Enzymatically Inactive, Exchange-Inert Co(III)–Carboxypeptidase A: Role of Inner Sphere Coordination in Peptide and Ester Catalysis†

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ABSTRACT: Catalytically inactive, exchange-inert Co(III)-carboxypeptidase A has been prepared by reaction of Co(II)-carboxypeptidase A with the active-site-directed oxidizing agent *m*-chloroperbenzoic acid. Co(III)-carboxypeptidase A, isolated by affinity gel filtration chromatography, has the same amino acid composition and molecular weight as the starting material and contains 0.95 g-atom/mol of cobalt and 0.01 g-atom/mol of zinc. Its electron paramagnetic resonance, circular dichroic, magnetic circular dichroic, and visible absorption spectra are consistent with those of octahedral Co(III) model complexes. Co(III)-carboxypeptidase A is essentially devoid of catalytic activity toward both peptide and ester substrates of the native enzyme, and stopped-flow fluorescence studies with dansylated substrates show that it binds peptides, but not esters. Furthermore, the protein does not react with either type of substrate to yield a single turnover. The implications of these findings to the mechanism of action of carboxypeptidase A are discussed in the light of the "metal-carbonyl" and "metal-hydroxide" hypotheses. Since Co(III)-carboxypeptidase A does not bind esters, inner-sphere coordination to the metal appears to be necessary for ester binding. All attempts to prepare Co(III)-carboxypeptidase A by treatment of Co(II)-carboxypeptidase A with hydrogen peroxide according to previously published procedures (Kang, E. P., Storm, C. B., & Carson, F. W. (1975) *J. Am. Chem. Soc. 97,* 6723) have been unsuccessful, and the present results do not confirm earlier reports that Co(III)-carboxypeptidase A exhibits esterase activity or that its activity is dependent on the method of preparation of the precursor Co(II)-carboxypeptidase A (Jones, M. M., Hunt, J. B., Storm, C. B., Evans, P. S., Carson, F. W. & Pauli, W. J. (1977) *Biochem. Biophys. Res. Commun. 75,* 253). These findings call for a reexamination of mechanistic conclusions based on the assumption that Co(III)-carboxypeptidase A is an active esterase.

Carboxypeptidase A, [(CPD)Zn],[1] contains one firmly bound zinc atom that is essential for both peptidase and esterase activities (Vallee & Neurath, 1955). Substitution for zinc by other metals with different properties variously affects the two activities allowing partial delineation of the role of the metal in catalysis (Coleman & Vallee, 1960; Auld & Holmquist, 1974). All such metallocarboxypeptidases that have been characterized thoroughly have contained exchange-labile metal atoms. Recently, however, the preparation of exchange-inert [(CPD)Co(III)] has been attempted by oxidation of [(CPD)Co(II)] with hydrogen peroxide (Kang et al., 1972, 1975). The catalytic properties of the product of the reaction, claimed to be [(CPD)Co(III)], were said to be dependent on the method of preparation of the precursor [(CPD)Co(II)] (Jones et al., 1977). Under certain circumstances, the product was found to be an active esterase.

Unfortunately, these studies did not present data which could demonstrate conclusively that [(CPD)Co(III)] was, indeed, the product. Based on these results, however, conclusions regarding the mechanism of action of [(CPD)Zn] have been drawn (Breslow & Wernick, 1977; Wells & Bruice, 1977; Cleland, 1977). Since these studies and their results have been thought to bear critically on the mechanism of action of carboxpeptidase A, we have prepared [(CPD)Co(III)] to extend and complete its chemical and physical characterization and to reexamine the effects on enzymatic activity of the replacement of zinc with an exchange-inert metal. The retention of either catalytic activity on oxidation would mitigate against inner sphere coordination of substrate to the metal during binding or hydrolysis, since exchange-inert Co(III) undergoes ligand exchange reactions very slowly (i.e., with a half-life greater than about 1 min) compared with the rate of enzymatic hydrolysis.

The present data show that the procedures for the preparation of [(CPD)Co(III)] reported previously yield a mixture of proteins rather than a single characterizable species. We have developed an alternative procedure which does yield monomeric, exchange-inert [(CPD)Co(III)] whose amino acid composition is the same as that of [(CPD)Zn], which contains a stoichiometric amount of cobalt, but essentially no zinc, and which exhibits spectral properties consistent with those of known octahedral Co(III) complexes. It is catalytically inactive toward both peptide and ester substrates regardless of the method of preparation of the precursor [(CPD)Co(II)]. These findings are clearly basic to the mechanism of action of carboxypeptidase A and obviate earlier conclusions (Kang et al., 1972, 1975; Jones et al., 1977).

Materials and Methods

Carboxypeptidase A, prepared by the method of Cox et al. (1964), was obtained as a crystal suspension (Sigma Chemical Co.) and purified by affinity chromatography (Peterson et al., 1976). [(CPD)Zn] was converted to [(CPD)Co(II)] by three

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[1] Abbreviations used: [(CPD)Me] refers to metallocarboxypeptidase A where (CPD) represents the apoenzyme and the brackets indicate the firm binding of metal, Me, where Me is Zn, Co(II), or Co(III); Bz, benzoyl; Dns, 5-dimethylaminonaphthalene-1-sulfonyl (dansyl); Cbz, carbobenzoxy; OPhe, 3-phenyllactic acid; Tris, tris(hydroxymethyl)aminomethane; *m*-CPB, *m*-chloroperbenzoic acid; *m*-CB, *m*-chlorobenzoic acid; CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; CABS, caproylaminobenzylsuccinyl-Sepharose.

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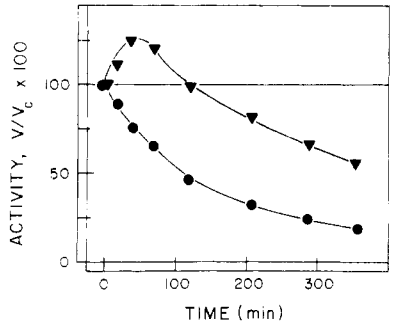

FIGURE 1: Effect of hydrogen peroxide on the peptidase (●) and esterase (▼) activities of [(CPD)Co(II)]. Activities are expressed as percentages of the unmodified controls. The enzyme, 1.08×10^{-4} M in 1 M NaCl, 0.1 M Tris-HCl, pH 7.5, was treated with 1 mM hydrogen peroxide at 22 °C, according to the procedure of Kang et al. (1975). (See also Figures 2 and 4 in Piras & Vallee, 1966a.)

different procedures. Unless stated otherwise, all studies reported herein were carried out with [(CPD)Co(II)] prepared by reconstitution of the crystalline apoenzyme with cobaltous ions (Auld & Holmquist, 1974). In a second procedure, [(CPD)Co(II)] was prepared by dialysis of [(CPD)Zn] in solution against cobaltous ions according to the procedure of Coleman & Vallee (1960). In the third procedure, [(CPD)-Co(II)] again was prepared by direct exchange of [(CPD)Zn] in solution against cobaltous ions, but according to the procedure of Jones et al. (1977). Radioactively labeled [(CPD)-^{60}Co(II)] was prepared by equilibrating ^{60}Co (New England Nuclear) with [(CPD)Co(II)] crystals and subsequently washing to remove unbound metal ions. Atomic absorption spectrometry (Fuwa & Vallee, 1963) served to establish the cobalt and zinc content of all metalloenzymes.

Metal-free conditions were maintained throughout by extracting all solutions of buffers, salts, and substrates with dithizone in carbon tetrachloride, using metal-free water and glassware (Thiers, 1957) and by treating solutions of phenol, hydrogen peroxide (Fisher certified), *m*-CPB (Aldrich), sodium dithionite (Fisher), and β-phenylpropionate (Eastman) with Chelex 100 resin. Contaminating *m*-CB was removed from *m*-CPB by washing the crystalline material with phosphate buffer, pH 7.5, and drying under reduced pressure (Fieser & Fieser, 1967).

