A Detailed Analysis of the Charge-Transfer Bands of a Blue Copper Protein. Studies of the Nickel(II), Manganese(II), and Cobalt(II) Derivatives of Azurin

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Abstract: The preparation and characterization of the manganese(II) and nickel(II) derivatives of the blue copper protein azurin (*Pseudomonas aeruginosa*) are described. The circular dichroic spectra of the nickel(II) and cobalt(II) derivatives are reported and are correlated with that of native azurin. The results of fluorescence, absorption, and competitive binding experiments indicate that all metals coordinate at the copper binding site. Ligand optical electronegativities are computed from the charge-transfer spectra of the cobalt(II), nickel(II), and copper(II) proteins, and transitions from a cysteine sulfur and a methionine sulfur are identified. Bands maximizing at 560 (130 M^{-1} cm^{-1}), 442 (3300 M^{-1} cm^{-1}), and 360 nm (1570 M^{-1} cm^{-1}) in the absorption spectrum of the nickel(II) derivative are assigned as πS(Cys) → Ni(II), σS(Cys) → Ni(II), and σS(Met) → Ni(II) charge-transfer bands, respectively. Corresponding assignments are proposed for bands of native azurin which appear at 820, 626, and 467 nm, respectively.

Introduction

The elucidation of the nature of the type 1, or blue, site in copper proteins has been a challenging problem to which various physical methods have been applied.[1-3] In the case of plastocyanin the picture has recently come into much sharper focus with the report of the analysis of a crystal structure at 2.7-Å resolution.[2] The latter has revealed that the copper is bound to a cysteine sulfur, a methionine sulfur, and two imidazole nitrogens, and that the ligands form an appreciably distorted tetrahedron about the copper. More recently, a low-resolution (3 Å) crystallographic model of azurin has been reported, and it indicates that the structure of the copper site is probably rather similar to that of plastocyanin.[3]

We have carried out studies of azurin using physical methods in conjunction with metal-replacement experiments. The method of metal replacement has been widely used in the study of metalloproteins as a means of probing the geometric structure of metal binding sites.[4] The characterization of metal-replaced derivatives can also provide information which may be useful in evaluating the faithfulness of synthetic analogues of the protein centers.[5,6] In previous work the substitution of Co(II) for Cu(II) proved helpful in distinguishing the d–d bands from the charge-transfer bands of the blue copper centers;[7,8] however, our understanding of the electronic properties of these systems remains incomplete. The preparation and characterization of additional derivatives of the blue site in azurin have allowed a more detailed analysis of the charge-transfer spectral region, the results of which are reported herein.

Experimental Section

Materials. The culture of *Pseudomonas aeruginosa* was obtained from the American Type Culture Collection (strain no. 10145). The metal ion solutions were made by volumetric dilution of 1000-ppm standard solutions (Matheson Coleman and Bell). The ammonium acetate buffers were prepared by the addition of glacial acetic acid to the appropriate amount of ammonia to give pH 8.0 or 9.0 buffers and were then passed through a Chelex-100 column to remove adventitious transition-metal ions. The sodium salt of *p*-hydroxymercuribenzoate (hereafter called pmb) was purchased from Sigma. All other chemicals used were reagent grade except the Triton X-100, which was scintillation grade.

Procedures. The bacteria were cultivated anaerobically in the medium described by Kaplan.[9] The native azurin was isolated from the cells by the method of Ambler,[10] and apoazurin was prepared by the method of Yamanaka.[11] The metal derivatives were prepared by the addition of 2 molar equiv of the metal ion to a sample of apoazurin which was then equilibrated at room temperature for 24-48 h. Prior to the analyses, samples of the azurin derivatives were passed through a G-25 desalting column, lypholized, dissolved in 1-2 mL of 0.05 M pH 9 ammonium acetate, and, finally, eluted from a G-75 column previously equilibrated with the pH 9 buffer.

