COBALT (III) CARBOXYPEPTIDASE A: PREPARATION AND ESTERASE ACTIVITY

E. P. Kang and C. B. Storm

Department of Chemistry, Howard University

Washington, D. C. 20001

Frederick W. Carson

Department of Chemistry, American University

Washington, D. C. 20016

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SUMMARY

Co(II) carboxypeptidase A has been oxidized to Co(III) carboxypeptidase A with hydrogen peroxide. The resultant metalloprotein has an absorption spectrum different from that of the Co(II) enzyme and the metal is no longer removable by dialysis. The Co(III) carboxypeptidase A retains esterase activity comparable to that of the Co(II) enzyme and has very low peptidase activity. This demonstrates that scission of a bond to the first coordination sphere of the metal is not necessary for the hydrolysis of ester substrates.

Carboxypeptidase A is a metalloenzyme known to require Zn(II) as an essential component of its active site in its native form. A wide variety of modifications of the enzyme has been made. These include replacement of the Zn(II) with a number of other metals and modification of reactive functional groups on the protein. The results of these modifications as well as the results of other approaches to the mechanism of action of carboxypeptidase A have been reviewed recently (1,2,3).

Zn(II) and all of the metal ions used until now to replace it in carboxypeptidase A belong to the exchange labile class of metal ions (4). Because the precise mechanistic role of the metal ion in the action of carboxypeptidase is unknown, we thought it would be interesting to prepare an analog with an exchange inert metal at the active site. The introduction of such a group presents a formidable problem unless inertness can be

conferred upon the metal after its incorporation into the active site. Fortunately, the Co(II)/Co(III) oxidation states comprise an exchange labile/exchange inert pair due to the change from a d^7 to a d^6 electronic configuration upon oxidation (4). Thus, it was anticipated that the known Co(II) carboxypeptidase A (5) might provide a convenient precursor to an exchange inert metalloenzyme.

Co(II) carboxypeptidase A was prepared by the method of Coleman and Vallee (5) from commercial native Carboxypeptidase A. The Co(II) enzyme, 4 mg/ml in 1 M sodium chloride, 0.01 M tris(hydroxymethyl)aminomethane, pH 7.5, was treated with a fourteen-fold excess (0.1 µl/ml of enzyme) of 30% hydrogen peroxide. After standing for six hours at room temperature, the solution was dialyzed for six hours each at 4° against two changes of a twenty-fold excess of buffer. In contrast to the Co(II) enzyme, the oxidized cobalt is no longer removable by dialysis against buffer, in agreement with the expected exchange inert character. Treatment of control samples of the native enzyme with hydrogen peroxide in a similar manner had no effect on either its esterase or peptidase activity. The visible spectral properties of the cobalt enzymes are given in Table I. Results for the Co(II) enzyme are similar to those found by Vallee and coworkers (5,6), while the oxidation product has quite different spectral characteristics.

The Co(III) carboxypeptidase A thus prepared was examined for peptidase activity using N-(N-benzyloxycarbonylglycyl)-L-phenylalanine (5,7) and for esterase activity using both the racemate and the L enantiomer of O-(N-benzoylglycyl)-β-phenyllactic acid (7). These results are presented in Table II. It was thought that if formation of a substrate bond to the first coordination sphere of the metal ion was required for the hydrolysis of either or both of these substrates, then the Co(III) enzyme would not be able to catalyze the hydrolysis. In fact, the esterase activity of the Co(III) carboxypeptidase A is comparable to that of the Co(II) protein,

Table I

Visible Spectral Maxima of Co(II) and Co(III) Carboxypeptidase A

Co(II) Carboxypeptidase A:	530 nm (ϵ = 205), 572 nm (ϵ = 195)
Co(III) Carboxypeptidase A:	503 nm (ϵ = 500)

Table II

Kinetic Constants for the Hydrolysis of Peptide and Ester Substrates by Zn(II), Co(II), and Co(III) Carboxypeptidase A at pH 7.5 and 22.0 ± 0.3° in 0.005 M Tris-HCl, 0.5 M NaCl Buffer

