Exchange-Inert Metal Ions as Probes of Enzyme Structure–Function Relationships. Cobalt(III), Cobalt(II), and Zinc(II) Azophenol Complexes as Models for Enzyme Azotyrosine Complexes

William I. White and J. Ivan Legg*

Contribution from the Department of Chemistry, Washington State University, Pullman, Washington 99163. *Received November 18, 1974*

Abstract: Exchange-inert cobalt(III) complexes with bidentate or tridentate azotyrosine-modified enzymes may be useful in studying enzyme mechanisms. Since cobalt may form either cobalt(II) or cobalt(III) complexes, it is necessary to determine whether cobalt(III) complexes of azophenols can be formed under conditions conducive to enzyme stability and whether the metal ion-perturbed azophenol spectrum changes in a manner characteristic of the oxidation state of the metal. As the literature contains considerable confusion with regard to the oxidation state of cobalt in azophenol and azonaphthol complexes, cobalt(III), cobalt(II), and zinc(II) complexes of 2-(tetrazolyazo)-*p*-cresol, 2-(thiazolylazo)-*p*-cresol, and 1-(2-pyridylazo)-2-naphthol have been prepared in solution, and most have been isolated in the solid state. The complexes have been characterized by visible spectroscopy, ^{1}H NMR, ESR, magnetic susceptibility, and conductivity measurements. It is clear that cobalt(III) complexes of these ligands are formed readily; moreover, spectral changes in the visible region are characteristic of the oxidation state of the complexed metal.

Exchange-inert complexes may be useful in the study of structure–function relationships in biologically active molecules. Indeed, the application of some classical cobalt chemistry may provide answers to a question of enzyme mechanism which has provoked considerable controversy over the past several years. Carboxypeptidase A is a metalloenzyme containing zinc(II) in the active site; it has both peptidase and esterase activity. The crystal structure has been determined,[1] and numerous kinetic studies have been conducted.[2] Both crystal structure studies of carboxypeptidase A and spectroscopic studies of the specifically modified enzyme, containing arsanilazotyrosine 248 (Figure 1a),[3-5] have suggested that Tyr-248 is at the active site during catalysis. Still, the role of Tyr-248 in catalysis remains obscure. Auld and Holmquist have found evidence indicating that this residue may be involved in peptidase, but not esterase, activity,[6] while others argue that hydrolysis of esters and peptides should proceed by the same mechanism.[2]

The best test of these hypotheses would be to modify Tyr-248 in such a way that it could not participate in catalysis. Thus, for example, if esterase activity remains and peptidase activity is eliminated,Tyr-248 is most likely required for peptidase, but not esterase, activity. The modification most likely to succeed is a complex in which the phenol oxygen of Tyr-248 forms a kinetically inert bond to a metal ion. Thus, the possibility is eliminated that the phenolate might come off the metal ion to participate in catalysis. Cobalt is ideally suited for such a study; the lability of cobalt(II) facilitates the insertion of the metal ion into the protein matrix, while the relative ease of oxidation allows attainment of the exchange-inert cobalt(III) oxidation state. It should be stressed from the outset that the final product of such a modification will contain a zinc(II) in the active site and a cobalt(III) ion coordinated to Tyr-248.

A cobalt(III) complex with monodentate phenol is not likely to form readily; hence, modifications which convert tyrosines to chelating agents are being investigated for their potential to complex cobalt(III). Such modifications have already been introduced by Vallee and coworkers. Diazotized arsanilic acid reacts with carboxypeptidase A to give almost exclusively the derivative containing arsanilazotyrosine 248 (Figure 1a), and the resulting enzyme has both peptidase and esterase activity.[7,8] Subsequently, Cueni has prepared carboxypeptidase A modified on the same residue with diazonium-1*H*-tetrazole (Figure 1b).[9] The high degree of specificity of these derivatives will permit the complexation and blocking of the phenol oxygen of only Tyr-248, an achievement which has not been possible with organic derivatives alone.

