Hydrolysis of Peptides by Carboxypeptidase A: Equilibrium Trapping of the ES_2 Intermediate†

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ABSTRACT: The cobalt absorption and electron paramagnetic resonance (EPR) spectra of cobalt carboxypeptidase undergo unique variations on formation of catalytic peptide and ester intermediates as previously recorded in cryoenzymologic experiments employing rapid-scanning spectroscopy and cryotrapping [Geoghegan, K. F., Galdes, A., Martinelli, R. A., Holmquist, B., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 2255–2262]. We here describe a means of stabilizing these intermediates, which we have termed "equilibrium trapping". It allows peptide intermediates to be observed for longer periods (≫1 min) at ambient as well as subzero temperatures. The reaction intermediate with the rapidly turned over peptide substrate Dns-Ala-Ala-Phe is trapped when the cobalt enzyme (>10 μM) has catalyzed the attainment of chemical equilibrium between high concentrations of the hydrolysis products Dns-Ala-Ala, 10 mM, and L-phenylalanine, 50 mM, and the product of their coupling Dns-Ala-Ala-Phe. Under these conditions, Dns-Ala-Ala-Phe is present in the equilibrated substrate–product reaction mixture at a level that exceeds the one predicted on the basis of K'_{eq} for hydrolysis of this substrate and is close to the enzyme concentration. Other pairs of peptide hydrolysis products yield similar results. Visible absorption and EPR spectra of the cobalt enzyme show that the synthesized peptide binds to the active site in the mode previously recognized as the ES_2 catalytic intermediate in peptide hydrolysis. Equilibrium trapping of the ES_2 intermediate allows analysis of its physicochemical properties by methods that could not be employed readily under cryoenzymological conditions, e.g., circular dichroic and magnetic circular dichroic spectra. Theoretical considerations and the present results suggest that similar trapping strategies should be applicable to intermediates of other hydrolytic enzymes.

Current mechanistic studies of carboxypeptidase A are designed to elucidate the nature of the intermediates formed during peptide and ester hydrolysis (Geoghegan et al., 1983; Auld et al., 1984; Galdes et al., 1986). Rapid kinetic studies with N-dansylated[1] substrates have recently provided the rate and equilibrium constants governing formation and breakdown of both a pre-steady-state and a steady-state intermediate, ES_1 and ES_2, respectively, both with peptides and depsipeptides (Galdes et al., 1983); thus, reactions with both types of substrate follow kinetic schemes that require two intermediates:

$$E + S \overset{K_1}{\rightleftharpoons} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \xrightarrow{k_3} E + P_1 + P_2$$

The structures and physicochemical properties of such transient intermediates can be obtained by methods that effectively operate within the catalytic time frame, i.e., employing rapid response times to counteract the short lifetimes of intermediates formed with substrates having high k_{cat}/K_m values. In monitoring syncatalytic spectral events in cobalt(II)-substituted carboxypeptidase A, we have coupled rapid-scanning spectrometry with a cryoenzymologic strategy, so that the lifetimes of intermediates are extended while data are collected in the briefest possible time. This approach has yielded the distinctive cobalt absorption and EPR spectra of reaction intermediates whose kinetic properties were earlier determined by radiationless energy-transfer methods under identical conditions (Galdes et al., 1983; Geoghegan et al., 1983; Auld et al., 1984).

The intermediates observed thus far in our studies of cobalt carboxypeptidase A at subzero temperatures exhibit unique absorption and EPR spectra that distinguish them from all other forms of the enzyme, including its complexes with inhibitors and pseudosubstrates. Knowledge of the distinctive spectral characteristics of these intermediates has now resulted in the discernment of conditions that stabilize the ES_2 intermediate of peptide hydrolysis for extended periods of time.

Materials and Methods

Cobalt carboxypeptidase A and dansyl oligopeptides were prepared as described previously (Galdes et al., 1983; Geoghegan et al. 1983). Carboxypeptidase A activity in enzyme–product mixtures was quenched by adding aliquots of the reaction mixtures to 3–5 volumes of 33% methanol containing 0.33 M TCA; this lowered the pH of the enzyme samples to below pH 2 and caused rapid precipitation of the protein. Following centrifugation in an Eppendorf microfuge, the peptide mixtures were analyzed on a Waters Assoc. gradient HPLC system using a Waters C_8 Radial-Pak column. Dns peptides were eluted with a gradient of 0.03 M sodium phosphate, pH 6.2–0.03 M sodium phosphate, pH 6.2/45% 2-propanol/5% acetonitrile over 35 min with a flow rate of

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[1] Abbreviations: Dns, dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; DED, monodansylethylenediamine; TCA, trichloroacetic acid; EPR, electron paramagnetic resonance; CD, circular dichroism; MCD, magnetic circular dichroism; peptide and depsipeptide intermediates, steady-state ES_2 intermediates recognized by their characteristic cobalt absorption and EPR spectra (Auld et al., 1984); Z, carbobenzoxy; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; RET, radiationless energy transfer.

