COBALT(III) CARBOXYPEPTIDASE A: AN ACTIVITY DEPENDING ON THE METHOD OF METAL ION REPLACEMENT

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SUMMARY

There are two different methods available for replacing the Zn(II) in carboxypeptidase A with Co(II). One involves direct metal ion exchange using a large molar excess of Co(II) while the other requires the preparation of the metal-free apoenzyme and reconstitution with Co(II). Oxidation of the product obtained by the first route gives Co(III) carboxypeptidase A which is inactive towards synthetic peptide substrates but active towards synthetic ester substrates. In contrast, oxidation of the Co(II) carboxypeptidase obtained by the second strategy gives a Co(III) carboxypeptidase A having neither peptidase nor esterase activity.

Substitution of metal ions at the active sites of metalloproteins has been used as a selective modification procedure in many instances. It is one of the mildest and most selective alterations that can be carried out. Such substitutions do not appear to cause significant changes in overall protein structure and the catalytic function of the protein is frequently preserved in the new metalloenzyme. Evidence is available that some metalloenzymes maintain activity even though a change in coordination geometry accompanies metal ion replacement, indicating that the conformation of the protein may be poised at nearly the ideal state by different metal ion coordination numbers and geometries.

For example, phosphoglucomutase is activated by Mg(II), Mn(II), Ni(II) and Co(II). Ray and Multani (1) have reported that the Mn(II) and Ni(II) are octahedral based on the Mn(II) electron spin resonance and the Ni(II) electronic spectra. On the other hand, the Co(II) form is thought to be either

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four- or five-coordinate and the visible absorption spectrum indicates that it is certainly not octahedral.

A number of different metal ion derivatives of carboxypeptidase A (EC 3.4.2.1)* have been prepared and they also appear to have different coordination geometries. Zn(II) CPA has a distorted tetrahedral geometry with His-69, Glu-72 and His-196 as the protein ligands and a water molecule as the fourth ligand in the resting state (2). Gray and co-workers have studied the spectral and magnetic properties of the Ni(II), Co(II) and Cu(II) derivatives (3,4). The Ni(II) derivative is octahedral and the Co(II) enzyme most likely is five-coordinate. The spectral properties of the Cu(II) derivative are consistent with a significant distortion away from square planar toward tetrahedral geometry. The Ni(II) and Co(II) CPA are fully active with both peptide and ester substrates. The only substrate so far found to be active with Cu(II) CPA is S-(trans-cinnamoyl)-L-α-mercapto-β-phenylpropionate (5).

Co(II) has been used as a replacement for the diamagnetic, colorless Zn(II) in CPA because of its paramagnetic and chromophoric properties accompanied by high hydrolytic activity. Two methods for making this substitution have been reported In one case native Zn(II) CPA is exposed to a large excess of Co(II) followed by dialysis against metal free buffer to a 1:1 Co(II):protein content. The other method requires preparation of metal-free apo CPA by dialysis against a chelating agent and subsequent reactivation with an essentially stoichiometric amount of Co(II). To this time all evidence has supported the idea that the Co(II) CPA's obtained by these two routes are identical. We have recently obtained results that demonstrate that the two proteins are not identical but have a "memory" of their method of preparation

We reported previously the preparation of Co(III) CPA by the oxidation of Co(II) CPA prepared by the direct exchange method (6) using hydrogen peroxide as the oxidant (7,8). Co(III) CPA prepared by this method has no peptidase activity towards carbobenzoxyglycyl-L-phenylalanine† but retains substantial esterase activity towards hippuryl-L-β-phenyllactic acid‡ and O-(trans-p-nitro-

* Abbreviated as CPA. † Abbreviated as CGP. ‡ Abbreviated as HPLA.

cinnamoyl)-L-β-phenyllactic acid* (7,8). In contrast, if one prepares Co(III) CPA by first making the apo enzyme, reactivating with Co(II) and then oxidizing with hydrogen peroxide, a Co(III) CPA is obtained that again has no peptidase activity towards CGP and has lost the esterase activity towards HPLA and NCPLA as well.

Materials and Methods: CPA_α was obtained from the Sigma Chemical Company and the CPA_γ from the Worthington Biochemical Corporation. Cobalt chloride solutions were prepared from weighed amounts of cobalt sponge (99.999% pure), obtained from K & K Laboratories, dissolved in metal-free HCl. The pH was then adjusted using metal-free base and the final solution was diluted to the desired concentration with metal-free buffer. All solutions were extracted with dithizone in either carbon tetrachloride or chloroform to remove any adventitious metal ion contamination. Polypropylene containers were used wherever possible.

The direct exchange of Co(II) for Zn(II) was carried out by dialyzing the native Zn(II) CPA against an equal volume of Co(II), 100 times more concentrated than the protein, in 1.0 M NaCl, 0.1 M Tris-HCl buffer at pH 8.0 for 48 hrs. at 4° (9). The dialysate was changed every 12 hrs. The excess cobalt ion was removed by dialyzing against a 20-fold excess of buffer for 36 hrs. with a change of buffer every 12 hrs. The apo enzyme was prepared by dialysis of the Zn(II) CPA, ca. 10^{-4} M in 1 M NaCl, 0.05 M Tris-HCl, pH 7.5, against 0.002 M o-phenanthroline (OP) in the same buffer at 4°. The chelating agent was removed by dialysis against 1M NaCl, 0.05 M Tris-HCl, pH 7.5 buffer at 4°. The Co(II) protein was obtained by adding one equivalent of Co(II) in metal-free buffer to the apo enzyme solution. The cobalt incorporation was followed using ^{57}Co label, which was monitored on the ^{14}C channel of a liquid scintillation counter. The apo CPA preparations were only carried out with the CPA_α. The Co(II) CPA was oxidized to Co(III) CPA with hydrogen peroxide as previously reported (7,8).

