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Rapid-Scanning Cryospectroscopy of Enzyme–Substrate–Inhibitor Complexes of Cobalt Carboxypeptidase A†

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Received February 20, 1986; Revised Manuscript Received April 2, 1986

ABSTRACT: Rapid-scanning cryospectroscopy of cobalt(II)-substituted carboxypeptidase A serves to identify and characterize ternary enzyme–substrate–inhibitor (IES) complexes formed by the interaction between the enzyme, a peptide substrate, and a noncompetitive inhibitor. A cobalt absorption spectrum distinct from any induced by peptide or inhibitor alone signals formation of the IES complex. Tight-binding noncompetitive inhibitors containing an aromatic ring, e.g., β-phenylpropionate, cause the IES complex to form much more slowly than simple binary complexes of the enzyme with either peptide or inhibitor. An inhibitor such as acetate, which binds more weakly and is less bulky, permits the IES complex to form relatively quickly. Remarkably, the cobalt spectra of the IES complexes match those previously found for the steady-state ester (depsipeptide) intermediates. Chemical quenching studies have demonstrated that in these ester intermediates the scissile bond is broken [Galdes, A., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 646–651]. This finding, in conjunction with the present studies, implies that a peptide and a noncompetitive inhibitor of its hydrolysis occupy the same binding loci as the hydrolytic products of a depsipeptide and further indicates that breakdown of an enzyme–biproduct complex is rate-determining for the turnover of depsipeptides.

Elucidation of an enzyme's mechanism requires detailed information about the transient structural changes that underlie its catalytic activity. Since these processes generally are very rapid, the response times of the methods applied to their detection must also be fast; stopped-flow and rapid-scanning spectroscopic techniques are particularly suitable (Auld, 1979). Application of such methods at subzero temperatures using an appropriate cryosolvent (Douzou, 1977) combines the advantages of shortening the analytical time frame with those of extending the duration of the process studied (Galdes et al., 1983). This approach has enabled the acquisition of absorption and EPR[1] spectra of the steady-state intermediates formed transiently during the hydrolysis of peptide and ester substrates by cobalt carboxypeptidase A, a fully active metallo derivative of this zinc protease (Geoghegan et al., 1983; Auld et al., 1984).

Kinetic studies of carboxypeptidase A inhibition have pointed to differences between the hydrolytic pathways for peptides and esters (Auld & Holmquist, 1974). Monocarboxylate anions exhibit two forms of inhibition toward carboxypeptidase substrates, one generally competitive with esters (Bunting & Myers, 1973, 1975) and one noncompetitive with peptides (Auld & Vallee, 1970; Auld & Holmquist, 1974). On the basis of the latter kinetic observation, such inhibitors are predicted to be capable of being cobound to the enzyme with a peptide substrate, forming an IES complex. The kinetics of the formation of such IES complexes and their structural basis are of interest from several viewpoints. Stopped-flow cryospectrometry has now made possible simultaneous measurements both of their rates of formation and of their absorption spectra.

Materials and Methods

Carboxypeptidase Aα (Sigma Chemical Co.) prepared by the method of Cox et al. (1964) was recrystallized and then converted to crystalline cobalt enzyme by a dialysis method (Geoghegan et al., 1983). The stopped-flow cryospectrometer has been described (Hanahan & Auld, 1980; Geoghegan et al., 1983). Scan times were 16.48 ms. Enzyme and substrate samples were prepared in 1 M NaCl for studies at 0 °C or in 4.5 M NaCl for studies at −15 to −20 °C. Precautions were taken to remove adventitious metal ions (Thiers, 1957). All solutions were buffered with 0.02–0.05 M MES. The pH values measured at +20 °C were corrected for the temperature coefficient of the buffer (Good et al., 1966).

† This work was supported in part by Grant-in-Aid 24967 from the National Institutes of Health of the Department of Health and Human Services to Harvard Medical School.
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[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; EPR, electron paramagnetic resonance.

