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Synthesis and Characterization of Cobalt(III) Ethylenediamine-*N*,*N*′-diacetate Complexes with Azo Dyes and Azo Amino Acids

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A series of ternary cobalt(III) complexes containing the quadridentate ligand ethylenediamine-*N*,*N*′-diacetate (EDDA) and bidentate azo ligands have been prepared and characterized as models for cobalt(III) azo protein derivatives. The azo ligands investigated are an azo naphthol dye, *p*-(2-hydroxy-1-naphthylazo)benzenesulfonic acid (OD-II = orange dye II), an azo phenol dye, 2-(4-carboxyphenylazo)-4,5-dimethylphenol (CDP), and azo amino acid derivatives, *N*-acetyl-3-(arsanilazo)-L-tyrosine (NA-MAT), *N*-acetyl-2-(arsanilazo)-L-histidine (NA-MAH(C-2)), *N*-acetyl-4-(arsanilazo)-L-histidine (NA-MAH(C-4)), and *N*-acetyl-2,4-bis(arsanilazo)-L-histidine (NA-BAH), which are direct analogues of the cobalt(III) azo protein derivatives. Two of the possible geometrical isomers for the OD-II and CDP complexes were separated and characterized by their ^1H NMR spectra. The visible electronic absorption spectra of the azo dyes and azo amino acids are distinctly and systematically altered upon cobalt(III) coordination, permitting the quantitative determination of cobalt(III) complexation to azo proteins. The absorption spectra vary little between isomers.

Introduction

We have developed a method for the complexation of substitution-inert Co(III) to histidine and tyrosine residues in proteins.[1-3] In order to direct and stabilize Co(III) coordination, we converted histidine or tyrosine residues to an azo histidine or azo tyrosine by diazo coupling with an aryl diazonium salt such as diazotized arsanilic acid. The chromophoric azo amino acids serve as bidentate chelating agents and are preferential sites for cobalt complexation in the proteins investigated. Substitution-labile Co(II) is introduced to the azo protein as the diaquo ethylenediamine-*N*,*N*′-diacetate (EDDA)[4] complex and is subsequently oxidized in situ to produce the exchange-inert Co^{III}(EDDA)(azo protein) complex. The octahedral, six-coordinate Co(III) complex is linked to the protein only through the bidentate azo amino acid. The resulting Co(III)-azo protein complexes are very stable to ligand exchange.[1-3]

This method has been employed to specifically label tyrosine 248 of carboxypeptidase A and constitutes the first modification of the enzyme which blocks only the phenolic oxygen of this active site residue.[1] This modification has provided information as to the potential role of tyrosine 248 in the hydrolysis of peptides and esters.[5] The Co(III) can be reduced with Fe^{II}EDTA, resulting in full return of the original azo protein properties. With the use of this technique, a radiolabeled derivative of insulin has been produced with ^{57}Co and employed in a radioimmunoassay.[3]

Successful production and characterization of the Co(III) azo protein derivatives is dependent on the synthesis and full characterization of Co(III) model complexes. Initial studies employed terdentate azo tyrosine analogues[6] and bidentate azo tyrosine analogues[7] as they relate more specifically to the Co(III) derivative of arsanilazotyrosine-248 carboxypeptidase.[1] The work presented here describes the preparation, isomer separation, and characterization of the OD-II and CDP[4] complexes of Co^{III}EDDA and the preparation and characterization of the Co^{III}(EDDA)(azo tyrosine and histidine) complexes which are direct analogues of the azo protein derivatives currently under investigation.[1-3] It is shown that distinct changes occur in the visible spectrum of the azo dyes or azo amino acids upon Co(III) coordination which are generally useful in determining the extent and specificity of Co(III) complexation to azo proteins. Importantly, it has been shown that the coordination modes of these ligands have little effect on the absorption spectrum. Thus it is not necessary to define the stereochemistry of the Co(III) complex in order to characterize a modified protein. The determination of cobalt coordination to the azo chromophores by spectral analysis is far more sensitive than in the absence of chromo-

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(4) Abbreviations used are as follows: EDDA, ethylenediamine-*N*,*N*′-diacetic acid; OD-II, orange dye II, *p*-(2-hydroxy-1-naphthylazo)-benzenesulfonic acid; CDP, 2-(4-carboxyphenylazo)-4,5-dimethylphenol; NA-MAH(C-2), *N*-acetyl-2-(arsanilazo)-L-histidine; NA-MAH(C-4), *N*-acetyl-4-(arsanilazo)-L-histidine; NA-BAH, *N*-acetyl-2,4-bis(arsanilazo)-L-histidine; NA-MAT, *N*-acetyl-3-(arsanilazo)-L-tyrosine; ISTEA, distilled 2-propanol and triethylamine bicarbonate buffer, pH 9.5.
(5) M. S. Urdea and J. I. Legg, *J. Biol. Chem.*, **254**, 11868 (1979).
(6) W. I. White and J. I. Legg, *J. Am. Chem. Soc.*, **97**, 3937 (1975).
(7) W. I. White and J. I. Legg, *Bioinorg. Chem.*, **6**, 163 (1976).

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phoric ligands since the ligand associated transitions of the Co(III)–azo dye complexes are some 10–100 times more intense than Co(III) d–d electronic transitions.