The substrates Cbz-Gly-L-Phe and Bz-Gly-L-OPhe were purchased from Sigma Chemical Co. and Fox Chemical Co., respectively, while Bz-$(Gly)_2$-L-Phe was synthesized according to the procedure of Auld & Vallee (1970), and Dns-$(Gly)_3$-L-Phe and Dns-$(Gly)_3$-L-OPhe were synthesized by that of Latt et al. (1972).

Peptidase activities toward Cbz-Gly-L-Phe were measured in 1 M NaCl, 0.5 M Tris-HCl, pH 7.5, 25.0 ± 0.1 °C at a substrate concentration of 0.02 M using a Technicon auto analyzer (Auld & Vallee, 1970). This technique was also used to assay [(CPD)Co(III)] for peptidase activity toward Bz-$(Gly)_2$-L-Phe in 1 M NaCl, 0.05 M Tris-HCl, 25.0 ± 0.1 °C at integral pH units between 6.0 and 10.0 at a substrate concentration of 1×10^{-3} M. Using Bz-Gly-L-OPhe as substrate, esterase activities were determined spectrophotometrically in 0.2 M NaCl, 0.05 M Tris-HCl, pH 7.5, 25.0 ± 0.1 °C at a substrate concentration of 5×10^{-4} M (Folk & Schirmer, 1963). The enzymatic activities of [(CPD)Co(II)] and [(CPD)Co(III)] were examined in the presence and absence of excess cobaltous ions.

Peptidase and esterase activities of [(CPD)Zn] and [(CPD)Co(III)] and their substrate binding properties toward Dns-$(Gly)_3$-L-Phe and Dns-$(Gly)_3$-L-OPhe, respectively, were examined by using a stopped-flow fluorescence technique (Latt et al., 1972) at 25.0 ± 0.1 °C with a Durrum-Gibson instrument equipped with the Durrum fluorescence accessory no. 16400, a 75-W xenon lamp and an EMI 9526B photomultiplier tube.

Protein concentrations were determined by measuring the absorbance at 278 nm and using a molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} for all metalloenzymes.

Slab gel electrophoresis was carried out under standard conditions (Weber et al., 1972) on 13.5% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate with 0.1% 2-mercaptoethanol in 0.1 M sodium phosphate buffer, pH 7.0. Gels were stained with Coomassie blue and destained in 7.5% acetic acid–5% methanol.

An affinity resin for [(CPD)Zn] and [(CPD)Co(II)] consisting of immobilized *p*-aminobenzylsuccinic acid (caproylaminobenzylsuccinyl-Sepharose, abbreviated CABS) was utilized in several of the purification procedures used herein. This resin binds [(CPD)Co(II)] and [(CPD)Zn] tightly in 1 M NaCl at pH 6.0 (Peterson et al., 1976). However, when the pH is raised to 7.5, these enzymes are eluted easily. [(CPD)-Co(III)] does not bind to CABS at either pH in 1 M NaCl.

Amino acid analyses were carried out with a Durrum D-500 amino acid analyzer. Samples were hydrolyzed in vacuo for 24 h with 6 M HCl at 110 °C. Tryptophan content was determined separately by MCD (Holmquist & Vallee, 1973).

Visible absorption spectra were obtained with a Cary Model 14 spectrophotometer equipped with a 0–0.1 absorbance slide wire. CD and MCD measurements were made with a Cary Model 61 spectropolarimeter equipped with a V4145 Varian superconducting magnet. Fluorescence measurements were carried out with a Perkin-Elmer MPF-3 fluorimeter. EPR spectra were obtained at 5 K using a Varian E9 EPR spectrometer equipped with an Air Products Helitran apparatus. The rate of γ emission of ^{60}Co was measured with a Searle Model 1185 γ counter.

The rate of loss of cobalt from [(CPD)^{60}Co(III)] and [(CPD)^{60}Co(II)] was measured with a Medical Research Apparatus pressure ultrafiltration cell according to the procedure of Paulus (1969) using Diaflo PM-10 filters that had been washed exhaustively to remove contaminating metal ions.

Results

Oxidation of [(CPD)Co(II)] with Hydrogen Peroxide. The preparation of [(CPD)^{60}Co(III)] was attempted by treating [(CPD)^{60}Co(II)] with hydrogen peroxide according to the procedure of Kang et al. (1975). The effect of this procedure on the peptidase and esterase activities of the enzyme toward Cbz-Gly-L-Phe and Bz-Gly-L-OPhe, respectively, as a function of time is shown in Figure 1. Peptidase activity decreases slowly in a monotonic fashion, but esterase activity undergoes an initial increase followed by a monotonic decrease. After 6 h, the peptidase and esterase activities of the reaction mixture were 19 and 55%, respectively, of that of the starting material.

This treatment with hydrogen peroxide also affects the spectral properties, metal content, molecular weight, and homogeneity of the enzyme. Soon after addition of hydrogen peroxide, the solution turns turbid. Over the same time interval, the visible absorption band of [(CPD)Co(II)], centered near 555 nm, decreases and concomitantly a strong end-absorbance from the ultraviolet develops. After 6 h, the EPR spectrum (not shown) of the reaction mixture decreases to about 40% of the initial signal characteristic of [(CPD)Co(II)].

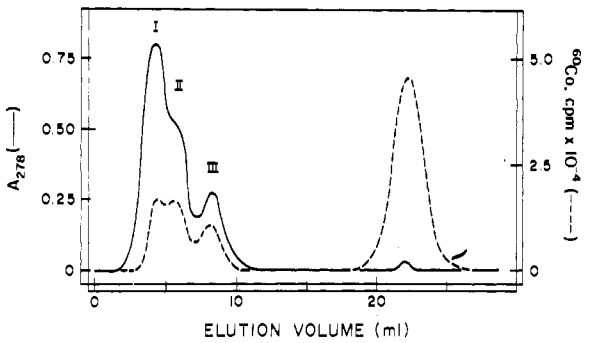

FIGURE 2: Gel filtration of [(CPD)^{60}Co(II)] treated with hydrogen peroxide for 6 h over Sephadex G-75 using 1 M NaCl, 0.1 M Tris-HCl, pH 7.5. Conditions of reaction as in Figure 1.

Gel filtration of an aliquot of the final reaction mixture over Sephadex G-75 yields an elution profile that indicates the presence of a heterogeneous mixture of proteins resolved into three bands (I, II, and III in Figure 2) and demonstrates that only about 40% of the cobalt is bound to protein. The cobalt content of fraction III, which emerges from the column in the same position as the [(CPD)Co(II)], is only 0.58 g-atom/mol. The two higher molecular weight fractions (I, II) also contain nonstoichiometric amounts of cobalt. On dialysis of these fractions against 0.1 mM zinc ions, the residual bound cobalt rapidly escapes from the dialysis bag, in contrast to the behavior reported previously (Kang et al., 1975).

The above data indicate that treatment of [(CPD)Co(II)] with hydrogen peroxide fails to yield a physiochemically homogeneous protein containing stoichiometric quantities of Co(III). Furthermore, the changes observed in enzymatic activity are attributable to the modification of amino acid residues of the protein (e.g., tyrosine, tryptophan, etc.), not to oxidation of Co(III) (see Discussion).

Oxidation of [(CPD)Co(II)] with m-Chloroperbenzoic Acid. To achieve the selective oxidation of the cobalt atom of [(CPD)Co(II)] without concomitant modification of amino acid residues, an active-site-directed oxidizing reagent would be most advantageous. Bunting & Myers (1975) have shown that benzoic acid and certain of its halo-substituted derivatives inhibit [(CPD)Zn]. Hence, it seemed that an aryl peroxy acid, such as *m*-chloroperbenzoic acid (*m*-CPB), might prove suitable. Initial studies of the reaction of [(CPD)Co(II)] with *m*-CPB indicated that the reagent rapidly (<1 min) oxidizes Co(II), as judged by EPR spectroscopy, and rapid gel filtration showed that the resultant Co(III) remains bound to the protein. While the G-75 elution pattern of material treated in this manner revealed considerable protein polymerization, this is virtually eliminated in the presence of phenol, a radical scavenger. Hence, the reaction in the presence of phenol was studied as a function of the concentrations of enzyme, buffer, and salt, temperature, pH, and time, and optimal conditions for the oxidation of cobalt and its retention by the enzyme were determined. The yield of [(CPD)Co(III)] increases from pH 6 to become maximal near pH 9. It is not dependent on the buffer concentration, the results being the same in 0.01 and 0.10 M Tris.