Previous studies suggest that it is possible to form more stable cobalt complexes with a tridentate azophenol, such as the tetrazolylazo derivative, than with a bidentate azophenol, such as the arsanilazo derivative.[10] Therefore, in support of studies of cobalt azoenzyme complexes, we have initiated model studies on similar ligands in order to determine criteria for the formation of cobalt complexes with such protein-bound chelating groups.

These model studies are vital to the enzyme studies, because the normal visible spectral methods for determining the oxidation state of cobalt in a complex are not applicable. The azophenol ligand chromophores have molar absorptivities in excess of 10^4 in the visible region, two orders of magnitude greater than the molar absorptivities associated with d–d transitions. This means that spectral changes in the ligand chromophore which accompany formation of cobalt(II) and cobalt(III) azophenol complexes must be related to the oxidation state of the cobalt by magnetic methods which are much more easily performed on model complexes than on enzymes. Although ESR measurements below 20°K on an enzyme–cobalt complex will establish the magnetic state of the cobalt,[11] easily applied visible spectral criteria will greatly facilitate the studies.

Although Vallee and coworkers have used tetrazolylazo(*N*-carbobenzoxy)tyrosine for model work,[3,12] we found 2-(tetrazolylazo)-*p*-cresol (TeAC), more useful for our purposes, since the TeAC yields simpler ^{1}H NMR spectra and exhibits better solubility in a range of solvents. Two commercially available ligands, 2-(thiazolylazo)-*p*-cresol (TAC) and 1-(2-pyridylazo)-2-naphthol (PAN), were also studied to determine to what extent the findings may be generalized (Figure 2).

Both TAC and PAN have been used as analytical reagents for the spectrophotometric determination of metal ions, such as zinc and cobalt.[13,14] Nevertheless, it appears that the cobalt(II) and zinc(II) complexes we have isolated as crystals are the first examples of solid zinc or cobalt(II)

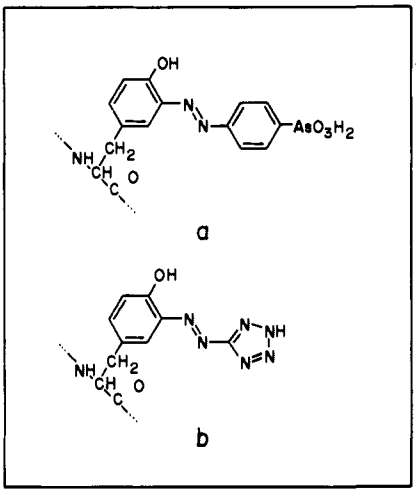

Figure 1. Tyr-248 of carboxypeptidase A modified with (a) diazotized arsanilic acid and (b) diazotized 5-aminotetrazole.

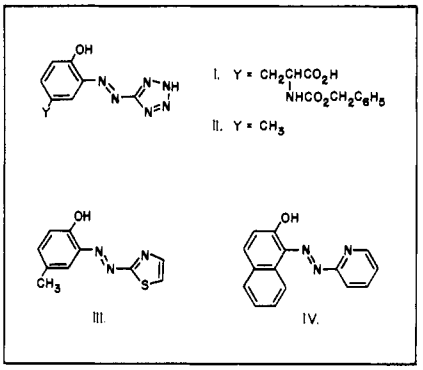

Figure 2. (I) Tetrazolylazo(*N*-carbobenzoxy)tyrosine, (II) tetrazolylazo-*p*-cresol (TeAC), (III) thiazolylazo-*p*-cresol (TAC), (IV) pyridylazo-2-naphthol (PAN).

complexes of these ligands. There is a great deal of misleading information in the analytical chemistry literature concerning cobalt complexes of tridentate azophenols, much of which might have been avoided if pure samples of the complexes in question had been isolated.

