Cryospectroscopy of Intermediates in the Mechanism of Carboxypeptidase A†

Kieran F. Geoghegan, Alphonse Galdes, Richard A. Martinelli, Barton Holmquist, David S. Auld, and Bert L. Vallee*

ABSTRACT: The electronic and paramagnetic spectral properties of cobalt carboxypeptidase A have served to identify transient intermediates in its catalysis of very rapidly hydrolyzed dansyl oligopeptides and their ester analogues at subzero temperatures. The visible absorption spectra of these intermediates are recorded with the same rapid-scanning, low-temperature, stopped-flow spectrometer that served to establish the kinetics of the previously unknown intermediate, ES_2; at −20 °C it forms in less than 500 ms and then is converted to products over a much longer period of time [Galdes, A., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 1888–1893]. The salt-dependent solubility of carboxypeptidase makes 4.5 M NaCl an ideal cryosolvent that alters neither the kinetic nor the spectral properties of cobalt carboxypeptidase. Both the absorption and EPR spectra of the peptide and ester intermediates, ES_2, differ significantly from one another and from those of the cobalt enzyme itself, its complexes with affinity labels, substrate analogue inhibitors, or products. The two types of substrates generate both different and new absorption bands and maxima of the visible spectrum. The corresponding EPR spectra differ most strikingly in the resolved hyperfine splitting of their g_1 resonances but also in their three apparent g values. The absorption, electron paramagnetic resonance, and magnetic circular dichroic spectra of the intermediates identify catalysis-related, dynamic alterations in the active site metal coordination sphere clearly distinct from those observed under static conditions. The systematic *changes* in the cobalt spectra of carboxypeptidase when forming the peptide and ester intermediates correlate with the formation of the ES_2 complex and thus render these data the first experimental test of the entatic-state hypothesis. Jointly, the present cryospectroscopic and previous cryokinetic studies of carboxypeptidase A show that peptides and esters form structurally distinct metallointermediates during hydrolysis. This is in accord with previous kinetic and chemical modification studies that indicated that the metal plays a critical but different role in the mechanisms of peptide and ester catalysis.

The chromophoric, paramagnetic cobalt(II) ion can be substituted readily for the colorless, diamagnetic zinc of carboxypeptidase A to yield a catalytically active, spectrally distinctive enzyme (Vallee et al., 1958; Coleman & Vallee, 1960; Vallee & Wacker, 1970; Latt & Vallee, 1971; Vallee & Holmquist, 1980; Geoghegan et al., 1983). Its unique absorption, CD,[1] MCD, and EPR spectra reflect the unusual, asymmetric environment that the enzyme creates for the cobalt atom (Vallee & Latt, 1970; Latt & Vallee, 1971; Kennedy et al., 1972; Holmquist et al., 1975) and are responsive to pH and the binding of inhibitors and pseudosubstrates (Latt & Vallee, 1971; Geoghegan et al., 1983). A relationship between the catalytic potential of the enzyme and the factors responsible for these unusual spectra has been postulated by Vallee & Williams (1968a,b).

As rapid kinetic approaches suitable to the study of enzyme intermediates have evolved, e.g., direct observation of ES complexes by RET (Auld, 1977; Lobb & Auld, 1980), the potential of these cobalt spectra to the recognition of the chemistry and structure of intermediates in metalloenzymes has been considered (Thompson et al., 1980). Yet, the documentation of spectral changes concomitant with catalysis has continued to present technical obstacles, leaving many of the features and detailed structural characteristics of metalloenzymes that result in catalysis experimentally inaccessible. Novel means for simultaneous rapid acquisition of kinetic and spectral data during catalysis are required to overcome these instrumental problems (Auld, 1979). Toward that end, we have combined a rapidly scanning spectrometer and a low-temperature stopped-flow instrument to allow simultaneous kinetic and spectral measurements at subzero temperatures in the same millisecond time frame. As a consequence, spectral changes can be recognized in the course of catalysis.

We here report the cryospectroscopic characterization of reaction intermediates that were first identified by cryokinetic studies of the carboxypeptidase A catalyzed hydrolysis of rapidly hydrolyzed peptide and ester substrates (Galdes et al., 1983). The absorption, EPR, and MCD spectra that characterize these intermediates are unlike those of complexes of this enzyme with inhibitors or pseudosubstrates and provide hitherto inaccessible information about an obligate step in the catalytic pathway. They show that formation of the catalytic intermediates is accompanied by characteristic changes in the spectral properties of cobalt carboxypeptidase that arise from alterations in the coordination sphere of the metal atom. Jointly, the cryospectroscopic and cryokinetic data indicate that peptides and esters form structurally distinct metallointermediates during hydrolysis and corroborate earlier indications that these two types of substrate are hydrolyzed through different mechanisms (Auld & Holmquist, 1974).

Materials and Methods

Bovine carboxypeptidase A (Sigma Chemical Co., St. Louis, MO), prepared by the method of Cox et al. (1964), was recrystallized and converted to cobalt carboxypeptidase crystals

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[1] Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; RET, radiationless energy transfer; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Cbz, carbobenzoxy; Dns, dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; OPhe, L-β-phenyllactate; OLeu, L-α-hydroxyisocaproic acid; metallointermediate, an intermediate formed during catalysis in which the metal coordination sphere is altered.

