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Cobalt(III) Carboxypeptidase A

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Abstract: Cobalt(III) carboxypeptidase A has been prepared by the oxidation of cobalt(II) carboxypeptidase A with hydrogen peroxide at pH 7.5. The oxidized enzyme shows negligible peptidase activity after dialysis against metal-free buffer or 1,10-phenanthroline while its esterase activity toward hippuryl-L-β-phenyllactate and *trans*-*p*-nitrocinnamoyl-L-β-phenyllactate is comparable to that of cobalt(II) carboxypeptidase A. Carbobenzyloxyglycyl-L-phenylalanine, toward which the cobalt(II) enzyme is active, competitively inhibits the activity of the cobalt(III) protein toward hippuryl-D,L-β-phenyllactate. The metal in the oxidized enzyme is no longer removable by dialysis against metal-free buffer, but may be reduced back to cobalt(II) and exchanged by dialysis against excess cobalt(II). Cobalt(III) carboxypeptidase A has a visible absorption maximum at 503 nm (ε 500). The pH dependence of ester hydrolysis by cobalt(III) carboxypeptidase A shows that catalysis requires the basic form of a group on the enzyme with pK_a 6.3–6.5 and the acidic form of a group with pK_a 9.1–9.5. These results have a bearing on the mechanism of action of carboxypeptidase A. In particular, they imply that the group of higher pK_a is not a water molecule bound to the metal and that ligation of substrates within the first coordination sphere of the metal does not occur during ester hydrolysis catalyzed by cobalt(III) carboxypeptidase A.

Carboxypeptidase A is a metalloenzyme with a molecular weight of about 34,500.[2,3] It catalyzes the hydrolysis of the C-terminal peptide bond of peptides as well as the corresponding ester bond of L-α-acyloxycarboxylic acids. Bovine pancreatic preparations have been obtained in several forms displaying some heterogeneity in their amino-terminal sequences.[4] It has been shown to require one atom of Zn(II) per molecule of protein for its normal peptidase activity.[5] Since the discovery of this metal ion involvement, there has been a great deal of effort put forth in attempting to elucidate both the manner in which Zn(II) is bound to the protein and the role the metal plays in the hydrolysis of substrate peptide or ester bonds. These results have been reviewed extensively in recent articles.[6-11] A large number of derivatives of carboxypeptidase A have been made in the course of these studies. Many of these have involved replacement of the Zn(II) with metals such as Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Cd(II), and Hg(II).[4,6] Although these metals differ in such physical properties as coordination geometry and ionic radius, they all belong to the exchange labile class of metal ions.[12] This exchange lability of the ligands in the first coordination sphere of the metal ion is necessary in order to introduce the ion into the protein. On the other hand, it would be quite useful, for both protein structure determination and mechanistic investigations, to have an exchange inert metal ion incorporated into a metalloenzyme. This property of exchange lability vs. exchange inertness is controlled largely by the d electron configuration of the metal ion. Some metals can be taken from one class to the other simply be changing their oxidation states. Such a situation exists with Co(II) (d^7), exchange labile, and Co(III) (d^6), exchange inert.

We report here the preparation of a metalloprotein containing an exchange inert metal ion, Co(III)-CPA.[13] A preliminary account of this work has appeared.[14] A similar oxidation of cobalt(II) carbonic anhydrase to the Co(III) enzyme has been reported.[15]

Experimental Section

Materials. Carboxypeptidase A (EC 3.4.12.2) prepared by the method of Anson[16] was obtained as an aqueous suspension of crystals, grade COA, from Worthington Biochemical Corporation. The substrates used in this study were obtained from the following sources: Gly-Gly-L-Phe, Nutritional Biochemical Co.; Z-Gly-L-Phe, Mann Research Laboratories; D,L-HPLA, Calbiochem Co.; L-HPLA, Cyclo Chemical Co.; and NCPLA, Eastman Organic Chemicals. Cobalt(II) solutions were prepared by dissolving cobalt sponge (99.999% pure), obtained from K and K Laboratories, in metal free hydrochloric acid. Radioactive cobalt solutions were prepared by adding $^{57}CoCl_2$, obtained from New England Nuclear, to nonradioactive solutions of $CoCl_2$. Special precautions against metal ion contamination included the use of polypropylene apparatus where possible, leaching glassware with 1:1 nitric acid: sulfuric acid, and extraction of buffers and other solutions with carbon tetrachloride containing 0.1 g/l. of diphenylthiocarbazone.[17]

Methods. Enzyme Preparations. All enzyme treatments except for concentration determinations and kinetic measurements were

carried out at 4° unless otherwise stated. Buffer refers to metal-free 1.0 *M* NaCl, 0.1 *M* Tris-Cl at pH 7.5 (measured at 22°) unless otherwise specified. Each enzyme preparation was chromatographed either as the native enzyme or after conversion to the Co(II) or Co(III) derivative as follows. About 10 ml of 10^{-5} to 10^{-4} *M* enzyme was applied to a Sephadex G-75 2.5 × 45 cm column equilibrated with buffer. The column was eluted with buffer at a flow rate of about 15 ml/hr and the protein concentration of each fraction was determined spectrophotometrically at 22°.