The work presented here indicates that bis tridentate azophenol complexes of zinc(II), cobalt(II), and cobalt(III) can be isolated and that the visible spectral properties are related in a straightforward manner to the oxidation state of the metal. Most important, these results indicate that cobalt(III) is the most stable oxidation state for cobalt in these complexes.

Experimental Section

Ligands. Tetrazolylazo-*p*-cresol (TeAC)[12] was prepared by coupling 5-diazonium-1*H*-tetrazole with *p*-cresol by the method of Sokolovsky and Vallee.[15] (*Caution: Concentrated solutions of diazonium tetrazole are highly explosive. The reagent must be handled with care and solutions above 0.2 M should not be employed*). The orange TeAC was separated from the purple bis coupling product and colorless unreacted phenol by chromatography on Sephadex LH-20, eluting with 0.01 *M* KOH. The orange solution was concentrated by evaporation, precipitated with concentrated hydrochloric acid, collected on a Buchner funnel, washed three times with 0.1 *M* HCl, and dried under vacuum. Thiazolylazo-*p*-cresol (TAC) was purchased from Dojindo Chemical Co. Pyridylazo-2-naphthol (PAN) was purchased from Baker Chemical Co.

Preparation of Bis[2-(tetrazolylazo)-*p*-cresolato]cobalt(III). TeAC (430 mg, 2.1 mmol) was dissolved in 100 ml of methanol. One milliliter of 1 *M* $CoCl_2$ solution (1.0 mmol) was added and the pH was adjusted to between 4 and 5 with concentrated sodium hydroxide solution. The solution was stirred in contact with air until it turned blue. It was then evaporated to dryness, redissolved in 50 ml of absolute methanol, filtered, and chromatographed on a column of Sephadex LH-20 (6 × 47 cm), eluting with methanol. The blue band was collected and evaporated to about 10 ml. Seventy milliliters of water was added and a further 10 ml of solution was evaporated. Dropwise addition of concentrated hydrochloric acid produced a blue precipitate, which was collected in a Buchner funnel and washed several times with 0.1 *M* HCl. The product was dried under vacuum in a drying pistol over boiling acetone. Anal. Calcd for $Co(C_8H_7N_6O)(C_8H_6N_6O)$: C, 41.4; H, 2.8; N, 36.2. Found C, 41.5; H, 3.0; N, 35.9.

Preparation of Bis[2-(thiazolylazo)-*p*-cresolato]zinc(II). TAC (220 mg, 1 mmol) was dissolved in 50 ml of methanol. Ten milliliters of 0.1 *M* $ZnSO_4$ solution (1 mmol) was added and then ca. 10 ml of concentrated ammonia; the solution was evaporated to dryness. The residue was triturated with several 100-ml portions of toluene; the combined toluene portions were evaporated to 75 ml, filtered, and allowed to evaporate slowly, producing small dichroic flakes. The crystals were collected on a Buchner funnel, washed with cold toluene and dried under vacuum in a drying pistol over boiling ethanol. Anal. Calcd for $Zn(C_{10}H_8N_3OS)_2$: C, 47.9; H, 3.2; N, 16.7. Found: C, 48.1; H, 3.3; N, 16.7.

Preparation of Bis[2-(thiazolylazo)*p*-cresolato]cobalt(II). TAC (440 mg, 2 mmol) was dissolved in 100 ml of methanol in a 2-l. separatory funnel. Two milliliters of 1 *M* $CoSO_4$ solution (2 mmol) was added. Five hundred milliliters each of water and benzene was added, together with ca. 50 ml of concentrated ammonia. The blue benzene layer was retained and concentrated by boiling to 200 ml. It was then cooled, filtered, and allowed to evaporate slowly, producing small, dichroic flakes, containing benzene of crystallization. The crystals were collected on a Buchner funnel, washed with cold benzene, and dried under vacuum in a drying pistol over boiling ethanol. Anal. Calcd for $Co(C_{10}H_8N_3OS)_2 \cdot \frac{1}{2}C_6H_6$: C, 51.7; H, 3.6; N, 15.7. Found: C, 51.8; H, 3.5; N, 15.6.

