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Infrared and Visible Circular Dichroism and Magnetic Circular Dichroism Studies on Cobalt(II)-Substituted Blue Copper Proteins

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Abstract: The near-infrared, visible, and near-ultraviolet circular dichroism (CD) and magnetic circular dichroism (MCD) spectra of the cobalt(II) derivatives of *Rhus vernicifera* stellacyanin, *Pseudomonas aeruginosa* azurin, and bean plastocyanin are reported. Bands attributable to transitions to $^4T_1(^4F)$ in a distorted tetrahedral Co(II) center have been observed in the near-infrared region. The centers of gravity of $^4T_1(^4F)$ are estimated to be 8250, 8375, and 8450 cm^{-1} above the $^4A_2$ ground state in the Co(II) derivatives of stellacyanin, azurin, and plastocyanin, respectively. In addition, one component of a split $^4A_2 \rightarrow {}^4T_2$ band has been observed at 5600 cm^{-1} in cobalt(II) stellacyanin. The average positions of the ligand field states are described satisfactorily by taking $Dq = 490$ and $B = 730$ cm^{-1} for all three cobalt(II)-substituted proteins. The ligand field analysis as well as MCD spectral comparisons suggest that the donor groups of the three blue copper sites are probably the same. It is proposed that each blue copper site involves a rather rigidly constrained ligand structure. The variation in the distortion of the site from tetrahedral symmetry in the three blue proteins is not large.

Our understanding of the structure of the blue (or type 1)[2] site in copper proteins has developed rapidly in recent years.[3-8] This understanding is based in part on spectroscopic[6,7] and magnetic[8] studies of the high-spin cobalt(II) derivatives of *Rhus vernicifera* stellacyanin, bean plastocyanin, and *Pseudomonas aeruginosa* azurin. The former experiments have been particularly revealing, as the electronic spectrum of a high-spin cobalt(II) center depends strongly on coordination geometry.[9-11] Our previous work was limited, however, to absorption measurements, and to the visible and ultraviolet spectral regions, where interpretation is complicated by overlap of the highest energy, spin-allowed d-d and the lowest energy charge transfer excitations. We have extended our absorption studies, therefore, to include circular dichroism (CD) and magnetic circular dichroism (MCD) measurements, as each spectroscopic method is associated with different selection rules and allows complementary information to be obtained. Further, the different selection rules associated with the dichroism methods have allowed us to investigate the low energy near-infrared ligand field transitions that often are obscured in absorption owing to protein and solvent vibrational overtone bands. From this investigation we have been able to reach definite conclusions regarding the geometry of the Co(II) binding site in the blue protein derivatives.

Experimental Section

Bean plastocyanin was purified by a standard procedure.[12] Stellacyanin was extracted and purified as described previously.[13] Azurin from *Pseudomonas aeruginosa* (strain no. 10145, American type culture collection) was obtained by a literature method.[14] The cobalt(II) derivatives of the three blue proteins were prepared as described earlier.[6,7] Protein samples for the near-infrared CD work were lyophilized and equilibrated twice with D_2O and finally dissolved in deuterated Tris buffer (0.025 M, pH 8.1). The protein concentrations used were in the range 0.5-1.0 mM.

Absorption spectra were obtained using a Cary 17 spectrometer. The CD and MCD spectra in the visible region were measured on a Cary 61 spectropolarimeter. Measurements in the near-infrared region were performed on a specially designed instrument that has been described previously.[15,16] The sign of the CD was determined using a nickel(II) tartrate solution. Magnetic circular dichroism measurements were made using a Varian superconducting magnet. Fields of approximately 40 kG were used in the experiments. CD and MCD are reported in $\Delta\epsilon$ and $[\theta]_M$ units, being normalized to a field of 10 kG in the case of MCD. The MCD spectra were corrected for the presence of natural optical activity.