Protein concentrations were determined by the biuret method of Goa,[12] calibrated by preparing a series of standard solutions of apoprotein (pH 9). The ϵ_{280} value of apoazurin (pH 9) was determined using apoazurin samples which had been eluted from a G-75 column, hydrolyzed in 6 M HCl, and calibrated against norleucine standards by amino acid analysis. The $\Delta\epsilon_{250}$ of the absorption of a solution of the apoazurin–pmb complex over that of a corresponding pmb solution[13] gave an analysis for uncomplexed apoazurin (pH 9). Copper,[14] nickel,[15] and cobalt[16] concentrations were determined using published procedures.

Instrumentation. The UV-visible absorption spectra were obtained with a McPherson EU-700 spectrophotometer, except for the low-temperature spectra, which were obtained on a Cary 14 equipped with a variable temperature cell holder. The CD spectra were obtained with a Cary 60 equipped with a Model 6002 circular dichroism accessory. The fluorescence measurements were made on a SPEX Fluorolog instrument. The pH was measured at room temperature with an Orion Model 601A digital ionalyzer. The amino acid analyzer was a Durrum D-500, and the EPR measurements were made on a Varian E-109 spectrometer.

Results

After the cyanide treatment and exhaustive dialysis against 0.05 M pH 8 or 0.05 M pH 9 ammonium acetate buffer, analysis showed that less than 3% of the original copper was present in the apoazurin samples. On introducing Cu(II) into the same, the characteristic blue color of native azurin rapidly developed, and the absorbance ratio (A_{626}/A_{280}) of the reconstituted protein Cu^{II}-Az was 0.5 or greater. Employing titrations with the mercurial reagent, we confirmed that apoazurin has one accessible sulfhydryl group, but that Cu^{II}-Az has none.[17]

On introducing Ni(II) into a solution of apoazurin, a yellow color developed, but much less rapidly than the coloration induced by Cu(II), while a faint bluish color appeared when Co(II) was used. Equilibration with Mn(II) resulted in a colorless solution. (In these experiments the concentration of apoazurin ranged between about 2×10^{-5} and 1×10^{-4} M.) Apoazurin solutions that had been equilibrated with Ni(II), Co(II), or Mn(II) evidenced either sharply reduced or negligible affinities for Cu(II) as well as for pmb. Analyses of the Ni(II), Co(II), or Mn(II) samples showed that significant quantities of the metal ions were retained after exhaustive

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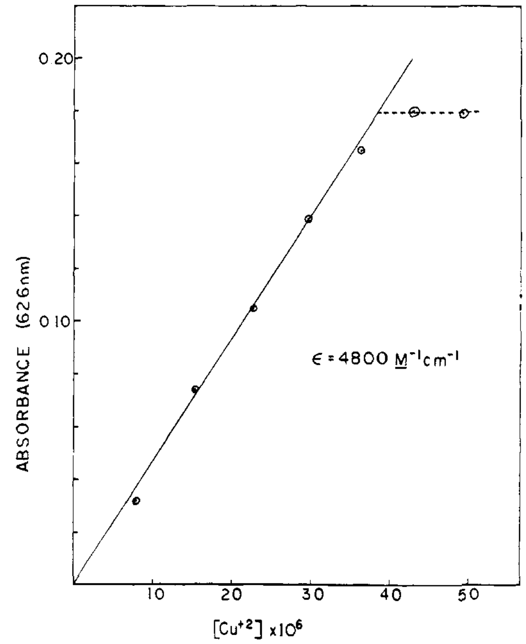


Figure 1. The titration of apoazurin with Cu^{2+} in 0.05 M pH 8.0 NH_4OAc buffer.

Table I. Stoichiometry Determinations

derivative	[M(II)] × 10^5 M	[apoazurin] × 10^5 M	[azurin] × 10^5 M
Ni^{II}-Az	2.12	0.88	2.95
	3.50	1.03	4.63
Co^{II}-Az	6.65	0.21	5.82
	4.64	0.62	4.67
Cu^{II}-Az	5.58	0.1	5.86

dialyses with 0.05 M pH 9 buffer and after gel filtration. As was the case with Cu(II)[17] and with Co(II),[7a] Mn(II) and Ni(II) induced a marked decrease in protein fluorescence at 310 nm. A twofold excess of Ni(II) gave approximately a 70% diminution, compared to that of apoazurin, whereas the analogous reaction with Mn(II) gave rise to only about a 60% diminution. Subsequent addition of Cu(II) to either of the above solutions produced little further change in the fluorescence intensity. The relative rates at which the fluorescence changes occurred followed the order Cu(II) > Co(II) ~ Ni(II) ≫ Mn(II). In the latter three cases at least, the fluorescence changes were more rapid at pH 9 than at pH 8.