Experimental Section

Materials. EDDA, CDP, and OD-II[4] were purchased from LaMont Laboratories, Alfred Bader Chemical Co., and Eastman Chemicals, respectively. The azo amino acids were synthesized according to Urdea et al.[8] $Co(EDDA)(H_2O)_2 \cdot H_2O$ was prepared by the method of Averill et al.[9] and stored under N_2 until used. The method of Kuroda and Watanabe[10] was used to prepare *s-cis*-$[Co(EDDA)(H_2O)_2]ClO_4$.

Preparation of Barium [*p*-(2-Hydroxy-1-naphthylazo)benzenesulfonato](ethylenediamine-*N,N'*-diacetato)cobaltate(III) ($Ba[Co(EDDA)(OD\text{-}II)]_2$). To a solution containing OD-II (1.10 g) and *s-cis*-$[Co(EDDA)(H_2O)_2]ClO_4$ (1.11 g) in 150 mL of water were added 30 mL of 0.1 N NaOH and blood charcoal (0.90 g). After the mixture was warmed at 75 °C for 3 h with stirring, the blood charcoal was removed by filtration and washed with a small amount of water. The filtrate and washings were combined and concentrated to dryness by rotary evaporation. The product was dissolved in 16 mL of 80% methanol and chromatographed in batches on Sephadex LH-20. For each separation 4 mL of the solution was applied to a column 5 × 50 cm and eluted with 80% methanol. Two major red bands developed. Four batches of the fast running band (isomer 1) were combined and evaporated to dryness in a stream of air. Then the solid was dissolved in a small amount of water and converted to the barium form by passing the solution through a cation-exchange column (Dowex 50W-X4, Ba^{2+} form). As in all ion-exchange conversions of counterions, at least a 10-fold excess of equivalents of resin-bound ion was employed. A flame test demonstrated that Na^+ was absent. After the eluate was concentrated to dryness by rotary evaporation, the solid was purified twice by reprecipitation from water by the addition of acetone. The purified product was washed with ether and dried under vacuum in a drying pistol over boiling ethanol; yield 60 mg. Anal. Calcd for $C_{44}H_{40}O_{16}N_8S_2Co_2Ba \cdot 6.5H_2O$: C, 38.48; H, 3.89; N, 8.16. Found: C, 38.46; H, 3.40; N, 7.80. Four batches of the slow running band (isomer 2) were also combined and evaporated to dryness in a stream of air. The solid was treated in the same way as isomer 1 to convert it into the barium salt. The crystalline product obtained by recrystallization from water was washed with acetone and then ether and dried as described above; yield 130 mg. Anal. Calcd for $C_{44}H_{40}O_{16}N_8S_2Co_2Ba \cdot 9H_2O$: C, 37.26; H, 4.12; N, 7.90. Found: C, 37.23; H, 4.13; N, 7.65. The products obtained prior to conversion to barium salts were used for ^1H NMR measurements.

Preparation of Barium [2-(4-Carboxyphenylazo)-4,5-dimethylphenolato](ethylenediamine-*N,N'*-diacetato)cobaltate(III) ($Ba[Co(EDDA)(CDP)]_2$). The complex was prepared according to the method of White and Legg.[7] Though isomer 1 isolated in the previous study was pure, isomer 2 was found to be contaminated by isomer 1. In this study it has been shown that the contamination was due to the instability of isomer 2. Isomer 2 has now been isolated in a pure state as detailed below. Separation of the two isomers was carried out by a homemade high-pressure liquid chromatograph.[11] After the reaction solution was filtered, the filtrate was concentrated to dryness by rotary evaporation. Then the material (50 mg) was dissolved in 3 mL of 75:25 ISTEA,[4,12] and a small amount of insoluble material was filtered. The filtrate was applied to a high-pressure liquid chromatograph silica column (4 × 36 cm) and eluted with 75:25 ISTEA, separating into two red bands. The fast running band on the column corresponds to the slow running band (isomer 2) observed on LH-20, and the slow running band to the first band (isomer 1).[7] Several batches of the fast running band (isomer 2) were combined and evaporated to dryness in a stream of air. The solid was treated in the same way as the OD-II complexes to convert the complex to its barium salt. The tiny flaky crystals (pure isomer) obtained by recrystallization from water were dried under vacuum in a drying pistol over boiling ethanol; yield 40 mg. Anal. Calcd for $C_{42}H_{44}O_{14}N_8Co_2Ba \cdot 11H_2O$: C, 37.69; H, 4.97; N, 8.38. Found: C, 37.74; H, 4.75; N, 8.13. This isomer can also be obtained by the LH-20 Sephadex column separation.[7] However, the isolation of the pure isomer is only possible when the compound is changed into barium salt by treating with cation-exchange resin as above. For ^1H NMR measurements the barium salt was changed into the sodium salt on a cation-exchange column (Dowex 50W-X4, Na^+ form). The procedure was performed in a cold room, and concentration of the solution was carried out by lyophilization.