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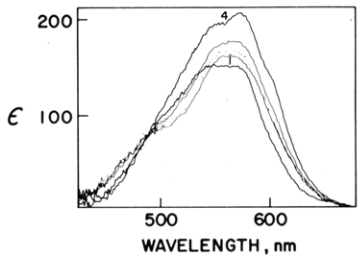

FIGURE 1: Changes in the absorption spectrum of cobalt carboxypeptidase A, 0.13 mM, upon mixing at 0 °C with Bz-Gly-Gly-Phe, 10 mM, in 50 mM MES and 1 M NaCl, pH 6.3. Spectra of the enzyme are shown at the following times after stopped-flow mixing: (1) 40 ms, (2) 1 s, (3) 5 s, and (4) 10 s. The 10-s spectrum (4) is that of the enzyme in the presence of the products of Bz-Gly-Gly-Phe hydrolysis, Bz-Gly-Gly and L-Phe. The spectrum of cobalt carboxypeptidase A under the same conditions of buffer and temperature is shown at the bottom in a heavy line.

Results and Discussion

Our combined cryokinetic (Galdes et al., 1983) and cryospectroscopic (Geoghegan et al., 1983) studies of zinc and cobalt carboxypeptidase have disclosed two intermediates in the hydrolysis of both peptides and depsipeptides (esters) and furnished all the rate and equilibrium constants for the reaction scheme:

$$E + S \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightarrow E + P$$

The chemical and kinetic data indicate that neither ES_1 nor ES_2 is an acyl enzyme intermediate. Both absorption and EPR spectra of ES_2 intermediates have consistently demonstrated (i) the formation of transient metal complexes and (ii) differences between the effects induced by peptides on the one hand and esters on the other (Auld et al., 1984).

Bz-Gly-Gly-Phe, 10 mM, is hydrolyzed rapidly by cobalt carboxypeptidase A, 0.1 mM, at 0 °C, with transient formation of a typical "peptide ES_2 intermediate" spectrum (Auld et al., 1984). Multiple rapid scans of the cobalt absorption spectrum during the reaction show that the intermediate forms within 40 ms of stopped-flow mixing of the enzyme and substrate (spectrum 1 of Figure 1). Its existence is signaled by a shift in the spectrum to a form that has a λ_{max} at 570 nm with intensity slightly increased relative to that of the free enzyme and with a distinctive shoulder near 480 nm on the high-energy side of the spectrum. The ES_2 intermediate remains at a constant steady-state concentration for at least 1 s, after which time it declines in concentration to zero over the next 10 s. After this time, the cobalt spectrum is that of the enzyme–phenylalanine product complex (K_i for L-Phe is 2.5 mM).

β-Phenylpropionate is a noncompetitive inhibitor of the hydrolysis of Bz-Gly-Gly-Phe; this implies that both the peptide and the inhibitor can be bound simultaneously to carboxypeptidase A. Verification of this cobinding and an understanding of its structural basis would clarify the mechanisms both of catalysis and of its inhibition.

At −17 °C in the presence of both Bz-Gly-Gly-Phe, 5 mM, and β-phenylpropionate, 0.5 mM, a transient cobalt carboxypeptidase A species exhibits an absorption spectrum (Figure 2A) that is distinct from those observed in the presence of either the substrate (Figure 1) or the inhibitor alone (Latt & Vallee, 1971). Similar spectral changes have been observed in 1 M NaCl at 0 °C, although they occur much more rapidly, as would be expected. Control experiments, not shown here, establish that this species is not obtained when Bz-Gly-Gly-Phe is replaced by equivalent concentrations by Bz-Gly-Gly, Bz-Gly, or either one of these products together with L-Phe. Thus, the new spectrum is that of a ternary enzyme–peptide–inhibitor

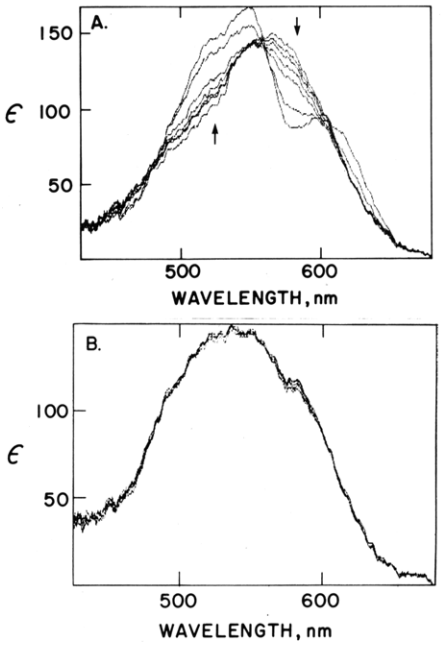

FIGURE 2: Changes in the absorption spectrum of cobalt carboxypeptidase A, 0.1 mM, upon mixing at −17 °C with a solution containing Bz-Gly-Gly-Phe, 5 mM, and β-phenylpropionate at (A) 0.5 mM and (B) 20 mM in 40 mM MES and 4.5 M NaCl, pH 7.0. Spectra of the enzyme are shown at the following times after mixing: (A) 80 ms, 150 ms, 230 ms, 480 ms, 1 s, 10 s, and 60 s; (B) 80 ms, 480 ms, 1 s, 10 s, 30 s, and 60 s. The arrows denote the direction of the changes in absorbance.