The effect of varying concentrations of *m*-CPB on the peptidase and esterase activities of [(CPD)Co(II)] and [(CPD)Zn] is shown in Figure 3. The enzyme concentration was 2.9×10^{-4} M in 0.1 M Tris-HCl, 1 M NaCl, pH 9, containing phenol equimolar to *m*-CPB. The reaction time was 30 s at 4 °C. There is a simultaneous and concomitant abolition of both the peptidase and esterase activities of [(CPD)Co(II)], but those of [(CPD)Zn] are not affected at all. When the reaction of *m*-CPB with [(CPD)Co(II)] is carried out in the presence of the inhibitor β-phenylpropionate, 0.1 M, the inactivation pattern is similar, except that higher concentrations of *m*-CPB are required to achieve the same degree of inactivation.

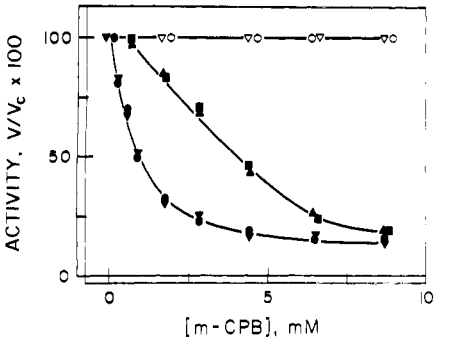

FIGURE 3: Effect of *m*-CPB on the peptidase and esterase activities of [(CPD)Zn] (▽, ○) and of [(CPD)Co(II)] in the absence (▼, ●) and presence (▲, ■) of 0.1 M β-phenylpropionate. The enzymes, 2.9×10^{-4} M in 0.1 M Tris-HCl, 1 M NaCl, pH 9, were treated with equimolar amounts of *m*-CPB and phenol at 4 °C for 30 s. Activities are expressed as percentages of the unmodified controls.

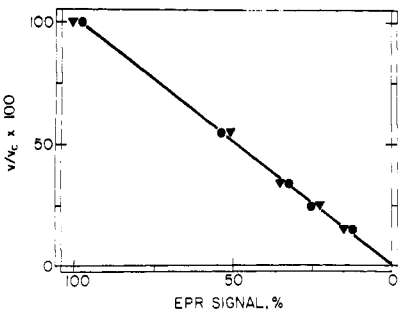

FIGURE 4: Correlation of decreases in the peptidase (●), esterase (▼) activities and the EPR signal of [(CPD)Co(II)] after treatment with *m*-CPB. Activities are expressed as percentages of the unmodified controls and the EPR signal is the percentage of intergrated area remaining after treatment.

The EPR spectrum of each of the samples with which the data in Figure 3 were obtained was examined immediately after reaction (Van Wart & Vallee, 1977). On reaction with increasing concentrations of *m*-CPB, a progressive decrease and ultimate abolition of the EPR signal characteristic of paramagnetic [(CPD)Co(II)] is observed. Since Co(III) is diamagnetic, this abolition indicates that oxidation has occurred. The loss of both peptidase and esterase activities correlates well with the reduction of the integrated area under the EPR signal (Figure 4). Furthermore, extrapolation to zero suggests that [(CPD)Co(III)] is completely inactive (<0.1% activity) and that the residual activity in the reaction mixture is due to unreacted [(CPD)Co(II)].

Isolation of Pure [(CPD)Co(III)]. Pure [(CPD)Co(III)] was isolated from the reaction mixture by a combination of affinity and gel filtration chromatography. Immediately after reaction of [(CPD)^{60}Co(II)] with *m*-CPB, the pH was adjusted to 6.0 and the reaction mixture passed over a column consisting of an upper layer of CABS affinity resin and a lower layer of Sephadex G-75. The upper layer binds unreacted [(CPD)Co(II)] (Peterson et al., 1976) while the lower one separates reaction by-products. The total time of chromatography is less than 3 min. The elution pattern (Figure 5) reveals that [(CPD)Co(III)] emerges as a single band, except for a very small shoulder due to polymerization. It contains 0.95

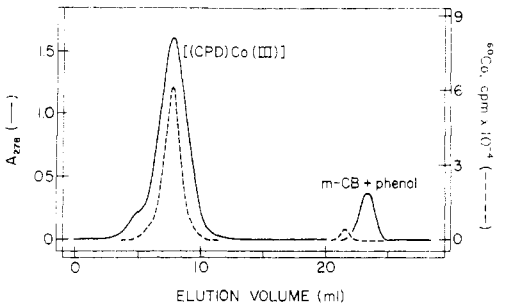

FIGURE 5: Isolation of homogeneous [(CPD)Co(III)] by CABS gel filtration chromatography using 0.01 M Tris-HCl, 1 M NaCl, pH 6, 22 °C.

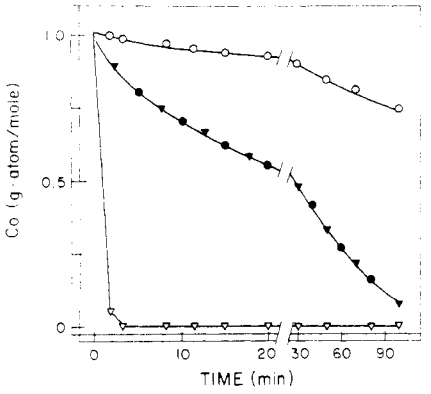

FIGURE 6: Rate of loss of ^{60}Co from [(CPD)^{60}Co(III)] in the presence (▼) and absence (●) of 0.1 mM Zn^{2+} and from [(CPD)^{60}Co(II)] in the presence (▽) and absence (○) of 0.1 mM Zn^{2+}. Rates were measured by pressure ultrafiltration, as described in the text.

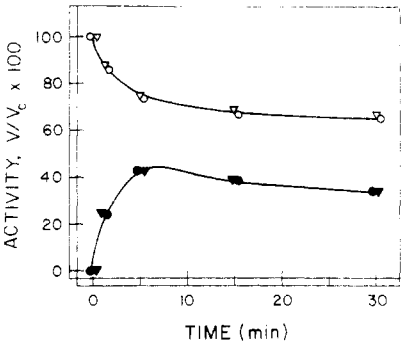

FIGURE 7: Effect of 0.15 M sodium dithionite on the peptidase (●, ○) and esterase (▼, ▽) activities of [(CPD)Co(II)] (○, ▽) and [(CPD)-Co(III)] (●, ▼). Activities are expressed as percentages of the unmodified controls.

TABLE I: Effect of Dithionite on the Peptidase and Esterase Activities of [(CPD)Co(II)] and [(CPD)Co(III)].

enzyme	$+S_2O_4^{2-}$ [a]	+CABS[b]	peptidase ($V/V_c \times 100$)	esterase ($V/V_c \times 100$)
[(CPD)Co(II)]			100	100
[(CPD)Co(II)]	+		72	75
[(CPD)Co(II)]	+	+	88	86
[(CPD)Co(III)]			<0.1	<0.1
[(CPD)Co(III)]	+		42	43
[(CPD)Co(III)]	+	+	96	92

[a] 0.15 M $S_2O_4^{2-}$ for 5 min. [b] CABS affinity chromatography to isolate [(CPD)Co(II)]; see text for details.

g-atom of cobalt and 0.01 g-atom of zinc per mol of protein, has the same amino acid composition as the starting material and is completely inactive. The remainder of the total protein is eluted from the column at pH 7.5 and consists solely of unreacted [(CPD)Co(II)]. Identical treatments of [(CPD)Zn] affect neither its activities, metal content, molecular weight, nor amino acid composition.