Preparation of Bis[2-thiazolylazo)-*p*-cresolato]cobalt(III) Chloride Monohydrate or Hydrogen Sulfate. TAC (462 mg, 2.1 mmol) was dissolved in 100 ml of methanol. One milliliter of 1 *M* $CoCl_2$ or $CoSO_4$ solution (1 mmol) was added and the pH adjusted to between 4 and 5 with concentrated sodium hydroxide solution. The solution was stirred exposed to air until it turned green. It was then evaporated to dryness, redissolved in 50 ml of methanol acidified with 1 drop of concentrated HCl for every 100 ml of methanol, filtered, and chromatographed on Sephadex LH-20, eluting with the acidified methanol. The green band was collected and evaporated to 50 ml. An equal volume of chloroform was added, and the solution was filtered. Crystals were obtained by slow evaporation from the solution exposed to the atmosphere. They were collected on a Buchner funnel, washed with a cold mixture of chloroform–methanol, and dried under vacuum in a drying pistol over boiling ethanol. Starting from $CoSO_4$, Anal. Calcd for $Co(C_{10}H_8N_3OS)_2HSO_4$: C, 40.5; H, 3.0; N, 14.2; Co, 9.9; S, 16.2. Found: C, 40.8; H, 2.9; N, 14.1; Co, 9.7; S, 15.9. Starting from $CoCl_2$, Anal. Calcd for: $Co(C_{10}H_8N_3OS)_2Cl \cdot H_2O$: C, 43.8; H, 3.3; N, 15.3. Found: C, 43.9; H, 3.2; N, 15.3.

Preparation of Bis[1-(2-pyridylazo)-2-naphtholato]zinc(II). This compound was made in the same manner as the bis[2-(thiazolylazo)-*p*-cresolato]cobalt(II), starting with 500 mg (2 mmol) of PAN. Anal. Calcd for $Zn(C_{15}H_{10}N_3O)_2$: C, 64.1; H, 3.6; N, 14.9. Found: C, 64.3; H, 3.7; N, 14.9.

Preparation of Bis[1-(2-pyridylazo)-2-naphtholato]cobalt(II). This compound was made in the same manner as bis[2-(thiazolylazo)-*p*-cresolato]cobalt(II), starting with 500 mg (2 mmol) of PAN. Anal. Calcd for $Co(C_{15}H_{10}N_3O)_2$: C, 64.9; H, 3.6; N, 15.1. Found: C, 65.1; H, 3.6; N, 15.0.

Preparation of Bis[1-(2-pyridylazo)-2-naphtholato]cobalt(III) Chloride. Two hundred fifty milligrams of PAN (1 mmol) was dissolved in ethanol and 0.5 ml of 1 *M* $CoCl_2$ solution was added. The solution was set in an air stream to evaporate. The solid product was dissolved and transferred to a 2-l. separatory funnel with a minimum of methanol. Five hundred milliliters each of chloroform and 0.1 *M* HCl was added, plus enough additional methanol to prevent formation of solid at the interface. The chloroform layer was saved and the upper layer extracted again with chloroform.

Table I. Cobalt and Zinc Azophenol and Azonaphthol Complexes

Complex	λ_{max}, nm ($\epsilon \times 10^4$)	Color	μ_{eff} (BM)	Λ (mho $\times 10^6$)
$Zn^{II}(TAC)_2$	387 (0.83), 605 (1.24)[a]	Blue	Diamag	Negligible
$Co^{II}(TAC)_2$	397 (0.82), 590 (1.24)[a]	Blue	3.4,[c] 4.0[d]	Negligible
$Co^{III}(TAC)_2$	400 (1.60), 670 (0.78)[b]	Green	Diamag	26
Zn^{II}–TeAC[e]	345, 511	Red		
Co^{II}–TeAC[e]	354, 510	Red		
$Co^{III}(TeAC)_2$	365 (1.31), 600 (0.52)[b]	Blue	Diamag	
$Zn^{II}(PAN)_2$	315 (0.94), 344 (0.87), 398 (0.63), 522 (2.35) 558 (2.85)[a]	Red	Diamag	Negligible
$Co^{II}(PAN)_2$	320 (1.21), 402 (0.68), 527 (1.59)[a]	Red	2.3[c]	Negligible
$Co^{III}(PAN)_2$	309 (1.18), 448 (1.15), 578 (1.02), 620 (0.88)[b]	Green	Diamag	31

