at four different carboxypeptidase A concentrations ranging from 2.7 to 10.2 μM. At each enzyme concentration, the intercept on the y axis represents the concentration of Dns-Ala-Ala at $t = 0$ (Figure 3). This value increases linearly, with a slope of 1.0, as enzyme concentration increases (insert to Figure 3), indicating 1.1 mol of product/mol of enzyme is formed instantaneously at all enzyme concentrations. At all four enzyme concentations, a turnover number in the range of 0.0083–0.0097 s^{-1} is obtained, again in good agreement with that measured from RET cryokinetic analysis.

The hydrolysis of 930 μM Dns-Ala-Ala-OPhe by 24 μM cobalt carboxypeptidase A was also followed over the temperature range from −20 to −50 °C in the ternary cryosolvent 40% ethylene glycol–20% methanol–40% water containing 0.25 M NaCl and 20 mM cacodylate, pH 7.5. Previous cryospectroscopic studies have established that intermediate ES_2 also accumulates in this ternary solvent (Geoghegan et al., 1983). As in the aqueous NaCl cryosolvent, a rapid formation of 0.9–1.1 mol of product/mol of enzyme is observed over the entire temperature range (Figure 4). At −50 °C, the liberation of the N-terminal product occurs during at 300-s time interval after enzyme and substrate are mixed, likely reflecting a decreased rate of formation for intermediate ES_2 at this temperature. The initial rate of Dns-Ala-Ala formation at −50 °C was estimated to be 0.6 μM s^{-1}. The turnover rate for subsequent product formation is at least 180 times slower than that of the initial rate (Figure 4). In agreement with previous cryokinetic studies (Galdes et al., 1983), the k_{cat} values calculated from HPLC product analyses decrease markedly with decreasing temperature and yield a linear Arrhenius plot, indicting that the rate-limiting step for the hydrolysis of this ester does not change over a 70 °C temperature range (insert to Figure 4).

In marked contrast, when the hydrolysis of the peptide Dns-Gly-Phe (174 μM) by cobalt carboxypeptidase A (18 μM)

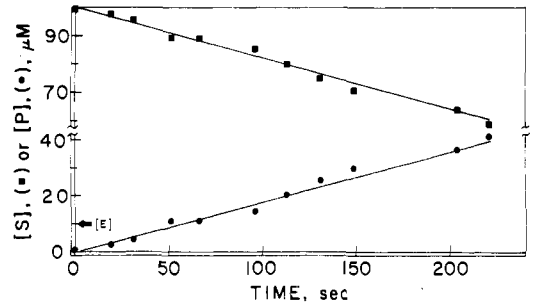

FIGURE 6: Time courses, determined from HPLC analyses, for the depletion of Dns-Ala-Ala-Phe (■) and the formation of Dns-Ala-Ala (●) during the hydrolysis of 100 μM Dns-Ala-Ala-Phe by 10 μM zinc carboxypeptidase A in 50% methanol–50% water containing 10 mM cacodylate and 0.25 M NaCl, pH 7.5 at −38.5 °C. The slopes yield a k value of 0.018 s^{-1}.

is followed by HPLC analyses, no rapid formation of product is seen at −20 °C in the cryosolvent 4.5 M NaCl (Figure 5). Under these conditions, RET cryokinetic studies establish a K_m value of 3.5 μM for the hydrolysis of Dns-Gly-Phe, so it can be calculated that the concentration of this steady-state intermediate is 17.3 μM during the initial turnover. Plots of [Dns-Gly-Phe] and [Dns-Gly] vs. time, however, clearly extrapolate to 0% product formation at $t = 0$ (Figure 5). The slope of these plots yields a turnover number of 0.03 s^{-1}, in close agreement to the value of 0.06 s^{-1} measured by RET cryokinetics. Similarly, when the hydrolysis of the peptides Dns-Gly-Phe (data not shown) and Dns-Ala-Ala-Phe (Figure 6) is followed in coorganic cryosolvents, no burst of product formation is discerned, and plots of product vs. time extrapolate to 0% product formed at $t = 0$.

Discussion

Our previous cryokinetic and cryospectroscopic studies on the mechanism of carboxypeptidase A have shown that hydrolysis of peptide and depsipeptide substrates proceeds via the rapid formation of a Michaelis complex, which then interconverts with a second intermediate, denoted by ES_2, whose breakdown is rate limiting (Galdes et al., 1983; Geoghegan et al., 1983; Auld et al., 1984). At the concentrations of peptides and depsipeptides employed for subzero spectroscopy, the peptide and ester ES_2 intermediates form maximally within 200 ms, at which time they constitute >95% of the total enzyme present (Auld et al., 1984). The subsequent breakdown of the intermediate to free enzyme plus products is much slower, 25–250 s, dependent on the substrate examined. The spectroscopic properties of the ES_2 intermediates can therefore be recorded readily at subzero temperatures with a rapid mixing and spectral scanning instrument (Geoghegan et al., 1983; Auld et al., 1984).

