Development of a Method for the Incorporation of Substitution-Inert Metal Ions into Proteins. Site-Specific Modification of Arsanilazotyrosine-248 Carboxypeptidase A with Cobalt(III)†

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ABSTRACT: This investigation demonstrates the use of substitution-inert metal ions as site-specific amino acid modifying reagents. The approach involves the production of a chelating agent at the site of interest with the subsequent in situ oxidation of substitution-labile cobalt(II) to exchange-inert cobalt(III) with H_2O_2. We have produced the chelate complex ethylenediamine-*N*,*N*′-diacetato(arsanilazotyrosinato-248 carboxypeptidase A)cobalt(III) [Co^{III}(EDDA)(AA-CPA-Zn)]. Model Co^{III}(EDDA)(azophenolate) complexes have helped to define the reaction conditions necessary to produce the enzyme derivative and have proved invaluable in the spectral analysis of the cobalt(III)–enzyme complex. The modified enzyme contains one *active-site* zinc and one *externally bound* cobalt per enzyme monomer. Circular dichroism and visible spectra of the derivative and apoenzyme substantiate the site-specific nature of the incorporation. Concomitant with Co^{III}EDDA incorporation, the enzyme loses its peptidase activity yet maintains its esterase activity. Reversal of the modification with Fe^{II}EDTA returns the original properties of the arsanilazotyrosine-248 enzyme.

Methods for incorporating substitution-inert metal ions, in particular cobalt(III), into proteins and peptides are currently under investigation (Legg et al., 1975; White & Legg, 1975, 1976; Legg, 1978; Urdea & Legg, 1978; Benson & Legg, unpublished experiments). A procedure has been devised which is based on the conversion of an amino acid in the protein of interest to a chelating agent, followed by in situ oxidation of cobalt(II) to cobalt(III) with H_2O_2. The following study demonstrates the potential of using Co(III) as a site-specific amino acid modifing reagent.

The zinc neutral exoprotease, bovine carboxypeptidase A (CPA-Zn)[1] (EC 3.4.2.1), was a particularly attractive test system. The enzyme has been specifically modified with diazotized arsanilic acid by Vallee and co-workers to yield the comparably active derivative, arsanilazotyrosine-248 carboxypeptidase A (AA-CPA-Zn) (Johansen & Vallee, 1973). The highly selective nature of the diazotization and the metal chelating capabilities of azophenols suggested the possibility of coordinating Co(III) specifically to this site. Since Co(III) complexation would involve the phenolic oxygen of tyrosine-248, it was anticipated that the necessity of this active-site component to the esterase and peptidase activities could be more precisely defined. Although the modification of the tyrosine residues of carboxypeptidase has been the subject of a large number of studies (Cueni & Riordan, 1978; Muszynska & Riordan, 1974; Vallee & Riordan, 1968), the derivative reported here is the first modification which blocks the phenolic oxygen of *only* tyrosine-248.

Water-soluble azodyes were employed in model studies to determine probable conditions which would result in enzyme modification and its reversal. The spectral and magnetic properties of these Co^{III}–azodye complexes were used in the characterization of the enzyme derivative.

Metal-catalyzed oxidative damage was found in both the models and the enzyme when free-radical scavengers were not used during hydrogen peroxide oxidations of cobalt. The pertinence of this observation to other studies will be discussed.

Experimental Procedures

Materials

Carboxypeptidase A prepared by the method of Cox et al. (1964) was obtained from Sigma Chemical Co. (Lot 56C-8100 and 16C-8205). Ethylenediamine-*N*,*N*′-diacetic acid (EDDA) and 2-(4-carboxyphenylazo)-4,5-dimethylphenol (CDP) were purchased from LaMont Laboratories and Alfred Bader Chemical Co., respectively. Eastman Chemicals supplied *p*-(2-hydroxy-1-naphthylazo)benzenesulfonic acid (orange dye II; OD-II). Bovine liver catalase was obtained from Boehringer-Mannheim. Metals were of "spec pure" grade purchased from Johnson Mathey Chemicals. Sigma Chemicals provided L-Bz-GP and DL-Bz-GOP. All other chemicals were the highest purity available. All buffers and solutions were extracted with 0.1% diphenylthiocarbazone in CCl_4, and glassware was leached with 50% nitric acid to prevent metal ion contamination (Latt & Vallee, 1971).

Methods

Synthesis of N-acetylmonoarsanilazotyrosine (NA-MAT) was carried out as described by Tabachnick & Sobotka (1959). Although the compound isolated had the same spectral properties reported by these authors, it was found that the reaction mixture contained three components (*N*-acetyltyrosine

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[1] The active-site Zn(II) is shown here in order to distinguish it from the externally bound Co(III). When the Zn is not shown, the apoenzyme is indicated from which the Zn(II), but not the Co(III), has been removed. CPA-Zn, bovine carboxypeptidase A; AA-CPA-Zn, arsanilazotyrosine-248 carboxypeptidase; Co^{III}(EDDA)(AA-CPA-Zn), ethylenediamine-*N*,*N*′-diacetato(arsanilazotyrosinato-248 carboxypeptidase A)cobalt(III); EDDA, ethylenediamine-*N*,*N*′-diacetic acid; CDP, 2-(4-carboxyphenylazo)-4,5-dimethylphenol; OD-II, orange dye II, *p*-(2-hydroxy-1-naphthylazo)benzenesulfonic acid; NA-MAT, *N*-acetylmonoarsanilazotyrosine; Co^{II}(EDDA)$(H_2O)_2$, diaquo(ethylenediamine-*N*,*N*′-diacetato)cobalt(II); Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; βPP, β-phenylpropionate. Co^{II}EDDA and Co^{II}(EDDA)$(H_2O)_2$ are used interchangeably. Unless otherwise stated, all metal–EDDA complexes discussed are the diaquo species. All Co^{III}EDDA complexes of the bidentate azodye ligands are represented as Co^{III}(EDDA)(azodye abbreviation). Bz-GP refers to *N*-benzoylglycyl-L-phenylalanine and Bz-GOP represents *N*-benzoylglycyl-L-phenyllactic acid.