Preparation of Cesium (*N*-Acetyl-3-(arsanilazo)-L-tyrosinato)-(ethylenediamine-*N,N'*-diacetato)cobaltate(III) ($Cs_3[Co^{III}(EDDA)(NA\text{-}MAT)]$). A mixture of $Co(EDDA)(H_2O)_2 \cdot H_2O$ (0.29 g) and NA-MAT (0.46 g) was stirred in 50 mL of water. The pH was adjusted to 9.5 with 1 N NaOH. The solution was stirred for 3 days at room temperature. The material was evaporated to dryness by rotary evaporation and dissolved in 50:50 ISTEA. The solution was then applied to a Whatman preparative PK1F TLC plate and eluted with the ISTEA buffer system.[12] The red complex ($R_f = 0.60$) was removed from the plate by scraping, dissolved in water, and centrifuged to remove the silica. After rotary evaporation, the solution was desalted on a G-10 Sephadex column and converted to the cesium form by chromatography on a Dowex 50W-X8 column that had been equilibrated with CsCl and thoroughly washed with water. The complex was then precipitated by the addition of 100 mL absolute ethanol which doubled the total solution volume. The precipitate was washed with ethanol and ether and was dried in a drying pistol at the temperature of boiling ethanol. Anal. Calcd for $C_{23}H_{24}O_{11}N_5AsCs_3Co \cdot 6H_2O$: C, 23.19; H, 3.13; N, 5.88. Found: C, 23.15, H, 3.50; N, 5.76.

Preparation of Barium (*N*-Acetyl-2-(arsanilazo)-L-histidine)-(ethylenediamine-*N,N'*-diacetato)cobaltate(III) ($Ba[Co(EDDA)(NA\text{-}MAH(C\text{-}2))]$). A mixture of *s-cis*-$[Co(EDDA)(H_2O)_2]ClO_4$ (1.05 g), $Ba_3[NA\text{-}MAH(C\text{-}2)]_2$ (2.00 g), and blood charcoal (0.5 g) in 50 mL of water was warmed at 80 °C for 10 min with stirring. The blood charcoal was removed by filtration and washed with a small amount of water. The filtrate and washings were combined, and the volume of the solution was increased to 150 mL by the addition of water. Approximately 150 mL of ethanol was then added, and the solution was kept at 4 °C overnight. The first precipitate (0.44 g) was separated by filtration and washed with ethanol and then ether. A second crop (0.98 g) was obtained by repeating this procedure. The first precipitate contained a small amount of impurity as evidenced by TLC,[12] while the second was apparently pure. The second precipitate was used for the elemental analysis and the spectroscopic measurements. The material was reprecipitated from water by the addition of ethanol. The isolated product was washed with 50% ethanol, ethanol, and then ether and dried under vacuum in a drying pistol at the temperature of boiling ethanol; yield 0.25 g. Anal. Calcd for $C_{20}H_{23}O_{10}N_7AsBaCo \cdot 4H_2O$: C, 27.78; H, 3.61; N, 11.34. Found: C, 27.76; H, 3.79; N, 11.01.

Preparation of Barium (*N*-Acetyl-4-(arsanilazo)-L-histidine)-(ethylenediamine-*N,N'*-diacetato)cobaltate(III) ($Ba[Co(EDDA)(NA\text{-}MAH(C\text{-}4))]$). A mixture of *s-cis*-$[Co(EDDA)(H_2O)_2]ClO_4$ (0.38 g), $Ca_3[NA\text{-}MAH(C\text{-}4)]_2$ (0.65 g), and blood charcoal (0.32 g) in 20 mL of water was warmed at 70–75 °C for 10 min with stirring. The blood charcoal was removed by filtration and washed with a small amount of water. The filtrate and washings were combined and concentrated by rotary evaporation. The solution was applied to a Sephadex G-25 column (2.5 × 65.5 cm) and eluted with water. The chromatography revealed a major brown band and a few lightly colored minor bands. The major brown band was collected and concentrated to dryness by rotary evaporation. The solid was dissolved in 20 mL of water and the pH adjusted to 8.0–8.2. A solution of $BaCl_2 \cdot 2H_2O$ (0.25 g) in approximately 7 mL of water was added. Subsequently 55 mL of ethanol was added, and the solution was kept at 4 °C overnight. The precipitate which formed was separated by filtration and washed with ethanol and then ether. The solid was reprecipitated by dissolving the material in 15 mL of water and adding 40 mL of ethanol. The solution was kept at 4 °C overnight, and the resulting precipitate was filtered and washed with 50% ethanol, ethanol, and then ether. The product was dried under vacuum in a drying pistol at the temperature of boiling ethanol; yield 0.36 g. Anal. Calcd for $C_{20}H_{23}O_{10}N_7AsBaCo \cdot 7H_2O$: C, 26.14; H, 4.06; N, 10.67. Found: C, 26.23; H, 4.01; N, 10.62.

(8) M. S. Urdea, K. Igi, G. J. Pielak, and J. I. Legg, submitted to *J. Biol. Chem.*
(9) D. F. Averill, J. I. Legg, and D. L. Smith, *Inorg. Chem.*, **11**, 2344 (1972).
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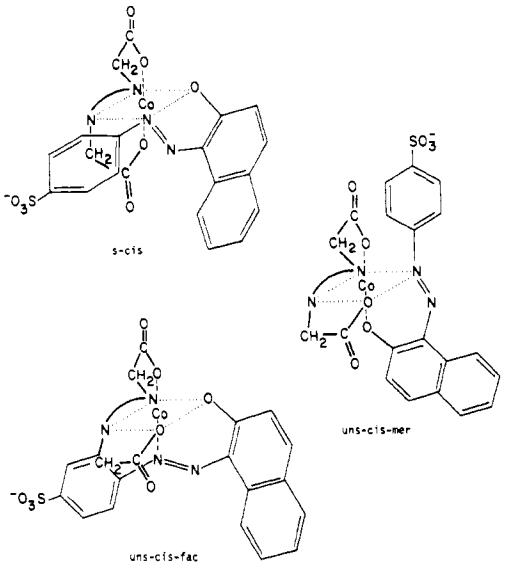

Figure 1. Three possible geometrical isomers for [Co(EDDA)(OD-II)]$^-$. Analogous isomers can be drawn for the corresponding CDP complex.