Zn(II)-CPA stock solutions were prepared by mixing commercial enzyme suspensions with enough buffer to give a protein concentration of about 10^{-4} *M* after standing for 24 hr. The solutions were then centrifuged at about 15,000*g* for 30 min and the supernatants were drawn off. Protein concentrations were monitored at 278 nm using ϵ 6.42 × 10^4 l. mol^{-1} cm^{-1} [18] for all metalloenzymes.[19]

Apocarboxypeptidase A was prepared by the method of Coombs et al.[20] Metal analysis by the procedure of Vallee and Gibson[21] indicated that the preparation contained 9% residual zinc.

Co(II)-CPA was prepared from stock Zn(II)-CPA solutions by the method of Coleman and Vallee.[23] Some samples were chromatographed on Sephadex G-75 as described above. The cobalt incorporation was followed by a combination of the method of Kitson[22] and determination of the γ emission rate of ^{57}Co with a Baird Atomic Model 530 spectrometer. The molar ratio of Co(II) to protein leveled off at 0.95–1.20 after dialysis against buffer to remove excess cobalt.

Co(III)-CPA was prepared by oxidation of 1.17 × 10^{-4} *M* Co(II)-CPA with 0.84–1.2 m*M* hydrogen peroxide for 6 hr at room temperature in 1.0 *M* NaCl, 0.1 *M* Tris-Cl buffer at pH 7.5. Then the solution was dialyzed for 6 hr each at 4° against two changes of a 20-fold excess of buffer. Some samples were subjected to gel chromatography as described above. Cobalt analysis as described above showed the product to have a cobalt/protein molar ratio of 0.95–1.20. Control solutions of Zn(II)-CPA subjected to the same treatment showed no loss of activity toward Z-Gly-L-Phe or D,L-HPLA.

Kinetics. All substrates were used as their sodium salts dissolved in metal-free buffer. Enzymatic hydrolyses at pH 7.5 in 0.5 *M* NaCl, 0.005 *M* Tris-Cl were followed spectrophotometrically using a Beckman DU spectrophotometer with a cell compartment thermostated at 22.0 ± 0.3° and a Gilford 2000 multiple absorbance recorder. Substrate at a given concentration was used to obtain a reference absorbance value, enzyme was added, and the absorbance change was followed to the completion of the reaction. The substrate concentrations, wavelengths, and molar absorptivity changes for each substrate were 1.56 × 10^{-3} *M* Gly-Gly-L-Phe, 222.5 nm ($\Delta\epsilon$ 1720); 0.52 × 10^{-3} to 7.57 × 10^{-3} *M* Z-Gly-L-Phe, 222.5 nm ($\Delta\epsilon$ 1370); 1.22 × 10^{-4} to 9.77 × 10^{-4} *M* D,L-HPLA, 254 nm ($\Delta\epsilon$ 592); 0.61 × 10^{-4} to 4.89 × 10^{-4} *M* L-HPLA, 254 nm ($\Delta\epsilon$ 592); and 0.255 × 10^{-4} to 1.53 × 10^{-4} *M* NCPLA, 300 nm ($\Delta\epsilon$ 4230). Initial velocities were calculated from the extrapolated initial slopes of absorbance vs. time recordings.

Studies of the pH dependence of kinetic parameters were performed in the manner described above, with the exception that 0.5 *M* NaCl, 0.005 *M* Tris-acetate buffers, prepared from tris(hydroxymethyl)aminomethane, sodium acetate, and acetic acid, were used in place of Tris-Cl.

The kinetic parameters k_{cat}, k_{cat}/K_m, and K_m and their standard deviations were calculated by computer fitting of initial velocities to the Lineweaver–Burk equation using a least-squares linear regression analysis program. The resultant plots of $1/v_0$ (reciprocal initial velocity) vs. $1/(S)_0$ (reciprocal initial substrate concentration) were linear at better than 95% confidence level.