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and both the mono- and bis-diazotized derivatives). The components were easily separated by TLC on Whatman/Quantum K-1 silica plates (Whatman) eluted with the isopropyl alcohol–triethylamine bicarbonate system (solvent system I; SSI) described by Warner & Legg (1979). (R_f of *N*-acetyl-Tyr = 0.96, monoazo-Tyr = 0.77, bisazo-Tyr = 0.58.) NA-MAT was isolated by preparative TLC on Whatman/Quantum PR1F plates eluted with SSI. The product was shown to be homogeneous in SSI and pyridine–acetic acid–H_2O (50:35:15) (SSII) (R_f = 0.91).

$Co^{II}(EDDA)(H_2O)_2 \cdot H_2O$ was prepared by the method of Averill et al. (1972) and stored under N_2 until used.

Preparative scale synthesis of $Co^{III}(EDDA)$(azodye) complexes was carried out by the air oxidation of an aqueous solution of 20 mM azodye and 20 mM $Co^{II}(EDDA)(H_2O)_2$ at pH 9.5 stirred vigorously at room temperature for 3 days. The CDP complex was isolated according to White & Legg (1976). Co^{III}(EDDA)(OD-II) was purified on Sephadex LH-20 (Pharmacia) equilibrated with 20:80 H_2O–methanol. About 20 mL of the crude reaction mixture was run down a 5 × 50 cm column. The major red band (band 2) was collected and evaporated to dryness in a bell jar under vacuum. The material was then dried in a drying pistol at the temperature of boiling methanol [Anal. Calcd for Na[Co(EDDA)-(OD-II)]·2.5H_2O: C, 42.18; N, 8.94; H; 3.86. Found: C, 42.23; N, 8.92; H, 4.08.] Preparative TLC of Co^{III}-(EDDA)(NA-MAT) as described for NA-MAT proved successful (R_f = 0.63), and the product was shown to be homogeneous in SSII (R_f = 0.56) and methanol–benzene–hexane (40:40:20) (SSIII) (R_f = 0.76).

Analytical Preparation of $Co^{III}(EDDA)$(azodye) Complexes. The following procedures were employed for all three complexes. A 1.0 mM solution of the azodye was prepared in 0.02 M veronal, 0.5 M NaCl, pH 8.5, and 0.1 M phenol. A stoichiometric quantity of $Co^{II}(EDDA)(H_2O)_2$ and a fivefold molar excess of H_2O_2 were then added. All H_2O_2 stock solutions were made up fresh just before use from a 30% solution (Mallinckrodt). The actual H_2O_2 concentration was determined by the absorbance at 240 nm (ϵ = 43.6). Aliquots were taken at various times, quenched with catalase (20 μg/mL), and chromatographed on the K-1 plates in SSIII. $Co^{III}(EDDA)(H_2O)_2^+$ was visualized by spraying with a 20% solution of $(NH_4)_2S$ while the azodye complexes and ligands could be seen directly. Similar studies were carried out with 0.1 mM solutions of the azodyes. In all cases, the products isolated exhibited the same R_f values as those obtained by air oxidation.

Reduction of the $Co^{III}(EDDA)$(azodye) Complexes. Fe^{II}EDTA was made up fresh by adding a stoichiometric amount of ferrous sulfate to a deaerated solution of 40 mM EDTA, pH 7.0. The solution was stirred at 4 °C under N_2 until the crystals had dissolved and then was used immediately. To a 0.1 mM solution of the cobalt complexes in 0.02 M Mes, pH 6.8, and 1 M NaCl was added a 50-fold molar excess of the iron complex. After 30 min at room temperature, the reaction was complete as evidenced by TLC. The ligands were recovered by removing excess Fe(III) and Co(II) on a Chelex 100 column (Bio-Rad) eluted with water.

CPA-Zn Purification. To purify the commercial enzyme, a sample of the crystalline suspension was washed with 0.02 M Mes, pH 7.0, centrifuged, and dissolved in 0.1 M Tris and 1.0 M NaCl, pH 7.5 (150 mg/20 mL). The solution was stirred for 5 h at 4 °C, centrifuged, and applied to a 5 × 30 cm Sephadex G-75 (Pharmacia) column equilibrated with the same buffer. It was found, as previously observed (E. T. Kaiser, personal communication), that the preparation contains some five components other than the major CPA-Zn band. The contamination comprised no more than about 7% of the total enzyme. Tubes containing the major band were combined and dialyzed against 2 L of 0.02 M veronal and 0.1 M NaCl, pH 8.0. Crystals appeared within 12 h. The dialysate was changed every 24 h for 2 days. At this time crystals were collected by centrifugation and suspended in 0.02 M Mes, pH 7.0 (yield, ~85%). If the enzyme was recrystallized (Petra, 1970) prior to Sephadex G-75 chromatography, a significant amount of contaminant was still found.

Diazotization of CPA-Zn was performed according to Johansen & Vallee (1971). Spectral analysis of the arsanil-azoenzyme indicated between 1.07 and 1.12 azotyrosines per enzyme and less than 3% azohistidine modification (Tabachnick & Sobotka, 1960). It is important to note that the 5–10% "over" modification was necessary to ensure complete derivatization of Tyr-248. Johansen et al. (1972) found that 95% of the incorporation occurred at Tyr-248 while the rest was probably modified at Tyr-19.

Metal analyses were all performed on a Perkin-Elmer Model 303 atomic absorption spectrophotometer equipped with a DCR1 time-averaging digital display.

Preparation of $Co^{III}(EDDA)$(AA-CPA-Zn). To a solution of AA-CPA-Zn in 0.02 M veronal and 0.05 M NaCl, pH 8.5, was added a 100-fold molar excess of phenol and a 25-fold excess of $Co^{II}(EDDA)(H_2O)_2$. The final concentration of the enzyme was 0.1 mM. To this was added a sufficient quantity of a freshly prepared H_2O_2 stock to achieve a 5:1 excess over the enzyme. After 7 h the reaction was quenched with catalase (2 μg/mL) and purified on a disposable Sephadex G-25M PD-10 column (Pharmacia) equilibrated with 1.0 M NaCl and 0.02 M Mes, pH 7.0 (up to 1 mL of sample could be applied).

Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories Technical Bulletin No. 1051) using CPA-Zn as a standard. No difference in absorbance was found between the dye complex of AA-CPA-Zn and that of CPA-Zn. The concentrations of these two enzymes were also obtained independently by using the molar absorptivities of 7.32×10^4 (Johansen & Vallee, 1975) and 6.42×10^4 M^{-1} cm^{-1} (Simpson et al., 1963), respectively, at 278 nm.