The electronic and paramagnetic spectral properties of cobalt carboxypeptidase A have served to identify these transient intermediates during catalysis. Both the absorption and electron paramagnetic resonance (EPR) spectra of the peptide and ester ES_2 interemdiates differ significantly from one another and from those of the cobalt enzyme itself (Geoghegan et al., 1983; Auld et al., 1984). These studies have established that peptide and ester substrates give rise to different ES_2 intermediates, both of which are indicative of metal complexes. The present work extends these studies by identifying the chemical nature of the reaction intermediates, in particular, the status of the scissile amide or ester bond for the ES_2 intermediates.

The chemical characterization of an intermediate can be obtained through two types of experiments. In rapid-quench experiments, the reaction is stopped as soon as the intermediate is formed and before the steady state is established, and the resulting quenched solution is subjected to chemical analysis. This approach is similar to classical "burst" experiments used to determine the rate-limiting step in proteases (Bender et al., 1962) except that it does not rely on spontaneous liberation of one of the products; instead, the intermediate is quenched immediately after its formation, before it undergoes significant breakdown. Such rapid-quench experiments have been used to identify the steady-state intermediates of several enzymes, e.g., arginine kinase (Barman et al., 1978) and hexokinase (Wilkinson & Rose, 1979).

In the second experimental approach, the reaction mixture is quenched at periodic intervals after the attainment of steady state and analyzed for appearance of products as a function of time. This procedure will give information on the chemical nature of intermediates that accumulate at steady state. Thus, extrapolation of a plot of product concentration vs. time to zero time will correct the extent of initial product formation for the contribution of turnover and yields the concentration of product formed before turnover. This technique was first used by Hartley and Kilby in 1954 in their study of the hydrolysis of p-nitrophenol esters by chymotrypsin to demonstrate that ester bond hydrolysis precedes enzymatic turnover, and it has subsequently been used in the study of many proteases (Fersht, 1984). Under these conditions, $[P_0]$ will be proportional to the total enzyme concentration, $[E_T]$ (Bender et al., 1966). Both types of experiment require that the quenching is rapid compared to turnover and that it does not cause interconversion or breakdown of the intermediates. As outlined under Materials and Methods, the first of these requirements is met during the quenching of carboxypeptidase A solutions with HCl; the second will be commented on further below.

The chemical identity of the intermediates of carboxypeptidase A hydrolysis is deducible from quench experiments done after steady state is established since the ES_2 intermediate is the major enzyme species present at steady state (Galdes et al., 1983; Auld et al., 1984). Extrapolation of the product concentration to zero time, $[P_0]$, measures the steady-state concentration of enzyme species in which bond cleavage has occurred. For the peptides Dns-Gly-Phe in 4.5 M NaCl (Figure 5) and Dns-Ala-Ala-Phe in 50% methanol and 0.25 M NaCl (Figure 6), the extrapolated $[P_0]$ values are zero, indicating that no product is generated prior to turnover and, therefore, that the steady-state intermediate ES_2 involves a complex between the enzyme and intact peptide substrate. These results are confirmed by studying the reaction from the reverse direction, i.e., peptide synthesis. After equilibrium is established for the reverse direction, the predominate enzyme species present has visible absorption and EPR spectra identical with those of the ES_2 intermediate formed in the hydrolytic direction (K. F. Geoghegan and A. Galdes, unpublished results). Under these conditions, chemical analyses clearly demonstrate that ES_2 represents a complex of intact peptide with the enzyme.

On the other hand, for the depsipeptide Dns-Ala-Ala-OPhe in both 4.5 M NaCl (Figures 2 and 3) and a ternary coorganic cryosolvent (Figure 4), $[P_0]$ extrapolates to 1 mol equiv of product/mol of enzyme over the entire temperature range from −20 to −50 °C, and $[P_0]$ is linearly proportional to $[E_T]$ (Figure 3). These results indicate that in depsipeptide hydrolysis the susceptible ester bond is already cleaved in intermediate ES_2 so that this species is more properly denoted by EP_1P_2, in which P_1 and P_2 are the products of the forward reaction (or the substrates for the reverse reaction).