Preparation of Calcium (*N*-Acetyl-2,4-bis(arsanilazo)-L-histidine)(ethylenediamine-*N*,*N'*-diacetato)cobaltate(III) (Ca_2[Co(EDDA)(NA-BAH)]). A mixture of *s-cis*-[Co(EDDA)$(H_2O)_2$]ClO_4 (0.74 g), Ca_2[NA-BAH)] (2.57 g), and blood charcoal (0.60 g) in 120 mL of water was warmed at 70–75 °C for 10 min with stirring. After the reaction solution cooled to room temperature, 52 mL of ethanol was added and the solution was cooled in an ice bath. The blood charcoal was removed by filtration, and the volume of the filtrate was increased to 280 mL by the addition of H_2O. To this solution was added 320 mL of ethanol, and the solution was cooled in an ice bath. The precipitate was filtered and washed with ethanol and then ether. The crude product was dissolved in 40 mL of water, and 13 mL of ethanol was added. After the solution cooled, a small amount of gummy precipitate formed which was removed by filtration. To the filtrate was added 200 mL of cold ethanol, causing immediate precipitation. The product was isolated by filtration and washed with ethanol and then ether. This process was repeated two more times. The product was dried in a $CaCl_2$ desiccator and then under vacuum in a drying pistol at the temperature of boiling ethanol; yield 0.73 g. Anal. Calcd for $C_{26}H_{26}O_{13}N_9As_2Ca_2Co\cdot 5H_2O$: C, 29.69; H, 3.45; N, 11.99. Found: C, 29.91; H, 3.65; N, 11.60.

Spectra. The electronic absorption spectra were recorded in water with either a Cary Model 14 or Varian Superscan 3 spectrophotometer. CD spectra were recorded on a Jasco model ORD/UV-5 with the SS20 CD modification (Sproul Scientific). Nuclear magnetic resonance spectra were recorded on a JEOL Model MH-100 spectrometer. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal reference.

Analyses. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, or Canadian Microanalytical Service Ltd., Vancouver, B.C., Canada.

Results and Discussion

Although the protein analogues can be synthesized by using a variety of methods as described here and previously,[6,7] many of these methods are unsuitable for the protein modifications due to the excesses in pH, temperature, and concentration employed. However, the studies with the models have been instrumental in establishing conditions most amenable to azo protein modification.[1-3] The preparation of the Co^{III}(EDDA)(azo dye or azo amino acid) complexes described here involves either the air oxidation of the Co^{II}(EDDA)(azo dye or azo amino acid) complexes or the substitution of H_2O on [Co^{III}(EDDA)$(H_2O)_2$]$^+$ by the azo dyes or azo amino acids.

The three possible geometrical isomers (*s*-cis, *uns*-cis-*fac*, *uns*-cis-*mer*) of [Co(EDDA)(OD-II)]$^-$ are shown in Figure 1.[13] The same types of isomers are also possible for [Co(EDDA)(CDP)]$^-$. Ligand field spectra are generally useful for distinguishing geometrical isomers of Co(III) complexes; however, the d–d electronic spectra are obscured by the strong ligand chromophores for these complexes so that the electronic absorption spectra cannot be used for determination of the structures. Fortunately, the ^{1}H NMR spectra could be employed to assign structure. The geminal proton coupling constants (J_{AB}) for the glycinate rings of EDDA, EDTA, and related Co(III) complexes are classified into two groups, those in the vicinity of 16 Hz for in-plane (G) rings (>N-CH_2CH_2N-$CH_2CO_2^-$ chelated meridionally) and those of 18 Hz for out-of-plane (R) rings (>NCH_2CH_2N$CH_2CO_2^-$ chelated facially).[14-17] It has been found that *s-cis*-[Co(EDDA)A_2] complexes show only one AB pattern (J_{AB} = 18 Hz) for the R rings and *s-cis*-[Co(EDDA)AB] complex two AB patterns (J_{AB} = 18 Hz) for the same R rings due to the

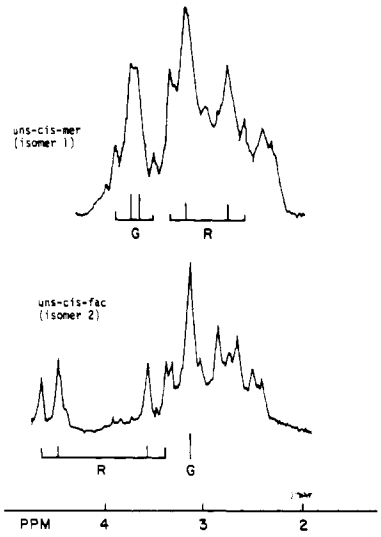

Figure 2. 100-MHz ^{1}H NMR spectra for the isomers of [Co(EDDA)(OD-II)]$^-$.