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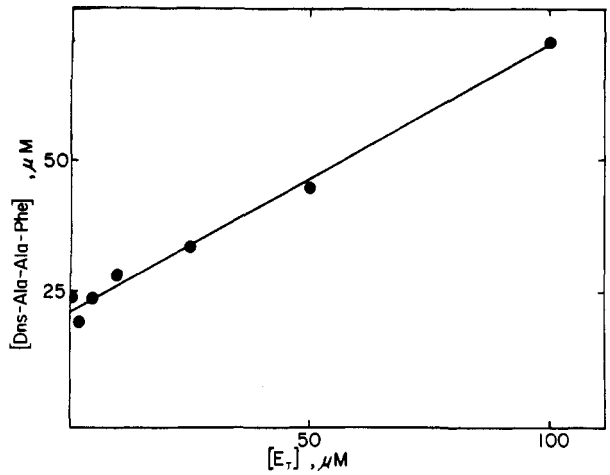

FIGURE 1: Effect of cobalt carboxypeptidase A concentration on amount of Dns-Ala-Ala-Phe produced following 1-min incubation of the enzyme with 50 mM L-Phe and 20 mM Dns-Ala-Ala at 0 °C, 1 M NaCl, and 50 mM Hepes, pH 7.25. Reactions were analyzed by HPLC (see Materials and Methods).

2 mL/min. The dansyl chromophore was monitored at 330 nm, and all samples contained Ala-Ala-DED ($\sim 6 \times 10^{-5}$ M) as an internal standard (Auld & Prescott, 1983).

Absorption spectra of cobalt carboxypeptidase A were recorded on a Cary 219 spectrophotometer in cells of 1-cm path length unless otherwise indicated. CD and MCD spectra were obtained on a Cary 61 spectropolarimeter, and EPR spectra were recorded with a Varian E-9 spectrometer as described previously (Geoghegan et al., 1983). Each instrument is interfaced to an Apple IIe computer via an ADALAB card (Interactive Microware Ltd., State College, PA) for data recording, storage, and manipulation. Spectra were replotted from the computer to a SP-600 X-Y plotter (Enter Computer Inc., San Diego, CA).

RESULTS

Carboxypeptidase A catalyzes peptide hydrolysis in vivo, a process that is favored thermodynamically in aqueous solution (K'_{eq} = 10–100 M, where K'_{eq} is the equilibrium constant measured in the presence of 55 M water; Fruton, 1982). Despite this one-sided equilibrium, enzyme-catalyzed peptide synthesis occurs in the presence of high concentrations of products by virtue of Le Chatelier's principle.

Thus, when 20 mM Dns-Ala-Ala and 50 mM L-Phe are incubated with 0.1 μM cobalt carboxypeptidase A at 0 °C in 1 M NaCl and 50 mM Hepes, pH 7.25, periodic HPLC analysis of acid-quenched aliquots of the reaction mixture reveals the appearance of Dns-Ala-Ala-Phe. Under these conditions, the initial rate of peptide synthesis is 5 μM min^{-1}. Equilibrium is established after about 30 min at which time 24 μM Dns-Ala-Ala-Phe is present. The initial rate of synthesis is in good agreement with that anticipated from the Haldane relationship (Segel, 1975), ≤12 μM min^{-1}, with values of K_m and k_{cat} of 3 μM and 5 s^{-1} respectively, as measured for the hydrolytic reaction. The rate of peptide synthesis increases as a function of enzyme concentration, and at 10 μM enzyme, equilibrium is reached in less than 1 min.

Over the enzyme concentration range 20–100 μM, HPLC analyses reveal a novel effect; the amount of Dns-Ala-Ala-Phe present in the reaction mixture at equilibrium is now in excess of that predicted on the basis of the equilibrium constant and increases with a linear dependence on the enzyme concentration (Figure 1). Since the enzyme concentrations are now *higher* than the equilibrium concentration of Dns-Ala-Ala-Phe,

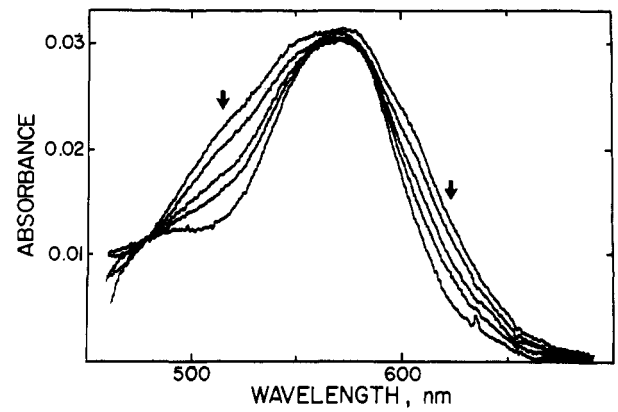

FIGURE 2: Visible absorption spectrum of cobalt carboxypeptidase A, 0.2 mM, in the presence of 50 mM L-Phe and increasing concentrations of Dns-Ala-Ala: 0, 0.35, 1.05, 1.75, and 10.5 mM. A fresh sample was prepared in 0.5 M NaCl and 35 mM Hepes, pH 7.0, for each Dns-Ala-Ala concentration and incubated 1 min at 20 °C before the spectrum was recorded. Arrows indicate the direction of the changes in the absorption as Dns-Ala-Ala concentration is increased. The spectra have been corrected for the tailing absorption of the high concentrations of Dns-Ala-Ala.