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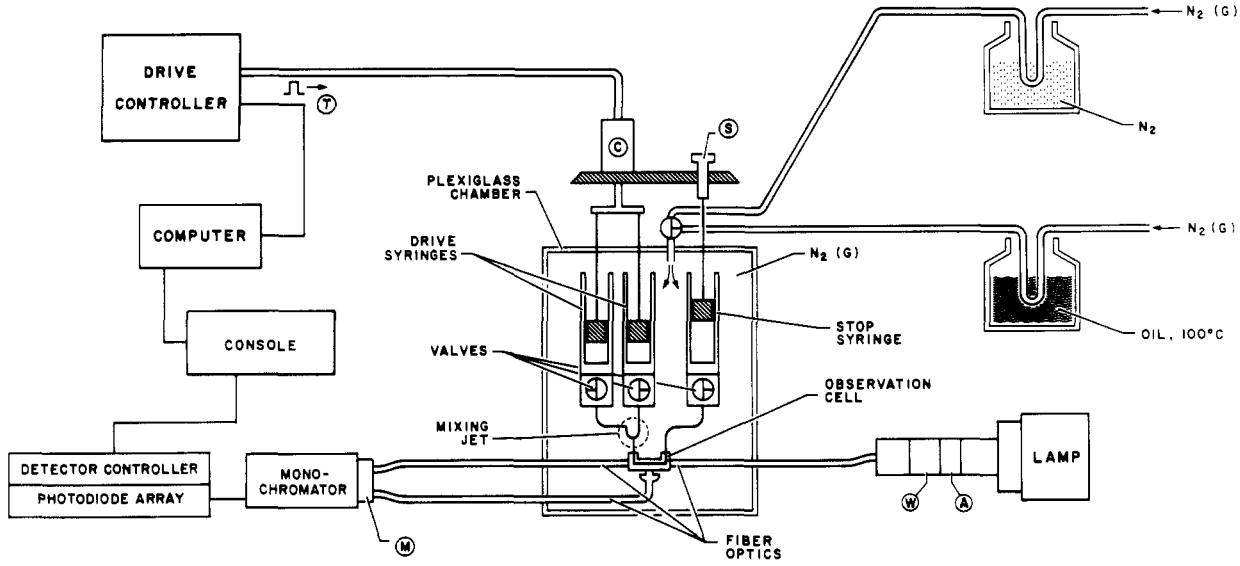

FIGURE 1: Schematic diagram of the low-temperature, stopped-flow, rapid-scanning spectrometer. Thermostated gaseous nitrogen is passed into the plexiglass environmental chamber that contains the quartz flow-through cell. The description of the arrangement of the light supply and detector system for measurement of fluorescence or single-wavelength transmittance has been reported (Hanahan & Auld, 1980). Quartz fiber optics are used to transmit light to the cell and from the cell to the monochromator/photodiode array. The light intensity is regulated by a combination aperture/diaphragm (A). A combination of optical and water filters removes ultraviolet and infrared radiation (W). A mode selector (M) allows scanning of either the transmittance or the fluorescence of the sample. On initiation of the reaction, the drive controller simultaneously triggers (T) the OM2 console to initiate recording and sends an air pulse to the pneumatic cylinder (C). The amount of solution mixed is controlled by the adjustable stop (S).

either as described by Auld & Holmquist (1974) or by successive dialysis of 2×10^{-4} M enzyme against 1 mM 1,10-phenanthroline in 1 M NaCl, 1 M NaCl containing 0.1 mM $CoCl_2$, 1 M NaCl, and 0.1 M NaCl, all containing 5 mM Hepes, pH 7.0. Crystalline cobalt carboxypeptidase prepared by these methods contained 0.93–1.0 g-atom of cobalt and less than 0.03 g-atom of zinc per mol of protein.

Oligopeptide and depsipeptide substrates were synthesized and characterized as previously described (Auld & Holmquist, 1974; Lobb & Auld, 1980). For most of the 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 ternary solvent 40% ethylene glycol–40% water–20% methanol containing 0.25 M NaCl–0.05 M sodium cacodylate was prepared according to the method of Douzou (1977). Deionized, glass-distilled water was used throughout. Precautions were taken to remove adventitious metal ions from all solutions (Thiers, 1957).

The visible absorption spectra of cobalt carboxypeptidase and its reaction intermediates were obtained by using a cryospectrometer consisting of a low-temperature stopped-flow instrument interfaced with a rapid-scanning spectrometer (Figure 1); the characteristics and performance of its stopped-flow module have been described (Hanahan & Auld, 1980).

The light source of the cryospectrometer is either a 200-W General Electric OC66A-T4CL quartz-iodine lamp or a 75-W Illumination Industries X75-2002 xenon lamp mounted in a LH 150 Schoeffel lamp housing equipped with a LH 150/1S UV-grade f/1.0 condenser to produce a collimated beam of light. Its intensity is controlled by an Ealing No. 22-3933 diaphragm with a 2–46-mm aperture coupled to an Alphax 46-mm shutter. Infrared radiation is removed by means of a cylindrical (4.5-cm diameter by 1.3-cm length) water filter containing optically flat quartz windows, and a Corning 305-nm cut-on filter eliminates UV radiation. A quartz lens focuses the collimated light on quartz fiber optics, which transmit the light to the 2-cm quartz observation cell in the low-temperature chamber. The light exiting the observation cell is transmitted through quartz fiber optics, dispersed by a JY 0.32-m Czerny-Turner monochromator (Model HR-320), and scanned by a EG & G PARC 1412 silicon photodiode array detector coupled to a 1218 detector controller. A Model 1215 OMA 2 console interfaced with a Digital Equipment PDP 11/34 computer provides for data manipulation and control of the system. The wavelength accuracy and resolution of the cryospectrometer were established with 5 mM aqueous solutions of neodymium and praseodymium nitrates. The Nd^{3+} absorption bands at 740.5, 575.5, and 522 nm and the Pr^{3+} band at 444.5 nm served for wavelength calibration. The λ_{max} values of 10 other bands measured between 340 and 800 nm at scan rates as high as 18 200 nm/s and at an absorbance of 0.01–0.05 were all in agreement to within 0.5 nm with those obtained by means of a deuterium line calibrated Cary 219 recording spectrophotometer.

For the spectral identification of short-lived intermediates described here, 16.48-ms spectral scans over the range from 400 to 700 nm were employed routinely. Signal to noise ratios were improved by computer-smoothing routines. All absorption spectra are corrected for background absorption due to protein and products. In conventional scanning on a Cary 219 spectrophotometer, this was achieved by placing the cobalt enzyme and the appropriate substrate in the reference cuvette followed by addition of 2 equiv of Zn(II) to form the zinc enzyme. Such compensation was achieved in rapid-scanning stopped-flow experiments by measuring and storing the spectrum of the zinc enzyme plus products for subsequent computer subtraction from the relevant spectrum of the cobalt enzyme plus substrate.