Time-Dependent Loss of Cobalt from [(CPD)Co(III)]. If the column chromatographic separation is not performed immediately after reaction, the resultant [(CPD)Co(III)] contains a variable, but always lower than stoichiometric amount of cobalt. To establish the basis for these observations, [(CPD)Co(III)], obtained after rapid (<3 min) isolation and containing 0.95 g-atom/mol of cobalt, was analyzed for bound cobalt as a function of time using the pressure ultrafiltration technique of Paulus (1969). Cobalt is lost from [(CPD)-Co(III)] with a half-life of about 30 min (Figure 6). Neither variation of pH, buffer, or salt concentration of the dialysis solutions, nor the type or concentration of buffer, salt, or pH of the solution in which the reactions with *m*-CPB were carried out prevents this loss of cobalt. Furthermore, the presence of 1,10-phenanthroline, ethylenediaminetetraacetic acid, zinc or cobaltous ions does not change this rate of loss, consistent with the exchange-inert character of Co(III). On the other hand, the rate of loss of cobalt from exchange-labile [(CPD)Co(II)] depends markedly on the presence of these agents in the solution, as shown in Figure 6 for zinc ions.

Under certain circumstances, Co(III) is known to catalyze the hydrolysis of coordinated amides (Buckingham et al., 1970). Hence, the possibility that the loss of cobalt from [(CPD)Co(III)] could be the consequence of peptide bond cleavage within the protein was investigated. Therefore, slab gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol was carried out on [(CPD)Co(III)] samples that had lost all bound metal. The material was found to be homogeneous and to have the same molecular weight (~35 000) as [(CPD)Co(II)], indicating that no peptide bonds within the protein had been cleaved.

Reduction of [(CPD)Co(III)] to [(CPD)Co(II)]. Reduction of [(CPD)Co(III)] to catalytically active [(CPD)Co(II)] was carried out using sodium dithionite. Since the cobalt atom of [(CPD)Co(III)] dissociates from the protein with a half-life of about 30 min, it is imperative to carry out the reduction very rapidly and with large excesses of reagent. Treatment of freshly prepared [(CPD)Co(III)] with 0.15 M dithionite for 5 min at pH 7.0 restores 43% of both peptidase and esterase activities (Figure 7). Identical treatment of [(CPD)Co(II)] lowers the activities of that enzyme to about 75%. After 5 min, both activities for both enzymes decrease at similar rates.

After treatment of [(CPD)Co(II)] and [(CPD)Co(III)] with dithionite for 5 min, an aliquot of each reaction mixture was adjusted to pH 6.0 and passed over the CABS affinity resin. For both mixtures, the breakthrough fraction eluting at pH 6.0 exhibited low (<3%) peptidase and esterase activities. The remainder of the protein eluted when the pH was changed to 7.5 and accounted for all of the activity in the original reaction mixture. With [(CPD)Co(II)], over 90% of the protein was isolated in the second fraction and exhibited peptidase and esterase activities of 88 and 86%, respectively. With [(CPD)Co(III)], about 50% of the protein was isolated in the second fraction and had peptidase and esterase activities that were 96 and 92%, respectively, of those of unreacted [(CPD)Co(II)]. Thus, sodium dithionite reduces [(CPD)-Co(III)] to [(CPD)Co(II)] with a yield of about 50%, regenerating its enzymatic activities. In addition, the visible ab-

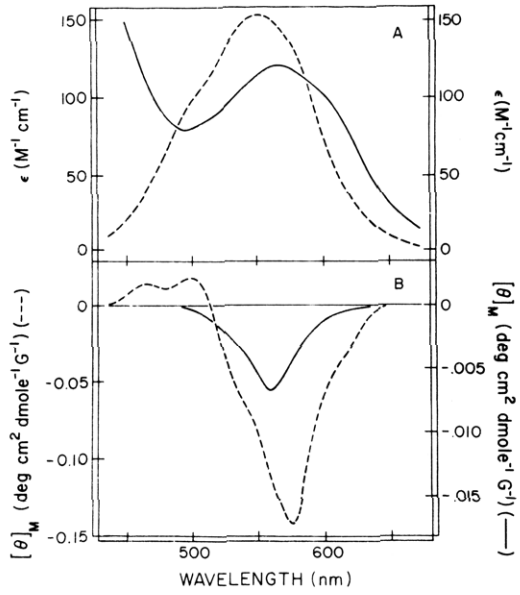

FIGURE 8: Spectral properties of [(CPD)Co(II)] (- - -) and [(CPD)-Co(III)] (—) at 4 °C. (A) Visible absorption spectra with [(CPD)Zn] in the reference beam; (B) MCD spectra.

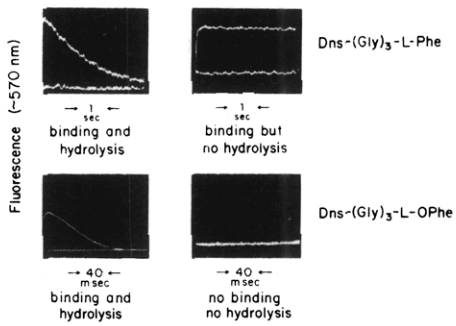

FIGURE 9: Stopped-flow fluorescence measurements of Dns-$(Gly)_3$-L-Phe and Dns-$(Gly)_3$-L-OPhe binding to [(CPD)Zn] and [(CPD)Co(III)] in 1 M NaCl, 0.02 M Tris-HCl, pH 7.5, at 25 °C. After mixing, the concentration of both proteins was 1×10^{-5} M and that of the peptide was 8.8×10^{-4} M and of the ester 3.8×10^{-4} M. Enzyme tryptophans were excited at 285 nm and Dns emission measured by means of a 430-nm cut-off filter.

sorption and EPR spectra of the resultant [(CPD)Co(II)] were identical with those of native [(CPD)Co(II)]. These results are summarized in Table I.

Spectral Properties of [(CPD)Co(III)]. The spectral properties of [(CPD)Co(III)] were examined using samples prepared by rapid (<3 min) affinity-gel filtration chromatography. [(CPD)Co(III)] does not exhibit an EPR spectrum at 5 K. Figure 8 shows the visible absorption and MCD spectra of [(CPD)Co(III)] obtained at 4 °C together with the corresponding spectra of [(CPD)Co(II)]. The absorption spectra were obtained by placing an identical concentration of [(CPD)Zn] in the reference beam. The intensities of the spectral bands of [(CPD)Co(III)] decrease with time at a rate corresponding to that of loss of cobalt from the protein (vide supra).

The visible spectrum of [(CPD)Co(III)] has a band at 565 nm with a molar absorptivity, ϵ (corrected for the end absorbance from the ultraviolet), of $\sim 100\ M^{-1}\ cm^{-1}$. The MCD spectrum of [(CPD)Co(III)] exhibits a negative extremum at 560 nm with a magnetic molar ellipticity, $[\theta]_M$, of ca. -0.006 deg cm^2 dmol^{-1} G^{-1}, a value substantially lower than that for [(CPD)Co(II)] which has a $[\theta]_M$ of ca. -0.15 deg cm^2 dmol^{-1} G^{-1} (Holmquist et al., 1975). The maxima, extrema, and magnitudes of the bands in the visible and MCD spectra and the absence of an EPR spectrum are all consistent with the known properties of octahedral Co(III) complexes (see Discussion). [(CPD)Co(II)] has a CD band at 538 nm with a molar ellipticity, $[\theta]$, of -500 deg cm^2 dmol^{-1}. [(CPD)-Co(III)] has a somewhat larger CD band centered at about 560 nm with $[\theta]$ of ca. -1000 deg cm^2 dmol^{-1}. These and other properties of [(CPD)Co(II)] and [(CPD)Co(III)] are summarized in Table II.

TABLE II: Comparison of Various Properties of [(CPD)Co(II)] and [(CPD)Co(III)].

property	[(CPD)Co(II)]	[(CPD)Co(III)]
metal content (g-atom/mol)		
Co	1.03	0.95
Zn	0.01	0.01
activity ($V/V_c \times 100$)[a]		
peptidase	100	<0.1
esterase	100	<0.1
substrate binding		
peptides	yes	yes
esters	yes	no
amino acid composition	same	same
mol wt[b]	~35 000	~35 000
absorption spectrum	500 (sh)	565 (~100)
λ, nm (ε, M^{-1} cm^{-1})	555 (150)	600 (sh)
	572 (150)	
CD spectrum	538 (−500)	~560 (−1000)
λ, nm (θ, deg cm^2 dmol^{-1})		
MCD spectrum	575 (−0.15)	560 (−0.006)
λ, nm ($[\theta]_m$, deg cm^2 dmol^{-1} G^{-1})		
EPR spectrum	yes	no

[a] Expressed as percent of unmodified [(CPD)Co(II)]. [b] By Na-DodSO$_4$ gel electrophoresis.