the excess Dns-Ala-Ala-Phe might exist in an enzyme-bound form. This is confirmed by spectral studies (see below). The plot of the concentration of Dns-Ala-Ala-Phe synthesized vs. enzyme concentration intersects the vertical axis at a value of 21 μM Dns-Ala-Ala-Phe (Figure 1), the concentration of Dns-Ala-Ala-Phe predicted to form at infinitely low enzyme concentration for the concentrations of products present.

L-Phe and Dns-Ala-Ala, the products of Dns-Ala-Ala-Phe hydrolysis, individually bind about a 1000-fold more weakly to the enzyme than the substrate. L-Phe occupies a single binding site with a K_i of 3 mM at pH 7.0, and the characteristic absorption and EPR spectra of its complex with the cobalt enzyme are quite unlike those of enzyme–substrate complexes (Latt & Vallee, 1971; Auld et al., 1984). Dns-Ala-Ala, at a concentration of 10 mM, does not perturb the visible absorption spectrum of the cobalt enzyme at all.

In the presence of high concentrations of both Dns-Ala-Ala and L-Phe, the spectral properties of the cobalt enzyme identify a state of the enzyme unique from that of its complexes with either L-Phe or Dns-Ala-Ala alone (Figure 2). Addition of Dns-Ala-Ala (1–10 mM) to the cobalt enzyme, 200 μM, in the presence of 50 mM L-Phe (16 times its K_i) systematically perturbs the absorption spectrum of the cobalt enzyme in the manner of a spectral titration (Figure 2). The spectral response becomes saturated when Dns-Ala-Ala reaches approximately 10 mM. Significantly, the final spectral line shape, λ_{max} (570 nm) and maximal intensity (near 150 M^{-1} cm^{-1}), coincides with that of the peptide intermediate formed from Dns-Ala-Ala-Phe, as identified in the forward[2] direction by rapid-scanning stopped-flow analysis at −20 °C (Geoghegan et al., 1983; Auld et al., 1984). The tailing absorption of the dansyl component complicates the acquisition of the absorption spectrum below 480 nm.

The decrease in absorbance at 520 nm that accompanies each incremental addition of Dns-Ala-Ala parallels the increase in Dns-Ala-Ala-Phe concentration measured by HPLC, closely linking formation of the intermediate spectrum to production of the dansyl tripeptide (Figure 3). Under these conditions, the Dns-Ala-Ala-Phe concentration is much higher

[2] In a substrate–product system such as Dns-Ala-Ala-Phe ⇌ Dns-Ala-Ala + Phe, we refer to hydrolysis of Dns-Ala-Ala-Phe as the forward reaction and to its synthesis as the reverse reaction.

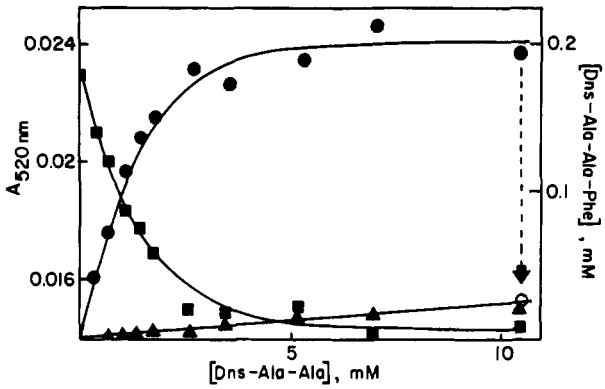

FIGURE 3: Decrease in absorbance at 520 nm (■) of cobalt enzyme, 0.2 mM, and increase in the concentration of Dns-Ala-Ala-Phe (●) as a function of Dns-Ala-Ala content. See Figure 2 for other conditions. (○) Dns-Ala-Ala-Phe detected in a sample containing 0.2 mM cobalt enzyme to which 0.7 mM L-benzyl succinate has been added following 1 min of preincubation and a further 1-min incubation has been allowed before quenching. (▲) Dns-Ala-Ala-Phe detected in samples containing cobalt enzyme, 2 μM.