The biuret analysis was calibrated over the range of 1.00×10^{-5} to 5.00×10^{-5} M with pH 9 solutions of apoazurin, for which ϵ_{280} was 8800 M^{-1} cm^{-1}. The accuracy of the biuret procedure was verified using a standard solution of Cu^{II}-Az at pH 8, the concentration of which was calculated independently from ϵ_{626} 4800 M^{-1} cm^{-1}, which was in turn measured by a titration of apoazurin with a standard Cu(II) solution (see Figure 1). By a similar procedure, the $\Delta\epsilon_{250}$ value for the pmb titration of apoazurin was found to be 8280 M^{-1} cm^{-1}. The results of the stoichiometry determinations are presented in Table I.

The UV-visible absorption and CD spectra of Ni^{II}-Az and Co^{II}-Az are presented in Figures 2 and 3, respectively, and the estimated molar absorptivities at the band maxima are compiled in Table II. Finally, we note that on dissolving Ni^{II}-Az in a pH 9.0, 0.05 M NH_4OAc/ethylene glycol solution, the relative absorbances at 355 and 442 nm were found to be independent of temperature in the range of −23 to 23 °C.

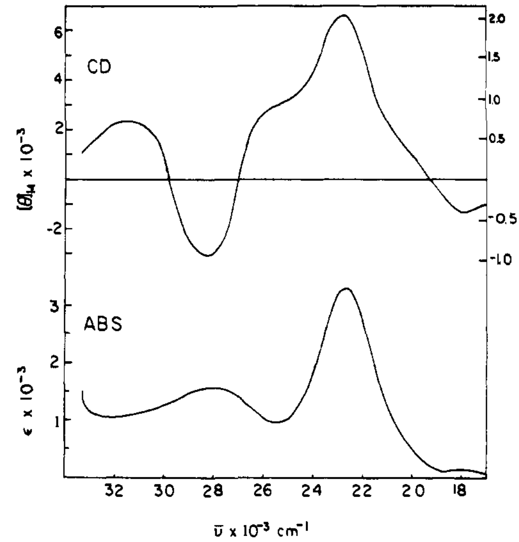


Figure 2. The CD and electronic absorption spectra of Ni^{II}-Az in the UV-visible region. The spectra were taken at 21 °C in 0.05 M pH 9.0 NH_4OAc buffer.

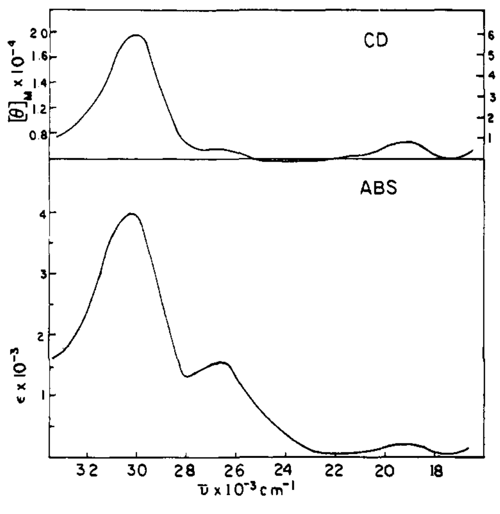


Figure 3. The CD and electronic absorption spectra of Co^{II}-Az in the UV-visible region. The spectra were taken at 21 °C in 0.05 M pH 9.0 NH_4OAc buffer.