Amino acid analyses were performed in triplicate at the Bioanalytical Laboratory, Washington State University, on a Beckman Model 120C amino acid analyzer by the method of Spackman et al. (1958). From these analyses, a molar absorptivity of 9.04×10^4 M^{-1} cm^{-1} was calculated for the Co^{III}(EDDA)(AA-CPA-Zn) derivative at 274 nm.

Tryptophan analyses were performed in triplicate on a Perkin-Elmer MPF-3L spectrofluorometer by the internal calibration method of Pajot (1976). Emission was set at 345 nm (slit 5.0 nm) with an excitation wavelength of 295 nm (slit 16 nm). The tryptophan content of all samples was maintained at between 3 and 8 μM.

Visible and CD Spectra. Visible spectra were taken on a Cary Model 14 spectrophotometer, and CD spectra were obtained on a Jasco Model ORD/UV-5 with the SS20 CD modification (Sproul Scientific) at a temperature of 23 °C. Concentrations and conditions are given in the figure captions.

Reduction of $Co^{III}(EDDA)$(AA-CPA-Zn) by $Fe^{II}EDTA$. A sufficient quantity of an Fe^{II}EDTA stock (prepared as previously described) was added to a 0.1 mM solution of the cobalt enzyme in 0.02 M Mes, pH 6.8, 1 M NaCl, and 25 mM βPP to give a 50-fold molar excess. After addition of Fe^{II}EDTA, 0.6-mL aliquots of the reaction mixture were

Table I: A Comparison of the Models and Enzyme Derivatives with Respect to Metal Content and Spectral Properties

	Co per molecule	Zn per molecule	major band in visible spectrum (nm)	pH dependence
NA-MAT, CDP, OD-II			485 (pH 10.5)	yes
Co^{III}(EDDA)-(azodyes)	1.0		510	no
apo-AA-CPA		<0.01	485 (pH 10.5)	yes
apo-Co^{III}(EDDA)-(AA-CPA)	1.07	<0.06	510	no
AA-CPA-Zn		0.98–1.02	485 (pH 10.5), 510 (pH 8.2)	yes
Co^{III}(EDDA)-(AA-CPA-Zn)	1.07–1.14	0.98–1.02	510	no
Fe^{II}EDTA-reduced AA-CPA-Zn	<0.01	0.99	485 (pH 10.5), 510 (pH 8.2)	yes

removed at various times, chromatographed on a PD-10 and then dialyzed against a 1000-fold excess of the same buffer for 24 h. Protein concentrations were determed by the Bio-Rad assay utilizing CPA-Zn as a standard. The change in absorbance at 510 nm at pH 7.0 is a direct measure of cobalt content as evidenced by parallel atomic absorption. Atomic absorption revealed that the enzyme contained between 0.95 and 1.03 g-atoms of zinc at all the times sampled.

Activity Measurements. Bz-GP hydrolysis was followed spectrophotometrically as reported by Folk & Schirmer (1963) on a Cary Model 14 spectrophotometer thermostated at 25 ± 0.1 °C ($\Delta\epsilon_{254}$ = 286). Assays were performed in 0.5 M NaCl and 0.05 M Tris, pH 7.5. Esterase activity toward DL-Bz-GOP was determined by titration of the protons released upon hydrolysis using a pH stat constructed in this laboratory (Warner et al., unpublished experiments). The assays were run in 0.1 mM Tris, pH 7.5, and 0.5 M NaCl. All activities are expressed in terms of the L isomer.

Results

Model Studies. The spectra of the azophenol dyes serve as excellent analogues for the arsanilazotyrosine enzyme. Both CDP and NA-MAT exhibit pH-dependent visible spectra characterized at low pH by a 320–340-nm peak and a 390-nm shoulder and in basic solution by a 485-nm absorption maximum (Figure 1 and Table I). The azonaphtholate, OD-II, is somewhat different, but it also possesses a 485-nm pH-dependent absorption. The behavior of the models is very similar to that of apo-AA-CPA as first reported by Johansen & Vallee (1973).

Characterization of Co^{III}(EDDA)(CDP) has been reported elsewhere (White & Legg, 1976). The diamagnetic 3+ oxidation state of cobalt is assured in the complex by the high-resolution ^1H NMR spectra and magnetic susceptibility data. Two geometrical isomers were isolated, and the visible spectra were found to be identical.

The crude reaction mixture of Co^{III}(EDDA)(OD-II) was separated into three red bands, each of which was shown to possess the same pH-independent visible spectrum. A 510-nm absorption maximum dominates the spectrum, the same as with the CDP complex. The major component (band 2) was collected and evaporated to dryness. Elemental analysis is consistent with the presumed composition.

Co^{III}(EDDA)(NA-MAT) was also prepared and then purified by preparative TLC. As is typical of the azodye complexes, a bathochromic shift of the 485-nm azophenolate maximum results in the 510-nm band (Figure 1A). Only one

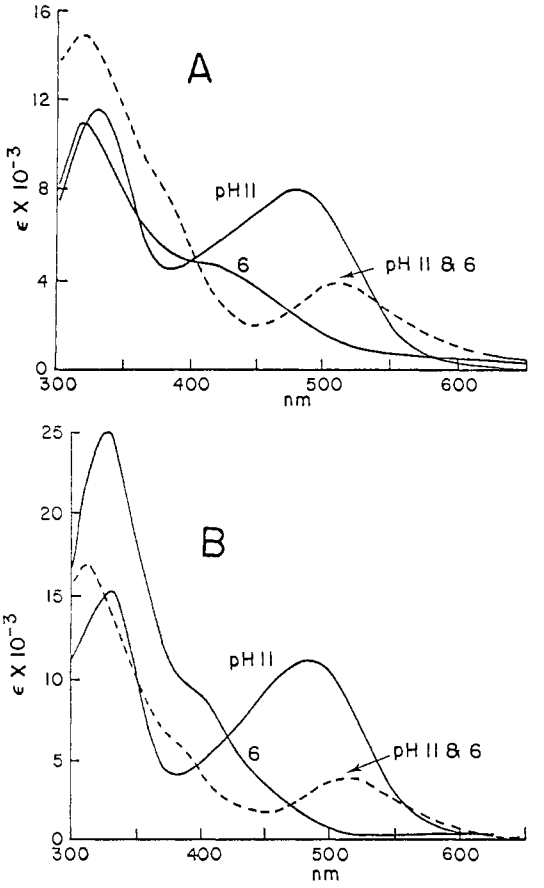

FIGURE 1: Visible spectra of azophenol ligands and Co^{III}(EDDA)-(azophenolate) complexes. (A) The pH-dependent spectrum of NA-MAT (—) and the Co^{III}(EDDA)(NA-MAT) complex (---). (B) CDP (—) and its Co^{III}EDDA complex (---).

product was found by TLC in a number of solvent systems.