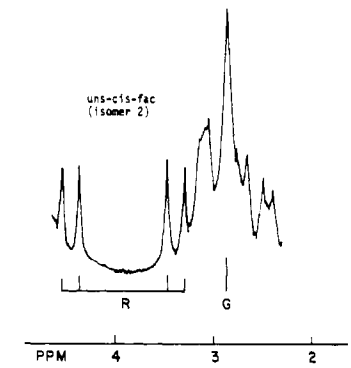

Figure 3. 100-MHz ^{1}H NMR spectra of *uns-cis-fac*-[Co(EDDA)(CDP)]$^-$.

(13) With respect to quadridentate EDDA and the unsymmetrical bidentate azo ligand, three geometrical isomers can be envisioned as shown in Figures 1 and 6. The *s*-cis and *uns*-cis designations refer to the mode of coordination of EDDA (see L. J. Halloran, A. L. Gillie, and J. I. Legg, *Inorg. Synth.*, **18**, 103 (1978)). For the complexes reported in this study the *fac* (facial) and *mer* (meridional) designations refer to the relative positions of the two EDDA nitrogents and the azo nitrogens in the *uns*-cis isomers. Linkage isomerism is possible which arises from the presence of two nitrogens in the azo group as discussed in ref 7.

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Table I. Glycinate Ring Methylene Proton Resonance Assignments[a]

complex	R ring[b]		G ring[c]	
	H_A	H_B	H_A	H_B
uns-cis-mer-[Co(EDDA)(OD-II)]$^-$ (isomer 1)	3.24	2.68	3.81	3.57
uns-cis-fac-[Co(EDDA)(OD-II)]$^-$ (isomer 2)	4.50	3.44	3.10	
uns-cis-mer-[Co(EDDA)(CDP)]$^-$ (isomer 1)[d]	3.25	2.66	3.73	3.56
uns-cis-fac-[Co(EDDA)(CDP)]$^-$ (isomer 2)	4.44	3.38	2.86	
uns-cis-mer-[Co(EDDA)(NA-MAH(C-2))]$^{2-}$	NA[e]		3.89	
uns-cis-mer-[Co(EDDA)(NA-MAH(C-4))]$^{2-}$	NA		3.99	
uns-cis-mer-[Co(EDDA)(NA-BAH)]$^{4-}$	NA		4.05	

[a] Values in ppm from DSS. [b] J_{AB} = 18 Hz. [c] J_{AB} = 16 Hz. [d] Reference 7. [e] Not assignable.

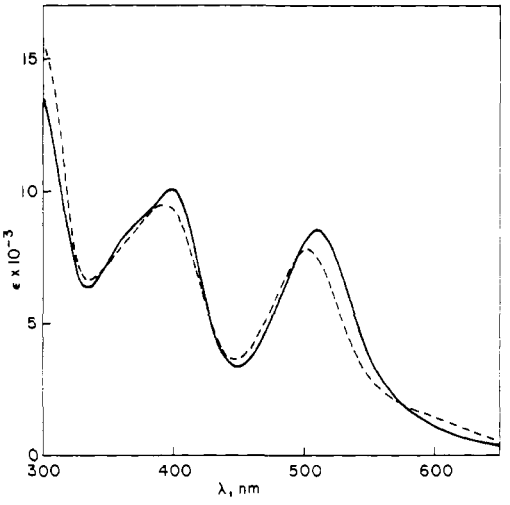

Figure 4. Absorption spectra for the isomers of [Co(EDDA)(OD-II)]$^-$, *uns-cis-mer* (—) and *uns-cis-fac* (- - -).

removal of the C_2 symmetry axis.[15,16] In contrast the *uns*-cis isomers of [Co(EDDA)A_2] show a single intense peak due to the G ring protons (J_{AB} = 16 Hz) superimposed on one AB pattern of the R ring protons. The singlet was considered to be an extreme case of an AB pattern in which the environments of the two protons are very similar.[15]

^{1}H NMR spectra for the isomers 1 and 2 (order of chromatographic elution) of [Co(EDDA)(OD-II)]$^-$ and isomer 2 of [Co(EDDA)(CDP)]$^-$ are shown in Figures 2 and 3. Isomer 1 of both the OD-II and CDP[7] complexes shows two AB patterns with J_{AB} = 18 Hz and J_{AB} = 16 Hz (Table I). Therefore, it is evident that these isomers are one of the two possible *uns*-cis isomers. White and Legg[7] assigned isomer 1 of the CDP complex to the *uns*-cis-*mer* isomer based on the observation that the AB pattern due to the R ring protons (J_{AB} = 18 Hz) is shifted dramatically upfield as compared with other previously reported complexes of this type[15] (Table I). In the case of *uns*-cis-*mer*, both H_A and H_B of the R ring are in the vicinity of one face of the benzoic acid ring and are expected to be shifted upfield.[18] Isomer 1 of the OD-II complex also shows an AB pattern J_{AB} = 18 Hz) located at much higher field (Table I) than expected for this type of complex.[15] Thus isomer 1 of the OD-II complex is also assigned to the *uns*-cis-*mer* isomer. In this case both H_A and H_B of the R ring are in the vicinity of one face of the benzenesulfonic acid ring (Figure 1). Isomer 2 of both the OD-II and CDP complexes shows an AB pattern with J_{AB} = 18 Hz due to the R ring protons and a sharp singlet due to the G ring protons superimposed on the complicated set of resonances due to the ethylene protons (Figures 2 and 3). In contrast to the