Discussion

Our results show that apoazurin binds Ni(II), Co(II), and Mn(II), as well as Cu(II), which is found in the native system. Several observations argue, albeit indirectly, that the metals utilize the same binding site. These include the facts that (1) each metal binds competitively with Cu(II) and with strict 1:1 stoichiometry, (2) in each case the binding interaction masks the sulfhydryl group titrated in apoazurin, and (3) in each case the binding induces a pronounced diminution in protein fluorescence. More directly, the analysis of the charge-transfer spectra of the Co(II), Ni(II), and Cu(II) derivitives, vide infra, demonstrates that these metals coordinate to at least some of the same ligands and in sites with related geometries.

The marked correspondence among the charge-transfer bands of the Co(II), Cu(II), and Ni(II) derivatives of azurin is most evident from a comparison of the CD spectra. The charge-transfer bands of interest lie in the visible region in the case of Cu(II), the d-d bands having been indentified at longer wavelengths.[18] Conveniently, the CD bands of Cu^{II}-Az in the visible region have been nicely resolved in the spectrum reported by Coleman and co-workers.[19] The CD spectrum of

Table II. Molar Absorptivities

derivative	$\bar{\nu}_{max}$, μm^{-1}	ε, M^{-1} cm^{-1}
Ni^{II}-Az	1.78	130
	2.28	3300
	2.82	1570
Co^{II}-Az	1.55	220
	1.92	550
	2.68	1540
	3.00	4000

Co^{II}-Az has been previously reported,[7b] but a feature at ~410 nm was overlooked, the weakly negative band in question being resolved in the spectrum reported in Figure 3. (Because of possible interference from nonmetal related protein bands, the spectrum of Co^{II}-Az below 330 nm has been excluded from the analysis.) Stick diagrams representing the relevant parts of the CD spectra are qualitatively drawn for the sake of comparison in Figure 4 and are aligned so that the positions of the most intense visible absorption band of Cu^{II}-Az and its analogues in the spectra of Co^{II}-Az and Ni^{II}-Az have been adjusted to 0.0 on the energy axis. The evident correspondence among the spectral features from derivative to derivative is remarkable in view of the fact that CD spectra are known to be extremely sensitive to effects of the local environment.[20] Indeed, the marked similarity of the spectra argue that the chromophores are located in rather similar environments. Inspection shows that four independent charge-transfer transitions are resolved in the spectrum of Cu^{II}-Az and five in the case of Ni^{II}-Az. Because of the background problem mentioned above, only the three low-energy bands are considered for Co^{II}-Az.

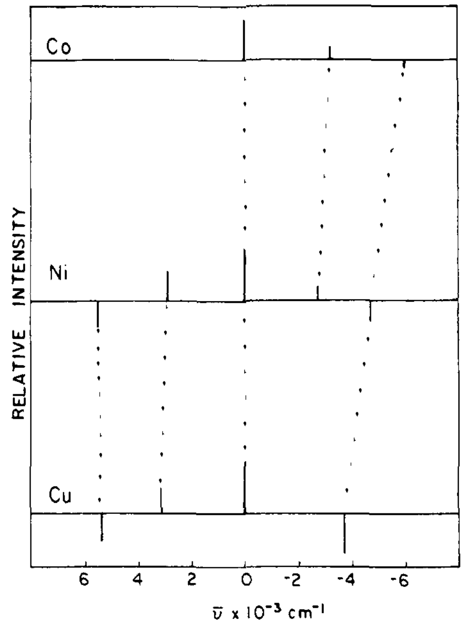


Figure 4. Representations of the CD spectra of Co^{II}-Az, Ni^{II}-Az, and Cu^{II}-Az. The σS(cys) → M(II) charge transfer transition of each spectrum has been adjusted 0.0 on the energy axis.

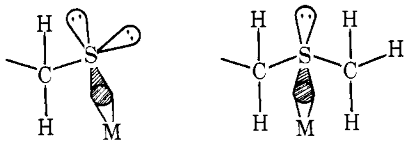
Table III. Estimation of X_L

system	$\bar{\nu}_{obs}$, μm^{-1}	$\bar{\nu}_{cor}$, μm^{-1}	X_M	X_L^{calcd}
Co^{II}-Az	2.68	1.51	1.9	2.40
Ni^{II}-Az	2.00	1.08	2.1	2.46
Cu^{II}-Az	1.22	0.40	2.4	2.53