The apparent acid dissociation constants of kinetically important groups on free Co(III)-CPA and on its complexes with ester substrates were obtained from the pH dependencies of k_{cat}/K_m and k_{cat} by the method of Massey and Alberty.[24]

The effect of Z-Gly-L-Phe on the esterase activity of Co(III)-CPA was determined by injecting varying amounts of this inhibitor into hydrolyzing samples of 2.44 × 10^{-4} *M* D,L-HPLA. The velocity immediately before injection, v, and the velocity immediately after injection of the inhibitor, v_i, were calculated from the extrapolated slopes of the resultant absorbance vs. time traces at 254 nm. A plot of v/v_i vs. v(Z-Gly-L-Phe)/(L-HPLA), where (L-HPLA) is the substrate concentration at the time of injection of the inhibitor, since D-HPLA is not hydrolyzed by Co(III)-CPA, was linear with unit ordinate intercept, indicating competitive inhibition.[25]

Least-squares fitting of the results to the equation $v/v_i = 1 + K_m v(\text{Z-Gly-L-Phe})/K_i k_{cat}(E)_0(\text{L-HPLA})$ allowed calculation of the enzyme inhibitor dissociation constant, K_i, from previously determined values of K_m and k_{cat}.

Amino Acid Analyses. One-milliliter samples of Zn(II)-CPA and Co(III)-CPA containing approximately 5 mg each of enzyme were added to 1-ml volumes of concentrated HCl in glass tubes. The tubes were sealed under vacuum and placed in a boiling water bath for 8 hr. The samples were subsequently dried under vacuum and subjected to amino acid analysis. The amino acid analysis was performed by D. von Endt at the Laboratory of Physical Anthropology, Smithsonian Institution, on a high speed single column analyzer built according to the design of E. Hare. The reproducibility of the instrument is ±1% for a particular amino acid.

Results

Striking changes in the hydrolytic activity and optical spectrum of Co(II)-CPA take place on oxidation with hydrogen peroxide. Zn(II)-CPA controls displayed no change in esterase or peptidase activity after similar treatment. This suggests that oxidation of Co(II) and not possible oxidation of amino acid residues is responsible for the activity changes in the peroxide-treated Co(II) enzyme.

The most characteristic change to be expected if the Co(II)-CPA were oxidized to Co(III)-CPA is that the metal would no longer be removable by extended dialysis. This was demonstrated by dialyzing ^{57}Co(III)-CPA against a 20-fold excess of buffer for 7 days, with a change in buffer every 12 hr. No significant radioactivity was found in any of the dialysates and no loss of radioactivity from the enzyme was observed. Under these conditions, Co(II)-CPA would have lost a substantial amount of its metal content.[23]

The oxidation of Co(II)-CPA is accompanied by distinct spectral changes. Co(II)-CPA has visible absorption maxima at 530 (ϵ 205) and 572 nm (ϵ 195).[17] In contrast, the visible maximum of Co(III)-CPA occurs at 503 nm (ϵ 500), Figure 1. The oxidation can be followed quite readily either at 503 nm or in the ultraviolet at 280 nm. The changes in absorbance at 280 and 503 nm as a function of time are shown in Figure 2. The oxidation may also be followed by the loss of peptidase activity, also shown in Figure 2. While the loss of peptidase activity coincides with the absorbance changes, the kinetics are complex and no attempt has been made to determine the rate law for the oxidation of Co(II)-CPA by hydrogen peroxide.

As with other modifications of the structure of carboxypeptidase A, the change in oxidation state of the cobalt brings about some dramatic changes in activity, which are summarized, along with kinetic constants for other derivatives for comparison, in Table I. The values reported for Co(III)-CPA all refer to enzyme samples that have undergone extensive dialysis against metal-free 1.0 *M* NaCl, 0.1 *M* Tris-Cl at pH 7.5 and 4°. Such treatment was necessary because the preparations showed low levels (5–12% of control Zn(II)-CPA) of activity toward Z-Gly-L-Phe if examined immediately after completion of the oxidation. Extended dialysis reduces the peptidase activity to less than 0.02% of that shown by the native enzyme. The residual peptidase activity found in this study is reminiscent of the 1–8% peptidase activity of apoCPA found by others[27-30] and attributed to Zn(II) or other adventitious metal ion contamination.

Such contamination is likely due to the fact that 9% residual zinc was measured in the apoenzyme used to prepare our cobalt derivatives. This prompted a study of the effect of 1,10-phenanthroline on freshly prepared Co(III)-CPA. It has been convincingly demonstrated that this chelating

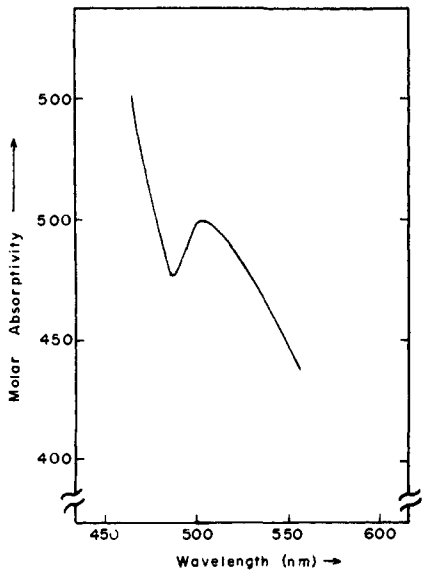

Figure 1. Visible absorption spectrum of cobalt(III) carboxypeptidase A in 1.0 *M* NaCl, 0.1 *M* Tris-Cl, pH 7.5, at room temperature.