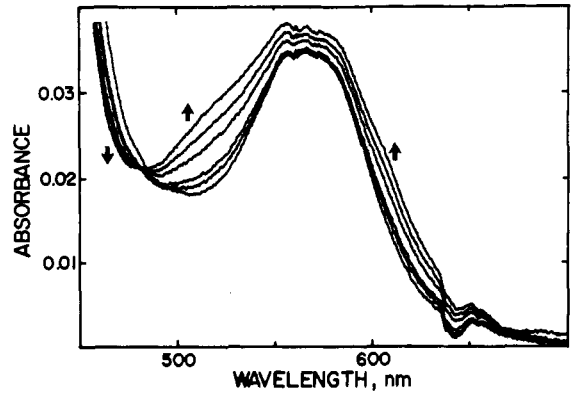

FIGURE 4: Time-dependent change in the absorption spectrum of the cobalt carboxypeptidase ES_2 intermediate formed from 10 mM Dns-Ala-Ala and 50 mM L-Phe in 0.5 M NaCl and 35 mM Hepes, pH 7.0, at 22 °C. Spectra taken at 1, 43, 51, 67, 77, and 116 min following addition of enzyme, 0.2 mM. The arrows indicate the direction of the absorbance change with time. These spectra are uncorrected for the near-UV tailing contribution of the Dns group.

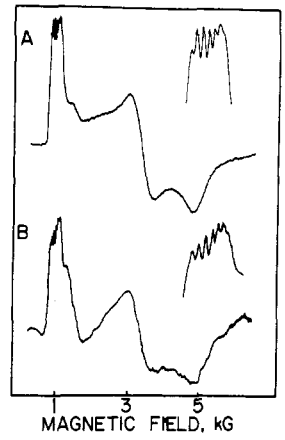

FIGURE 5: Comparison of EPR spectra of cobalt carboxypeptidase A intermediates formed at substrate–product equilibrium (A) and as a transient in the forward reaction (B). In (A), cobalt enzyme, 0.6 mM, in the presence of 50 mM L-Phe and 10 mM Dns-Ala-Ala in 1 M NaCl and 50 mM Hepes, pH 7.0, at 20 °C. In (B), Dns-Ala-Ala-Phe, 10 mM, rapidly mixed with enzyme, 0.6 mM, in 4.5 M NaCl and 10 mM Hepes, pH 7.5, at −20 °C and reaction quenched in liquid nitrogen.

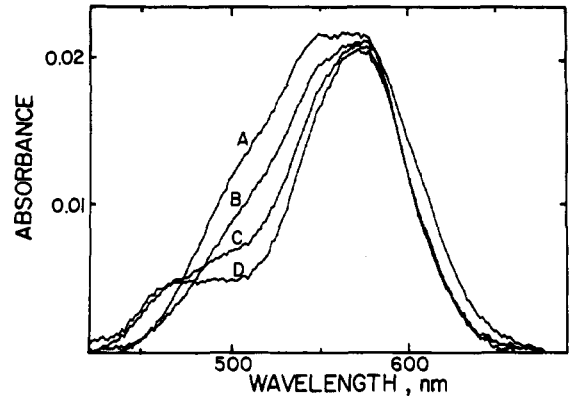

FIGURE 6: Visible absorption spectrum of cobalt carboxypeptidase, 35 μM, in the presence of 2.5 mM Z-Sarc-Ala and 50 mM (A) L-Ala, (B) L-Val, (C) L-Leu, and (D) L-Phe. Samples were prepared at 20 °C in 15 mM Hepes and 0.4 M NaCl, pH 7.0, and spectra recorded in a cuvette with a path length of 5 cm.

than that predicted from simple equilibria and, in fact, is nearly equivalent to that of the enzyme. This indicates that the peptide intermediate-type spectrum is generated by a 1:1 complex of Dns-Ala-Ala-Phe with the cobalt enzyme.

If the enzyme is present at a high concentration, 200 μM, but made unavailable for peptide binding by addition (following the equilibration period) of a 3-fold excess of the strong competitive inhibitor L-benzyl succinate (K_i = 0.5 μM; Byers & Wolfenden, 1973), within 1 min the observed concentration of Dns-Ala-Ala-Phe drops to a level detected when the enzyme is present only at a concentration of 2 μM (Figure 3). Under these conditions, the cobalt absorption spectrum of the enzyme becomes that of its 1:1 complex with L-benzyl succinate (not shown).

The ES_2 peptide intermediate spectrum formed from 200 μM enzyme in the presence of 10 mM Dns-Ala-Ala and 50 mM L-Phe at 20 °C persists for over 30 min before changing to a spectrum characteristic of an L-Phe enzyme complex (Latt & Vallee, 1971) (Figure 4). HPLC analysis of samples withdrawn during this time shows that the basis of the ultimate disappearance of the ES_2 peptide intermediate spectrum is slow hydrolysis of one of the equilibrium components, Dns-Ala-Ala, to Dns-Ala and L-Ala.