Using the semiempirical optical electronegativities of Jørgenson,[21] it is possible to analyze the charge-transfer bands in some detail. The method has previously been used in conjunction with absorption data to assign the most intense band of Cu^{II}-Az in the visible region.[8] Using the information revealed by the CD spectra, however, a more complete analysis is possible. Briefly, the analysis entails relating the energies of the charge-transfer bands to empirically derived parameters characteristic of the centers involved. The equation

$$\bar{\nu}_{cor} = 3.0(X_L - X_M) \qquad (1)$$

relates X_L, the optical electronegativity of the ligand, and X_M, the optical electronegativity of the metal, to $\bar{\nu}_{cor}$, a corrected transition energy expressed in μm^{-1}. The quantity $\bar{\nu}_{cor}$ is obtained from the experimentally observed transition energy $\bar{\nu}_{obsd}$ by correcting for certain interelectron repulsion effects and effects of ligand field splitting.[21] The relevant correction factors have already been developed[8] and will be assumed for the purposes of this analysis. The lowest energy relatively intense charge transfer transition is found at 2.68 μm^{-1} in the absorption spectrum of Co^{II}-Az and at 1.22 μm^{-1} in the case of Cu^{II}-Az.[19] In the case of Ni^{II}-Az, the analogous band is poorly resolved in the absorption spectrum, but it can be assigned an energy of 2.00 μm^{-1} from the CD spectrum. From these data, the correction factors,[8] the X_M values of Co(II), Ni(II), and Cu(II),[21] and eq 1, an optical electronegativity, X_L^{calc}, can be computed as seen in Table III. The average value of 2.46 is clearly well below the values of 2.9[22] and 2.8[8] which have been calculated for thioether donors such as a methionine sulfur. Previously, X_L for cysteine sulfur was estimated using the spectral data of spinach ferredoxin;[8] however, a more reliable estimate can be obtained from the spectral data of rubredoxin, for which detailed assignments have been made.[23] Using the previously cited correction factors,[8] a X_M value for Fe(III) of 2.4, and the $\pi t_1 \rightarrow e$ charge transfer band at 1.35 μm^{-1},[23] we find that $\bar{\nu}_{cor}$ = 0.23 μm^{-1} and that X_L = 2.48. The striking agreement between this value and that derived from the spectra of the azurin derivatives firmly establishes the assignment of the low-energy charge-transfer bands of the azurin derivatives as πS → metal transitions involving non-σ-bonding "lone pairs" of a cysteine sulfur. Only one band is resolved in this region in the case of Cu^{II}-Az whereas two bands are resolved in the CD spectra of Ni^{II}-Az and Co^{II}-Az (see Figure 4). Two effects are evident which could give rise to the splitting in the latter derivatives.[24] One is that the d^7 (Co(II)) and d^8 (Ni(II)) metals have more than one unfilled d orbital, and, in noncubic symmetry, transitions terminating in the different metal orbitals may occur with different energies. However, unless a very fortuitous overlapping of components occurs in the spectra of *both* Co^{II}-Az and Ni^{II}-Az, the magnitude of this effect would appear to be small since analogous splittings of the higher energy charge-transfer bands are not observed. An alternative explanation can be based on the fact that two πS → metal transitions are possible from a cysteine sulfur. Crudely, as an aid to visualizing the orbitals involved, one can imagine that a transition can occur from either of two "lone pairs" of the cysteine sulfur, which have approximate π symmetry with respect to the metal σ bonding orbital and which may have different energies depending on the local environments. The π "lone pairs" are illustrated in the diagram below, where the



shaded orbital is involved in the σ bond. The fact that the splitting is not observed in the case of Cu^{II}-Az could obtain because of small differences in its site geometry, or possibly