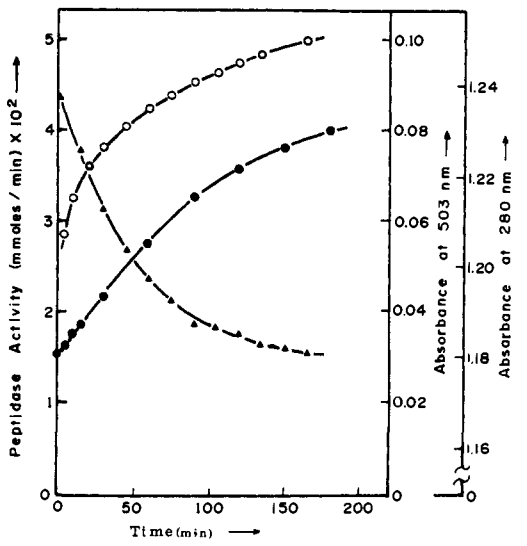

Figure 2. Absorption and peptidase activity changes during the oxidation of cobalt(II) carboxypeptidase A in 1.0 *M* NaCl, 0.1 *M* Tris-Cl at pH 7.5 and 22°: (●) absorbance of 1.63×10^{-4} *M* enzyme and 9.81×10^{-3} *M* hydrogen peroxide at 503 nm; (○) absorbance of 1.36×10^{-5} *M* enzyme and 3.27×10^{-3} *M* hydrogen peroxide at 280 nm; (▲) activity of aliquots of the 1.36×10^{-5} *M* enzyme sample (above) assayed with 1.36×10^{-4} *M* Z-Gly-L-Phe and 1.36×10^{-7} *M* enzyme in 0.5 *M* NaCl, 0.005 *M* Tris-Cl at pH 7.5 and 22.0°.

agent inhibits Zn(II)-CPA by removing the metal from the enzyme.[30] A sample of 1.63×10^{-6} *M* Co(III)-CPA that showed low peptidase activity was incubated with 2.00×10^{-3} *M* 1,10-phenanthroline in 1 *M* NaCl, 0.1 *M* Tris-Cl, pH 7.5, at 4°. An aliquot withdrawn and assayed within 1 min after mixing showed a reduction of peptidase activity toward Z-Gly-L-Phe of 59% while the esterase activity toward D,L-HPLA was 87% of the original value. No peptidase activity could be observed after 30 min of incubation, while the esterase activity remained constant at 87% of the original value for the next 8 hr. This is good evidence that the small, variable, residual peptidase activity of freshly prepared Co(III)-CPA is due to adventitious metal ion contamination.

A catalytically inactive protein component of commercial Zn(II)-CPA was detected and removed as follows. A sample of ^{57}Co(III)-CPA prepared from unpurified native enzyme was chromatographed on Sephadex G-75. The radioactivity from the ^{57}Co coincided with the absorbance at 280 nm of two protein components, Figure 3. Chromatography of the commercial Zn(II)-CPA under identical conditions also gave two absorbance maxima at the same fractions, the second peak being much smaller than the first. In both cases the first peak was enzymatically active while the second peak was not. Co(III)-CPA prepared from Zn(II)-CPA from which the minor component had been removed by chromatography displayed only a single peak on gel chromatography.

Co(III) complexes may be exchange labile in the presence of Co(II).[31,32] This lability is induced by electron transfer between the Co(II)/Co(III) centers. The rate at which electron transfer takes place between Co(II) and a Co(III) complex is strongly dependent on the structures of the two metal centers and the presence of catalysts in solution such as oxygen, hydroxide, and chloride. For instance, the presence of chloride or hydroxide accelerates electron transfer between Co(III) hexamine and $Co^{II}(NH_3)_n$ some 10^6- to 10^7-fold. Also, Tris buffer would probably favor the electron exchange as it is known to be a good complexing agent for cobalt species.[33]

The possibility of inducing such exchange in this case was investigated by dialyzing ^{57}Co(III)-CPA against a Tris-Cl buffer containing cold Co(II). Figure 4 shows the appearance of ^{57}Co counts outside of the dialysis bag and the return of peptidase activity to the protein inside the dialysis bag. These results are consistent with the added Co(II) reducing the ^{57}Co(III)-CPA, thus labilizing the ^{57}Co to exchange and restoring the peptidase activity by formation of Co(II)-CPA. The activity restored after 24 hr in the presence of a 50-fold excess of Co(II) was even higher than that of protein in the presence of an equimolar amount of Co(II) as expected, due to the significant dissociation constant of Co(II)-CPA.[23]