As opposed to the azodyes prior to Co(III) coordination, all of the Co^{III}(EDDA)(azodye) complexes are characterized by pH-independent visible spectra (Table I). This implies that Co(III) is coordinated to the azodye ligands throughout the pH range studied, substantiating the inert nature of the complexes.

Although all the Co^{III}(EDDA)(azodye) complexes could be synthesized by air oxidation, H_2O_2 oxidation with a free-radical scavenger also proved successful. Air oxidation was not applicable to the enzyme system due to the time and vigorous stirring required. The reactions were performed on an analytical scale (1.0–0.1 mM) and were followed by TLC. Cobalt complexes with the same R_f values and spectral properties as the complexes produced by the air oxidation method were obtained for all three azodyes.

A "bleaching" of the dyes was noted if a scavenger was not used during the H_2O_2 oxidation. The decoloration was presumably due to the formation of an oxidized product of the azodyes, e.g., azoxy derivatives. The reaction was found to be Co(II)-catalyzed since the addition of H_2O_2 and Co^{II}EDDA rapidly bleached the dyes ($t_{1/2} \sim 1$ min) while the addition of Co^{II}EDDA or H_2O_2 alone had no such effect. Phenol completely prevented bleaching. Co^{III}EDDA and H_2O_2 also brought about decoloration but at a much slower rate ($t_{1/2} \sim$ 30 min).

The characteristic spectrum of the Co^{III}(EDDA)(azodye) complexes is attainable only when oxidation of Co(II) has taken place. The addition of a 25-fold molar excess of Co^{II}EDDA to a 0.1 mM solution of OD-II, CDP, or azotyrosine does not alter significantly their spectral properties.

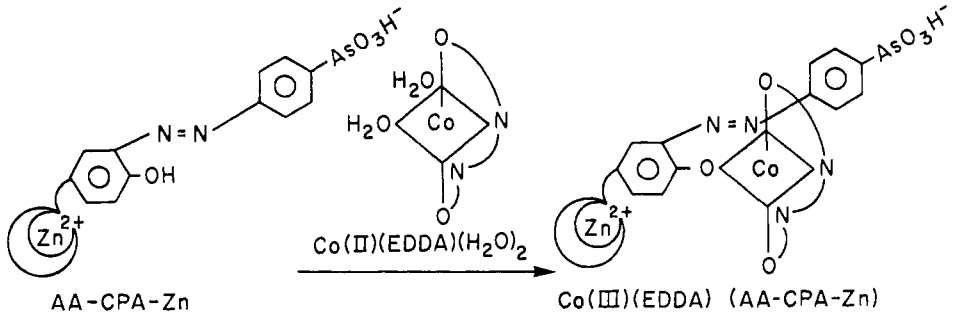

FIGURE 2: Synthesis of $Co^{III}(EDDA)(AA\text{-}CPA\text{-}Zn)$ from AA-CPA-Zn and $Co^{II}(EDDA)(H_2O)_2$. Conditions for the synthesis: 2.5 mM $Co^{II}(EDDA)(H_2O)_2$, 10.0 mM phenol, 0.5 mM H_2O_2, and 0.10 mM AA-CPA-Zn in 0.02 M veronal, pH 8.5, and 0.5 M NaCl for 7 h at 21 °C. At pH 8.5 free azotyrosine is in equilibrium with coordinated tyrosine, the latter form predominating, but for clarity the ligand is shown as the free azophenol.

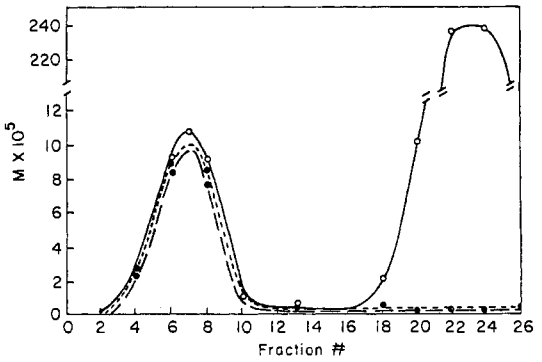

FIGURE 3: Sephadex G-25 elution profile of the $Co^{III}(EDDA)$-(AA-CPA-Zn) reaction mixture, after 7 h (Co (—); Zn (– –); AA-CPA-Zn (- - -)). The AA-Tyr to enzyme ratio was 1.07.

Addition of H_2O_2 to oxidize the Co(II) initiates a gradual change in the azodye spectrum which shifts the absorption maximum to 510 nm. Monitoring by TLC suggests that this shift is a direct measure of the extent of Co(III) complex formation.

A 50-fold molar excess of $Fe^{II}EDTA$ quantitatively reduced the Co(III) complexes in about 30 min. Upon purification, a full return of the pH-dependent visible spectra for each of the dyes was found. Cobalt to azodye stoichiometry was determined by atomic absorption analysis for cobalt and from the concentration of azodye as calculated from the spectrum after reduction.

Enzyme Modification. The visible and CD spectra for the arsanilazotyrosine-248 enzyme reported here (inserts of Figures 4 and 5, respectively) are in excellent agreement with those reported by Johansen & Vallee (1973), substantiating the presumed identity of the modifications.