As with the absorption, the EPR spectrum of the cobalt enzyme in the presence of individual peptide reaction products does not resemble that of a peptide intermediate (Geoghegan et al., 1983). However, when both products are present at high concentrations, the cobalt EPR spectrum becomes very similar to that of the peptide intermediate formed from Dns-Ala-Ala-Phe in the direction of hydrolysis (Figure 5). The resonance at g_1 = 6.8 exhibits hyperfine splitting, a characteristic of both peptide and depsipeptide intermediate spectra, and the apparent g values and hyperfine coupling constants for the enzyme in the presence of the product mixture are close to those detected transiently for the intermediate of Dns-Ala-Ala-Phe hydrolysis under cryospectrokinetic conditions. Thus, the intermediate-like spectral properties of the cobalt enzyme that arise from the enzyme–product reaction are identifiable both in its EPR and in the visible absorption spectrum.

The preceding data were obtained with a single pair of products. Intermediate-like absorption spectra (Figure 6) are also obtained with other N-blocked dipeptides and other amino acids. Thus, Z-Sarc-Ala, 2.5 mM, one of a class of peptide substrates that are hydrolyzed particularly slowly (Snoke & Neurath, 1949), in the presence of L-Phe, 50 mM, results in an absorption spectrum characteristic of a fully formed peptide intermediate (Figure 6). HPLC analysis confirms the formation of Z-Sarc-Ala-Phe. Similarly, the EPR spectrum of the cobalt enzyme in the presence of Z-Sarc-Ala and L-Phe

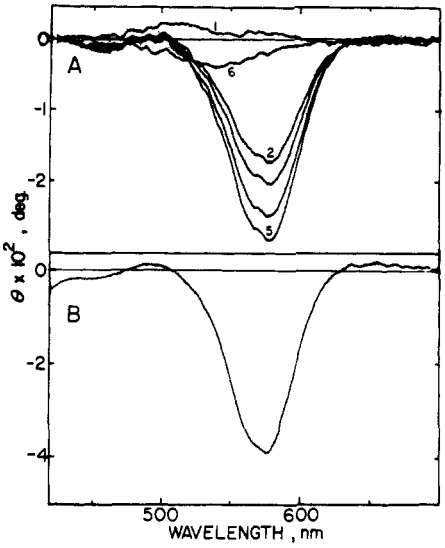

FIGURE 7: (A) CD spectral titrations of cobalt carboxypeptidase, 0.4 mM, in the presence of 50 mM L-Phe and increasing concentrations of Z-Sarc-Ala in 2 M NaCl. The path length of cuvette was 2 cm; other conditions were as for Figure 6: 1, cobalt enzyme plus L-Phe; 2–5, 1 plus Z-Sarc-Ala concentrations of 1.0, 1.4, 2.9, and 6.9 mM, respectively; 6, cobalt enzyme alone. (B) CD spectrum obtained with cobalt enzyme, 0.4 mM, in the presence of 20 mM Dns-Ala-Ala and 50 mM L-Phe.

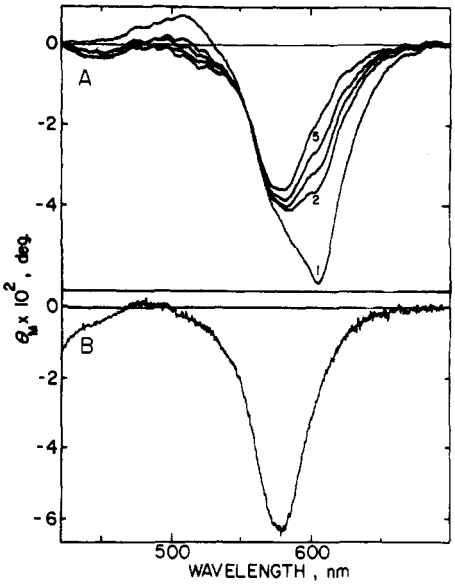

FIGURE 8: (A) MCD spectral titration of cobalt carboxypeptidase in the presence of 50 mM L-Phe and Z-Sarc-Ala corresponding to the CD titrations shown in Figure 7A. The spectrum of the free cobalt enzyme is omitted for clarity. The MCD spectra are corrected for CD. (B) MCD spectrum corresponding to the CD spectrum shown in Figure 7B.

is that of a peptide intermediate (not shown). Importantly, use of the carbobenzoxy N-blocking group confirms the existence of the 480-nm transition characteristic of the absorption spectrum of the peptide intermediate (Auld et al., 1984). Replacement of L-Phe by L-Leu, L-Val, or L-Ala results in formation of the intermediate-like enzyme–peptide complex but in decreasingly lesser fractions of the total enzyme, likely due to higher dissociation constants for the peptides synthesized (Figure 6).