Table I. Kinetic Constants for Zn(II)–CPA, Co(II)–CPA, and Co(III)–CPA[a]

Substrate	Enzyme	10^3K_m	$10^{-3}k_{cat}$ (min^{-1})
Z-Gly-L-Phe	Zn(II)–CPA	4.4 ± 0.4	6.3 ± 0.3
	Co(II)–CPA	3.9 ± 0.9	19.0 ± 2.0
	Co(III)–CPA	*b*	*b*
Gly-Gly-L-Phe	Co(III)–CPA	*c*	*c*
D,L-HPLA[d]	Zn(II)–CPA	0.009 ± 0.004	29.7 ± 0.4
	Co(II)–CPA	0.14 ± 0.01	41.0 ± 1.0
	Co(III)–CPA	0.35 ± 0.11	60.0 ± 8.0
L-HPLA	Zn(II)–CPA	0.09 ± 0.02	27.0 ± 0.2
	Co(II)–CPA	0.137 ± 0.007	40.1 ± 0.7
	Co(III)–CPA	0.32 ± 0.06	67.0 ± 7.0
NCPLA	Zn(II)–CPA[e]	0.099 ± 0.002	13.80 ± 0.06
	Co(III)–CPA	0.18 ± 0.01	5.7 ± 0.2

[a] All kinetic constants reported here for this study were determined at pH 7.5 in 0.5 *M* NaCl, 0.005 *M* Tris-Cl at 22.0 ± 0.3°. [b] The apparent activity at a substrate concentration of 0.0063 *M* was about 21,500 times lower than that of Co(II)–CPA. [c] No apparent activity. [d] The substrate concentration is determined by the L-enantiomer only. [e] Tomalin et al.;[26] determined at pH 7.48 in 0.500 *M* NaCl, 0.005 *M* Tris-Cl at 25.0°.

One test of whether replacement of Zn(II) by Co(III) changes the CPA structure or catalytic function markedly is to examine the effect of pH on kinetic constants for ester hydrolyses. Results of such an investigation of the pH dependence of k_{cat}, K_m, and k_{cat}/K_m for the hydrolysis of both D,L-HPLA and NCPLA catalyzed by Co(III)-CPA are given in Figures 5 and 6. These results were analyzed in the manner detailed by Carson and Kaiser.[34] Values of the acid dissociation constants of ionizing groups affecting cat-

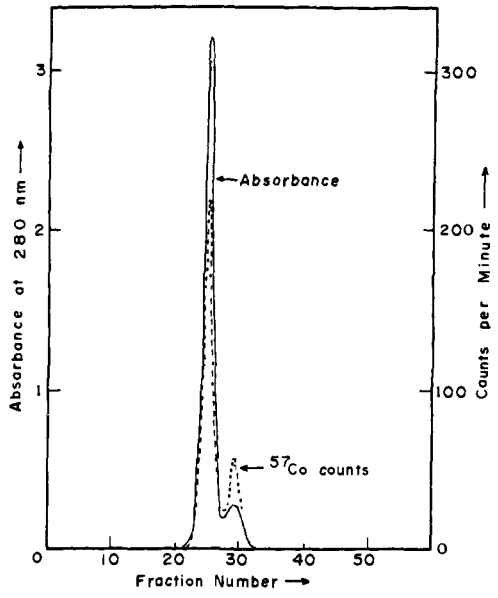

Figure 3. Gel chromatography of 1.2×10^{-4} *M* ^{57}Co(III) carboxypeptidase A on a Sephadex G-75 2.5 × 45 cm column using 1.0 *M* NaCl, 0.1 *M* Tris-Cl, pH 7.5, as the eluent at 4°. Approximately five 3.0-ml fractions per hour were collected.

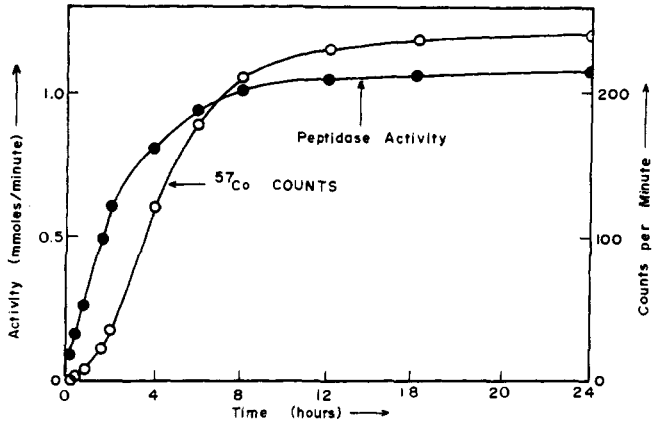

Figure 4. Peptidase activity gained and radioactivity released during equilibrium dialysis of 2.19×10^{-5} *M* ^{57}Co(III) carboxypeptidase A against an equal volume of 2.15×10^{-3} *M* $CoCl_2$ in 1.0 *M* NaCl, 0.1 *M* Tris-Cl at pH 7.5 and 4°: (●) activity of aliquots of enzyme assayed with 3.27×10^{-3} *M* Z-Gly-L-Phe and 7.28×10^{-8} *M* enzyme in 0.5 *M* NaCl, 0.005 *M* Tris-Cl at pH 7.5 and 22°; (○) radioactivity appearing in the dialysate during equilibrium dialysis.