Synthesis of $Co^{III}(EDDA)(AA\text{-}CPA\text{-}Zn)$ was accomplished as summarized in Figure 2. After 7 h, the reaction was quenched with catalase and chromatographed on Sephadex G-25 to remove unreacted $Co^{III}(EDDA)(H_2O)_2^+$ from the protein fraction. The elution profile of the $Co^{III}EDDA$–enzyme (Figure 3) shows the presence of equimolar Co, Zn, and azotyrosine in the enzyme fraction. Unreacted $Co^{III}EDDA$ and the scavenger were effectively removed by this procedure. Further chromatography or dialysis did not result in a decrease of the cobalt to enzyme ratio. The Co(III)–enzyme is homogeneous on Sephadex G-100, eluting with the same V_e as the native enzyme, and contains 0.98–1.02 g-atoms of Zn, 1.07–1.14 g-atoms of Co (Table I), and 1.07–1.12 arsanilazotyrosines per enzyme molecule.

Analysis by spectrofluorometry in 6 M guanidine hydrochloride and 30 mM β-mercaptoethanol demonstrated that the tryptophans in the Co(III) derivative were unaltered by the modification. The amino acid analyses presented in Table II further substantiate the lack of oxidative damage to the protein.

Table II: Amino Acid Analysis of CPA-Zn and $Co^{III}(EDDA)(AA\text{-}CPA\text{-}Zn)$

	CPA-Zn[a]	CPA-Zn[b]	$Co^{III}(EDDA)$-(AA-CPA-Zn)[b]
Asp	27.9	28.0	28.0
Thr[c]	27.8	27.0	27.5
Ser[c]	33.0	34.0	32.4
Glu	25.0	25.5	25.1
Pro	9.7	9.6	10.1
Gly	22.5	22.6	22.5
Ala[d]	20.0	20.0	20.0
Val	15.8	16.0	16.0
Met	3.0	3.2	3.0
Ile	20.3	20.3	20.0
Leu	23.2	22.8	22.7
Tyr	19.2	19.3	17.8[g]
Phe	16.0	16.3	16.1
Lys[e]	15.0	15.0	15.0
His	8.2	7.5	8.0
Arg	11.1	11.4	11.3
Trp[f]	8	7.5	7.5

[a] Bargetzi et al. (1963). [b] This study. [c] Values corrected to zero time according to Bargetzi et al. (1963). [d] Long-column results calculated in terms of Ala. [e] Short-column values calculated in terms of Lys. [f] Trp concentration determined by spectrofluorometry. [g] Arsanilazotyrosine is lost during hydrolysis.

Model studies have shown that both cobalt(III)– and zinc(II)–monoazophenolate complexes have characteristic 510-nm ligand-associated transitions of roughly equal intensity ($\epsilon_{510nm} \simeq (3\text{–}8) \times 10^3$) (White & Legg, 1976; Johansen & Vallee, 1973). The presence of this band, although indicative of metal coordination, does not allow differentiation of Co(III) vs. Zn(II) complexation since the Co(III) d–d transitions are obscured ($\epsilon_{octahedral}$ is typically less than 200). The similarity between the Co(III) and Zn(II) spectra complicates the assignment of the enzymic spectral properties since arsanilazotyrosine-248 forms a complex with the active-site Zn(II) (Johansen & Vallee, 1973). However, the substitution inertness of the cobalt(III)–arsanilazotyrosine complex permits a distinction to be made. Whereas the active-site complex is labile as evidenced spectrally (Johanson & Vallee, 1973) (insert, Figure 4), the Co(III) complex exhibits an absorption spectrum which is essentially invariant with pH (Figure 4) (see Table I). The small change in absorbance at the absorption maximum implies that less than 1% of the azotyrosines are not coordinated to Co(III), if, indeed, this change can be attributed to the existence of unmodified AA-CPA-Zn.

AA-CPA-Zn is also characterized by an intense CD spectrum, which, like the visible spectrum, changes significantly with pH (Johanson & Vallee, 1973) (insert, Figure 5). A negative extremum at 510 nm ($[\Theta]_{510nm}^{pH8.5} = -43\,000$) due

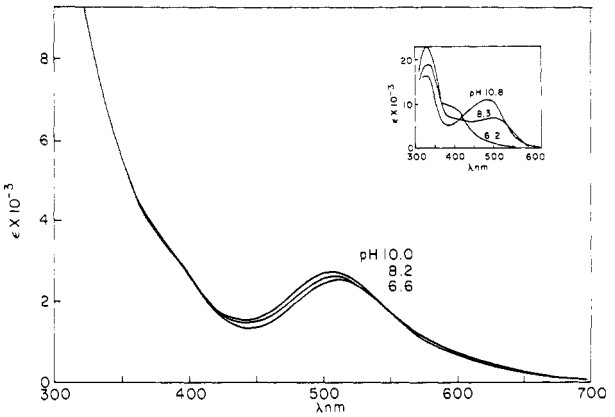

FIGURE 4: Effect of pH on the visible spectrum of Co^{III}(EDDA)-(AA-CPA-Zn). The insert shows the effect of pH on the visible spectrum of AA-CPA-Zn.

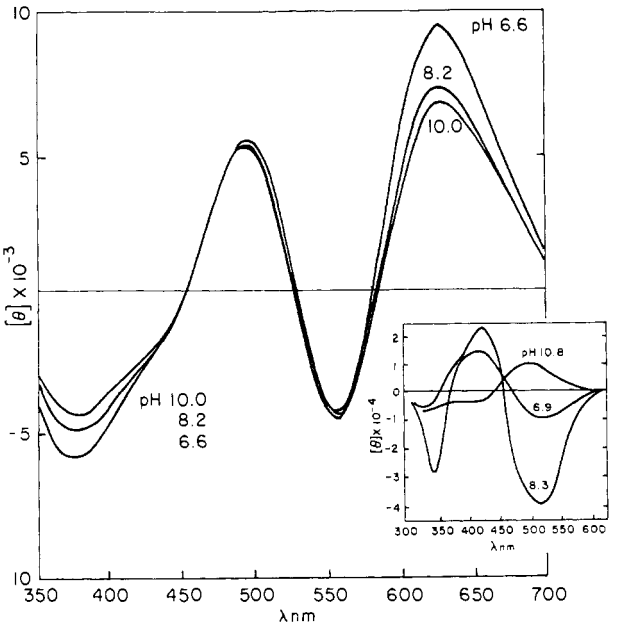

FIGURE 5: Effect of pH on the CD spectrum of Co^{III}(EDDA)-(AA-CPA-Zn). The insert shows the effect of pH on the CD spectrum of AA-CPA-Zn.

to the zinc–azophenolate interaction shifts with increasing pH to a positive band at 485 nm ($[\theta]_{485nm}^{pH10.8} = +10\,500$), indicative of the "free" azophenolate. Although separated by only 25 nm, these two environmental states of the arsanilazotyrosine are clearly distinguished in the CD spectrum due to the difference in sign.