Slow-scanning (1–10 nm/s) cryospectroscopy was performed in a KM-611772 dewar cell (Kontes Glass, Vineland, NJ) adapted to a Cary 219 spectrophotometer. The dewar was filled with methanol, which was chilled by flowing cold nitrogen gas through an immersed copper coil. Temperature was monitored with a copper–constantan thermocouple immersed in the methanol and controlled by adjusting the nitrogen flow. The dewar cell was modified to permit rapid

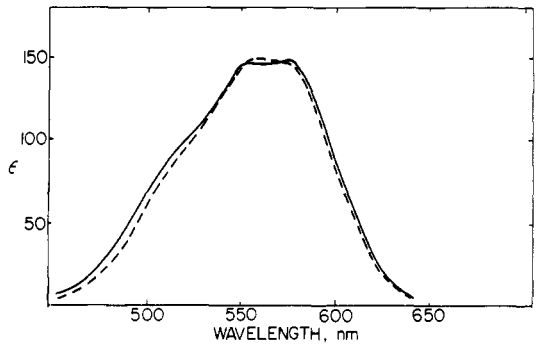

FIGURE 2: Absorption spectra of cobalt carboxypeptidase, 1.0×10^{-4} M, recorded on conventional and rapid-scanning spectrometers at 20 and −20 °C: spectrum at 20 °C, 1 M NaCl–50 mM Hepes, pH 7.5 (—), obtained on a Cary 219 spectrometer at a scan rate of 1 nm/s; spectrum at −20 °C, 4.5 M NaCl–10 mM Hepes buffer, pH 7.5 (--), obtained on the rapid-scanning spectrometer in 16.48 ms at a scan rate of 18 200 nm/s.

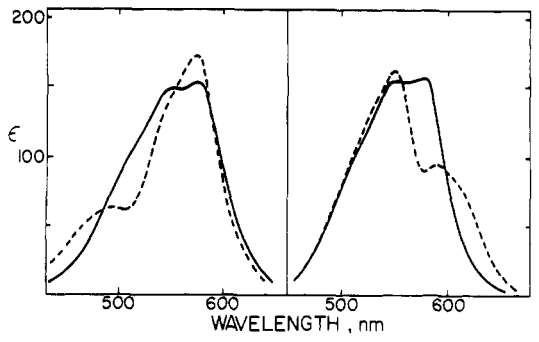

FIGURE 3: Absorption spectra of cobalt carboxypeptidase A reaction intermediates (ES_2) with peptide and ester substrates. Spectra were recorded on the rapid-scanning stopped-flow spectrometer, with cobalt enzyme, 1.0×10^{-4} M, and each substrate dissolved in 4.5 M NaCl–10 mM Hepes, pH 7.5, −20 °C. (Left panel) Spectrum recorded 250 ms after mixing cobalt enzyme with the peptide Dns-Gly-Phe, 2.0×10^{-4} M (--), and that recorded after hydrolysis is complete, 40 s (—). (Right panel) Spectrum recorded 500 ms after mixing cobalt enzyme with the ester Dns-Ala-Ala-OPhe, 1.0×10^{-4} M (--), and when hydrolysis is complete, 200 s (—).

addition of substrate to enzyme and manual mixing of the sample with a Teflon rod.

Cryostabilized intermediates for EPR studies were prepared directly in EPR tubes and trapped by rapid freezing in liquid nitrogen. For this purpose, aliquots of the cobalt enzyme, 275 μL, in 4.5 M NaCl–50 mM Hepes, pH 7.5, were cooled to −20 °C in standard EPR tubes, and 25 μL of substrate in 50% methanol was added and mixed rapidly by means of an extended-length positive-displacement Drummond pipet. After a period of time, usually 5–10 s, the tube was plunged into liquid nitrogen to quench the reaction. The sample was then kept at −195 °C until measurement at 4 K on an X-band Varian E-9 EPR spectrometer equipped with an Air Products Helitran apparatus for temperature control. The field was calibrated with Varian strong pitch, $g = 2.0028$ (Poole, 1967).

MCD spectra were measured on a Cary 61 spectropolarimeter equipped with a Varian V4145 superconducting magnet at a field of 4 T (40 000 G). A brass cell holder was designed to hold a 2-mL cuvette with a 2-cm light path within the bore of the superconducting solenoid, similar to the cell holder previously described (Holmquist & Vallee, 1978). The cell was maintained at −40 °C by cold nitrogen gas and the temperature monitored by a microthermocouple cemented to the cell holder in thermal contact with the cuvette. Both the cuvette and the cell holder were open at the top to allow addition of substrate to initiate reactions. The sample compartment of the instrument was purged with dry nitrogen to prevent water condensation. Cobalt enzyme, 2×10^{-4} M, in the ternary solvent was pipetted into the cuvette maintained at −40 °C in the brass cell holder. When the sample temperature had reached −40 °C, the substrate in methanol was added and brought to a final concentration of 1 mM and mixed, and the assembly was then inserted into the solenoid. At −40 °C, the intermediate formed with Dns-Ala-Ala-OPhe has a half-life for conversion to products of >15 min, allowing sufficient time for measurements of complete MCD spectra at a scan rate of 4 nm/s.

Results

The absorption spectrum of cobalt carboxypeptidase A, 1×10^{-4} M in 4.5 M NaCl–10 mM Hepes, pH 7.5, at −20 °C, recorded in 16.48 ms on the rapid-scanning cryospectrometer, exhibits a broad shoulder near 500 nm and two maxima of equal intensity at 555 and 575 nm ($\epsilon = 155$); it is virtually identical with that obtained in 1 M NaCl at 20 °C on a Cary 219 spectrophotometer (Figure 2) (Latt & Vallee, 1971). The spectrum of the enzyme at −40 °C in the ternary solvent, a cryosolvent also employed in this study to cryostabilize intermediates, is essentially the same as those in NaCl at 20 and −20 °C (Figure 7). Thus, neither the temperature nor the cryosolvent significantly alter the spectrum of cobalt carboxypeptidase from that in 1 M NaCl at 20 °C.