Substrate Binding to [(CPD)Co(III)]. [(CPD)Co(III)] hydrolyzes neither peptides nor esters. To determine whether this inactivity is due to the inability to bind substrate or to hydrolyze bound substrate, the binding of Dns-$(Gly)_3$-L-Phe and Dns-$(Gly)_3$-L-OPhe to [(CPD)Co(III)] was investigated using the stopped-flow fluorescence method of Latt et al. (1972). This procedure uses Förster electronic energy transfer between the dansyl group of bound substrate and tryptophanyl residues of the protein to visualize substrate binding.

Figure 9 shows the oscilloscope tracings for the mixing of [(CPD)Zn] or [(CPD)Co(III)] with Dns-$(Gly)_3$-L-OPhe or Dns-$(Gly)_3$-L-Phe. With either substrate, the patterns observed for [(CPD)Zn] show rapid binding during the mixing time of the instrument, followed by hydrolysis. In contrast, the pattern for the interaction of [(CPD)Co(III)] with Dns-$(Gly)_3$-L-Phe indicates that the protein binds, but does not hydrolyze this peptide. In successive experiments with a given preparation of [(CPD)Co(III)], this fluorescent signal decreases with time and eventually vanishes as cobalt dissociates from the protein; the resultant apoprotein does not bind the peptide, in contrast to the apoprotein obtained by direct removal of zinc from [(CPD)Zn]. On the other hand, the ester Dns-$(Gly)_3$-L-OPhe is neither bound nor hydrolyzed. Hence, the inactivity of [(CPD)Co(III)] toward peptides is due to its failure to hydrolyze bound substrate, while for esters it is its inability to bind substrate.

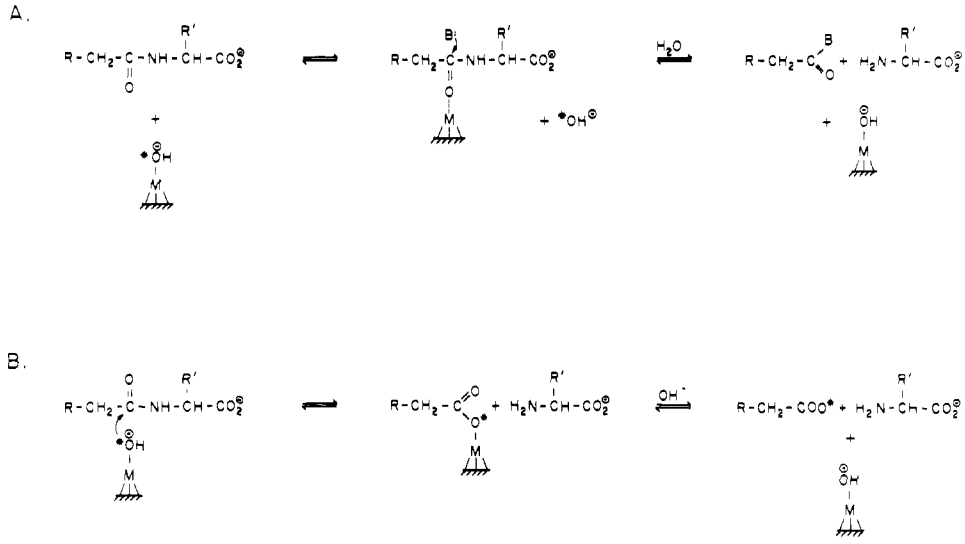

FIGURE 10: Simple representation of the (A) "metal-carbonyl" and (B) "metal-hydroxide" mechanisms of peptide catalysis by [(CPD)Zn]. See text for explanation.

Possibility of Residual Binding of m-Chlorobenzoate and Phenol to [(CPD)Co(III)]. If *m*-CB and/or phenol were to remain bound to [(CPD)Co(III)], this might conceivably account for the failure of the protein to bind esters or hydrolyze peptides. To investigate this possibility, [(CPD)Co(II)] was reacted with *m*-CPB in the presence of phenol and the reaction mixture was chromatographed (as in Figure 5) to separate [(CPD)Co(III)] from unbound reaction by-products. Two main peaks were obtained: one for the protein (I) and another for the small molecules (II). After several hours when all of the cobalt in [(CPD)Co(III)] had dissociated, peak I was treated with dithionite to reduce all cobalt to the (II) state. The pH was then adjusted to 9 and the solution extracted with ethyl acetate to remove any phenol present followed by adjustment of pH to 3 and extraction with ethyl acetate to remove any *m*-CB present. The same extraction procedures were applied to peak II. Both the *m*-CB and phenol in the extracts were determined quantitatively from the absorbance in the 250–280-nm region. All of the *m*-CB and phenol were present in peak II; none was found in peak I. In a separate control experiment it was established that, when *m*-CB and phenol *are* present together with protein, this extraction procedure *does* remove them. Hence, neither one of these agents remains bound to [(CPD)Co(III)].

Possibility of Single Turnover of Peptides and Esters by [(CPD)Co(III)]. The possibility was examined that peptide and/or ester substrates interact with [(CPD)Co(III)] turning over only once, such that part of the substrate remains attached to the enzyme. This might occur if, e.g., substrate were to undergo attack by metal-bound hydroxide (see Figure 10B). Since Co(III) is exchange-inert, such an attack would leave the N-terminal portion of the substrate bound to the metal. To examine this possibility, in separate experiments, Dns-$(Gly)_3$-L-Phe or Dns-$(Gly)_3$-L-OPhe was added to freshly prepared [(CPD)Co(III)]. After 1 min, each mixture was gel filtered to separate bound and unbound materials. In both experiments, fluorescence analysis of the protein fractions at 570 nm upon excitation at either 285 or 320 nm indicates that no *dansyl* chromophore is bound to the protein. Hence, neither substrate interacts with [(CPD)Co(III)] to result in such a single turnover.

Oxidation of [(CPD)Co(II)] Prepared by Different Methods. Jones et al. (1977) have reported that the catalytic properties of the product resulting from the treatment of [(CPD)Co(II)] with hydrogen peroxide are a function of the method of preparation of [(CPD)Co(II)]. When [(CPD)-Co(II)] is prepared by reconstitution of the apoenzyme with cobaltous ions and then reacted with hydrogen peroxide, the product is reported to have neither peptidase nor esterase activity. On the other hand, when the reaction is carried out on [(CPD)Co(II)] prepared by the direct exchange of cobalt for zinc via equilibrium dialysis, the resulting material is said to have esterase, but not peptidase activity. Although our results show (vida supra) that treatment of [(CPD)Co(II)] with hydrogen peroxide does not produce appreciable quantities of [(CPD)Co(III)], we nevertheless compared the effects of the hydrogen peroxide treatment of Kang et al. (1975) and the *m*-CPB treatment described here on samples of [(CPD)-Co(II)] prepared by three different procedures.

The results obtained with [(CPD)Co(II)] prepared by reconstitution of the apoenzyme with cobaltous ions have been reported already (see above). [(CPD)Co(II)], prepared by dialysis of [(CPD)Zn] against excess cobaltous ions according to the method of Jones et al. (1977), contained 0.40 g-atom/mol cobalt and 0.59 g-atom/mol zinc. This mixture was subjected to hydrogen peroxide and *m*-CPB, respectively. In addition, the mixture of [(CPD)Zn] and [(CPD)Co(II)] was separated by CABS affinity chromatography (Bazzone, T. J., Cueni, L. C., Sokolovksy, M., & Vallee, B. L., in preparation), and the latter was oxidized by the two procedures in analogous fashion. Finally, [(CPD)Co(II)] prepared by the direct-exchange procedure of Coleman & Vallee (1960) was oxidized with both reagents.