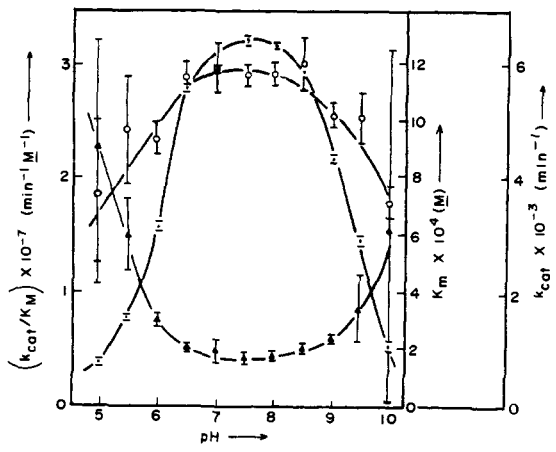

Figure 5. Effect of pH on kinetic parameters for the hydrolysis of 2.55×10^{-5} to 1.53×10^{-4} *M* *trans*-*p*-nitrocinnamoyl-L-β-phenyllactate catalyzed by 1.22×10^{-8} *M* cobalt(III) carboxypeptidase A in 0.5 *M* NaCl, 0.005 *M* Tris-acetate buffer at 22°: (I) twice the standard deviation of a parameter; (▲) K_m; (○) k_{cat}; (●) k_{cat}/K_m.

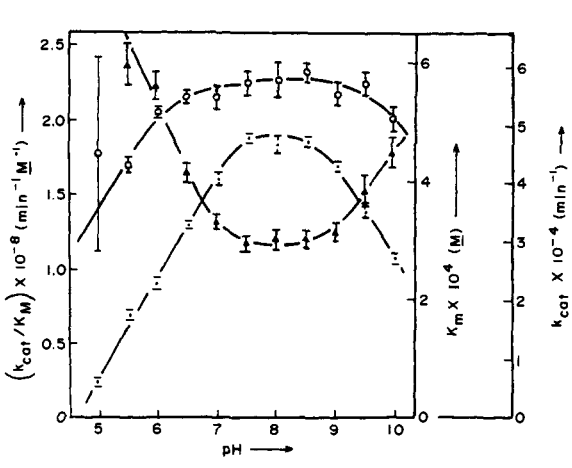

Figure 6. Effect of pH on kinetic parameters for the hydrolysis of 1.22×10^{-4} to 9.77×10^{-4} *M* hippuryl-D,L-β-phenyllactate catalyzed by 4.62×10^{-9} *M* cobalt(III) carboxypeptidase A in 0.5 *M* NaCl, 0.005 *M* Tris-acetate buffer at 22°: (I) twice the standard deviation of a parameter; (▲) K_m; (○) k_{cat}; (●) k_{cat}/K_m.

alytic activity on the free enzyme, K_{aE}'s, and on the enzyme-substrate complex, K_{aES}'s, may be determined from the variations of k_{cat}/K_m and k_{cat}, respectively, with pH. These were obtained as pK_a values by the graphical method of Massey and Alberty[24] and are listed in Table II.

It seemed possible that the lack of peptidase activity in Co(III)-CPA might be due to an inability of the enzyme to bind the peptide substrate. However, Z-Gly-L-Phe was found to be an excellent competitive inhibitor of the esterase activity of this enzyme toward D,L-HPLA, with a K_I of $(1.07 \pm 0.06) \times 10^{-3}$. This is of the same order of magnitude as the K_m values of 4.4×10^{-3} and 3.9×10^{-3} for Z-Gly-L-Phe hydrolyses catalyzed by Zn(II)-CPA and Co(II)-CPA, respectively. This result is similar to the finding of Auld and Holmquist[35] that k_{cat} is sharply decreased and K_m not much affected for the peptidase activity of Cd(II)-CPA compared to Zn(II)-CPA.

Acid hydrolyses of both Zn(II)-CPA and Co(III)-CPA were carried out and the hydrolysates were analyzed on an amino acid analyzer. The results and a comparison with literature analyses are given in Table III.