Cobalt carboxypeptidase A exhibits a weak CD spectrum with a single negative band centered at 538 nm (500 deg cm^2/dmol) (Latt & Vallee, 1971). L-Phe perturbs this spectrum as shown in Figure 7A, changing the line shape in a characteristic fashion that has been reported (Latt & Vallee, 1971), whereas 2.5 mM Z-Sarc-Ala has no effect. The CD spectrum of the intermediate formed from 6.9 mM Z-Sarc-Ala and 50 mM L-Phe is marked by a 10-fold enhancement in negative ellipticity at 578 nm (Figure 7A). The CD spectrum of the intermediate resulting from 20 mM Dns-Ala-Ala and 50 mM L-Phe is nearly identical (Figure 7B).

Formation of the peptide intermediates for Z-Sarc-Ala-Phe (Figure 8A) and Dns-Ala-Ala-Phe (Figure 8B) is also accompanied by distinctive effects on the cobalt enzyme MCD spectrum. Addition of Z-Sarc-Ala to the enzyme–L-Phe mixture brings about a shift in maximal ellipticity from 604 to 578 nm; an isosbestic point at 592 nm indicates that the titration corresponds to conversion of the initial species directly to the final species (Figure 8A).

Discussion

The physical and chemical detection and characterization of intermediates in enzymatic catalysis is most often accomplished by rapidly mixing enzyme with substrate and observing transient intermediates during their conversion to products. This requires rapid measurements or suitable quenching procedures. We have previously used both approaches to characterizied peptide and ester intermediates of carboxypeptidase A (Galdes et al., 1983, 1986; Geoghegan et al., 1983; Auld et al., 1984).

With certain enzymes, however, productive enzyme–substrate complexes may be observed even when the reaction is at equilibrium, providing excellent opportunities for their characterization (Knowles & Albery, 1977). The present data are the first to show that this approach is applicable to the intermediates of carboxypeptidase A.

An enzyme must catalyze its specific reaction in either direction with equal facility, and by the same mechanism. At equilibrium, these forward and reverse reactions proceed at equal rates. Subject to the requirement that equilibrium be maintained, however, there can be variation in the disposition of the majority of the enzyme present in the equilibrated substrate–product mixture. Thus, the enzyme could exist mostly either as free enzyme or as an enzyme–product complex. However there may also be conditions under which an enzyme–substrate complex is the predominant species at equilibrium, as shown here.

HPLC analysis of acid-quenched enzyme–product mixtures may be used to follow the "reverse" action of carboxypeptidase A as it catalyzes the attainment of substrate–product equilibrium, e.g., the production of Dns-Ala-Ala-Phe in a solution originally containing L-Phe and Dns-Ala-Ala. The experimental proof involves an acid-quenching step, which both inactivates the enzyme and releases bound substrate (Galdes et al., 1986); hence, the substrate concentration measured by HPLC includes both free substrate and that bound to the enzyme. At catalytic enzyme concentrations, i.e., ≤ 1 μM, the equilibrium levels of substrate and products (Figure 1) are in good agreement with those predicted on thermodynamic grounds (Carpenter, 1960), and the concentration of substrate synthesized is independent of the enzyme concentration.

At higher enzyme concentrations, however, the amount of substrate detected exceeds that predicted by simple equilibrium considerations (Figure 1), suggesting that some physical or chemical agent is serving to "trap" Dns-Ala-Ala-Phe. As we observe no precipitation or aggregation of the peptide, the remaining possibility is that the Dns-Ala-Ala-Phe in excess of the amount predicted by K'_{eq} is bound to the enzyme. The observation that no excess Dns-Ala-Ala-Phe is detected when L-benzyl succinate occupies the active site following product

and substrate equilibration confirms this.

The spectral data show that the ES complex in identical with the one previously identified as a transient catalytic intermediate when the reaction is examined in the forward direction (Auld et al., 1984). Its appearance, concomitant with the generation of Dns-Ala-Ala-Phe, shows that this intermediate is a complex of the enzyme with intact peptide rather than an enzyme–product complex, in agreement with a rapid-quench study of the hydrolysis of peptides (Galdes et al., 1986).

Schematically, the situation can be represented as follows:

$$S \rightleftharpoons P_1 + P_2; \quad K'_{eq} = [P_1][P_2]/[S] \tag{1}$$

$$E + S \rightleftharpoons ES_T \rightleftharpoons E + P_1 + P_2; \quad K_{app} = [E_T\text{–}ES_T][S]/[ES_T] \tag{2}$$

$$[S_T] = [S] + [ES_T] = [S] + [E_T]/(1 + K_{app}/[S]) \tag{3}$$

where $[E_T]$, $[ES_T]$, and $[S_T]$ represent the total enzyme, ES complexes, and substrate concentrations, respectively, E, S, P_1, and P_2 represent the free enzyme, substrate, and products, K'_{eq} is the apparent overall equilibrium constant in the presence of 55 M water, and K_{app} is the apparent K_m for S in the presence of P_1 and P_2 (Breslow & Wernick, 1977).