(IES) complex. Investigation of its rate of formation at −17 °C in 4.5 M NaCl permits resolution of successive stages of the process.

In the first 100 ms following stopped-flow mixing of the enzyme with a solution of the substrate plus inhibitor, the cobalt absorption spectrum becomes quite similar to that initially observed when the enzyme is mixed with Bz-Gly-Gly-Phe alone (Figures 1 and 2A). However, between 0.20 and 30 s the spectrum progressively changes to a new form unlike either of those seen with the substrate or inhibitor alone. This spectrum has a band with maximal absorptivity near 551 nm, a shoulder at 520 nm, a local minimum at 596 nm, and a broad peak centered at 610 nm. Remarkably, the line shape and intensities closely resemble those of the type of spectrum that typifies the steady-state intermediate in the hydrolysis of depsipeptides by cobalt carboxypeptidase A (Geoghegan et al., 1983; Auld et al., 1984).

At −17 °C, the enzyme's spectrum does not change further for many minutes following formation of the depsipeptide intermediate like complex. The half-time for disappearance of this complex is about 30 min at −17 °C. Thus, the inhibitor permits binding of the substrate Bz-Gly-Gly-Phe while effectively retarding its breakdown, as would be expected for a noncompetitive inhibitor.

Carboxypeptidase A binds β-phenylpropionate in more than one mode (Latt & Vallee, 1971). In addition to the noncompetitive mode examined here, it also binds in a second, weaker mode that competitively inhibits peptide hydrolysis (Auld et al., 1972). At pH 6, β-phenylpropionate binds more tightly in the noncompetitive mode, with a K_i value of approximately 10 μM, while the competitive mode is apparently due to binding of a second molecule, with a K_i' value of approximately 1 mM. The EI and IEI complexes are readily distinguished by their cobalt spectra. The first β-phenylpropionate molecule binds without affecting the cobalt spectral line shape but causes the maximal intensity of the spectrum

to increase by 30%; in contrast, binding of the second molecule causes a significant spectral shift and lowers the maximal intensity below that of the free enzyme (Latt & Vallee, 1971). On this basis, inhibitor concentrations greater than 1 mM would be predicted to result in increasing competition between the peptide substrate and β-phenylpropionate for a site on the enzyme.

Rapid mixing of cobalt carboxypeptidase A, 0.1 mM, with Bz-Gly-Gly-Phe, 5 mM, and β-phenylpropionate over a wide concentration range, from 0.5 to 20 mM, confirms this expectation (Figure 2). With the inhibitor at 0.5 mM giving an $[I]/K_i$ of 50 and $[I]/K_i'$ of 0.5, the IES complex forms fully (Figure 2A). Higher concentrations of β-phenylpropionate, however, cause partition of the enzyme between this IES and the IEI species, in which the second inhibitor molecule competes with Bz-Gly-Gly-Phe for the overlapping portion of their binding sites. With the inhibitor at 20 mM, giving an $[I]/K_i'$ of 10, the enzyme's spectrum is fully in the form denoting the IEI species throughout the time range of 50 ms–60 s (Figure 2B), demonstrating that Bz-Gly-Gly-Phe is now totally excluded from the active site.