Both at room temperature and at −20 °C, the C-terminal and N-terminal products of the carboxypeptidase-catalyzed hydrolysis of the peptide and ester substrates used in this study have a minimal effect on the absorption spectrum of cobalt carboxypeptidase when added in concentrations comparable to those of substrates in the reaction mixture (data not shown).

The entire visible spectrum of the cobalt enzyme can be scanned within milliseconds after mixing with substrate to determine changes generated during catalysis. Our pre-steady-state kinetic studies of the carboxypeptidase A catalyzed hydrolysis of a number of peptides and esters by RET at −20 °C established the presence of a previously unknown intermediate, ES_2, which forms in less than 500 ms and then is converted to products over a time period[2] from 20 to 200 s as hydrolysis proceeds (Galdes et al., 1983). Here we will be concerned exclusively with the spectral properties observed coincident with the presence of this ES_2 intermediate, which is the predominant species during the steady state. These spectra, typical of the peptide and ester intermediates, differ significantly both from that of the resting enzyme and from one another.

The absorption spectrum of the cobalt enzyme in the presence of Dns-Gly-Phe, both 1×10^{-4} M, was recorded successively during the course of hydrolysis at −20 °C (Figure 3). Within 250 ms after mixing enzyme with substrate, i.e., the period during which ES_2 accumulates to its maximal concentration, the absorbance near 525 nm decreases while that at 575 nm increases to result in a new spectrum characteristic of the ES_2 intermediate with a λ_{max} at 570 nm. Simultaneously, the shoulder near 500 nm is replaced by a new maximum near 480 nm. Subsequently, as hydrolysis goes to completion, this spectrum is converted to that of the cobalt enzyme plus products, Dns-Gly and Phe.

The spectrum of the ES_2 intermediate formed with the ester, Dns-Ala-Ala-OPhe, differs markedly both from that of the resting enzyme and of the peptide ES_2 intermediate (Figure

[2] The decay time of ES_2 depends on both the enzyme and the identity of the substrate and on their concentrations. The range of times given is for the hydrolysis of a number of different substrates, by carboxypeptidase A, all 1×10^{-4} M at −20 °C, pH 7.5.

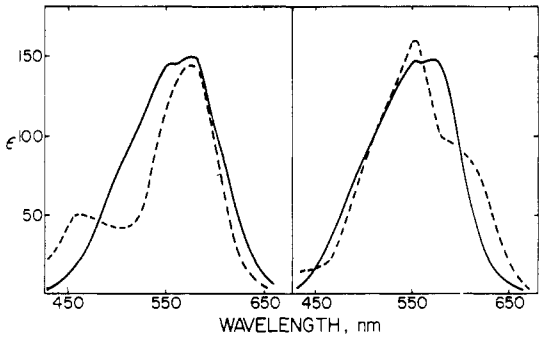

FIGURE 4: Rapid-scanning stopped-flow spectra of the intermediate ES_2 formed in the reaction of cobalt carboxypeptidase, 1×10^{-4} M, with peptides and esters: (left panel) Dns-Gly-Ala-Phe, 2.7×10^{-4} M, recorded at 250 ms (--) and 60 s (—); (right panel) Dns-Gly-Ala-OPhe, 1.2×10^{-4} M, recorded at 250 ms (--) and 10 s (—). Conditions for both are −20 °C and 4.5 M NaCl–10 mM Hepes, pH 7.5. In both instances, the last spectrum is that of enzyme plus products.

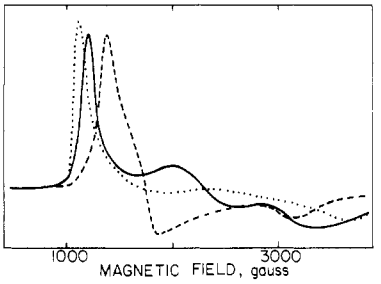

FIGURE 5: EPR spectra of cobalt carboxypeptidase, 5×10^{-4} M (—), and of its complexes with Phe, 10 mM (···), and OPhe, 10 mM (--), formed by addition of concentrated inhibitor to the enzyme in 4.5 M NaCl–50 mM Hepes, pH 7.5. The EPR spectra were recorded at 4 K.

3). Within 500 ms of mixing, a double-banded spectrum forms that has two distinct maxima at 550 and 600 nm and a shoulder near 500 nm. Over a subsequent period of 200 s, the spectrum of the ester ES_2 intermediate is converted to that of the enzyme plus products, Dns-Ala-Ala and OPhe. The rates of appearance and disappearance of these spectral intermediates correlate with those for formation of ES_2 and products, respectively, as determined by concomitant kinetic studies (Galdes et al., 1983).

Figure 4 shows the spectra obtained for the ES_2 intermediates of the tripeptide Dns-Gly-Ala-Phe and its exact ester analogue Dns-Gly-Ala-OPhe in 4.5 M NaCl, pH 7.5 at −20 °C. Again, they clearly differ from one another and also from that of cobalt carboxypeptidase alone but are remarkably similar to those obtained with Dns-Gly-Phe and Dns-Ala-Ala-OPhe, respectively. Thus, the spectrum of the peptide ES_2 intermediate has an absorption maximum at 575 nm and a new band near 470 nm, while that of the ester exhibits two bands of unequal intensity with maxima at 550 and 600 nm and a prominent shoulder at 520 nm. Though not shown here, the spectral properties of the steady-state intermediates formed during the hydrolysis of the peptides Dns-Ala-Ala-Phe, Dns-Glu-Ala-Phe, Cbz-Gly-Gly-Leu, and Cbz-Gly-Gly-Phe are similar to those of Dns-Gly-Phe and Dns-Gly-Ala-Phe. Similarly, the spectra of the intermediates formed with the esters Cbz-Gly-Gly-OVal and Cbz-Gly-Gly-OLeu are all nearly the same as those of the esters in Figures 3 and 4. In each case the ES_2 intermediate is formed rapidly, <500 ms, and then breaks down to yield a spectrum characteristic of the enzyme plus product mixture as hydrolysis goes to completion.