Substrate	Enzyme Derivative	$K_m \times 10^2$	$K_{cat} \times 10^{-4}$ (min^{-1})
N-(N-Benzyloxycarbonylglycyl)-L-phenylalanine	Zn(II)	0.44 ± 0.04	0.63 ± 0.03
	Co(II)	0.39 ± 0.09	1.9 ± 0.2
	Co(III)	----[a]	----[a]
O-(N-Benzoylglycyl)-D,L-phenyllactic Acid [b]	Zn(II)	0.0099 ± 0.0004	2.97 ± 0.04
	Co(II)	0.014 ± 0.001	4.1 ± 0.1
	Co(III)	0.035 ± 0.011	6.0 ± 0.8
O-(N-Benzoylglycyl)-L-phenyllactic Acid	Zn(II)	0.0089 ± 0.002	2.70 ± 0.02
	Co(II)	0.0137 ± 0.0007	4.01 ± 0.07
	Co(III)	0.032 ± 0.006	6.7 ± 0.7

[a]The Co(III) enzyme at a concentration of 3.66×10^{-6} M gave 1.11 times as much hydrolysis in 1230 min as the Co(II) enzyme gave at a concentration of 1.89×10^{-7} M over a 1 min time span using a substrate concentration of 0.0063 M. Thus, the apparent peptidase activity of the Co(II) derivative was about 21,500 times higher than that of the Co(III) enzyme. It is possible that this very low residual activity may have been due to contaminants.

[b]The substrate concentration is determined by the L enantiomer only.

while the peptidase activity is almost eliminated. That the N-(N-benzyloxycarbonylglycyl)-L-phenylalanine can still bind to the Co(III) carboxypeptidase A is demonstrated by the fact that it is an excellent competitive inhibitor

of esterase activity, K_I = 0.00107 ± 0.00006 under the conditions given in Table II. This inhibition constant is comparable to the K_m for the hydrolysis of this peptide catalyzed by the Co(II) enzyme, Table II. Thus, both forms of cobalt carboxypeptidase A bind N-(N-benzyloxycarbonylglycyl)-L-phenylalanine strongly.

The most popular current suggestions for the mechanism of action of carboxypeptidase A involve either (a) action of the metal ion as a Lewis acid to polarize the carbonyl group prior to attack by a nucleophile or (b) nucleophilic attack on the carbonyl group by the oxygen of a water molecule coordinated to the metal atom (3,8-12). Mechanism (a) requires substitution into the first coordination sphere of the metal involved or participation of a coordinatively unsaturated species. Both are unlikely with Co(III) under our reaction conditions. Mechanism (b) has been demonstrated in Co(III) model systems utilizing a coordinated hydroxide group as an intramolecular nucleophile (11). This gives rise to an intermediate with a cobalt-oxygen bond, which should be stable and persist for a considerable length of time. One would have to invoke an attack on this coordinated carboxylate by some other nucleophile, such as Glu_{270} or an activated water molecule, in order to obtain productive catalysis. Thus, the first of these mechanisms is incompatible with the observed esterase activity of Co(III) carboxypeptidase A while the second remains an open possibility.

Recent affinity labeling experiments have shown that the Glu_{270} at the active site of Zn(II) carboxypeptidase A has strong nucleophilic character (13-16). It appears that this group may be a sufficiently good nucleophile to attack an ester carbonyl group without the necessity of covalent bonding of the carbonyl oxygen to the nearby metal atom.

The results reported here are similar to several others obtained with carboxypeptidase A derivatives. For example, the Cd(II) and Hg(II) derivatives retain esterase activity while losing peptidase activity (7)

and the same is true of the acetylated Zn(II) enzyme (17). These results with various modified enzymes and observations on the effects of pH have led to the suggestion that carboxypeptidase A catalyzes the hydrolysis of esters by a mechanism different from that for peptides (17,18,19). The results we report here require either that the two types of substrates are hydrolyzed by different mechanisms or that a common mechanism is involved in which scission of a bond within the first coordination sphere of the metal ion does not occur.

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