The spectral effects observed here with Bz-Gly-Gly-Phe and β-phenylpropionate are quite analogous to those seen with other peptide substrates and carboxylic acid inhibitors. IES complexes that are spectrally similar form with peptides such as Z-Gly-Gly-Phe, Z-Sar-Phe, and Dns-Gly-Phe and inhibitors such as β-phenyl-L-lactate, β-phenyl-D-lactate, 2-phenylacetate, indole-3-acetate, 2-hydroxyisocaproate, and acetate. A number of these inhibitors are noncompetitive inhibitors of peptide hydrolysis (Auld & Vallee, 1970; Auld & Holmquist, 1974), while all of them are competitive with ester hydrolysis (Bunting & Myers, 1973; Kaiser & Kaiser, 1969). A carboxyl group is the common structural feature of all these compounds; the presence or absence of an α-hydroxyl group or an aromatic ring does not prevent them from forming a distinctive IES spectrum. In marked contrast, the presence of an α-amino group does prevent the participation of the inhibitor in such a ternary complex; neither L-phenylalanine nor D-phenylalanine is able to produce an IES complex with a peptide, consistent with kinetic demonstrations that they are competitive inhibitors of peptide hydrolysis (Auld et al., 1972).

The formation of an IES complex is a relatively slow process, as observed for Bz-Gly-Gly-Phe and β-phenylpropionate at −17 °C. Slow formation of an IES complex is also observed at 0 °C when the cobalt enzyme, 0.1 mM, is mixed with the substrate Z-Sar-Phe, 5 mM, and the inhibitor β-phenyl-L-lactate, 0.2 mM (Figure 3A). Following initial formation of a peptide intermediate type spectrum within 30 ms, over the next 30 s the enzyme's cobalt spectrum slowly shifts to the form that characterizes the ternary IES complex. In contrast, replacement of the relatively bulky β-phenyl-L-lactate by the smaller and more weakly binding inhibitor acetate, 50 mM, allows formation of a spectrally similar complex in less than 150 ms under otherwise equivalent conditions of enzyme and substrate concentrations and at the same pH and temperature conditions (Figure 3B). Although the inhibitors are used at unequal concentrations, each is present at a value close to its K_i; thus, the large difference in rates of formation of the two spectrally similar complexes is probably due in major part to the different molecular sizes of the two inhibitors.

Initial formation of a peptide intermediate spectrum is not an invariable feature of the spectral changes that follow mixing of the cobalt enzyme with a combination of a peptide and an inhibitor. In experiments conducted with a molar ratio of β-phenyl-L-lactate to Z-Sar-Phe higher than that used in

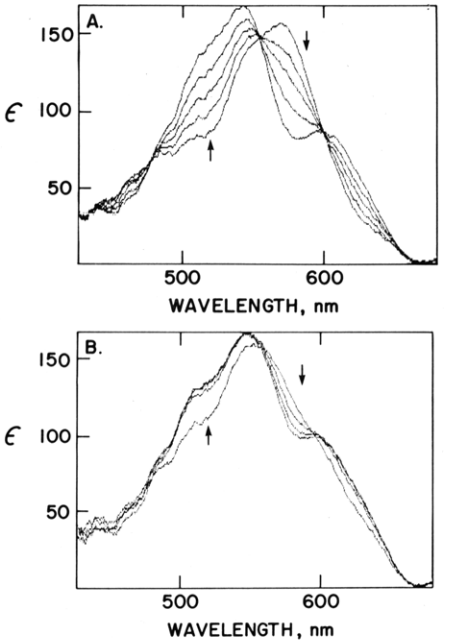

FIGURE 3: Changes in the absorption spectrum of cobalt carboxypeptidase A, 0.1 mM, following mixing at 0 °C with Z-Sar-Phe, 5 mM, and either (A) β-phenyl-L-lactate, 0.2 mM, or (B) acetate, 50 mM. Spectra of the enzyme are shown at the following times after mixing: (A) 30 ms, 480 ms, 1 s, 2 s, and 30 s; (B) 70 ms, 100 ms, 130 ms, and 180 ms. The buffer is the same as in Figure 1.

Figure 3A, the enzyme is observed first to have a cobalt spectrum similar to that of its complex with the inhibitor alone, which then gives way to the spectrum of the IES complex. The present studies indicate that the first step in the formation of the IES complex is likely a rapid reversible formation of an EI or ES complex, which then in a reversible process slowly converts to the IES complex.

Rapid cryospectroscopy allows monitoring of structural changes within an enzyme active site within the time frame in which the enzyme performs its functions. This study, which focuses on the mechanism of noncompetitive inhibition, has yielded spectral data that constitute the first demonstration of the formation of IES complexes of carboxypeptidase A based on direct measurement of the cobalt spectrum, a structure-related property. The absorption spectrum that typifies these species differs markedly from that of the free enzyme, indicating direct interaction of the metal atom with at least one of the jointly bound ligands.