Equation 3 predicts that at fixed product concentrations the total substrate concentration in the reaction mixture will linearly depend on the enzyme concentration. This linear relationship was indeed found for the synthesis of Dns-Ala-Ala-Phe from Dns-Ala-Ala and L-Phe (Figure 1); the y intercept of a plot of [Dns-Ala-Ala-Phe] vs. [carboxypeptidase A] yields a value of 48 M for K'_{eq}, in reasonable agreement with the value of 30 M predicted by the theory of Carpenter (1960). Equation 3 also implies that significant accumulation of substrate above its equilibrium concentration will only occur optimally when $K_{app} \leq [S]$, i.e., for tight-binding substrates. In fact, Dns-Ala-Ala-Phe is one of the tightest binding peptide substrates known for carboxypeptidase A ($K_m \simeq 3$ μM with the cobalt enzyme), which leads to the high levels of synthesized peptide detected in these experiments. In contrast, when the experiment is conducted with 20 mM Dns-Gly-Gly and 50 mM L-Phe, the measured concentration of synthesized Dns-Gly-Gly-Phe is fairly constant (20.7 ± 4 μM; K_{eq} = 48 M) over the enzyme concentration of 1–100 μM. The K_m for Dns-Gly-Gly-Phe is 400 μM (Latt et al., 1972), and hence, since $[S] \ll K_{app}$, it does not accumulation above its equilibrium concentration.

Rapid-mixing cryospectroscopic studies earlier showed that two sequential ES complexes, denoted ES_1 and ES_2, are formed during the hydrolysis of Dns-Ala-Ala-Phe and other peptide substrates by carboxypeptidase A (Galdes et al., 1983; Geoghegan et al., 1983; Auld et al., 1984). The kinetic constants governing the interconversion of these two intermediates predict that ES_2 will predominate at equilibrium. For Dns-Ala-Ala-Phe, it has been calculated that $[ES_2]/[ES_1] \simeq 200$ (Auld et al., 1984). This is in good agreement with the present observation of the cobalt enzyme absorption and EPR spectra previously assigned to the ES_2 intermediate for peptides in the equilibrated mixture of enzyme, products, and substrate. The spectral properties of ES_1 await delineation.

The present observations extend the time frame feasible for experimental analysis of the ES_2 peptide intermediate from milliseconds to several minutes at ambient temperatures or even longer if the temperature is lowered. This offers obvious opportunities to obtain further knowledge of this species by applying experimental techniques that employ time averaging or are otherwise not particularly suited to rapid operation at low temperature. The CD and MCD spectra of the peptide intermediate are two instances in which this has been accomplished (Figures 7 and 8).

The rates of formation and breakdown of the ES_2 intermediate of peptide hydrolysis have now been obtained for a number of substrates (Galdes et al., 1983). Further, the relevant cobalt absorption, EPR, CD, and MCD spectra (Geoghegan et al., 1983; Galdes et al., 1983; Auld et al., 1984; see above) are now known, as is the fact that this intermediate is an enzyme–substrate rather than an enzyme–product complex (Galdes et al., 1986). The MCD spectrum (Figure 8) suggests pseudotetrahedral coordination geometry for the metal atom in the intermediate, since in general its line shape corresponds to that of many tetrahedral cobalt complex ions (Holmquist et al., 1975). The CD spectrum, however, lacks an obvious parallel with those of any enzyme–inhibitor complexes of the enzyme (Latt & Vallee, 1971); this indicates that it arises from a ligand field about the cobalt atom that is generated only when a substrate is bound to the enzyme.

The present experiments further suggest that this equilibrium approach can be optimized to stabilize intermediates in a time frame of hours. Hydrolysis of Dns-Ala-Ala by the high enzyme concentrations used limited the length of time for which the enzyme–Dns-Ala-Ala-Phe complex could be studied. However, the very slowly hydrolyzed and weakly binding peptide substrate Z-Sarc-Ala with $k_{cat} < 1$ s^{-1} at 20 °C and $K_m > 25$ mM (K. F. Geoghegan, unpublished observations) largely overcomes this effect, since peptides whose penultimate peptide bond is N-methylated are hydrolyzed very slowly by carboxypeptidase A (Snoke & Neurath, 1949). The absorption and EPR spectra of the ES_2 intermediate of Z-Sarc-Ala-Phe show it to be a tightly bound substrate of the enzyme. The intermediate is less fully formed when the L-Phe is replaced by an equivalent concentration of L-Leu or L-Val and is scarcely detectable when L-Ala is the amino acid product (Figure 6). It seems likely that the use of slowly hydrolyzed components such as Z-Sarc-Ala in conjunction with the use of subzero temperatures will provide authentic intermediates of peptide hydrolysis for periods long enough to permit structure analysis by almost any experimental method (e.g., NMR or EXAFS).