The results (Table III) show that the resultant activities of the three preparations are characteristic of these two oxidizing agents. In all instances, treatment with hydrogen peroxide lowers peptidase to a greater extent than esterase activity, but treatment with *m*-CPB uniformly lowers both peptidase and esterase activities in parallel and to the same extent. We find no evidence to suggest that the activity of [(CPD)Co(III)] depends upon the method of preparation of the precursor, [(CPD)Co(II)].

Discussion

The replacement of the active-site metal of a metalloenzyme with another that has a different coordination geometry, atomic radius, etc., has long been a successful means to study the involvement of the metal in the mechanism of action of the

TABLE III: Effect of Hydrogen Peroxide[a] and *m*-CPB[b] on the Activities of [(CPD)Zn] and Different Preparations of [(CPD)Co(II)].

enzyme	method of preparation	starting metal content (g-atom/mol) Zn	Co	act. after H_2O_2 treatment[a] ($V/V_c \times 100$) peptidase	esterase	act. after *m*-CPB treatment[b] ($V/V_c \times 100$) peptidase	esterase
[(CPD)Zn]		1.02	0.00	98	120	100	100
[(CPD)Co(II)]	reconstitution of (CPD)[c]	0.01	1.03	19	55	21	20
[(CPD)Co(II)]	direct-exchange procedure of Jones et al.[d]	0.59	0.40	62	78	53	54
[(CPD)Co(II)]	direct-exchange procedure of Jones et al.,[d] plus CABS chromatography	0.00	0.98	24	49	19	20
[(CPD)Co(II)]	direct-exchange procedure of Coleman & Vallee[e]	0.02	0.98	28	52	20	20

[a] Hydrogen peroxide treatment consists of reacting 1.2×10^{-4} M enzyme in 0.1 M Tris-HCl, 1 M NaCl, pH 7.5, with 1 mM H_2O_2 for 6 h at 22 °C. [b] *m*-CPB treatment consists of reacting 2.9×10^{-4} M enzyme in 0.1 M Tris-HCl, 1 M NaCl, pH 9, containing 3 mM phenol with 3 mM *m*-CPB for 30 s at 4 °C. [c] Auld & Holmquist, 1974. [d] Jones et al., 1977. [e] Coleman & Vallee, 1960.

enzyme (Vallee & Riordan, 1968). Since certain metal atoms can assume more than one stable oxidation state, each with different properties, it should be possbile to change one metal atom into an essentially new one by oxidation or reduction in situ. The goal of the present study was the preparation of [(CPD)Co(III)] by oxidation of [(CPD)Co(II)]. Since Co(II) is labile, but Co(III) is inert to ligand-exchange reactions within the first coordination sphere, the effect of this transformation on the catalytic properties of the enzyme should help decide whether inner sphere coordination between the metal and substrate occurs during binding or hydrolysis.

The interpretation of the changes in functional properties of an enzyme undergoing chemical modification requires that the reaction be site-specific, preferably affecting only a single group and be free of side reactions that alter the metal content, molecular weight or any other property of the enzyme critical to binding, catalysis or structural stability. It is essential that the changes in functional properties can be correlated with the extent of modification of the specific group in question. For the present objective, a suitable oxidizing reagent and reaction conditions are required to oxidize selectively the cobalt atom of [(CPD)Co(II)].

When carried out in the presence of phenol, the oxidation of [(CPD)Co(II)] to [(CPD)Co(III)] with *m*-CPB satisfies all the above criteria. The observed changes in peptidase and esterase activities correlate with the oxidation of the cobalt atom, as judged by EPR spectroscopy. The reaction selectively oxidizes only the cobalt atom of [(CPD)Co(II)] as is apparent from the fact that it does not affect the properties of [(CPD)Zn] in any discernible way. Amino acid analysis shows conclusively that none of the residues of the protein are altered or destroyed, and the reaction does not affect its molecular weight or metal content. Further, sodium dithionite reduces [(CPD)Co(III)] to restore virtually fully active [(CPD)-Co(II)]. This demonstrates that the oxidation reaction has not altered the functional capacity or any other relevant property of the enzyme and, further, that the cobalt atom resides at the same site before and after oxidation.

m-CPB exhibits many of the characteristics of an active-site-directed reagent toward [(CPD)Co(II)]. Its structure resembles that of many competitive inhibitors and substrates of the enzyme. Figure 3 shows that with increasing concentrations of *m*-CPB, the percent of inactivation observed per 30 s reaction time reaches a plateau. This is the behavior expected for an active-site-directed, irreversible inactivation in which the enzyme, E, and reagent, R, form a reversible complex, E·R, prior to reaction (Baker, 1967).

$$R + E \leftrightarrow E \cdot R \rightarrow E' + R' \quad (1)$$

Here, R, R', E, and E' are *m*-CPB, *m*-CB, [(CPD)Co(II)], and [(CPD)Co(III)], respectively. Furthermore, the shift in the pattern of inactivation in the presence of β-phenylpropionate shows that it protects [(CPD)Co(II)] from oxidation by *m*-CPB—the behavior expected for an active-site-directed reaction in the presence of a competitive inhibitor (Segel, 1975). *m*-CPB oxidizes [(CPD)Co(II)] to [(CPD)Co(III)] at an extremely high rate compared with hydrogen peroxide. In contrast, *m*-CPB does not similarly oxidize Co(II) *E. coli* alkaline phosphatase (Van Wart & Vallee, 1977), whereas hydrogen peroxide does so readily (Anderson & Vallee, 1975). Hence, *m*-CPB appears to have a particular affinity for the active-site of [(CPD)Co(II)], apparently due to the *m*-chlorobenzoyl moiety of the reagent.

The reason that *m*-CPB does not completely oxidize [(CPD)Co(II)] to [(CPD)Co(III)] (Figure 3) may likely be due to the fact that the *m*-CB produced by the reaction binds to, and hence protects, [(CPD)Co(II)] from *m*-CPB. Even in the absence of enzyme, *m*-CPB decomposes rapidly to *m*-CB at pH 9; therefore, the use of increasingly high concentrations of *m*-CPB cannot overcome the protective effect of *m*-CB on unreacted [(CPD)Co(II)]. Moreover, there is a limit to the amount of *m*-CPB to which the enzyme can be subjected before amino acid side chains of the molecule are oxidized non-specifically.

The rapid isolation of pure, monomeric [(CPD)Co(III)] containing a nearly stoichiometric amount of cobalt has been made possible through the use of the CABS affinity resin. Under the conditions where [(CPD)Co(II)] binds tightly, [(CPD)Co(III)] passes through the resin, possibly because the binding of the enzyme to benzyl succinate requires inner sphere coordination to the metal. Neither *m*-CB nor phenol remain bound to [(CPD)Co(III)] after oxidation, leaving the active-site region free for the binding of potential substrates. In spite of this, pure [(CPD)Co(III)] is catalytically inactive toward all peptides and esters examined over the pH range 6–10.