[a] Spectra taken in benzene solution. [b] Spectra taken in methanol solution. [c] Determination on solid crystals. [d] Determination in benzene solution. [e] Stoichiometry uncertain, as solid complexes could not be isolated. Spectra taken in methanol:water (1:1), 0.1 M $KHCO_3$ buffer at pH 8.

The two green chloroform layers were combined and washed with more 0.1 M HCl, again with the addition of methanol to prevent solid formation. The chloroform layer was filtered into an erlenmeyer flask and allowed to evaporate slowly to produce prismatic crystals. The crystals were collected on a Buchner funnel, washed with cold methanol–chloroform, and dried under vacuum in a drying pistol over boiling ethanol. Anal. Calcd for $Co(C_{15}H_{10}N_3O)_2Cl$: C, 61.0; H, 3.4; N, 14.2. Found: C, 60.8; H, 3.5; N, 14.0. This complex was prepared by a different method by Liu.[16]

Physical Measurements. Visible spectra were recorded on a Cary Model 14 spectrophotometer. Solutions with a ligand concentration of 2.5×10^{-5} M and a path length of 1 cm gave spectra identical with those from solutions 5×10^{-4} M in ligand and a path length of 0.05 cm. Nuclear magnetic resonance spectra were recorded on either a Varian A60 or Varian T60 spectrometer. Magnetic measurements were performed at room temperature on the solids by the Faraday method and in benzene solution by the Evans method.[17] The ESR spectra were recorded on a Varian E9 ESR spectrometer at room temperature and below 20°K. Cobalt(III) complexes showed no absorption at any temperature while $Co(TAC)_2 \cdot \frac{1}{2}C_6H_6$ gave g values of 4.34 and 2.04 and $Co(PAN)_2$ gave $g = 2.01$. Frozen benzene solutions (ca. 10^{-4} M) were employed. Conductivity measurements were made with an Industrial Instruments Model RC 16B2 conductivity bridge using dimethyl sulfoxide as a solvent. TAC complexes were studied at a concentration of 1.0×10^{-3} M; PAN complexes were studied at a concentration of 5.0×10^{-4} M. Analyses were determined by D. Harsh, Department of Chemistry, University of Idaho, Moscow, Idaho, and by Galbraith Laboratories, Inc., Knoxville, Tenn. Activation analysis for cobalt was done at the Nuclear Research Laboratory, Washington State University.

Results and Discussion

The conductivities, magnetic moments, and absorption spectral data obtained for the various complexes prepared in this study are summarized in Table I. The visible absorption spectra are of particular interest to us, both to compare with previously reported azotyrosine enzyme complexes, and, especially, to distinguish the oxidation state of cobalt in azotyrosine enzyme complexes currently under investigation in this laboratory. Therefore, these spectra are reproduced in Figures 3–5.

Cobalt(II) Complexes. There have been many reports in the literature where the formation of cobalt(II) complexes of heterocyclic azo dyestuffs in solution has been claimed.[13,14] Some workers have assumed that because a cobalt(II) salt is used, a cobalt(II) complex is produced in solution.[18] Others have assumed that if studies are performed in the presence of a reducing agent, the complex formed will be a cobalt(II) complex.[19] Despite observations published by Iwamoto and Fujimoto more than a decade ago, which showed clearly the fallacious nature of these assertions,[20] new work containing these assumptions continues to be published. Two otherwise helpful review articles are misleading on this subject.[13,14]