Discussion

A major consideration in this study was whether hydrogen peroxide acts solely to oxidize the Co(II) of Co(II)-CPA to Co(III) or whether other functional groups in the protein which are susceptible to oxidation were also undergoing change. The oxidation of Co(II)-CPA reported here was carried out under very mild conditions, the molar ratio of hydrogen peroxide to protein being about 10 and the temperature 22°. For comparison, Neumann[37] reported that hydrogen peroxide concentrations of 0.1-0.5 *M* were required for oxidation of 0.1-1.0% protein solutions at pH below 4 and 25-30°. At pH 8 or higher, 0.005 *M* oxidant was adequate, but 22 hr or more were required to complete the oxidation of Met-192 in α-chymotrypsin. Piras and Vallee[38] reported the oxidation of approximately six tryptophans in Zn(II)-CPA using 0.015 *M* hydrogen peroxide in carbonate-dioxane solutions at pH 8.4. This brought about an initial increase in esterase activity and a decrease in peptidase activity. The hydrogen peroxide concentrations used

Table II. pK_{aE} and pK_{aES} Values for the Hydrolysis D,L-HPLA and NCPLA Catalyzed by Co(III)–CPA[a]

Substrate	pK_{aE1}	pK_{aE2}	pK_{aES1}	pK_{aES2}
D,L-HPLA	6.5	9.1	5.9	9.5
NCPLA	6.5	9.1	6.3	9.1

[a] Kinetics were determined in 0.5 *M* NaCl, 0.005 *M* Tris-acetate buffer at 22.0 ± 0.3°.

preparatively in this study were about 15 times lower than those used by Piras and Vallee. The fact that Zn(II)-CPA controls showed unchanged peptidase and esterase activities argues strongly for the primary effect of the oxidant being on Co(II) and not on amino acid residues of catalytic importance. The amino acid analyses of Zn(II)-CPA and Co(III)-CPA shown in Table III further demonstrate that the integrity of the protein is maintained, both cystine and methionine residues being unaffected by the oxidant. It is impossible to say at this time whether any tryptophan has been oxidized since only acid hydrolysis of the protein has been carried out.

In many situations electron transfer reactions take place readily between Co(II) and Co(III) complexes. This leads to the creation of a new Co(III) complex and permits what was the original Co(III) complex to undergo ligand exchange reactions characteristic of its new Co(II) oxidation state.[31] The conditions under which these electron transfer reactions take place can be very mild. When ^{57}Co(III)-CPA is dialyzed against cold Co(II), two effects on the metalloprotein are readily observable: the radioactive cobalt becomes exchange labile and the peptidase activity returns. The time courses of these processes, shown in Figure 4, differ remarkably. The peptidase activity returns first, following a simple hyperbolic curve. In contrast, the release of ^{57}Co follows a sigmoid curve, demonstrating an induction period. This type of complex behavior is what would be expected *if* the Co(III) in the protein is first reduced to Co(II), giving immediate restoration of peptidase activity, followed by slow exchange of the ^{57}Co(II) with the excess cold Co(II) in solution. If any amino acid residue in the protein had been oxidized by the hydrogen peroxide, it would not likely undergo reduction under these mild conditions.

The effect of 1,10-phenanthroline on Co(III)-CPA is also consistent with the exchange inert character expected of the metal. This chelating agent does not influence the esterase activity or metal content of Co(III)-CPA samples that have been subjected to extensive dialysis. This is in sharp contrast to the case with other metallocarboxypeptidases. 1,10-Phenanthroline reversibly inhibits crystalline glutaraldehyde-cross-linked Zn(II)-CPA without removal of the metal under certain conditions,[39] while it causes rapid inhibition and removal of the metal ion from dissolved or cross-linked Zn(II)-CPA under other conditions.[30,40]

The pK_a values listed in Table II show that ester hydrolyses catalyzed by Co(III)-CPA depend on the basic form of a group on the enzyme with $pK_a \sim 5.9$-6.5 and the acidic form of a group with $pK_a \sim 9.1$-9.5, assuming that the same functionalities affect catalysis in both E and ES. These values are about the same as those found for hydrolysis of *trans*-cinnamoyl-L-β-phenyllactate, a reactive ester substrate, catalyzed by Zn(II)-CPA (pK_{aE1}, pK_{aES1} = 6.2-6.5 and pK_{aE2} = 9.4)[41] and Mn(II)-CPA (pK_{aE2} = 9.3).[42] They are also similar to those found in peptide hydrolyses catalyzed by Zn(II)-CPA (pK_{aE1}, pK_{aES1} = 6.1-6.3 and pK_{aE2} = 9.0-9.1), Mn(II)-CPA (pK_{aE1} = 6.4; pK_{aE2} = 8.9), and Co(II)-CPA (pK_{aE1}, pK_{aES1} = 5.3-5.6 and pK_{aE2} = 9.1).[43]

