EXCHANGE INERT Co(III)-CARBOXYPEPTIDASE A: A CATALYTICALLY INACTIVE METALLOCARBOXYPEPTIDASE

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Summary: Exposure of cobalt (II) carboxypeptidase A_α, [(CPD)Co(II)], to small molar excesses of the oxidizing agent m-chloroperbenzoate rapidly destroys (< 30 sec) both its peptidase and esterase activities in parallel. Concomitantly, the characteristic Co(II) electron paramagentic resonance (EPR) signal is abolished. [(CPD)Co(III)], isolated from the reaction mixture, has the same molecular weight and amino acid composition as [(CPD)Co(II)], contains 0.95 g-atom of Co and 0.01 g-atom of Zn per mole of protein, does not exhibit an EPR spectrum and is catalytically completely inactive towards both peptide and ester substrates. Identical treatment of the native zinc enzyme affects neither its catalytic activity nor its metal content. The reaction of m-chloroperbenzoate with [(CPD)Co(II)] follows saturation kinetics and is prevented by the inhibitor β-phenylpropionate. Furthermore, under the conditions found to oxidize [(CPD)Co(II)] effectively, there is no reaction with Co(II) *E. coli* alkaline phosphatase. Thus, m-chloroperbenzoate has the characteristics of an active-site directed oxidizing reagent for [(CPD)Co(II)].

Introduction: Substitution of various metals for the essential zinc atom of carboxypeptidase A, [(CPD)Zn], markedly changes its peptidase and esterase activities (1,2). Almost all such metal replacements have utilized exchange-labile metal atoms. Recently, however, Kang *et al.* (3,4) have reported that carboxypeptidase A containing exchange-inert Co(III) can be prepared by direct oxidation of [(CPD)Co(II)] with hydrogen peroxide to yield a product with negligible peptidase activity, but which retains esterase activity comparable to that of [(CPD)Co(II)]. This observation has led to speculations regarding the overall mechanism of action of the enzyme and, more specifically, the role of the metal atom in the catalytic process (3,4). In particular, from the retention

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of esterase activity on oxidizing [(CPD)Co(II)] to [(CPD)Co(III)], it has been inferred that ester substrates are not liganded within the first coordination sphere of the metal during hydrolysis, since Co(III) is inert to ligand exchange reactions (3,4). The implications of these interpretations led to the present experiments intended as a prelude to more detailed studies of the catalytic properties of [(CPD)Co(III)]. However, we find this species completely inactive and, hence, fail to confirm the results and conclusions of the earlier reports (3,4).

MATERIALS AND METHODS: [(CPD)Zn] (Sigma) was converted to [(CPD)Co(II)] according to previously published procedures (5). Metal-free conditions were maintained throughout by treating hydrogen peroxide (Fisher certified) and m-chloroperbenzoate (m-CPB) (Aldrich) solutions with Chelex 100 resin, by extracting all buffers, salts and substrates with dithizone in CCl_4 and by using metal-free water and glassware (2). Atomic absorption spectrometry (6) served to establish the zinc and cobalt content of the respective enzymes. Peptidase and esterase activities were determined using carbobenzoxyglycyl-L-phenylalanine and hippuryl-L-β-phenyllactate as substrates (5). The electron paramagnetic resonance (EPR) spectra of the samples were examined at 5° K using a Varian E9 EPR spectrometer equipped with an Air Products Helitran apparatus (7). Amino acid analyses were carried out with a Durrum 500 amino acid analyzer. Samples were hydrolyzed in vacuo for 24 hrs with 6 N HCl at 110°. Tryptophan content was determined separately by magnetic circular dichroism using a Cary Model 61 spectropolarimeter equipped with a Varian superconducting V4145 magnet (8).

RESULTS AND DISCUSSION: Attempts to prepare homogeneous, monomeric [(CPD)Co(III)] by oxidation of [(CPD)Co(II)] with hydrogen peroxide according to published procedures (3,4) proved unsuccessful. Invariably, the EPR spectrum of the product revealed that a substantial fraction of the enzyme was still in the paramagnetic [(CPD)Co(II)] state. Moveover, gel filtration on Sephadex G-75 resulted in a heterogeneous mixture of proteins containing nonstoichiometric quantities of cobalt and exhibiting variably reduced peptidase and esterase activities.