Our previous subzero RET kinetic studies of the zinc and cobalt enzymes and the electronic absorption and EPR spectral studies of the ES_2 reaction intermediates consistently demonstrated (i) the formation of transient metal complexes, (ii) differences between the effects induced by peptides and esters, and (iii) strong similarities between those induced by all peptides on the one hand and all esters on the other. The marked alterations of the cobalt spectra likely reflect the coordination of a substrate carboxyl and/or carbonyl group to the metal at a critical step in the course of catalysis.

In conjunction with our previous cryokinetic and cryospectroscopic studies, the rapid quench experiments described above unequivocally demonstrate that amide bond hydrolysis occurs during or after the rate-limiting step and is preceded by coordination of the substrate to the metal. On the other hand, the results obtained with depsipeptides strongly suggest that ester bond hydrolysis precedes the rate-limiting step. For the depsipeptides, therefore, another alternative must be considered, namely, that ES_2 represents an acyl or a metallotetrahedral intermediate that is cleaved to products by the acid during the quenching step. The first of these possibilities is dismissed easily, since our previous cryokinetic studies and chemical trapping experiments demonstrated that ES_2 does not represent a covalent acyl-enzyme intermediate as has been observed in α-chymotrypsin (Galdes et al., 1983; Lobb & Auld, 1979). The present acid quench experiments are also inconsistent with an acyl intermediate and therefore reinforce our previous conclusions. Thus, the high methanol content of the quench solution provides a potent alternative nucleophile to water so that the reaction of an acy intermediate in the denatured enzyme with solvent would be expected to result in the formation of substantial amounts of methyl ester. In spite of this, no traces of Dns-Ala-Ala-OMe could be detected in any of the quenched reactions.

The second alternative cannot be as easily dismissed by the present data, since a metallotetrahedral intermediate could collapse to products without incorporation of methanol during acid quenching. However, this would require exclusive conversion to products, rather than partitioning to a mixture of substrate and product, which is an unlikely situation. Moreover, spectroscopic studies demonstrate that the spectra of depsipeptide ES_2 intermediates can be mimicked by those of peptides in the presence of noncompetitive carboxylate inhibitors, even when the latter lack an alcohol group and hence cannot give rise to the postulated tetrahedral species (K. F. Geoghegan, A. Galdes, D. S. Auld, and B. L. Vallee, unpublished observations). It therefore appears that for depsipeptides ester bond cleavage precedes the rate-limiting step, and ES_2 represents a metallocomplex formed between the enzyme and the cleaved substrate. The rate-limiting step for depsipeptides thus involves release of products, which may occur directly or through a mandatory conformational change followed by rapid product release.

The marked difference in chemical state of the intermediates of peptide and ester hydrolysis suggests a plausible explanation for a puzzling feature of the zinc protease catalyzed hydrolysis of amide and ester bonds. The best studied zinc proteases, carboxypeptidase, thermolysin, and angiotensin converting enzyme, all display an equal of greater catalytic efficiency toward peptides than toward the corresponding ester analogues (Auld & Holmquist, 1974; Homquist & Vallee, 1976; Keung et al., 1980). This enzymatic leveling of activity is not observed in serine proteases nor is it expected since an ester bond is much more labile than an amide bond (Bruice & Benkovic, 1966). The results can be rationalized, however, if product release is rate limiting for the esters but not for amides. In this case, the hydrolytic step for catalysis of esters would occur earlier in the pathway and thus could still be faster than cleavage of the amide bond, since it is easier to hydrolyze an ester bond than an amide bond.

The change in rate-determining step observed here for a metalloprotease differs completely in type from that observed for serine proteases. In the latter case, it is generally believed that the rate-limiting step for peptides is formation of an acyl intermediate while the rate-limiting step for esters is deacylation of such an intermediate. Hydrolysis of peptides by carboxypeptidase A does not proceed through nucleophilic catalysis resulting in a covalent acyl species as is observed in serine or cysteine proteases, but more likely through general base catalysis of a metal-bound intermediate. This mechanism may be a general feature of zinc proteases, as studies on thermolysin also indicate that peptide hydrolysis involves a metal-bound intermediate and general base catalysis (Hangauer et al., 1984).