The spontaneous and irreversible loss of cobalt from [(CPD)Co(III)] implies that the environment of the metal atom in the protein is thermodynamically unstable. Co(III) prefers octahedral coordination geometry and nitrogen over oxygen donors. Assuming that the ligands to cobalt in [(CPD)Co(II)] are the same as those for Zn in [(CPD)Zn], two nitrogens (one each from His-69 and His-196) and two oxygens (one each from Glu-72 and water) constitute the donor set. Hence, on oxidation to Co(III), it seems likely that the two oxygen donors remain. Furthermore, constraints imposed by the tertiary structure of the protein may well place the Co(III) atom in a distorted octahedral geometry. Jointly, these two factors likely account for the thermodynamic instability of the

TABLE IV: Electronic Absorption Spectra of Some Co(III) Complexes.

compound	donor set	$^1T_{1g} \leftarrow {}^1A_{1g}$ λ, nm (ε, M^{-1} cm^{-1})
$[Co(en)_3]^{3+}$	N_6	464 (88)[a]
$[Co(NH_3)_6]^{3+}$	N_6	472 (56)[a]
$[Co(NH_3)_5H_2O]^{3+}$	N_5O_1	491 (49)[b]
$[Co(NH_3)_5C_2O_4H]^{2+}$	N_5O_1	505 (~75)[b]
$[Co(NH_3)_5Cl]^{2+}$	N_5Cl	~530 (~50)[c]
$[Co(en)_2C_2O_4]^+$	N_4O_2	500 (113)[d]
c-[Co(en)EDDA]$^+$	N_4O_2	495 (169)[e]
		554 (sh)
t-[Co(en)EDDA]$^+$	N_4O_2	448 (sh)[e]
		529 (87)
c-$[Co(NH_3)_2EDDA]^+$	N_4O_2	500[e]
$[Co(NH_3)_4C_2O_4]^+$	N_4O_2	510 (81)[d]
c-$[Co(en)_2Cl_2]^+$	N_4Cl_2	530 (78)[d]
$[Co(NH_3)_4Cl_2]^+$	N_4Cl_2	540 (45)[d]
$[Co(EDTA)]^-$	N_2O_4	538 (347)[f]
$[Co(EDTP)]^-$	N_2O_4	556 (253)[f]
$[Co(IDA)_2]^-$	N_2O_4	562 (152)[f]
$[Co(H_2O)_6]^{3+}$	O_6	606[a]
$[Co(C_2O_4)_6]^{3-}$	O_6	602 (125)[a]

[a] Lever, 1968. [b] van Eldick & Harris, 1975. [c] McCaffery et al., 1967. [d] Collman & Schneider, 1966. [e] Legg & Cooke, 1965. [f] Tait & Busch, 1976.

Co(III)-protein complex in which the active-site-directed oxidation of [(CPD)Co(II)] has temporarily "trapped" the Co(III) atom in this site.

The kinetics of metal loss from [(CPD)Co(III)] are consistent with those expected for an exchange-inert metalloprotein. The spontaneous rate of loss of cobalt from the protein is slow, qualifying as an exchange-inert rate [i.e., one whose half-life is <1 min (Cotton & Wilkinson, 1972)]. Furthermore, this rate is not influenced by the presence of other metal ions or of chelating agents. Apparently, the rupture of the Co(III)-protein ligand coordination bonds is the rate-limiting step for loss of metal. Indeed, if Co(III) were exchange-labile, the dissociation from the protein would be rapid and [(CPD)Co(III)] could not be isolated on the time scale required to perform steady-state kinetic assays.

The spectral properties of [(CPD)Co(III)] are consistent with those of octahedral Co(III) model complexes. Oxidation of [(CPD)Co(II)] with *m*-CPB abolishes the EPR signal characteristic of the paramagnetic (d^7) Co(II) atom and the resultant [(CPD)Co(III)] does not itself exhibit an EPR spectrum. Since virtually all known discrete Co(III) complexes are octahedral,[2] this strongly suggests that the d^6 Co(III) atom is spin-paired in an octahedral-like environment ($e_g^6t_{2g}^0$). The Co(III) atom does not remain in the distorted tetrahedral environment of the Co(II) enzyme since, if it did, it would be paramagnetic (its electronic configuration being approximated by either $e^4t_2^2$ in a strong field or $e^3t_2^3$ in a weak field) and thus exhibit an EPR spectrum.

Two principal absorption bands corresponding to the spin-allowed $^1T_{1g} \leftarrow {}^1A_{1g}$ and $^1T_{2g} \leftarrow {}^1A_{1g}$ transitions are expected for octahedral Co(III) complexes. The wavelength ($\lambda_{max} \cong$ 565 nm) and intensity ($\epsilon \cong 100$) of the visible absorption band of [(CPD)Co(III)] (Figure 8) are appropriate for the $^1T_{1g} \leftarrow {}^1A_{1g}$ transition; the shoulder near 600 nm might be attributable to the splitting of this transition due to an environment of lower than O_h symmetry. The $^1T_{2g} \leftarrow {}^1A_{1g}$ transition occurs at shorter wavelength and would be obscured by the intense absorption of the protein.

The energy of the visible $^1T_{1g} \leftarrow {}^1A_{1g}$ transition in Co(III) model complexes shows a dependence upon the nature of donor atoms that is in accord with the spectrochemical series. The wavelength of maximum absorbance (λ_{max}) of a large number of spin-paired octahedral Co(III) complexes with different donor sets has been tabulated and representative examples are listed in Table IV. The approximate wavelengths (in nm) observed for the $^1T_{1g} \leftarrow {}^1A_{1g}$ transition shift further to the red as oxygen or chloride donors replace nitrogen donors, as follows: N_6, 460-470; N_5O, 490-500; N_5Cl, ~530; N_4O_2, 460-540; N_4Cl_2, 530-540; N_2O_4, 540-560; O_6, ~600 nm. Although there is some spread in these wavelengths, especially for N_4O_2 where the transition is often split, there is a fairly good correlation between the λ_{max} and the strengths of these ligands in the spectrochemical series.

These data can be used to approximate the donor set of the Co(III) in [(CPD)Co(III)] from its wavelength of maximal absorbance, which is 565 nm. This is consistent with either an N_2O_4 or N_2O_3Cl donor set. Since in the [(CPD)Co(II)] enzyme, cobalt is initially bound to an N_2O_2 donor set, oxidation to Co(III) results in the acquisition of two oxygen or one oxygen and one chloride donor. The oxygen donor is most likely water (or hydroxide), but could also conceivably come from an amino acid side chain, such as Glu, Tyr, etc. Since the oxidation is not dependent on the Tris concentration, it is unlikely that Tris is incorporated into the coordination sphere. Chloride, if involved, would come from the solvent. The spontaneous loss of cobalt from [(CPD)Co(III)] is probably due to the thermodynamic instability of this donor set.

While [(CPD)Co(II)] exhibits a weak CD band at 540 nm, $[\theta] \cong -500$ deg cm^2 $dmol^{-1}$ and a moderately strong MCD band, $[\theta]_M \cong -0.15$ deg cm^2 $dmol^{-1}$ G^{-1}, both the CD and MCD bands of [(CPD)Co(III)] are weak. The low value of the magnetic molar ellipticity, $[\theta]_M$, for [(CPD)Co(III)] compared with [(CPD)Co(II)] is consistent with the change from a distorted tetrahedral to an octahedral environment. The estimated value of $[\theta]_M$ of about −0.006 deg cm^2 $dmol^{-1}$ G^{-1} for [(CPD)Co(III)] is in the range −0.001 to −0.006 deg cm^2 $dmol^{-1}$ G^{-1} characteristic of octahedral Co(III) complexes (McCaffery et al., 1967).

The reaction of [(CPD)Co(II)] with hydrogen peroxide does not satisfy the criteria enumerated earlier for a successful chemical modification and apparently does not produce appreciable quantities of [(CPD)Co(III)]. The biphasic effect of this reaction on esterase activity and the differential effects on peptidase and esterase activities (Figure 1) strongly suggest that at least two groups undergo modification, reminiscent of results obtained earlier by ultraviolet irradiation of carboxypeptidase A (Piras & Vallee, 1966a,b). Moreover, the changes in activity do not correlate with the change in oxidation state of the cobalt atom. Further, the elution pattern of Figure 2 shows that this reaction results in polymerization of the protein and significantly alters its cobalt content. Interestingly, identical treatment of [(CPD)Zn], on the other hand, affects its activities much less.

The basis for these observations can be understood in terms of a reaction between amino acid residues of the enzyme and free radicals produced as a result of the decomposition of hydrogen peroxide. Metal ions that undergo one-electron transfer are known to catalyze the decomposition of peroxides (Pryor, 1966), as shown for cobalt:

$$HO\text{-}OH + Co(II) \rightarrow HO^{-}\cdot + HO^- + Co(III) \quad (2)$$

[2] Only the extremely weak field octahedral $[(Co(H_2O)_3F_3]$ and $[Co(F)_6]^{3-}$ complexes, not possible for [(CPD)Co(III)], are known to be paramagnetic (Cotton & Wilkinson, 1972).