Table III. Amino Acid Composition of Zn(II)–CPA_γ and Co(III)–CPA_γ

Amino acid	Bradshaw et al.[2] Zn(II)–CPA_γ	Bargetzi et al.[36] Zn(II)–CPA_γ	This study Zn(II)–CPA_γ	This study Co(III)–CPA_γ
Asn	16	26–27	25.3	25.6
Asp	12			
Thr	24	23–24	23.0	24.0
Ser	31	30–31	34.9	33.6
Gln	11	24–25	23.4	24.4
Glu	16			
Pro	10	10	11.2	11.3
Gly	23	22.5	21.1	22.7
Ala	19	19	20.8	20.6
Cystine	1	1	1.0	1.0
Val	16	16	18.8	18.6
Met	3	3	3.2	2.9
Ile	20	20	18.6	19.2
Leu	23	23	19.3	21.4
Tyr	19	19	19.9	19.7
Phe	15	15	18.1	18.6
Lys	15	15	16.2	16.4
His	8	8	14.7[a]	15.1[a]
Arg	10	10	12.2	10.2
Trp	7	8	*b*	*b*
NH_3			27.7	29.3

[a] An overlapping peak caused by tris(hydroxymethyl)aminomethane accounts for the high apparent His values. [b] Trp decomposed during acid hydrolysis of the proteins.

Auld and Vallee[43] pointed out that deprotonation of a water molecule bound to the metal atom is unlikely to give rise to the pK_{aE2} seen in peptide hydrolyses because of the latter's invariance over this range of metallocarboxypeptidases. This conclusion may extend to the esterase activity of this enzyme as well, particularly if the same group gives rise to the higher pK_{aE} seen in both ester and peptide hydrolyses. A water molecule attached to Co(III) should have a pK_a value that is displaced markedly to the acid side from one attached to Co(II) if both metals have ligands of the same charge. Although the precise identification of the catalytically important ionizing groups in carboxypeptidase A remains in doubt for both types of catalysis, Glu-270 continues to present an attractive possibility for the acid of lower pK_a on the basis of X-ray[44] and chemical modification studies.[11,45-47]

The very fact that Co(III)-CPA displays esterase activity also has some bearing on the mechanism of action of Zn(II)-CPA and its derivatives. Most mechanisms proposed for either peptidase or esterase activity involve participation of the metal ion in a catalytic capacity. Recent suggestions invoke either a Lewis acid function in which the carbonyl oxygen of the group being hydrolyzed binds within the first coordination sphere of the metal[3,6,8,44] or action of a ligand in that first coordination sphere as a nucleophile.[44,48,49]

Neither mechanism is likely in the hydrolysis of esters catalyzed by Co(III)-CPA if cleavage of a metal-ligand bond is involved because such bonds are normally kinetically inert. For example, cobalt(III) pentaamine derivatives such as $Co(NH_3)_5H_2O^{3+}$, $Co(NH_3)_5Cl^{2+}$, $Co(NH_3)_5O_2CCH_3^{2+}$, and $Co(NH_3)_6^{3+}$ have hydrolytic first-order rate constants at 25° in acid of 5.8×10^{-6}, 1.7×10^{-6}, 1.2×10^{-7}, and 10^{-10} sec^{-1}, respectively.[31] These compare to k_{cat} values of about 100-1000 sec^{-1} for Co(III)-CPA catalysis of ester hydrolyses, Table I. For ligand exchange of the first coordination sphere of the Co(III) to be involved would require ligands to be entering and leaving that sphere at rates that are much higher than is consistent with known Co(III) chemistry.

An alternative mechanism involving Co(III) which would avoid this difficulty may be considered. The first step would be nucleophilic attack of a group within the first coordination sphere of Co(III) on the carbonyl group of the susceptible ester bond with displacement of the terminal α-hydroxy acid of the substrate. If the resultant intermediate were attacked at the carbonyl group by an external nucleophile, it could result in displacement of the first nucleophile, still ligated to Co(III). Such a pathway is unlikely since hydrolysis of $Co(NH_3)_5O_2CCF_3^{2+}$ goes primarily by cobalt–oxygen bond cleavage at low hydroxide ion concentrations,[50] $Co(NH_3)_5O^-$ being the worst oxygen leaving group known.[51] Thus, neither acid- nor base-catalyzed mechanisms involving inner sphere coordination of an ester substrate to the metal ion of Co(III)-CPA seem likely and the metal may be serving a structural rather than a catalytic role in this case.

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(13) Abbreviations used are: apoCPA, apocarboxypeptidase A; M, metal ion; M-CPA, a metallocarboxypeptidase A; D,L-HPLA, hippuryl-D,L-β-phenyllactic acid sodium salt; L-HPLA, hippuryl-L-β-phenyllactic acid sodium salt; NCPLA, *trans*-*p*-nitrocinnamoyl-L-β-phenyllactic acid sodium salt; Tris-Cl, tris(hydroxymethyl)aminomethane–hydrochloric acid buffer.
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