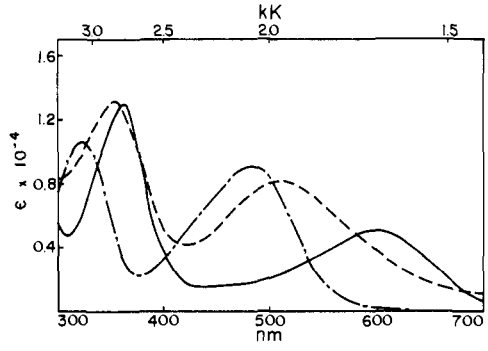

Figure 3. Spectra of tetrazolylazo-*p*-cresol (TeAC) and its complexes with cobalt in a 50% solution of methanol in water: (- —) TeAC anion, pH 12; (- - -) Co^{II}–TeAC, pH 8; (—) $Co^{III}(TeAC)_2^-$. The molar absorptivity indicated is per ligand unit. To obtain molar absorptivities for the 1:2 metal:ligand complexes, the ordinate values must be multiplied by two.

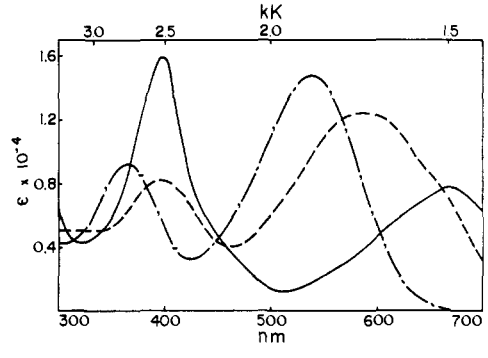

Figure 4. Spectra of thiazolylazo-*p*-cresol (TAC) and its complexes with cobalt: (- —) TAC anion, pH 12, 50% solution of methanol in water; (- - -) $Co^{II}(TAC)_2$ in benzene; (—) $Co^{III}(TAC)_2^+$ in methanol. The molar absorptivity indicated is per ligand unit. To obtain molar absorptivities for the 1:2 metal:ligand complexes, the ordinate values must be multiplied by two.

Although Iwamoto and Fujimoto were able to demonstrate that the red complex of cobalt with PAN was paramagnetic, magnetic susceptibility measurements of that complex in solution indicated that oxidation was taking place during the course of the experiment.[20] The preparation of cobalt(II) complexes of both TAC and PAN are the first examples of the isolation of pure cobalt(II) complexes of azophenols and the determination of their magnetic moments in the solid state (Table I). Liu obtained a solid sample of cobalt(II)–PAN which had a μ_{eff} slightly lower than the value we obtained, but did not have good elemental analyses.[16] A low-spin cobalt(II) complex of 2-thiazolylazoresorcinol was reported, but the purity of the preparation was not clear.[21]

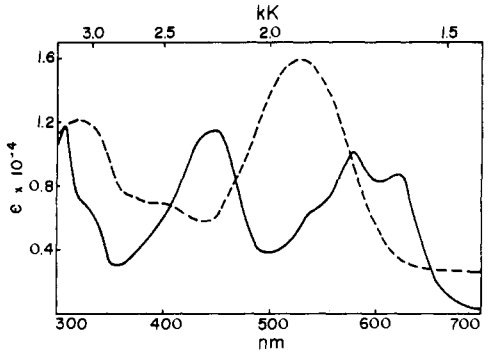

Figure 5. Spectra of pyridylazo-2-naphthol (PAN) complexes with cobalt: (- - -) $Co^{II}(PAN)_2$ in benzene; (—) $Co^{III}(PAN)_2^+$ in methanol. The molar absorptivity indicated is per ligand unit. To obtain molar absorptivities for the 1:2 metal:ligand complexes, the ordinate values must be multiplied by two.