Upon Co^{III}(EDDA) coordination of arsanilazotyrosine-248, a distinctly different CD spectrum is obtained (Figure 5). In the visible region two pH-independent bands of opposite sign ($[\theta]_{555nm} = -4600$ and $[\theta]_{490nm} = +5200$) are observed. Both the spectrum and its behavior as a function of pH contrast markedly with that of AA-CPA-Zn where the intense pH-dependent negative band at 510 nm associated with Zn(II) chelation is observed (insert, Figure 5). The intensities of the broad negative 450–350-nm band and the positive 625-nm band in the Co(III) derivative are somewhat dependent upon pH and suggest a change in the environment of the cobalt(III)–azophenolate complex. This is not surprising since the microenvironment of the enzyme-bound Co(III) complex would be expected to change with the protonation state of the protein itself, as does the azotyrosine in apo-AA-CPA. It is important to note that, as opposed to AA-CPA-Zn, these are changes in intensity and not in band position. Furthermore,

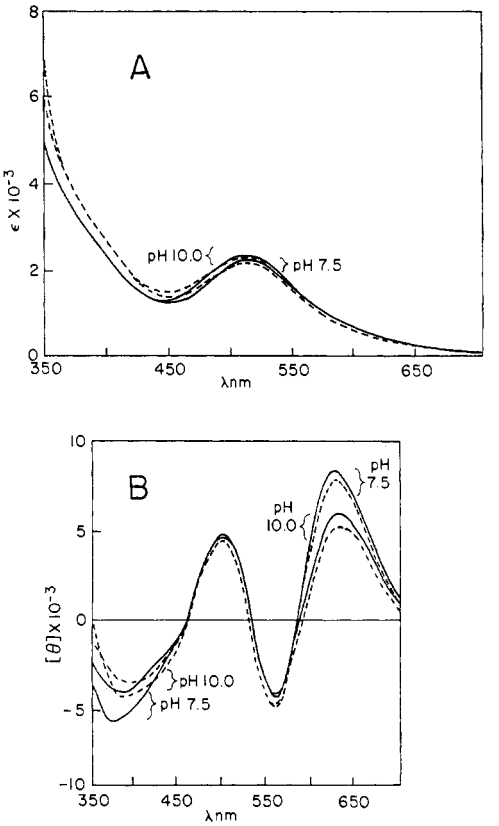

FIGURE 6: Comparison of the visible and CD spectral properties (A and B, respectively) of Co^{III}(EDDA)(AA-CPA-Zn) (—) and apo-Co^{III}(EDDA)(AA-CPA) (---).

the changes in intensity are less than 5% of those observed for AA-CPA-Zn.

The changes in the visible absorption and CD spectra are a direct measure of the extent of cobalt incorporation during the modification. Samples were taken at various times throughout the modification, quenched with catalase, and purified by Sephadex G-25. Spectra and cobalt analyses were obtained for each sample. Cobalt uptake was proportional to the change in absorbance at 510 nm. At pH 7.0, the visible and CD spectra of the samples exhibited isosbestic behavior at 468 and 454 nm, respectively, during Co(III) incorporation.

The visible absorption and CD spectral behavior of Co^{III}(AA-CPA-Zn), particularly the invariance of the spectra in the 480- and 560-nm region, implies virtually 100% coordination of the arsanilazotyrosine-248 to Co(III). The great similarity between the absorption spectral properties of the models and those of the Co(III)-modified enzyme (Figures 1 and 4) further substantiates this conclusion. Both possess the characteristic pH-independent 510-nm absorption maximum and a 380–390-nm shoulder.

Dialysis of the cobalt(III)–enzyme against 1,10-phenanthroline resulted in an apo derivative that contains 0.06 g-atom of Zn and 1.07 g-atoms of Co. The CD and visible spectral properties of apo-Co^{III}(EDDA)(AA-CPA) were almost identical with those of the enzyme before removal of the Zn (Figure 6, upper and lower panel). Importantly, the spectra for apo-AA-CPA did not possess a 510-nm contribution (Johansen & Vallee, 1973) whereas for apo-Co^{III}(EDDA)-(AA-CPA) the 510-nm visible absorption maximum which typifies azophenolate–metal coordination was still present. Thus, the spectra observed upon Co(III) incorporation must result from a cobalt(III)–azophenolate complex.

It is evident that substitution-inert Co(III) as opposed to exchange-labile Co(II) is incorporated into the enzyme. The

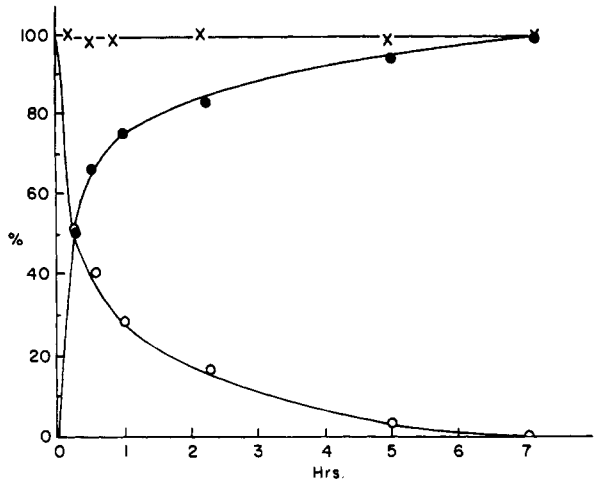

FIGURE 7: Loss of peptidase activity toward 1 mM Bz-GP (O) as a function of $Co^{III}EDDA$ incorporation into AA-CPA-Zn. Absorbance at 510 nm, pH 7.0, divided by the total absorbance change upon complete modification is indicated by (●). The esterase activities toward 0.9 mM Bz-GOP at various times are given by (×). All activities are with reference to an AA-CPA-Zn control.