The EPR spectrum of cobalt carboxypeptidase in 4.5 M NaCl (Figure 5) is identical with that obtained in 1 M NaCl

Table I: Apparent g Values and Hyperfine Coupling Constants (A) for Cobalt(II) Carboxypeptidase[a]

sample	g_1	g_2	g_3	A ($\times 10^4$ cm^{-1})
Co(II) enzyme	5.65	2.98	2.03	unresolved
Co(II) enzyme + Phe	6.09	*b*	*b*	unresolved
Co(II) enzyme + OPhe	4.94	3.87	2.12	unresolved
ES_2 (Dns-Gly-Phe)	6.78	2.02	1.41	127
ES_2 (Dns-Ala-Ala-OPhe)[c]	6.99	2.08	1.44	171

[a] Solutions prepared in 4.5 M NaCl–50 mM Hepes, pH 7.5. Spectra measured at 4 K. [b] g values not assigned due to the weak and broad nature of the signals. [c] Assignments tentative due to complexity of spectrum which is under investigation.

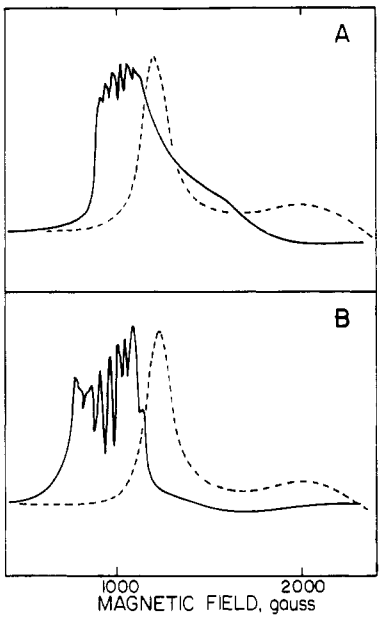

FIGURE 6: EPR spectra of cobalt carboxypeptidase, 5×10^{-4} M (--), and the ES_2 intermediates formed in its reaction with (A) Dns-Gly-Phe (—) and with (B) Dns-Ala-Ala-OPhe (—), both 2×10^{-3} M prepared in 4.5 M NaCl–50 mM Hepes, pH 7.5, −20 °C (see Materials and Methods). The spectra were recorded at 4 K.

(data not shown). It exhibits three resonances: a prominent one at $g_1 = 5.65$ and weaker ones at $g_2 = 2.98$ and $g_3 = 2.03$ (Table I). The spectra of the product complexes formed with Phe and OPhe (Figure 5) differ from that of the free enzyme, primarily in minor shifts of the g values that characterize the resonances (Table I).

The EPR spectra of the ES_2 intermediates are obtained by rapidly freezing the reaction mixture upon their formation (see Materials and Methods). The EPR spectra of the peptide and ester ES_2 intermediates differ from one another and from those of the resting cobalt enzyme and its complexes with substrate-analogue inhibitors or affinity labels in regard to the three apparent g values (Table I) and most strikingly in the well-resolved hyperfine splitting of the g_1 resonance (Figure 6). Moreover, the values of the hyperfine coupling constants, A, for the Dns-Gly-Phe and Dns-Ala-Ala-OPhe ES_2 intermediates differ. Thus, for the peptide ES_2 intermediate the hyperfine structure of g_1 centered at $g = 6.78$ has an A value of 127×10^{-4} cm^{-1} while that for the ester intermediate centered at $g = 6.99$ has an A value of 171×10^{-4} cm^{-1}. Raising the temperature of the cryotrapped peptide and ester ES_2 intermediates to 20 °C allows hydrolysis to go to completion and restores the EPR spectrum characteristic of the enzyme plus products (Figure 5).

As will be reported elsewhere, extensive investigations of these and other peptide and ester substrates demonstrate that

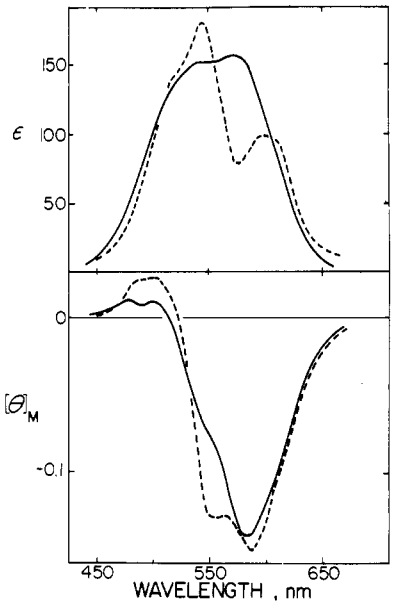

FIGURE 7: Absorption and MCD spectra of cobalt carboxypeptidase A, 2.0×10^{-4} M (—), and the intermediate ES_2 (--) formed during its reaction with Dns-Ala-Ala-OPhe (1 mM) at −40 °C, in a cryosolvent consisting of 40% ethylene glycol–20% methanol–40% water–0.05 M sodium cacodylate–0.25 M NaCl, pH 7.5. Units of MCD are deg·cm^2·dmol^{-1}·G^{-1}. The spectrum of the intermediate was measured within 2 min of mixing.

such hyperfine splitting is characteristic only of the ES_2 intermediates. Further, affinity labels, reversible substrate-analogue inhibitors, or products do not induce hyperfine splitting under the present conditions.