The enzyme activity assays were done at room temperature in 1 M NaCl, 0.05 M Tris-HCl, pH 7.5. In cases where adventitious metal ion contamination was a potential problem the assays were done in the presence of 10^{-5} M EDTA. It has been demonstrated that up to 10^{-4} M EDTA does not affect the activity of the native Zn(II) CPA toward either peptide or ester substrates (10). For the peptidase assay the substrate concentration was from 10^{-4} to 10^{-5} M at a protein concentration of about 3×10^{-9} M; for the esterase activity the substrate concentration was from 10^{-5} to 10^{-6} M and the protein concentration was about 3×10^{-10} M. We have found pyridine-2,6-dicarboxylate to be the best reagent for removal of adventitious metal ion contamination in Co(III) CPA (11).

Results and Discussion: Co(III) CPA prepared by oxidation of Co(II) CPA obtained by activating apo CPA with one equivalent of Co(II) has neither esterase nor peptidase activity. This contrasts with Co(III) CPA prepared by oxidation of Co(II) CPA obtained by direct exchange of Co(II) for Zn(II), which does not have peptidase activity but shows substantial esterase activity (7,8). The k_{cat}/K_M values and relative specific esterase activities for several different CPA derivatives are summarized in Table 1.

* Abbreviated as NCPLA.

Table 1

The k_{cat}/K_M and relative specific esterase activity with HPLA for several CPA derivatives.

	$k_{cat}/K_M \times 10^{-8}$ [a]	relative specific activity $\times 10^{-4}$ [b]	
Zn(II) CPA	3.60 ± 0.17 (6)	2.78	100%
Co(II) CPA (DE)[c]	4.15 ± 0.31 (4)	2.37	85%
Co(II) CPA (apo)[d]	4.25 ± 0.77 (3)	3.00	108%
Co(III) CPA (DE)	1.43 ± 0.12 (2)	0.98	35%
Co(III) CPA (apo)[f]	less than 1% activity		
Zn(II) CPA (apo)	3.24 ± 0.73 (2)	2.59	93%
Co(II) CPA (apo, DE)[e]	4.55 ± 0.39	3.02	108%
Co(III) CPA (apo, DE)	0.27 ± 0.04	0.32	12%
apo CPA[f]	less than 1% activity		

a. Taken from the slope of the E/V vs. 1/S plots over a substrate concentration range of 3×10^{-5} - 5×10^{-4} M at an enzyme concentration of 3×10^{-10} M and are in units of 1 $moles^{-1}$ min^{-1}. The figure in brackets is the number of runs averaged for the table entry.
b. The relative specific activity at an HPLA concentration of 2×10^{-4} M. The rates are reported as moles of substrate hydrolyzed per min. per mole of CPA.
c. A metallo CPA prepared from the Zn(II) CPA by the direct exchange method.
d. A metallo CPA prepared from apo CPA by adding one equivalent of metal ion.
e. A cobalt CPA obtained by first making the apo protein, reactivating with Zn(II) and then introducing the Co(II) by direct exchange, followed by oxidation with hydrogen peroxide in the Co(III) case.
f. Assayed at 10^{-9} M protein.

A sample of Co(III) CPA was prepared by the direct exchange method and chromatographed on Sephadex G-50 in 1.0 M NaCl, 0.1 M Tris-HCl, pH 7.5. A peak fraction from the Co(III) CPA peak, 10^{-4} M, assayed for 43% esterase activity and 18% peptidase activity, relative to the native protein. Atomic absorption analysis for zinc showed that the preparation contained 18% zinc contamination. This preparation was then treated with excess pyridine-2,6-dicarboxylic acid in buffer. When the protein was diluted directly into the assay mixture, both the peptidase and esterase activities were less than 1% compared to native CPA. When the protein was diluted into the buffer mixture and allowed to stand for

20 min., followed by addition of substrate, the peptidase activity remained at less than 1%, but the esterase activity returned to 26% of that of the native enzyme.

When the apo protein was prepared and reactivated with Zn(II), and then Co(II) introduced by direct exchange followed by oxidation, the resulting Co(III) CPA retained about one-third the esterase activity of the protein prepared by direct exchange, Table 1. Treatment of either native Zn(II) CPA (7,8) or Zn(II) CPA obtained by reactivation of apo CPA with one equivalent of Zn(II) with hydrogen peroxide, under the same conditions used to oxidize Co(II) CPA, has no effect on its specific activity towards HPLA.

In Zn(II) metallo proteins having histidine ligands to the metal ion the imidazole may be coordinated by either the N-1 or N-3 nitrogens. In the case of the insulin hexamer all three imidazole ligands of both Zn(II) centers are coordinated by the N-3 nitrogens of the B-10 histidines (12). In carbonic anhydrase His-93 and 95 are bound by N-3 while His-117 is bound by N-1 (13). In native Zn(II) CPA both His-69 and 196 are attached to the metal ion by N-1 (2). These observations suggest a possible explanation for our result. That is, when Co(II) is introduced into CPA by direct exchange the metal ion binds to the His N-1, as in the Zn(II) case. But when the Co(II) is introduced into the apo CPA one or both of the histidines may be bonded by the N-3 of the imidazole. The active site may well be flexible enough to accomodate this at the Co(II) level, as evidenced by the toleration of different metal ion coordination geometries (3,4). However, when the octahedral Co(III) geometry is imposed on the protein rather different results would be expected and are reflected in the different esterase activities observed.

Experiments designed to elucidate the exact cause of these different esterase activities are being pursued.

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