The combined use of cryokinetic and cryospectroscopic techniques allows a more direct approach to a long-standing issue in carboxypeptidase A catalysis, the issue of whether the enzyme hydrolyzes peptide and esters by the same or different pathways. A large number of studies has shown that the two types of substrates are influenced differently by chemical modification and metal substitution, as well as by other factors [reviewed in Vallee et al. (1983)]. The present study indicates that the rate-limiting step for esters is different from that for peptides, so different metallointermediates are being observed at steady state. If the substrates are following the same pathway, the metallointermediate observed in peptide hydrolysis should be observable in the pre-steady-state time interval during ester hydrolysis. So far, no such common species has been detected by rapid-scanning cryospectroscopy (Geoghegan et al., 1983; Auld et al., 1984).

Registry No. Dns-Ala-Ala-OPhe, 84623-94-9; Dns-Gly-Phe, 37922-97-7; Dns-Ala-Ala-Phe, 84623-92-7; carboxypeptidase A, 11075-17-5.

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Kinetic Studies Suggest That Light-Activated Cyclic GMP Phosphodiesterase Is a Complex with G-Protein Subunits†

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ABSTRACT: Cyclic GMP phosphodiesterase (PDE) in rod disk membranes has three subunits of molecular weight 88 000 (α), 84 000 (β), and 13 000 (γ). Physiological activation of the enzyme by light is mediated by a GTP binding protein (G protein). The enzyme can also be activated by controlled digestion with trypsin, which destroys the γ subunit, leaving the activated enzyme as $PDE_{\alpha\beta}$ [Hurley, J. B., & Stryer, L. (1982) *J. Biol. Chem. 257*, 11094–11099]. Addition of purified γ subunit to $PDE_{\alpha\beta}$ inhibited the enzyme fully. This suggested the possibility that G protein could also activate PDE by removing the γ subunit and leaving the active enzyme in the form of $PDE_{\alpha\beta}$. Should this be true, the properties of light- and trypsin-activated enzymes should be comparable. We found this not to be the case. The K_m of light-activated enzyme for cyclic GMP was about 0.9–1.4 mM while that of trypsin-activated enzyme was about 140 μM. The cyclic AMP K_m was also different for the two enzymes: 6.7 mM for light-activated enzyme and 2.0 mM for trypsin-activated enzyme. The inhibition of both enzymes by the addition of purified γ subunit also differed significantly. Trypsin-activated enzyme was fully inhibited by the addition of about 200 nM γ, but light-activated enzyme could not be fully inhibited even with 2600 nM inhibitor subunit. The K_i of the trypsin-activated enzyme for γ was 15 nM and of the light-activated enzyme 440 nM. These studies suggest that unlike trypsin-activated enzyme, light-activated PDE is not $PDE_{\alpha\beta}$ but possibly a complex with G protein as $PDE_{\alpha\beta}$·G or $PDE_{\alpha\beta}$·G·γ where the γ subunit is not removed from the complex but just displaced.

Photolyzed rhodopsin in vertebrate rod disk membranes (RDM) activates GTP binding protein (Godchaux & Zimmerman, 1979) (G protein) by catalyzing the exchange of GTP for GDP on this protein (Fung & Stryer, 1980; Leibman & Pugh, 1982). Activated G protein then activates cyclic GMP phosphodiesterase (PDE) (Uchida et al., 1981; Fung et al., 1981). G protein and PDE are peripheral membrane proteins (Godchaux & Zimmerman, 1979; Baehr et al., 1979, 1982), and both are multisubunit protein complexes (Baehr et al., 1979, 1982). G protein has three subunits of M_r 39 000, 37 000, and 6000 (Godchaux & Zimmerman, 1979; Baehr et al., 1982; Wheeler et al., 1977; Kuhn, 1980), and PDE has three subunits of M_r 88 000, 84 000, and 13 000 (Baehr et al., 1979; Kohnken et al., 1981) designated respectively as α, β, and γ. The functions of the individual subunits of these two proteins and whether some of them leave the membrane and become "solubilized" during the activation process are a subject of investigation in several laboratories. The M_r 39 000 subunit of G protein carries the GTP binding site though all three subunits appear essential for the rhodopsin-catalyzed nucleotide exchange (Fung & Stryer, 1980; Fung et al., 1981; Fung, 1983). Addition of nucleotide-bound M_r 39 000 subunit alone to dark RDM activated PDE (Fung et al., 1981).

The nature of the changes in PDE subunit interactions caused by activated G protein is not clear. Hurley & Stryer (1982) showed that controlled digestion with trypsin removed the M_r 13 000 subunit from PDE, resulting in maximal activation of the enzyme. Addition of purified M_r 13 000 subunit to the trypsin-activated PDE inhibited the enzyme completely (Hurley & Stryer, 1982). If the physiological activation of

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