Lowering the temperature and changing the cryosolvent greatly prolongs the lifetime of the ES_2 intermediates. Thus, at −40 °C in 40% ethylene glycol–20% methanol–40% water containing 0.25 M NaCl–50 mM sodium cacodylate, pH 7.5, the half-life for the conversion of the Dns-Ala-Ala-OPhe ES_2 intermediate to products is increased greatly to approximately 40 min. Yet, the absorption spectrum of this ester intermediate at −40 °C in this ternary solvent is virtually the same as that at −20 °C in 4.5 M NaCl (Figure 7), indicating that neither temperature nor solvent alters it. The extended lifetime of this intermediate under these conditions permitted the measurement of its MCD spectrum (see Materials and Methods).

The MCD spectrum of cobalt carboxypeptidase exhibits one major negative extremum at 577 nm, a shoulder at 537 nm, and two positive extrema at 503 and 478 nm. It closely resembles the MCD spectra of Co(II) complex ions with tetrahedral coordination, as determined by X-ray diffraction analysis (Holmquist et al., 1975). The MCD spectrum of the ES_2 intermediate formed with Dns-Ala-Ala-OPhe at −40 °C reflects a spectral response analogous to that of the corresponding absorption spectrum (Figure 7). Instead of the single negative extremum of the cobalt enzyme alone, that of the intermediate exhibits two prominent negative extrema centered at 575 and 548 nm, respectively. Warming restores the MCD spectrum characteristic of the enzyme plus products in the ternary solvent, as would be expected on hydrolysis of the substrate.

Discussion

While mechanistic conclusions are frequently derived from kinetic studies, the structural basis of enzymatic activity is generally deduced from time-averaging procedures applied to systems at equilibrium (Weber, 1975). At room temperature the time interval for catalytic and structural studies differs widely, and the delineation of the structure of short-lived enzymatic intermediates in the course of catalysis has been difficult. Subzero temperatures, which decrease the rates of enzymatic reactions, combined with rapid mixing and rapid scanning instrumentation allow acquisition of the spectra of intermediates in metalloenzyme catalysis. Thus, contraction of the time frame required for spectral analysis of an intermediate and expansion of its lifetime can be combined so that kinetics and spectra can be observed simultaneously.

For carboxypeptidase A, absorption, MCD, and EPR spectroscopies have proven particularly effective in examining the coordination and oxidation state of the enzymatically essential cobalt atom substituted at the active site. The spectral features of cobalt have made it a most effective chemical probe with which to investigate the functional and structural features of the metal in zinc metalloenzymes. The present study examines the interaction of substrates with cobalt carboxypeptidase A in the course of catalytic events at subzero temperatures.

Our earlier experiments in supercooled water explored the effect of temperature and other experimental variables on the spectra and activity of carboxypeptidase and helped define suitable conditions for further studies (Thompson et al., 1980). The native enzyme and two of its chromophoric derivatives, cobalt carboxypeptidase and the monoarsanilazotyrosine-248–zinc enzyme are enzymatically active, and their catalytic rates decrease predictably over the range of temperatures in which the aqueous phase of the supercooled water in oil emulsion medium remains liquid. Further, the absorption spectra remain unchanged in emulsions from 38 to −38 °C. Thus, studies of structure and function of the enzyme can be carried out at temperatures low enough to allow accumulation of possible intermediates of good substrates and with sufficient amounts of enzyme to permit their detection. However, there are problems inherent in the use of the supercooled water in oil emulsion when combining spectroscopy with rapid kinetics. On the one hand, light scattering and the relatively low absorptivity of the cobalt enzyme make definitive spectral characterization in such emulsions difficult. On the other hand, the high viscosity of such supercooled emulsions interferes with stopped-flow measurements.

In devising a suitable experimental system for such work at subzero temperatures, the salt-dependent solubility of carboxypeptidase A (Putnam & Neurath, 1946) proves to be a particularly important and convenient intrinsic property of this enzyme (Galdes et al., 1983) that facilitates studies in homogeneous, nonviscous solutions. As this enzyme is much more soluble in the presence of salt, virtually all previous kinetic studies of carboxypeptidase have been performed in solutions containing 1 M NaCl. An increase in the concentration of NaCl to 4.5 M minimally alters the kinetic (Galdes et al., 1983) and spectral properties of the enzyme (Figure 2) but lowers the freezing point of the medium significantly; owing to this circumstance, both kinetic and spectral measurements can therefore be performed at −20 °C. Further, the effects and suitability of other cryosolvents for spectroscopy and kinetics of carboxypeptidase A at subzero temperatures can be compared to those of 4.5 M NaCl, which, henceforth, can serve as a cryosolvent standard. In fact, the spectrum of cobalt carboxypeptidase in the aqueous-organic cryosolvent at −40 °C (Figure 7) is remarkably similar to spectra in 1 M NaCl at 20 °C or in 4.5 M NaCl at −20 °C (Figure 2).

Though a slow enzymatic rate is necessary, this is not sufficient to allow decisive definition of intermediates when formed in the course of the catalytic event. This requires

temporal resolution of, e.g., spectral changes at scanning speeds sufficient to discriminate spectral details at discrete stages of the catalytic process as identified by kinetic measurements (Galdes et al., 1983). The intermediates formed with peptide and ester substrates that exhibit k_{cat}/K_m values of 10^7 M^{-1} s^{-1} are too transient for characterization at room temperature, but they are readily detected at −20 °C. RET studies of carboxypeptidase using dansylated peptide and ester substrates at −20 °C have demonstrated the formation and breakdown of a noncovalent intermediate, ES_2, subsequent to the Michaelis complex, ES_1 (Galdes et al., 1983). Such studies have led to the definition of appropriate conditions and time intervals for the detection and characterization of this intermediate by spectral methods. The detailed kinetics of the cobalt-substituted enzyme are closely similar to those of the native zinc enzyme (B. L. Vallee, D. S. Auld, B. Holmquist, A. Galdes, K. F. Geoghegan, and R. A. Martinelli, unpublished results).