Table IV. Detailed Band Assignments

system	energy, μm^{-1}	assignment
Cu^{II}-Az	1.24	$\pi S(Cys) \rightarrow Cu(II)$
	1.61	$\sigma S(Cys) \rightarrow Cu(II)$
	1.92	$\pi S(Met) \rightarrow Cu(II)$
	2.14	$\sigma S(Met) \rightarrow Cu(II)$
Ni^{II}-Az	1.78	$\pi S(Cys) \rightarrow Ni(II)$
	2.00	$\pi S(Cys) \rightarrow Ni(II)$
	2.28	$\sigma S(Cys) \rightarrow Ni(II)$
	2.55	$\pi S(Met) \rightarrow Ni(II)$
	2.82	$\sigma S(Met) \rightarrow Ni(II)$
Co^{II}-Az	2.45	$\pi S(Cys) \rightarrow Co(II)$
	2.68	$\pi S(Cys) \rightarrow Co(II)$
	3.00	$\sigma S(Cys) \rightarrow Co(II)$

because the other component is hidden under another transition. In either case, or if a combination of the two effects is operative, it should be noted that the fundamental band assignment remains $\pi S(Cys) \rightarrow$ metal.

We may now shift our attention to the higher energy charge-transfer bands. Based on energy and relative intensity considerations,[7a,21] the intense bands associated with the transitions which appear at the zero of energy in Figure 4 are readily assigned as the $\sigma S \rightarrow$ metal transitions from cysteine.

For the donor involved in the next lowest energy transition (1.92 μm^{-1} in Cu^{II}-Az and 2.55 μm^{-1} in Ni^{II}-Az) we calculate an average optical electronegativity of 2.82. This value is in good agreement with the estimates of the optical electronegativity of methionine sulfur cited above, and therefore the bands may be assigned to S(Met) → metal charge transfer. In principle, as can be appreciated from the diagram above, both $\sigma S \rightarrow$ metal and $\pi S \rightarrow$ metal components are expected from a thioether donor; hence the possibility arises that the bands at 1.92 and 2.14 μm^{-1} in the spectrum of Cu^{II}Az could both be associated with methionine sulfur. In previous work the 2.14-μm^{-1} transition has been assigned as a π(amide) → copper[18] and as a copper → π^*(tyrosine)[26] transition, and an apparently analogous band in ceruloplasmin has been assigned as tyrosine → copper charge transfer.[27] There is, however, no evidence to suggest that these groups are involved in the type 1 copper site.[28] Instead, NMR[29-31] results and the X-ray work[3] suggest that two imidazole nitrogens from histidine residues are involved. Since charge-transfer bands involving the latter should occur well into the UV region of the spectrum,[26] it seems most reasonable to assign the bands in question as the π and the σ components of S(Met) → Cu(II) charge transfer. The detailed assignments that we propose are listed in Table IV and we emphasize that *all of the bands considered are reasonably assigned and correlated with optical electronegativities invoking only the involvement of a cysteine sulfur and a methionine sulfur.*

It is interesting that in the spectrum of Cu^{II}-Az the charge-transfer bands assigned to the methionine sulfur are considerably less intense than those assigned to the cysteine sulfur. (This pattern is less pronounced in the spectrum of Ni^{II}-Az; see Figure 2.) In this regard it must be remembered that the intensities of these transitions will in general be very sensitive to orbital overlap patterns, relative bond distances, etc. Moreover, the chromophores can interact with each other, altering the "intrinsic" intensity patterns associated with each. In the case of a dipole-dipole coupling, the intensity pattern is very sensitive to the relative orientation of the oscillators.[32] Alternatively, the coupling can be mediated through bonds involving metal orbitals.[33] In any case the detailed intensity

As far as the geometrical arrangement of the donors is concerned, the evidence suggests that distorted tetrahedral geometry obtains, at least for Cu^{II}-Az, Co^{II}-Az, and Ni^{II}-Az. That distorted tetrahedral geometry is involved in the case of Cu^{II}-Az is shown by the analysis of the ligand field bands.[18] The large molar absorptivities observed for the ligand field bands of Co^{II}-Az (see Table II) strongly supports the proposal that the Co(II) is found in a distorted tetrahedral environment.[7a] In the case of Ni^{II}-Az the resonance Raman spectrum has been found to have bands related to those of Cu^{II}-Az and has been shown to be consistent with a pseudo-tetrahedral coordination environment.[34] Finally, it can be noted that the excellent correlation of optical electronegativities reported herein supports the idea that the different derivatives are characterized by related geometries about the metal centers.