However, the conversion of [(CPD)Co(II)] to [(CPD)Co(III)] was successful using m-chloroperbenzoate (m-CPB), an oxidizing reagent with structural features similar to those of certain inhibitors of carboxypeptidase A. The reaction was investigated as a function of enzyme, buffer, and salt concentrations, temperature, pH and time. Optimal conditions for the oxidation

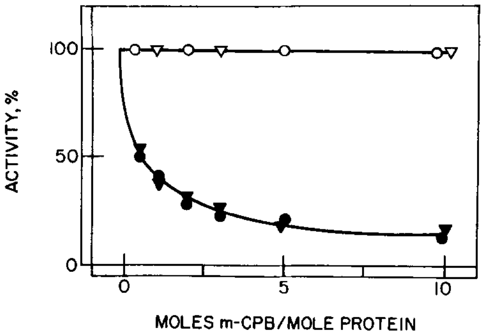

Figure 1: Peptidase (○) and esterase (▽) activities of [(CPD)Zn] and those of [(CPD)Co(II)] (●) (▼) on reaction with increasing concentrations of m-chloroperbenzoate (m-CPB). Activities are expressed as percentages of the unmodified controls.

of cobalt and its retention by the enzyme were determined. Preliminary studies showed that Co(II) oxidation occurred readily, but was accompanied by the production of free radicals that resulted in polymerization of the enzyme. However, in the presence of the radical scavenger phenol the oxidation proceeded without polymerization.

In a typical experiment, m-CPB (0.1 M in ethanol) is added to 2.9 x 10^{-4} M enzyme in 0.1 M Tris·HCl, 1 M NaCl, pH 9, 4° containing phenol, equimolar to the m-CPB. After reaction for 30 seconds, an aliquot of the sample is transferred to an EPR tube, frozen immediately in liquid nitrogen and then scanned on the EPR spectrometer. The remainder is diluted into 0.1 M Tris·HCl buffer containing 0.1 mM Co(II) to quench the reaction and assayed immediately for peptidase and esterase activities. The effect of varying concentrations of m-CPB on the catalytic activities of [(CPD)Zn] and [(CPD)Co(II)] are shown in Fig. 1 and on the EPR spectrum of [(CPD)Co(II)] in Fig. 2. Oxidation of [(CPD)Co(II)] reduces both peptidase and esterase activities in parallel fashion and simultaneously abolishes the characteristic [(CPD)Co(II)] EPR spectrum. On the other hand, identical treatment does not affect the activities of [(CPD)Zn].

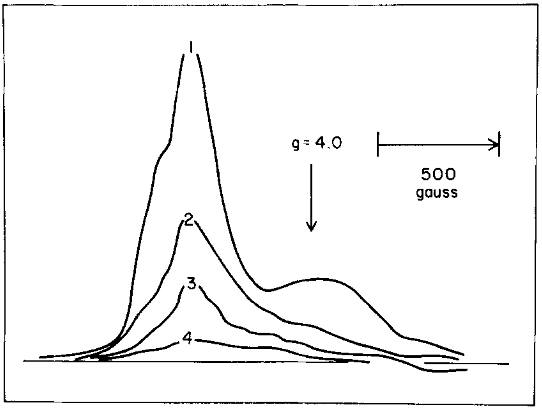

Figure 2: Changes in the EPR spectrum of [(CPD)Co(II)] on reaction with increasing concentrations of m-chloroperbenzoate (m-CPB). The ratios of moles m-CPB/mole protein are (1) 0.0 (2) 0.5 (3) 2.0 and (4) 10.0, i.e., those at which the activity measurements in Fig. 1 were performed. Microwave power, 20 mW; modulation frequency, 100 kHz; microwave frequency, 9.40 GHZ; 5°K.