While kinetic studies predicted the existence of such IES species, the fact that their absorption spectra match those of depsipeptide intermediates could not be anticipated and promises to reward further study with significant insight into the mechanism by which the enzyme turns over these substrates. It is noteworthy that both L- and D-α-hydroxylic monocarboxylic inhibitors as well as nonhydroxylic inhibitors are competent to participate in the complexes whose spectra match those of depsipeptide steady-state intermediates. This finding strongly suggests that no ester bond is need to generate this species and is consistent with the postulate that the depsipeptide intermediate is formed after cleavage of the ester link. This implies that product dissociation must be the rate-determining step in the turnover of these substrates.

This postulate is consistent with the results of a separate rapid-quenching study, which showed that carboxypeptidase A rapidly releases 1 mol of product per mole of enzyme before steady state is established for a depsipeptide substrate (Galdes et al., 1986). It also provides a rational explanation for the observation that in many cases carboxypeptidase A turns over

peptides and their exact depsipeptide analogues at nearly equal rates despite the inherently more labile character of the esters (Bruice & Benkovic, 1966). If product release is rate-determing for the depsipeptide, then cleavage of the scissile bond may occur as a rapid early step in catalysis. The rate of completing turnover would then be determined by the subsequent slower product release step that could occur at rates comparable to those for peptide cleavage.

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Detection of a Transient Enzyme–Steroid Complex during Active-Site-Directed Irreversible Inhibition of 3-Oxo-Δ^5-steroid Isomerase†

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Received March 3, 1986; Revised Manuscript Received April 25, 1986

ABSTRACT: The reaction of the active-site-directed irreversible inhibitor (17*S*)-spiro[estra-1,3,5(10),6,8-pentaene-17,2′-oxiran]-3-ol (**5**β) with 3-oxo-Δ^5-steroid isomerase has been monitored by repetitive scanning ultraviolet spectroscopy of a solution of **5**β plus isomerase against a blank containing only **5**β. Upon initial mixing of **5**β with the isomerase an absorbance maximum at ca. 250 nm appears. With time, this peak decreases and is replaced with a new peak near 280 nm. These results directly demonstrate the existence of a transient enzyme–steroid intermediate in the inactivation reaction. The ultraviolet spectrum suggests that the steroid in the transient complex resembles the ionized phenol, while the phenolic group in the irreversibly bound complex is un-ionized. These spectral studies support our previous proposal that there are two enzyme–steroid complexes that are related by a 180° rotation about an axis perpendicular to the plane of the steroid nucleus. This hypothesis offers an explanation for the reaction of 17β-oxiranes with the same residue (Asp-38) that is thought to be involved in the catalytic mechanism. Two new oxiranes, (17*S*)-spiro[estra-1,3,5(10)-triene-17,2′-oxiran]-3β-ol (**6**β) and (17*S*)-spiro[5α-androstane-17,2′-oxiran]-3-one (**8**β), were also found to be potent active-site-directed irreversible inhibitors of the isomerase (k_3/K_I = 31 M^{-1} s^{-1} and 340 M^{-1} s^{-1}, respectively). The relationship of these results to the nature of the active site of the isomerase is discussed.

Active-site-directed irreversible inhibitors of steroid-metabolizing enzymes and affinity labels of steroid receptors have yielded significant insights into the molecular mechanism of biologically important processes (Gronemeyer, 1985; Katzenellenbogen, 1977, 1984; Benisek et al., 1982; Batzold et al., 1976). Fundamental to the design and utilization of such compounds is the existence of a high-affinity stereospecific steroid–protein interaction. However, there is recent evidence that several steroid-transforming enzymes may bind steroids in more than one orientation (Sweet & Samant, 1980; Bevins et al., 1980; Strickler et al., 1980; Adams & McDonald, 1981; Kayser et al., 1983; Waxman et al., 1983). Clearly, consideration must be given to the existence of multiple binding modes in the interpretation of the nature of binding of both substrates and inhibitors to the active site. In addition, it is possible that multiple binding modes of natural ligands may be biologically significant.

† Supported by a grant from the National Institutes of Health (GM 33059). Part of this work has been submitted by C.L.B. to the University of Maryland Baltimore Graduate School as partial fulfillment of the requirements for the Ph.D. degree.

0006-2960/86/0425-5159$01.50/0