Determination of the magnetic susceptibility is a straightforward means of demonstrating the existence of a cobalt(II) complex. In addition, there are methods for indicating whether a cobalt complex in solution has been oxidized; these have been neglected in some previous studies. If the complex is stable to acid hydrolysis, this suggests the presence of exchange-inert cobalt(III). If a sample of the complex can be isolated, a ^1H NMR spectrum can be obtained. The existence of a diamagnetic ^1H NMR spectrum for the complex is a strong indicator of diamagnetic cobalt(III).

Our success in preparation of these cobalt(II) complexes stems primarily from the choice of solvent. Cheng and Bray reported that the cobalt(II)–PAN complex is stable in absolute ethanol.[22] In our studies, however, the red cobalt(II)–PAN complex turned to the green cobalt(III) complex before useful measurements could be made in any polar solvent. Moreover, in many polar solvents, the labile nature and apparent low stability of the cobalt(II) complexes lead to dissociation of metal from ligand, and the spectra reported for cobalt(II) complexes are on occasion actually composite spectra of cobalt complex plus ligand. Some combination of these factors probably explains the difference between the cobalt(II)–PAN complex spectrum shown in Figure 5 and that previously published.[20] It also explains why the colors previously reported for cobalt(II) and zinc(II) complexes of TAC are not the same as those reported here.[23] By using nonpolar solvents, such as benzene and carbon tetrachloride, we have avoided both problems mentioned above. Because both oxidation and dissociation would create more ionic species, this environment favors the retention of neutral, associated cobalt(II) complexes. The negligible conductivities found for the cobalt(II) complexes even in dimethyl sulfoxide (Table I) indicate their nonionic nature.

Unfortunately, the highly polar nature of the tetrazole ring prevents utilization of this approach; TeAC and its complexes will not dissolve in benzene. Furthermore, the red cobalt(II) complex of TeAC is oxidized very readily to the blue cobalt(III) complex making quite difficult the isolation of the red complex. However, it was possible to establish the nature of the cobalt(II) complex spectrum by recording the spectrum at pH 8 immediately after mixing a solution of the ligand with a tenfold excess of cobalt(II) in order to ensure formation of the complex. The spectrum of the zinc complex could also be obtained in solution in a similar manner.

It is appropriate to comment on the unusual magnetic moments found for the two cobalt(II) complexes that were isolated (Table I). The good chemical analyses and reproducible values for μ_{eff} obtained from one preparation to another suggest that there cannot be significant contamination by a cobalt(III) complex. The cobalt(II)–PAN complex is not sufficiently soluble in benzene to permit a magnetic susceptibility determination by the Evans method. However, the fact that the μ_{eff} in solution for the cobalt(II)–TAC complex is higher than the solid state moment suggests that a lack of magnetic dilution may contribute to the low magnetic moment. Another contributing factor is likely to be a spin pairing equilibrium. A large number of octahedral cobalt(II) complexes have been found with anomalously low magnetic moments.[24] The ligand in such cases is always a tridentate, meridional, aromatic, nitrogen-containing species. In a temperature study for one such compound, Busch and coworkers were able to account for all of the magnetic data in terms of a spin pairing equilibrium.[25] On the other hand, Hogg and Wilkins report that the temperature dependence of the anomalously low magnetic moments of cobalt(II) terpyridine complexes does not permit a straightforward interpretation in terms of spin-pairing alone.[26] In this latter case, the fact that complexes with larger counteranions have larger magnetic moments suggests that a lack of magnetic dilution may also be a factor in the terpyridine complexes. Cobalt(II) complexes of ligands such as 1,2-di(6-methyl-2-pyridyl)1,2-diaza-2-propene also had anomalously low magnetic moments, but no attempt was made to explain them.[27] The presence of an ESR signal below 20°K but not at room temperature for the cobalt(II)–TAC and cobalt(II)–PAN complexes confirms high spin Co(II).