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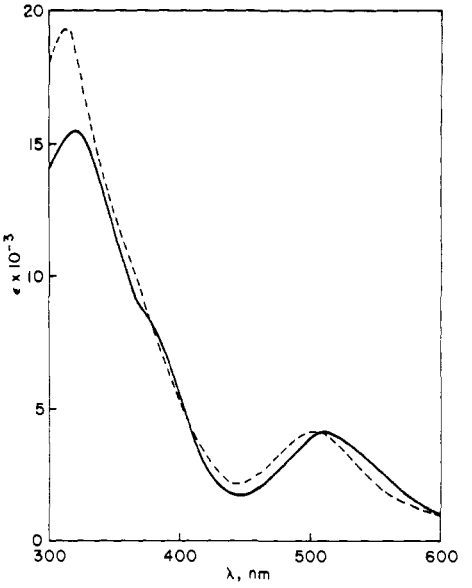

Figure 5. Absorption spectra for the isomers of [Co(EDDA)(CDP)]$^-$, *uns-cis-mer* (—) and *uns-cis-fac* (- - -).

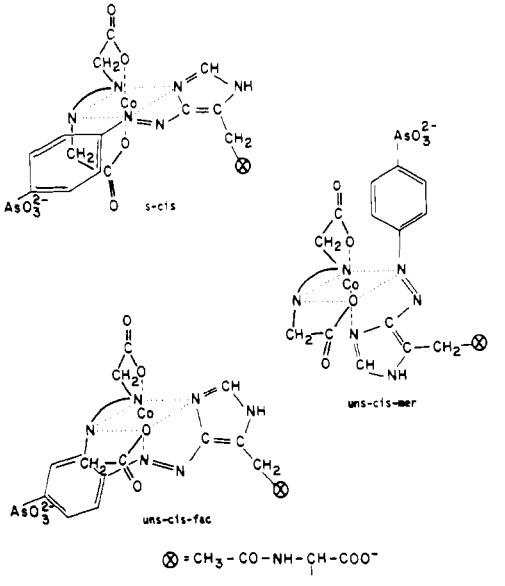

Figure 6. Three possible geometrical isomers for [Co(EDDA)(NA-MAH(C-4))]$^{2-}$.

uns-cis-*mer* isomers, the G ring proton signal is at much higher field than expected[15] (Table I). Thus, isomer 2 of both the OD-II and the CDP complexes can be assigned the *uns*-cis-*fac* configuration since the H_A and H_B protons of the G ring are in the vincinity of one face of the benzenesulfonic acid or benzoic acid ring (Figure 1).

The electronic absorption spectra for both isomers of the OD-II and CDP complexes are shown in Figures 4 and 5. The shapes of the two curves for each set of isomers are very similar showing two bands with similar maxima and molar absorptivities in the visible region. These electronic absorption spectra are derived from intraligand transitions as modified by Co(III) coordination to the phenolate or naphtholate.[1,6,7] Therefore, little information on geometrical isomerism can be obtained from these spectra.

The three possible geometrical isomers (*s*-cis, *uns*-cis-*mer*, *uns*-cis-*fac*) for [Co(EDDA)(NA-MAH(C-4))]$^{2-}$ are shown in Figure 6. The complexes [Co(EDDA)(NA-MAH(C-2))]$^{2-}$ and [Co(EDDA)(NA-BAH)]$^{4-}$ can also have analogous geometrical isomers.[19] ^{1}H NMR spectra for the isolated

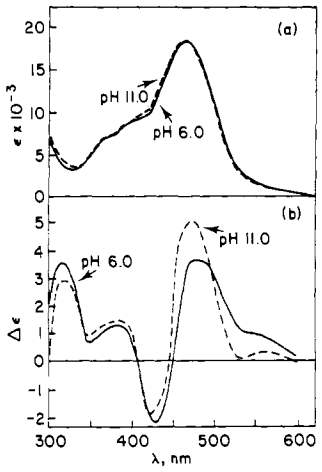

Figure 7. Absorption spectrum (a) and CD spectrum (b) of *uns-cis-mer*-[Co(EDDA)(NA-MAH(C-2))]$^{2-}$ at pH 6.0 and 11.0.

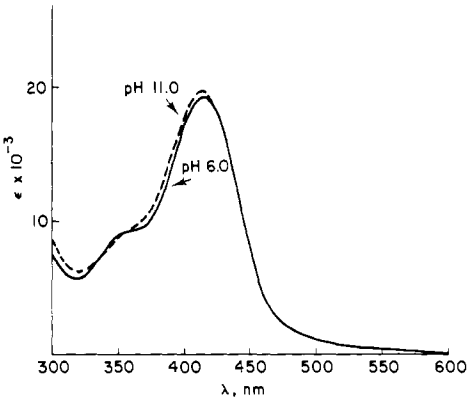

Figure 8. Absorption spectrum of *uns-cis-mer*-[Co(EDDA)(NA-MAH(C-4))]$^{2-}$ at pH 6.0 and 11.0.