The cobalt absorption spectra of the ES_2 intermediates have been obtained by means of a low-temperature stopped-flow instrument (Auld, 1979; Hanahan & Auld, 1980) that has been interfaced with a rapid-scanning spectrometer (Figure 1). Since this equipment scans the spectral range from 400 to 700 nm in <17 ms, the absorption spectra can be obtained within the lifetime of the ES_2 intermediates, i.e., in the course of hydrolysis even of substrates that are turned over very rapidly.

The absorption, MCD, and EPR spectra of the cobalt enzyme at subzero temperatures are virtually the same as those at 20 °C. In contrast, those typical of the peptide and ester intermediates differ remarkably from one another and from that of the resting enzyme or its complexes with products. The absorption spectra of all peptide intermediates are similar to each other as, in turn, are all ester intermediates (Figures 3 and 4).

In metalloenzymes the coordination sphere of catalytically active metal atoms has been found to be highly asymmetric and distorted. As a consequence the spectra of catalytically active metalloenzymes generally do not resemble those of metal complex ions and are "atypical" as compared with those of conventional metal complex ion models. It has been suggested that the active site of metalloenzymes is "poised for catalysis", its intended biological function, a situation referred to as the *entatic state* (Vallee & Williams, 1968a,b).

The entatic-state hypothesis then further directs attention to the correlation and interdependence of enzymatic function with atypical spectra of (transition) metalloenzymes. "Entasis" in this context denotes a condition of tension or stress at the active sites of these metalloenzymes *prior* to their interaction with substrates. Unfortunately, methods or approaches to extend the basis of this postulate experimentally have not been available. In particular, the means were not at hand to observe the predicted changes in the coordination sphere and spectra of an active metalloenzyme engaged in catalysis (Vallee & Williams, 1968a). The present cryospectrometer stopped-flow instrument provides that opportunity. The precise correlation of the formation and breakdown of the ES_2 complexes with the remarkable, systematic *changes* of the cobalt spectra of carboxypeptidase when forming peptide and ester intermediates is the first experimental test of this hypothesis and strongly supports it (Figures 3, 4, 6, and 7). Past chemical efforts to eludicate this question were limited to static systems, and the procedures available could only be directed to their presumed coordination geometry on the basis of their comparison with that of models; those, however, lack the inherent potential to reveal dynamic changes correlated with catalysis itself. The spectral alterations in its course here described clearly identify characteristic, catalysis-related changes in the active site metal-coordination sphere. Whatever structural formulations will emerge from future investigations both of the statics and dynamics of metal binding sites of metalloenzymes, henceforth attention can focus on activity-related chemical transformations during catalysis.

However, while delineating the dynamics of the coordination sphere during catalysis, the past data on the geometry and symmetry of the static metal binding site of cobalt carboxypeptidase in the absence of substrate must be recalled for reference. The intensities, energies, and patterns of electronic absorption bands of many Co(II) complex ion spectra have been assigned in terms of donor groups, symmetry, and overall coordination geometry on the basis of X-ray crystallographic analysis (Latt & Vallee, 1971; Rosenberg et al., 1975; Holmquist et al., 1975). The corresponding MCD spectra signal overall metal-coordination geometry (Holmquist et al., 1975; Vallee & Holmquist, 1980).

The paramagnetism of the d^7 Co(II) ion allows characterization of such complexes by EPR, and their spectra are particularly sensitive to distortions in the symmetry and geometry of the cobalt coordination sphere, which are reflected in the g values and degree of hyperfine splitting (McGarvey, 1966; Pilbrow, 1978; Bencini et al., 1981). Thus far, Co(II)-substituted enzymes generally have been found to be high-spin complexes, i.e., with three unpaired electrons, though their EPR spectra and those of models have been reported relatively infrequently compared to those of, e.g., copper and iron enzymes; perhaps, this is due to the extremely low temperatures, 4–7 K, required for such experiments. Jointly, the absorbance, MCD, and EPR spectra of cobalt carboxypeptidase have been thought to be most consistent with an asymmetrically coordinated cobalt atom, bound firmly in a distorted tetrahedral-like environment[3] (Latt & Vallee, 1971; Holmquist et al., 1975), and this is in agreement with the X-ray structure of the zinc enzyme (Lipscomb et al., 1968), which also indicates that the metal coordination of the active site is distorted.

X-ray diffraction analyses of a substantial number of yet other metalloenzymes have confirmed spectral predictions that the ligand field of their metal binding sites distorts their electronic structures, and unusual bond lengths and angles of the ligands that are part of the metal coordination sphere have been described in some such instances (Williams, 1971; Jensen, 1974; Colman et al., 1978; Lipscomb, 1980). Low symmetry due to these and other features of enzyme structures are thought to be reflected in their atypical spectra, which are always abolished by denaturation (Vallee & Williams, 1968a,b; Vallee & Galdes, 1983). While the effect of inhibitors on the spectra of cobalt enzymes has been studied under equilibrium conditions, obviously there is no information on the characteristics of transient intermediates in their catalysis, since they have not been observed heretofore. Although at present spectral details of transient catalytic intermediates cannot identify definitive structures (see above), certain trends can be discerned. The shift of the principal band in the electronic cobalt spectrum to higher and lower wavelengths

[3] It has been emphasized here and elsewhere that cobalt and other metalloenzyme spectra cannot be interpreted unambiguously on the basis of those of Co(II) complex ions (Vallee & Williams, 1968a,b). We have therefore referred to the mode of coordination of metalloenzymes, including carboxypeptidase and its complexes with substrates, products, and/or inhibitors, as "tetrahedral- and pentacoordinate-like" or quasi-tetrahedral etc., implying that the assignment is empirical. This convention is intended to deemphasize formal coordination geometry.

with peptide and ester substrates, respectively, suggests that in one there is a weakening and in the other a tightening of the metal coordination sphere.

The considerations pertinent to the interpretation of the EPR spectra of the intermediates are analogous to those of absorption spectra. Their most significant—and remarkable—feature is the induction and loss of hyperfine splitting in g_1 concomitant with the formation and breakdown, respectively, of the steady-state intermediate, ES_2. Thus far, we have not seen hyperfine splitting in the metalloenzyme or in its complexes with substrate-analogue inhibitors, products, or affinity labels.