Since peroxides are two-electron oxidizing agents, this reaction produces a hydroxide free radical. Hence, the reaction of [(CPD)Co(II)] with hydrogen peroxide probably produces hydroxide free radicals whose subsequent attack on the protein is responsible for the observed changes in the catalytic properties of the enzyme. Reaction of hydrogen peroxide with [(CPD)Zn], on the other hand, cannot produce radicals by way of route 2, since zinc does not undergo one-electron transfer. In this case, free radicals can be produced either by simple homolytic cleavage or by one-electron oxidation of other groups of the enzyme. For the zinc enzyme, the net result is the production of fewer radicals and similar, albeit much less pronounced, effects on the activity of the enzyme (Table III).

The effect of hydrogen peroxide on the activities, molecular weight, and metal content of [(CPD)Co(II)] closely resembles that observed previously for [(CPD)Zn] treated either with hydrogen peroxide in carbonate–dioxane or with ultraviolet light (Piras & Vallee, 1966a,b). In the case of ultraviolet irradiation, the changes in activity have been shown to be due to the destruction of tyrosyl, tryptophanyl, and histidyl residues. Since it is well known that ultraviolet irradiation also produces free radicals (Pryor, 1966), the element common to all three of these procedures is most likely the production of free radicals and their subsequent attack on the protein.

It is of interest to consider the basis for the loss of the peptidase and esterase activities of [(CPD)Co(II)] on conversion to [(CPD)Co(III)] in terms of two major proposals for the mechanism of action of the enzyme. In the "metal–carbonyl" mechanism (Figure 10A), the carbonyl group of the scissile peptide (or ester) bond coordinates to the metal, is polarized, and thus activated to attack by a nucleophile (represented in Figure 10A by "B"), e.g. OH^- or Glu-270. In the "metal–hydroxide" mechanism (Figure 10B), the substrate undergoes nucleophilic attack by metal-coordinated hydroxide ion. Both of these two mechanisms are possible for either peptide or ester hydrolysis, but they are shown schematically in Figure 10 for peptide hydrolysis only.

The transformation from an exchange-labile to an exchange-inert metal atom should affect these two mechanisms differently. It would effectively inactivate an enzyme operating by the "metal–carbonyl" mechanism, since the first step requires that the substrate enter the inner coordination sphere of the metal, and this ligand exchange reaction for an exchange-inert metal is much slower than observed catalytic rates. However, the first step for an exchange-inert metalloenzyme operating by the "metal-hydroxide" mechanism—attack of substrate by metal-coordinated OH^-—is possible leading to the formation of a "metal–carboxyl anhydride" intermediate (Figure 10B). This species would generally be expected to have a long lifetime, since breakage of the M–O bond is a slow process. Hence, this would lead to a single turnover, leaving the N-terminal portion of the substrate bound to the protein. An alternative suggested recently (Wells & Bruice, 1977) is that this "metal–carboxyl anhydride" could undergo C–O bond cleavage upon subsequent nucleophilic attack to liberate the N-terminal portion of the substrate and regenerate metal-bound hydroxide to be used over again for catalysis. If this mechanism were to occur, an enzyme containing an exchange-inert metal atom could retain activity. Based on such considerations, the catalytic behavior of [(CPD)Co(III)] can serve to militate for or against one of the two mechanisms of hydrolysis shown in Figure 10, while omitting yet other possible alternatives, of course.

[(CPD)Co(III)] binds, but does not hydrolyze peptides, even for a single turnover. Assuming that the oxidation of [(CPD)Co(II)] to [(CPD)Co(III)] does not reorient or otherwise disturb active site residues critical to catalysis, this behavior cannot be rationalized in terms of the "metal–hydroxide" mechanism. If this were the mechanism, [(CPD)Co(III)] would either be active or exhibit a single turnover (vide supra). Since peptides still bind, the conversion of Co(II) to Co(III) apparently does not change the active site significantly. It could be argued that the change to octahedral coordination geometry might reorient the metal-bound hydroxide relative to the substrate, thereby preventing hydrolysis. However, there is evidence to indicate that a substantial degree of freedom in the geometry about the metal is possible while activity is retained. For example, the Zn (distorted tetrahedral) (Quiocho & Lipscomb, 1971), Co(II) (distorted tetrahedral or five-coordinate) (Holmquist et al., 1975; Rosenberg et al., 1973), Ni (octahedral) (Rosenberg et al., 1973) and VO(II) (square-bipyramidal) (DeKoch et al., 1974) enzymes all exhibit peptidase activity, even though their coordination geometries differ widely.

On the other hand, the behavior of [(CPD)Co(III)] toward peptides *is* consistent with the "metal–carbonyl mechanism." [(CPD)Co(III)] and the apoenzyme, both of which bind but do not hydrolyze peptides, share the inability to interact with substrate via inner sphere coordination to metal. This suggests that inner sphere coordination is necessary for hydrolysis, but not for binding. It is not possible to completely eliminate the possibility that, upon oxidation, a residue critical to hydrolysis becomes coordinated to Co(III). Glu-270 and Tyr-248, both thought to serve catalytic roles (Lipscomb et al., 1968), are two possible candidates. If such coordination were to occur, it could then account for the failure of the protein to hydrolyze peptides. The incorporation of Tyr-248 into the coordination sphere is not likely to be the reason for the inactivation since, when oxidation is carried out at pH 6 where the tyrosine is protonated and not a ligand, the results are the same as at pH values where it is deprotonated. The coordination of Glu-270 is also unlikely if it is as far away (about 6 Å away from the metal in the native enzyme) as shown by x-ray structure analysis (Lipscomb et al., 1968). Hence, among the two proposals discussed, the results for [(CPD)Co(III)] are more consistent with the "metal–carbonyl" mechanism of peptide hydrolysis, although this cannot be thought to constitute verification of this hypothesis.

Unfortunately, the behavior of [(CPD)Co(III)] toward esters cannot be used in similar fashion to distinguish between the mechanisms of Figure 10 for ester substrates, since [(CPD)Co(III)]—like the apoenzyme—does not even bind esters. This suggests that inner sphere coordination to the metal, possibly through either the carbonyl group of the scissile bond or the terminal carboxyl group, is necessary for ester binding. Since esters do not bind, little information regarding the role of the metal in the catalytic step can be abstracted.

The data shown here for exchange-inert [(CPD)Co(III)] point to a differential role of the metal in peptide vs. ester hydrolysis, in agreement with earlier studies (Auld & Holmquist, 1974) with a series of exchange-labile metallocarboxypeptidases (Me = Zn, Co(II), Mn, Cd). Metal substitution at the active site affects k_{cat}, but not K_M, for peptides. Conversely, for esters, metal substitution affects K_M, but not k_{cat}. These results were interpreted to indicate that the metal atom interacts with the carboxyl or carbonyl group of esters during binding but with the carbonyl group of peptides during hydrolysis. The present results with [(CPD)Co(III)] are consistent with these conclusions.

The properties of [(CPD)Co(III)] prepared by *m*-CPB oxidation are the same whether the precursor [(CPD)Co(II)] is reconstituted from (CPD) with Co(II) or prepared by direct

exchange of Co(II) for the Zn of [(CPD)Zn]. Furthermore, treatment of all preparations of [(CPD)Co(II)] with hydrogen peroxide fails to yield detectable quantities of [(CPD)Co(III)], but consistently yields enzymes whose peptidase and esterase activities both are decreased to varying degrees (see above). The present results do not confirm reports that treatment of [(CPD)Co(II)] with hydrogen peroxide yields [(CPD)-Co(III)], that [(CPD)Co(III)] is an active esterase (Kang et al., 1972, 1975) or that the method of preparation of the precursor [(CPD)Co(II)] affects the outcome of the reaction (Jones et al., 1977). Thus, our data obviate the need to consider the suggestion that [(CPD)Co(II)] prepared by different methods contains Co(II) coordinated to different nitrogen atoms of the histidyl ligands of the protein. As a consequence, a reexamination of mechanistic conclusions (Breslow & Wernick, 1977; Wells & Bruice, 1977; Cleland, 1977) based on these earlier reports is indicated.

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