NA-MAH(C-2) and NA-MAH(C-4) complexes are very complicated in the methylene proton region. However, a rather sharp singlet which integrates to two protons is observed at 3.89 ppm for the NA-MAH(C-2) complex and at 3.99 ppm for the NA-MAH(C-4) complex (Table I) as is often observed for the G ring methylene protons of *uns*-cis isomers.[15-17] The resonances from the R ring methylene protons overlap the backbone ethylene protons, showing a complicated spectrum between ~2.1 and ~3.8 ppm which can not be definitely assigned. The upfield shift of the R ring methylene protons is caused by the location of H_A and H_B in the vicinity of one face of the arsanilic acid ring (Figure 6) as observed for the *uns*-cis-*mer* isomers of [Co(EDDA)(azo dye)] (vide supra). Thus the isolated NA-MAH(C-2) and NA-MAH(C-4) complexes probably also have the *uns*-cis-*mer* configuration (Figure 6). The ^{1}H NMR spectrum for the isolated NA-BAH complex is also very similar to the above two complexes, showing a sharp singlet at 4.05 ppm (Table I). By the same reasoning, this complex most likely has the *uns*-cis-*mer* geometry.

Electronic absorption spectra for *uns-cis-mer*-[Co(EDDA)(NA-MAH(C-2))]$^{2-}$, *uns-cis-mer*-[Co(EDDA)(NA-MAH(C-4))]$^{2-}$, *uns-cis-mer*-[Co(EDDA)(NA-BAH)]$^{4-}$, and [Co(EDDA)(NA-MAT)]$^{3-}$ at pH 6.0 and 11.0 are shown in Figures 7a, 8, 9, and 10a. Free ligand spectra are compared

(19) In addition, NA-MAH(C-2) and NA-BAH complexes could exhibit linkage isomerism for a total of two and three isomers for each geometrical isomer, respectively. Each of these in turn consists of a diastereomeric pair. Therefore a total of 12 and 18 isomers could exist for NA-MAH(C-2) and NA-BAH complexes, respectively. As might be expected from the close similarity between these isomers, no separation was observed by the various chromatographic means employed.

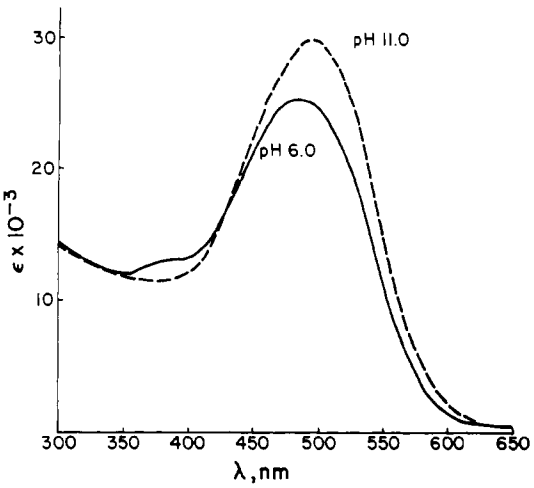

Figure 9. Absorption spectrum of *uns-cis-mer*-[Co(EDDA)(NA-BAH)]$^{4-}$ at pH 6.0 and 11.0.

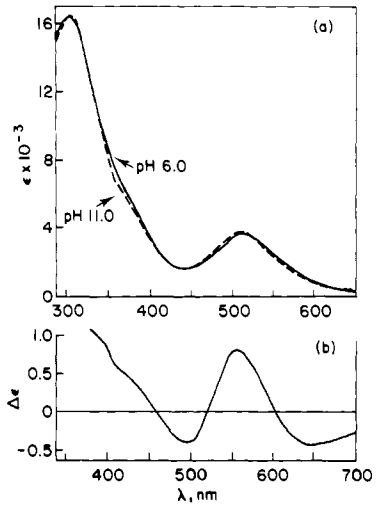

Figure 10. Absorption spectrum (a) and CD spectrum (b) of [Co(EDDA)(NA-MAT)]$^{3-}$ at pH 6.0 and 11.0.

Table II. Wavelength Maxima and Molar Absorptivities for the Azo Amino Acids and Their Cobalt(III) Complexes

compd[a]	λ_{max}[b] (ϵ)[c] pH 6.0	pH 11.0
NA-MAH(C-2)	387 (2.64)	389 (2.54)
[Co(EDDA)(NA-MAH(C-2))]$^{2-}$	468 (1.86)	467 (1.88)
NA-MAH(C-4)	352 (2.77)	352 (2.75)
[Co(EDDA)(NA-MAH(C-4))]$^{2-}$	415 (1.92)	414 (1.97)
NA-BAH	419 (3.18)	498 (4.09)
[Co(EDDA)(NA-BAH)]$^{4-}$	483 (2.56)	493 (2.98)
NA-MAT	325 (2.54), 390 (0.96) sh	328 (1.61), 485 (1.20)
[Co(EDDA)(NA-MAT)]$^{3-}$	304 (1.73), 512 (0.38)	310 (1.74), 512 (0.39)

[a] Spectral properties for the azo amino acids are reported in ref 8; the composition of the salts of the cobalt complexes are given in the Experimental Section. [b] Wavelengths given in nm. [c] Molar absorptivity $\times 10^{-4}$ M^{-1} cm^{-1}.

to complexed ligand spectra in Table II. Circular dichroism (CD) spectra for *uns-cis-mer*-[Co(EDDA)(NA-MAH(C-2))]$^{2-}$ and [Co(EDDA)(NA-MAT)]$^{3-}$ are shown in Figures 7b and 10b. The NA-MAH(C-2), Na-MAH(C-4), and NA-MAT complexes exhibit absorption spectra which are essentially invariant with pH, whereas the absorption spectrum of the NA-BAH complex is somewhat dependent upon pH. The invariance of the spectra imply that the azo dye ligands are coordinated to Co(III) throughout the pH range, sub-

stantiating the inert nature of the complexes. The pH dependence of the NA-BAH complex was anticipated since the azo imidazolate pK_a is significantly lower for NA-BAH (pK_a = 8.8) as compared to NA-MAH(C-2) or NA-MAH(C-4) ($pK_a \leq 11.5$).[8] Therefore, in the pH range of 6.0–11.0, the deprotonation of the bisazo ligand would be expected to result in a spectral change (as is the case with the ligand itself; see Table II). The azo imidazolate pK_a's of the monoazo histidine ligands are not appreciably lowered upon coordination since at pH 11.0 the spectra of the cobalt complexes are not significantly changed from the spectra of pH 6.0 (Figures 7a and 8).