The binding site of azurin appears to an S_2N_2 donor set with distorted tetrahedral geometry, and hence is closely related to the binding site found in plastocyanin. Not surprisingly, charge-transfer bands having energies similar to those of Cu^{II}-Az are found in the spectrum of plastocyanin,[18] and, accordingly we suggest that analogous assignments can be made for them. Similar bands are also found in the spectrum of stellacyanin, which has a cysteine residue, *but no methionine residue.*[35] Evidently, another group having σ and π optical electronegativities similar to those of a thioether group is involved. We suggest that the donor is probably a disulfide sulfur from a *cystine* residue.

Less information was available from the manganese derivative than was the case with the other derivatives, largely because no charge-transfer bands were observed in its spectrum. The spin pairing energy[21] is large for charge transfer transitions involving an $S = \frac{5}{2}$ system and the optical electronegativity of manganese is a relatively small value.[21] For these reasons the charge-transfer bands would be expected at high energies, too high to observe in this case. We also searched for an EPR signal from Mn^{II}-Az, but the only signals we found were very weak and quite similar to those we observed for buffer solutions of Mn(II).

A final point of interest concerns the qualitative trend in the rate of uptake of the different metal ions by apoazurin—especially the fact that Cu(II) reacts considerably more rapidly than does Co(II), Ni(II), or Mn(II). As discussed by Silva and Williams,[36] kinetic factors can operate in living systems to effect the selective uptake of essential elements, including metal ions. Thus, the fact that apoazurin reacts more rapidly with Cu(II) than with other metal ions may be a significant factor in the synthesis of azurin in vivo.

After this manuscript was submitted, a report of the X-ray absorption spectrum of Cu^{II}-Az appeared.[37] The results indicated that two nitrogens and two sulfurs are probably coordinated to the copper, consistent with our results and the report of the crystal structure of azurin.[3]

Note Added In Proof. After our manuscript was submitted, a paper appeared which also considered the possibility that the ~450-nm band of stellacyanin could be assigned as S(cystine) → Cu(II).[41]

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Electrochemical Studies on a Series of Manganese(III) Complexes Containing Symmetrical Pentadentate Ligands

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Abstract: The redox properties of a series of manganese(III) complexes incorporating dianionic pentadentate Schiff-base ligands have been examined using cyclic voltammetry. Substituents on the aromatic portion of the ligand and changes on the central donor atom were shown to cause changes in the reduction potentials of the Mn(III) ion. Electron-withdrawing substituents on the aromatic portion of the ligand resulted in a lower reduction potential relative to complexes possessing ligands with electron-donating groups. Changes in the reduction potential were also dependent upon the type of central donor atom and its substituent. Correlation of electrochemical data on these complexes with previously observed reactivity patterns of several of the same complexes with dioxygen and nitric oxide is described.

Introduction

The role that manganese plays in the photosynthetic liberation of dioxygen from water and the electron-transfer reactions of mitochondrial superoxide dismutase are undoubtedly related to the ability of the manganese ion to function as a redox catalyst.[2] The capacity of the metal ion to change between oxidation states can be related in part to the ligand field produced about the metal ion.[3] In this regard the oxidation state accessibility of manganese has been explored[4,5] using a variety of macrocyclic ligands. A dinegatively charged unsaturated macrocyclic ligand was observed to stabilize Mn(III) relative to a ligand that is uncharged and saturated. Ring size was also shown in this study to be important in stabilizing certain manganese oxidation states.

The extent and nature of dioxygen reactivity with manganese(II) chelates is also dependent in part on the latter's redox properties. Two reports have recently appeared which deal with the electronic effect of various ligand aromatic substituents on the reactivity of certain manganese(II) complexes toward dioxygen and nitric oxide.[6,7] Complexes with electron-withdrawing substituents were shown to differ significantly in their dioxygen reactivity pattern when compared with complexes possessing electron-donating substituents. In two cases (3-NO_2, 5-NO_2) ligand oxidation was inhibited sufficiently so that several manganese(III) products were obtained depending

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