After reaction of [(CPD)Co(II)] with a two-fold molar excess of m-CPB, the pH is adjusted to 6.0 and the reaction mixture passed over a column consisting of an upper layer of affinity resin (9), known to bind [(CPD)Co(II)], and a lower layer of Sephadex G-75. This column acts both to remove unreacted [(CPD)Co(II)] and to separate reaction by-products from the desired [(CPD)Co(III)]. The protein eluted by the starting buffer is homogeneous, contains 0.95 g-atom of cobalt and 0.01 g-atom of zinc per mole of protein, is devoid of catalytic activity toward both peptides and esters and has the same molecular weight and amino acid composition as the starting material. Furthermore, no EPR spectrum is detected, as would be expected for Co(III) which is generally diamagnetic. The remainder of the total protein from the reaction mixture elutes from the column at pH 7.5, and consists only of unreacted [(CPD)Co(II)]. Reaction of [(CPD)Zn] under identical conditions followed by gel filtration and metal analysis confirms that m-CPB affects neither its metal content nor its molecular weight (Table I). All of the above data are consistent with the conclusion that m-CPB oxidizes

TABLE I: Effect of reaction of m-Chloroperbenzoate on the Enzymatic Activities and Metal Content of Cobalt(II) and Zinc Carboxypeptidase A and Cobalt (II) Alkaline Phosphatase[a]

	Before Reaction		After Reaction				
Enzyme	Zn g-atom/mole	Co g-atom/mole	Zn g-atom/mole	Co g-atom/mole	Activity,[b] %		
					Peptidase	Esterase	Phosphohydrolase
[(CPD)Co(II)][c]	0.01	1.03	0.01	0.95	< 0.1	< 0.1	-
[(CPD)Co(II)] + β-phenylpropionate, 0.1 M[d]	0.01	1.03	0.01	1.03	100	100	-
[(CPD)Zn][d]	1.03	0.0	1.03	0.0	100	100	-
Co(II) - E. coli alkaline phosphatase[d]	0.05	2.1	0.05	2.1	-	-	100

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[a] Conditions for all enzymes: [Enzyme] = 2.9×10^{-4} M in 1 M NaCl, 0.1 M Tris·HCl, pH 9, 4°. Equal molar amounts of, first, phenol and, next, m-CPB, each two-fold over the concentration of metal, were added and the reaction quenched after 30 seconds.

[b] Expressed as percentages of the unmodified controls.

[c] After reaction, the unreacted [(CPD)Co(II)] and unbound Co were removed by affinity-gel filtration chromatography.

[d] After reaction, unbound metal ions were removed by gel filtration.

[(CPD)Co(II)] to catalytically inactive [(CPD)Co(III)] and that oxidation of the metal atom -- rather than of amino acid residues of the protein -- is responsible for the loss of enzymatic activity.

Figure 1 shows that with increasing concentrations of m-CPB the percent of inactivation observed per 30 second reaction time reaches a plateau. This behavior is reminiscent of saturation kinetics, and suggests the formation of a m-CPB-enzyme complex prior to inactivation. When the reaction with a two-fold excess of m-CPB is carried out in the presence of β-phenylpropionate, a potent inhibitor of [(CPD)Co(II)] (10), no oxidation is observed. Presumably, β-phenylpropionate protects the Co atom in the enzyme from oxidation by blocking the active site. Furthermore, Co(II) _E. coli_ alkaline phosphatase, which is known to be readily converted into an inactive Co(III) derivative by treatment with hydrogen peroxide (11), is unaffected by a two-fold excess of m-CPB (calculated on the basis of 2 Co per mole). Together, these results indicate that m-CPB has a particular affinity for [(CPD)Co(II)] and, thus, exhibits the characteristics of an active site directed oxidizing reagent.

Co(III) differs from Co(II) in that it is inert to substitution reactions, prefers octahedral coordination and nitrogen donors. The loss of catalytic function upon oxidation of [(CPD)Co(II)] to [(CPD)Co(III)] may be related to one or all of these differences, and studies designed to differentiate between these possibilities are currently underway. Our efforts to reproduce that data of Kang _et al._, by repetition of all experimental details reported (3,4), have failed to yield a physiochemically homogeneous enzyme containing 1 g-atom of Co(III) with the enzymatic characteristics described by these authors. Thus, the differences in the catalytic properties of the [(CPD)Co(III)] enzyme prepared by us and by Kang _et al._ (3,4) remain unexplained.

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