As is typical of the Co(III)–azo dye complexes,[6,7] complexation of NA-MAH(C-2) and NA-MAH(C-4) induces a bathochromic shift of ca. 80 and ca. 60 nm, respectively, and decreases the intensity of absorption bands by 30% in both cases (Table II). A bathochromic shift of 64 nm is observed for BAH at pH 6 on complexation (Table II). On the other hand, at pH 11 the absorption maximum of the NA-BAH complex is nearly the same as that of the free ligand. This behavior can be accounted for by the difference in protonation state of the ligand as previously discussed. Complexation of NA-BAH also decreases the intensity of the absorption maximum 20–30% (Table II). Similarly NA-MAT shows a bathochromic shift of 25 nm upon coodination to Co(III), and the intensity also is lowered. Similar shifts are observed for the corresponding Co(III) azo enzyme and hormone derivatives.[1-3]

The Na-MAH(C-2) complex possesses an intense CD spectrum (Figure 7a), yet the NA-MAH(C-4) and NA-BAH complexes show very weak CD spectra (not shown). Whether or not the NA-MAH(C-2) ligand stereoselectively coordinates to Co^{III}EDDA is not clear. It is doubtful that the CD spectrum of the NA-MAH(C-2) complex arises simply from the vicinal effect due to the presence of an asymmetric α-carbon on the azo histidine ligand since the NA-MAH(C-4) and NA-BAH complexes would be expected to exhibit a CD spectrum as well if this were the primary factor giving rise to the detectable CD spectrum. The NA-MAT complex also possesses a distinct CD spectrum (Figure 10b). It is significant that this CD spectrum is nearly identical in band position and relative band intensity with the Co^{III}(EDDA)(azo tyrosine 248 carboxypeptidase A) complex described by Urdea and Legg.[1] This CD pattern appears to be characteristic of azophenol complexes when placed in asymmetric environments.[20]

The NA-MAH(C-2), NA-MAH(C-4), and NA-MAT complexes are very stable within the pH range of 6–10. At pH 11.0, however, the monoazo histidine complexes slowly decompose. As judged by the change in the visible spectrum after the solutions set for 1 week at pH 11.0 and 25 °C, the C-2 and C-4 monoazo histidine complexes decompose by about 15 and 10%, respectively. The NA-BAH complex slowly decomposes at pH 6.0 and 11.0, yet appears to be stable at pH 8.0 and 25 °C. This behavior relates to the stability expected for the corresponding protein derivatives.

The present study indicates that distinct changes in the visible spectrum of the azo dyes or azo amino acids occur upon Co(III) coordination. The coordination modes of these azo ligands have little effect on the absorption spectrum. Thus, the spectra can be used with reasonable certainty to characterize the protein derivatives without concern for the presence of isomers. However, without further analysis it does not appear that circular dichroism can be applied in such a straightforward manner. The spectral properties are being used to establish the extent and specificity of Co(III) incorporation into azo proteins.[1-3] The Co^{III}(EDDA)(azo protein) visible spectra are very similar in all respects to the model complexes. This is suggested by the studies of Urdea et al.[2] which demonstrate that the visible spectrum of a number of azo proteins can be simulated by simply combining the proper proportions of the model azo amino acids calculated to be present in the azo protein; that is, microenvironmental effects due to the protein matrix do not alter the visible spectrum of the azo chromophores. Therefore, a similar situation is anticipated for the coordinated azo amino acids.

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Registry No. *uns-cis-mer*-[Co(EDDA)(OD-II)]$_2$Ba, 78128-64-0; *uns-cis-fac*-[Co(EDDA)(OD-II)]$_2$Ba, 78184-57-3; *uns-cis-mer*-[Co(EDDA)(CDP)]$_2$Ba, 78128-65-1; *uns-cis-fac*-[Co(EDDA)(CDP)]$_2$Ba, 78184-58-4; *uns-cis-mer*-[Co(EDDA)(NA-MAH(C-2))]Ba, 78149-45-8; *uns-cis-mer*-[Co(EDDA)(NA-MAH(C-4))]Ba, 78166-86-6; *uns-cis-mer*-[Co(EDDA)(NA-BAH)]Ca_2, 78149-46-9; [Co(EDDA)(NA-MAT)]Cs_3, 78166-84-4; *s-cis*-[Co(EDDA)$(H_2O)_2$]ClO_4, 32715-40-5; Co(EDDA)$(H_2O)_2$, 42573-16-0.

(20) J. I. Legg, K. Igi, G. J. Pielak, B. D. Warner, and M. S. Urdea, *ACS Symp. Ser.*, No. 119, 195 (1980).