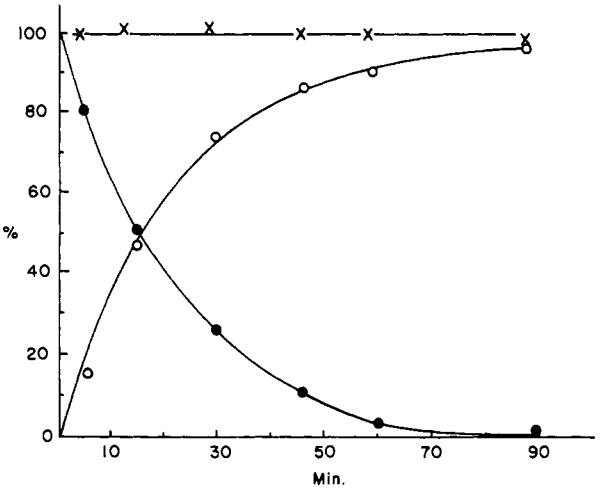

FIGURE 8: Return of peptidase activity toward 1 mM Bz-GP as a function of cobalt removal. Symbols are defined as described in the legend in Figure 7. Conditions for the $Fe^{II}EDTA$ reduction are given under Methods.

cobalt–azotyrosine coordination is unperturbed by gel chromatography, pH changes, urea denaturation, or dialysis against the chelating agents 1,10-phenanthroline or EDTA. Unless H_2O_2 is added to the reaction mixture, cobalt is not detected in the enzyme fractions after Sephadex G-25 gel chromatography. A comparison of the visible spectra obtained for the models and the cobalt–enzyme also demonstrates Co(III) coordination since the visible spectra of the models and $Co^{III}(EDDA)$(AA-CPA-Zn) are remarkably similar. Only the Co(III) complex of the models gives rise to a pH-independent ligand-associated transition at 510 nm and only upon Co(II) oxidation is this pH-independent spectrum produced. In the models as in the enzyme, the change in the visible spectrum is a direct measure of the extent of Co(III) complexation.

Reversal of the Modification. As with the models, reduction of the enzyme-bound cobalt complex was achieved with a 50-fold molar excess of $Fe^{II}EDTA$. Preliminary studies indicated that Zn(II) was partially removed from the active site during reduction, but this problem was alleviated by adding a 25-fold molar excess of the carboxypeptidase inhibitor, β-phenylpropionate (βPP). One binding mode of βPP is coordination to the active-site Zn(II) which results in the formation of a ternary βPP–Zn–CPA complex (Coleman & Vallee, 1964; Steitz et al., 1967). This complexation prevents Zn(II) removal by chelating agents (Coleman & Vallee, 1964). It was anticipated that βPP would prevent Zn(II) removal during the reduction, and the reduced enzyme prepared in this manner contained 0.98–1.02 Zn and <0.01 Co per enzyme molecule. $Fe^{III}EDTA$ and $Co^{II}(EDDA)(H_2O)_2$ were removed by Sephadex G-25 chromatography. Upon removal of the cobalt, the visible spectrum characteristic of AA-CPA-Zn returns and possesses the same pH-dependent behavior as the enzyme before cobalt modification. Likewise, the CD spectrum of AA-CPA-Zn is regenerated, indicating that the microenvironment of the arsanilazotyrosine is apparently indistinguishable from that which it possesses before cobalt modification.

Activities. Activities of the enzyme toward the peptide Bz-GP and the structurally matched ester Bz-GOP were monitored as a function of $Co^{III}EDDA$ incorporation (Figure 7). Cobalt incorporation is concomitant with the loss of peptidase activity, while the apparent activity toward 0.9 mM Bz-GOP is unaltered as compared to that of the arsanilazoenzyme. Upon treatment with $Fe^{II}EDTA$, the peptidase activity is restored to 95% that of the AA-CPA-Zn control (Figure 8).

When AA-CPA-Zn or CPA-Zn was treated with a fivefold molar excess of H_2O_2, no apparent alterations in the spectral or kinetic properties are observed after 7 h at 21 °C. In contrast, when a sample of AA-CPA-Zn or CPA-Zn is incubated with a 5-fold molar excess of H_2O_2 and a 25-fold molar excess of $Co^{II}(EDDA)(H_2O)_2$ without phenol to scavenge free radicals, the enzymes are irreversibly inhibited and display distinctly altered UV spectra, suggesting oxidative damage. The addition of Cu^{II}- or $Fe^{II}EDDA$ and H_2O_2 also results in structural damage, whereas $Zn^{II}EDDA$ and H_2O_2 show no effects, which implies that an oxidizable metal ion is required.

Discussion

Stabilization of a Co(III) complex requires careful regulation of the coordination environment. A set of six ligands of sufficient ligand field strength (ligands relatively high in the spectrochemical series) must be provided. Thus, although it is possible to produce $Co^{III}(H_2O)_6^{3+}$ electrochemically, it is very unstable whereas $Co^{III}(NH_3)_6^{3+}$ is very stable to reduction since ammonia is considerably higher than water in the spectrochemical series (Cotton & Wilkinson, 1972). An octahedral coordination geometry is also necessary. Although five-coordinate Co(III) complexes have been reported, they are invariably isolated in the solid state (Cotton & Wilkinson, 1972). If found in solution, a five-coordinate Co(III) complex would not be expected to exhibit substitution inertness since this is essentially the transition state for ligand exchange in a six-coordinate Co(III) complex. The difference in stability between five- and six-coordinate Co(III) can be attributed to differences in crystal field stabilization energy (Basolo & Pearson, 1968).

Although native metal binding sites in proteins may meet the criteria for stabilization of the 3+ vs. 2+ oxidation state of cobalt, it is difficult to be certain that Co(II) oxidation has occurred at the original binding site. For instance, in alkaline phosphatase it appears that a Co(II) located in the structural site can migrate to the catalytic site prior to H_2O_2 oxidation (Anderson & Vallee, 1977). Successful oxidation of cobalt in a metalloprotein containing a tetrahedral or five-coordinate

site almost certainly requires that the site is flexible enough to become octahedral six coordinate. The necessary change in coordination number complicates matters since new ligands must be provided, potentially from other amino acids in the protein. Even then, the complex may be labile due to the weak ligand field of the ligands provided.