New routes to the detection, stabilization, and characterization of enzymatic intermediates continue to be sought as valuable approaches to mechanistic questions. The present data show that carboxypeptidase A can serve as an "equilibrium trap" for one of its own very highly specific substrates. This suggests that intermediates of other proteases may be susceptible to trapping by the same means as shown here. Endoproteases, which catalyze reactions more thermodynamically favored for peptide synthesis than those of exopeptidases (Carpenter, 1960), are particularly susceptible to such approaches. Nonetheless, validation of the relevance to catalysis of a spectrally defined state of an enzyme by means of rapid syncatalytic analysis will continue to be a primary requirement.

ACKNOWLEDGMENTS

We kindly thank Dr. Richard E. Martinelli for the EPR data on the Dns-Ala-Ala-Phe intermediate in the hydrolytic direction.

Registry No. L-Ala, 56-41-7; L-Val, 72-18-4; L-Leu, 61-90-5; Z-Sarc-Ala, 66378-07-2; L-Phe, 63-91-2; Dns-Ala-Ala-Phe, 84623-92-7; Dns-Ala-Ala, 102830-37-5.

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Carbohydrate Substrate Specificity of Bacterial and Plant Pyrophosphate-Dependent Phosphofructokinases†

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ABSTRACT: Pyrophosphate-dependent phosphofructokinase from the facultative anaerobic bacterium *Propionibacterium freudenreichii* and from the mung bean *Phaseolus aureus* has been purified to homogeneity. Potential utilization of carbohydrate substrate analogues for each enzyme was initially screened by using Fourier transform ^{31}P NMR at pH 8 and 25 °C and monitoring the appearance of the phosphate resonance in the direction of D-fructose 6-phosphate phosphorylation (forward reaction direction) and, with the bisphosphate analogues, the appearance of the pyrophosphate resonance in the direction of phosphate phosphorylation (reverse reaction direction). Both enzymes are strict in their requirements for the sugar phosphate substrate, with only D-fructose 6-phosphate, D-sedoheptulose 7-phosphate, and 2,5-anhydro-D-mannitol 6-phosphate, or their respective bisphosphates in the reverse reaction direction, utilized as substrates at detectable levels. The dissociation constants for D-psicose 6-phosphate, D-tagatose 6-phosphate, and L-sorbose 6-phosphate are an order of magnitude larger than that for D-fructose 6-phosphate, indicating a stringent steric requirement for the D-threo (trans) configuration at the two nonanomeric furan ring hydroxyl groups. These results strongly suggest that the anomeric, epimeric, and tautomeric form of the sugar phosphate substrates favored by both enzymes is the β-D-fructofuranose form. Dissociation constants for nonsubstrate analogues were used to provide information on the nature of the active site. Competitive inhibition patterns vs. fructose 1,6-bisphosphate were obtained for a series of 1,*n*-alkanediol bisphosphates (where $n = 2$–9). The bacterial enzyme binds compounds with $n = 6$, 7, and 8 more tightly ($K_i \cong 200$ μM) than any of the others tested. The plant pyrophosphate-dependent phosphofructokinase, however, binds all analogues less tightly than the bacterial enzyme with 1,8-octanediol bisphosphate ($K_i \cong 650$ μM) binding tighter than any of the other analogues in the alkanediol bisphosphate series. Thus, although the active sites for the two enzymes are similar, there are distinct differences.

Pyrophosphate-dependent phosphofructokinase (pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90) catalyzes the reversible reaction:[1]

$$F6P + MgPP_i \rightleftharpoons FBP + P_i + Mg^{2+} \quad (1)$$

The enzyme has a very strict requirement for pyrophosphate as the phosphoryl donor with as yet no detectable rates reported for analogues of pyrophosphate or for tri- and tetrapolyphosphates (Reeves et al., 1974; O'Brien et al., 1975; Carnal & Black, 1979; Sabularse & Anderson, 1981; Bertagnolli & Cook, 1984; Wood & Goss, 1985). However, the carbohydrate substrate (phosphoryl acceptor) for this enzyme

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[1] Abbreviations: ATP-PFK, ATP-dependent phosphofructokinase; PP_i-PFK, pyrophosphate-dependent phosphofructokinase; ATP, adenosine 5′-triphosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; F6P, fructose 6-phosphate; T6P, tagatose 6-phosphate; FBP, fructose 1,6-bisphosphate; Taps, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino]-1-propanesulfonic acid; F2,6P, fructose 2,6-bisphosphate; G6P, glucose 6-phosphate; S7P, sedoheptulose 7-phosphate; SBP, sedoheptulose 1,7-bisphosphate; Pipes, 1,4-piperazinediethanesulfonic acid; C_nP_2, *n*-alkanediol 1,*n*-bisphosphate; ADP, adenosine 5′-diphosphate; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, NAD phosphate.

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