Spurred by increasing interest in Co(II)-substituted enzymes, EPR studies have established the range of g values and hyperfine constants expected for 4-, 5- and 6-coordinate Co(II) complex ions while revealing the sensitivity of the EPR parameters to small changes in the symmetry of the metal atom [e.g., Benicini et al. (1979a,b, 1980, 1981) and Horrocks et al. (1980)]. Since they generally exhibit a high degree of anisotropy ($g_1 \neq g_2 \neq g_3$), geometry cannot be assigned on the basis of EPR parameters alone. However, their sensitivity can be employed to detect changes in the Co(II) coordination sphere. It has been claimed that hyperfine splitting in EPR spectra of cobalt(II) ions relates directly to geometry and that the magnitude of A_1 progresses in the order tetrahedral < trigonal bipyramidal < square pyramidal $\simeq$ octahedral in the systems studied (McGarvey, 1966; Bencini et al., 1980). If one were to accept this premise and believe that it would pertain to entatic sites, the present findings for the intermediates would be inconsistent with an assignment of square pyramidal and/or hexacoordinate geometry, but they could be reconciled with both distorted tetrahedral-like or pentacoordinate-like models.

Hyperfine splitting as observed for the intermediates frequently attends low symmetry of Co(II) complex ions, and in the intermediates it could result from distortion of symmetry accompanying the transient interaction of a substrate atom with the metal's coordination sphere. The g_1 hyperfine structure differs significantly for peptides and esters (see Table I), implying that both of them alter the symmetry of the resting enzyme's metal coordination sphere to an extent characteristic of each. Jointly, however, their EPR, absorption, and MCD spectra eliminate 6-coordination of the metal atom as a likely configuration for these transients. More detailed examination of such spectra of intermediates should resolve some of the remaining ambiguities.

In summary, whatever the ultimate structural assignments, the available data offer important novel insight into the mechanism of the enzyme. The present cryospectroscopic and the concomitant cryokinetic studies (Galdes et al., 1983) show directly that both peptides and esters form metallointermediates prior to the rate-determining step, contrary to popularly held mechanistic views in which peptides are speculated to form such intermediates only after the rate-limiting step (Cleland, 1977; Rees & Lipscomb, 1981). The spectra demonstrate further that the metallointermediates of peptides and esters are different, in accord with previous results of chemical modifications and kinetics (Vallee et al., 1970; Riordan, 1973; Auld & Holmquist, 1974). These studies indicated that the metal plays a critical but different role in the mechanisms of peptide and ester hydrolysis [reviewed in Vallee et al. (1983)]. The definition of the precise structural relationships of these metallointermediates to other chemical events in the course of catalysis will add new and valuable dimensions to the study of carboxypeptidase A and enzyme mechanisms in general.

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Interaction of Chick Oviduct Progesterone Receptor with the 2′,3′-Dialdehyde Derivative of Adenosine 5′-Triphosphate†

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ABSTRACT: Avian oviduct progesterone receptor was treated with the 2′,3′-dialdehyde derivative of ATP (oATP) in an attempt to demonstrate the presence of nucleotide binding sites on the receptor. oATP, when added to cytosol, inhibited binding by transformed receptor to ATP–Sepharose, DNA–cellulose, phosphocellulose, or isolated nuclei in an irreversible manner. oATP did not disrupt the steroid–receptor complex, but it did alter the ionic properties of the receptor. This was demonstrated by an increased affinity of receptor for DEAE-cellulose and for hydroxylapatite. oATP mimicked the effect of ATP on progesterone receptor with regard to two properties: it altered the rate of receptor inactivation that occurs in the absence of progesterone, and it promoted receptor conversion from an 8S complex to lower sedimenting forms (4–6 S). The action of oATP on the receptor could be blocked by the addition of pyridoxal 5′-phosphate, which has been shown previously to interact with the progesterone receptor. A partial interference of oATP action was also observed when ATP was added. These results indicate that oATP interacts with the progesterone receptor and may be used as an affinity-labeling agent for receptor characterization.

Previous work in this laboratory has established that a relationship exists between the presence of ATP and the observed properties of the avian progesterone receptor. First, it was found that the receptor binds ATP–Sepharose (Moudgil & Toft, 1975, 1977) through a reaction that is reversed by high ionic strength or free ATP. The receptor must be in the transformed state[1] in order to bind to the ATP–Sepharose (Miller & Toft, 1978), and receptor transformation by a variety of methods including high ionic strength, elevated temperatures, increased pH, or dilution will yield the ATP binding form of the receptor (Toft et al., 1980). Additionally, high concentrations of ATP (greater than about 3 mM) enhance the temperature-induced transformation reaction while lower concentrations of ATP (less than 1 mM) inhibit this transformation reaction (Toft et al., 1977). Also, higher concentrations of ATP alone (about 10 mM) will tranform receptor to the ATP–Sepharose binding form in the absence of increased temperature (Moudgil et al., 1981; V. Moudgil et al., unpublished observations).

Several enzyme systems that interact with nucleotides have been characterized with respect to their nucleotide binding site(s) by use of the 2′,3′-dialdehyde derivative of the nucleotide as an affinity-labeling compound that binds covalently at the nucleotide binding site (Bragg et al., 1981; Easterbrook-Smith et al., 1976; Fayat et al., 1978; Gregory & Kaiser, 1979; King & Carlson, 1981; Kumar et al., 1979; Malcolm & Moffatt, 1978; Westcott et al., 1980). A survey of the data from these studies indicates that the usual effect of the dialdehyde on the enzyme is one of inhibition of the enzymatic activity.

While the avian progesterone receptor is affected by ATP, as mentioned above, it is not known whether the ATP exerts its effects directly on the receptor or whether other cytosolic

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[1] Transformation refers to the process by which steroid-bound receptor is converted from the cytoplasmic form to its nuclear counterpart. Inactivation refers to the process by which steroid-free receptor is converted to a form incapable of binding steroid.

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