A different approach which can be applied to metalloproteins and nonmetalloproteins alike is to alter an amino acid to a chelating agent prior to cobalt introduction. The problems associated with in-site oxidation of Co(II) substituted in metalloproteins are obviated by the approach taken in this study. The conversion of an amino acid to a chelating agent provides a metal coordination site that can be used to direct and stabilize metal complexation. A number of methods have been reported for the production of chelating agents in proteins, but diazotization holds a number of distinct advantages over these methods. Diazotization is simple and often quite specific (Fairclough & Vallee, 1970; Gorecki et al., 1978; Johansen et al., 1972). Most importantly, the derivatives are intensely colored and serve as spectral probes for monitoring metal coordination. The ligand-associated transitions provide 10–100 times greater sensitivity than can Co(III) d–d transitions. Specificity and extent of incorporation can be determined since metal coordination results in predictable alterations of the azodye spectra.

To produce the Co(III) complex, four more ligands must be added to compliment the bidentate azodye. It was found that the tetradentate ligand EDDA, whose Co(III) complexes have been extensively studied (Brubaker et al., 1971; Halloran & Legg, 1974), was ideal for this purpose. A solution of $Co^{II}(EDDA)(H_2O)_2$ is easily prepared and stable to oxidation for several days even without the rigorous exclusion of oxygen, and yet cobalt oxidation takes place readily in the presence of a good bidentate chelating agent such as an azophenol. The solution stability and relative ease with which Co^{II}EDDA complexes can be oxidized to the corresponding Co(III) complexes make tetradentate EDDA ideally suited to these studies. Other tetradentate chelating agents were investigated, but EDDA was found to be distinctly superior for the desired protein modification. The use of tetradentate EDDA in conjunction with the protein-bound bidentate azodye ensures that the only amino acid involved in coordination to the Co(III) is that which has been modified to give the bidentate azodye since all six permissable sites on the octahedral Co(III) are occupied by these two ligands.

The specificity of diazotization in CPA-Zn and the thoroughly characterized azotyrosine-248 spectra (Johansen & Vallee, 1973) provided an excellent opportunity to apply this approach to protein modification. Since tyrosine-248 has been implicated in the activity of the enzyme and since cobalt complexation would block the phenolic oxygen, this modification was potentially valuable in determining the necessity of this amino acid for activity.

Co^{III}EDDA incorporation results in the loss of peptidase activity toward Bz-GP (<0.1% of a CPA-Zn or AA-CPA-Zn control), but the enzyme maintains its capacity to hydrolyze the ester, Bz-GOP. These results suggest that tyrosine-248 is required for the peptidase but not esterase activity of the enzyme. More extensive kinetic studies are being conducted.

The importance of the model investigations cannot be too greatly stressed. Conditions for the modification and reversal as well as the spectral characterization came directly from work with the azodye ligands. The potential for metal-catalyzed oxidative damage was also recognized during the model studies.

As predicted by the models, the visible spectrum of Co^{III}(EDDA)(AA-CPA-Zn) possesses a 510-nm maximum indicative of metal coordination, and, as opposed to AA-CPA-Zn, the spectrum is essentially invariant with pH. The 480–600-nm region of the CD spectrum is also pH invariant whereas a $\Delta[\theta]$ of nearly 55000 is found upon raising the pH from 8.0 to 10.5 in AA-CPA-Zn (Johansen & Vallee, 1973). This change in AA-CPA-Zn has been assigned to the dissociation of the zinc(II)–azotyrosine complex to give the free azotyrosinate (Johansen & Vallee, 1973). Since no such change is noted in the Co^{III}EDDA derivative, it appears that 100% of the azotyrosine population is fully coordinated to a metal throughout this pH range. Unambiguous assignment of these spectra to Co(III) and not Zn(II) coordination is possible since removal of the active-site zinc does not significantly alter the CD or visible spectra.

It is evident that Zn(II) remains in the active site of the Co^{III}EDDA derivative since the enzyme functions as an esterase showing K_m values comparable to those of the native enzymes (M. S. Urdea, unpublished experiments). In both the native apoenzyme (Coleman & Vallee, 1964; Auld & Holmquist, 1974) and the enzyme with Co(III) in the active site (Van Wart & Vallee, 1978), esters bind much more poorly and neither functions as an esterase.

Fe^{II}EDTA fully reverses the modification, returning the original CD, UV, and visible spectra of AA-CPA-Zn. EDTA alone has no effect. This substantiates the lack of other alterations, e.g., free-radical damage, and confirms the assignment of the distinctly different spectra obtained upon Co(III) coordination of arsanilazotyrosine-248. Consistent with this suggestion, the Fe^{II}EDTA reversal returns 95% of the peptidase activity.

Denaturation, pH changes, and chelating agents do not reverse the cobalt modification. Although not a direct proof of Co(III) and not Co(II) coordination, this is a direct proof of inertness, and it is this characteristic of Co(III) which makes it a useful probe. It is difficult to propose a Co(II) complex of azotyrosine since oxidation is required to produce the modification and only reduction can reverse it.

It is important to note the necessity of free-radical scavengers to this modification since metal-catalyzed oxidative damage is detected without the addition of phenol. In the absence of phenol during Co(II) oxidation, Fe^{II}EDTA does not return the spectral or kinetic properties of the enzyme. Since oxidative damage has been observed during unscavenged in situ Co(II) to Co(III) oxidation with H_2O_2, the presumption that Co(III) incorporation into an enzyme is alone responsible for resultant changes in behavior may not be generally valid. That is, oxidative damage from free radicals may occur despite the fact that incubation of the protein with H_2O_2 in the absence of exogenous metal ions and scavengers has no effect. However, in this study the ability to return the original properties of the enzyme upon cobalt removal argues strongly against metal-catalyzed structural damage.

The method described here should be applicable in a number of instances. The diazotization and subsequent cobalt modification of proteins are easily accomplished with inexpensive commercially available compounds. The six-coordinate cobalt complex produced is of sufficient kinetic stability to assure that the metal ion will remain on the protein during subsequent studies. Although of somewhat limited value in the study of structure–function relationships, the extension to radiolabeling of proteins and peptides is far reaching, and such studies are currently under investigation in this laboratory. A preliminary report of the modifications of insulin, glucagon,

and luteinizing hormone-releasing hormone has appeared elsewhere (Urdea et al., 1979).

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