Zinc(II) Complexes. The zinc complexes of these ligands were prepared for two reasons. These complexes permit an additional check on the oxidation state of the metal ion in the compounds assigned as cobalt(II) complexes, since it was anticipated that the oxidation state and radius of the metal ion would be the primary factors determining the nature of the metal ion-perturbed azophenol spectrum. Certainly, in the cases we have observed, the zinc complexes have almost the same colors and spectra as the corresponding cobalt(II) complexes (Table I). This relationship holds very closely for the phenol-containing ligands. With PAN, the zinc produces a much brighter red than does cobalt(II), and the absorption bands are much sharper than the corresponding bands observed for the cobalt(II) complex.

The second reason for examining the zinc complexes is that carboxypeptidase A is a zinc-containing enzyme. Distinct spectral changes are observed in the azophenol chromophore on complexation of arsanilazotyrosine 248 to zinc(II) at the active site of arsanilazocarboxypeptidase,[3,4] and it will be necessary to distinguish these spectra from those generated when cobalt(III) complexes this residue.

Cobalt(III) Complexes. Chromatography on lipophilic Sephadex proved to be particularly helpful in the preparation of cobalt(III) complexes. Although the cobalt(III)–PAN complex was not prepared using chromatography, subsequent experiments indicate that chromatography may well provide a better separation than the liquid–liquid extraction method reported.

The method of preparation and elemental analysis of the cobalt(III) TeAC complex indicates that, in solution, the tetrazole ring loses its proton at pH above about 3. At neutral pH, the complex has a net negative charge. Addition of acid neutralizes this charge and the complex falls out of solution, with one of the two TeAC molecules still deprotonated. A variety of derivatives of tetrazole have pK values in the range of 1 to 6 with most of the values between 3.5 and 5.[28] As the coordinated cobalt(III) ion would be expected to increase the acidity of that proton, the behavior of the cobalt(III) complex of TeAC is not at all surprising.

Although many azophenol and azonaphthol complexes of cobalt have been reported as cobalt(II) complexes, it is clear from descriptions of their stability to acid and generally irreversible behavior that they are in fact cobalt(III) complexes. It is worthwhile to point out that determination of the stoichiometry of such complexes in solution by commonly employed equilibrium methods is not appropriate, and the determination of stoichiometry may be done properly only through isolation of the solid complex.

Isolation of solid complex also permits facile determination of the oxidation state of the cobalt through a variety of methods, all of which were applied to the complexes described here. Diamagnetic ^1H NMR spectra of the complexes suspected to contain cobalt(III) provided strong evidence for the absence of unpaired electrons. The lack of magnetic moments in the solid state and in solution, as well as the lack of an ESR signal at room temperature and below 20°K confirmed the presence of cobalt(III).

Implications for Enzyme Studies. There are several important ramifications of this work for studies of azo-modified enzymes particularly with regard to the studies of carboxypeptidase A. The spectral changes which occur upon complexation of these azophenols with cobalt(II) and zinc(II) are quite similar to those reported to occur for complexation of arsanilazotyrosine 248 with these and other divalent metal ions in carboxypeptidase A.[3-5] Furthermore, it is apparent that cobalt(III) complexes of tridentate azophenols form with ease, and that the spectral changes associated with the azophenol in changing from uncomplexed ligand to the cobalt(II) complex and to the cobalt(III) complex are distinct and follow a pattern. In general, the maxima shift to lower energy during these changes, and decrease in molar absorptivity as shown particularly in Figures 3 and 4. Distinct color changes are associated with these spectral changes as indicated in Table I. These dramatic spectral changes should make it possible to identify the azophenol environment in the modified enzyme when cobalt(II), cobalt(III), and zinc(II) may be present. The correlation of magnetic data with the visible spectral data demonstrates the usefulness of these complexes as models for the enzyme work, as the magnetic data are not readily obtainable for the corresponding enzyme complexes.

Studies underway in this laboratory of the interaction of cobalt with modified carboxypeptidase A support this conclusion.[29] In addition, as anticipated, models are proving to be valuable in establishing the precise conditions under which the desired cobalt(III) complex of modified carboxypeptidase A can be obtained.

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