Results and Discussion

Figures 1-3 present the visible-uv absorption, CD, and MCD spectra of cobalt(II) derivatives of stellacyanin, plastocyanin, and azurin. The absorption and MCD spectra are seen to be quite similar for the three proteins, whereas the CD curves exhibit substantial differences. Through a comparison of the absorption and MCD spectra, the charge transfer manifold can be distinguished from the ligand field region. The charge trahsfer transitions exhibit a characteristic MCD spectrum, which shifts over the series independently of the ligand field bands at lower energy. No d $\rightarrow$ d transitions are found to be at energies above 20 500 cm^{-1}, a region in each absorption spectrum that is obscured by the charge transfer bands. The lack of a d-d band centered at ~22 000 cm^{-1} rules against a square pyramidal structure, as such cobalt(II) complexes are expected to exhibit a $^4A_2 \rightarrow {}^4A_2$ transition at approximately this energy.[17]

Further, the MCD spectra in the region of 12 500-20 500 cm^{-1} (Figures 1-3) may be compared to those of Co(II) model complexes.[18-21] These comparisons allow us to exclude trigonal bipyramidal Co(II) complexes as models for the metal-binding site. Although such complexes exhibit two absorption bands[17] in the visible region, their MCD spectra show large negative

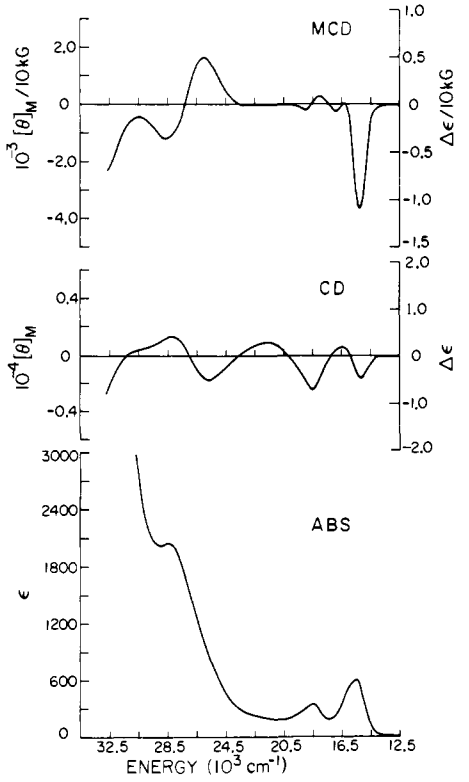

Figure 1. Electronic absorption (below), CD, and MCD (above) spectra of cobalt(II) stellacyanin in the uv-visible region. Spectra were taken at 25 °C in Tris buffer (pH 8).

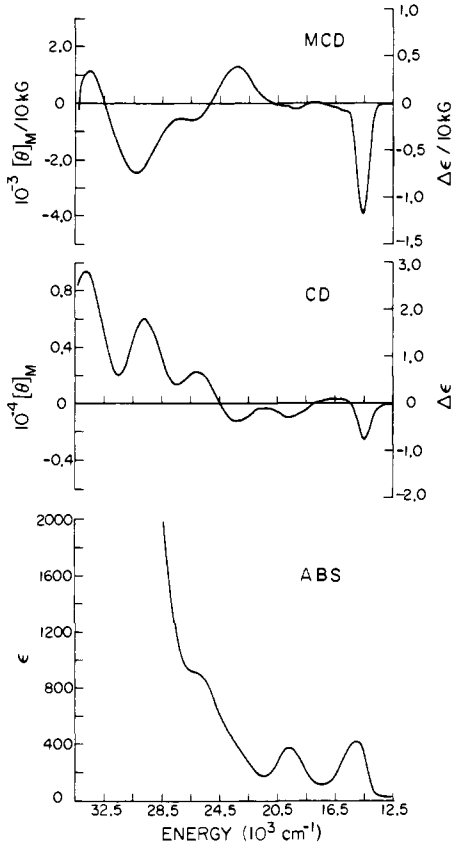

Figure 2. Electronic absorption (below), CD, and MCD (above) spectra of cobalt(II) plastocyanin in the uv-visible region. Spectra were taken at 25 °C in Tris buffer (pH 8).

peaks[18] that contrast with the relative lack of MCD activity associated with the highest energy d-d band in each of the cobalt(II) proteins. The latter behavior, however, is consistent

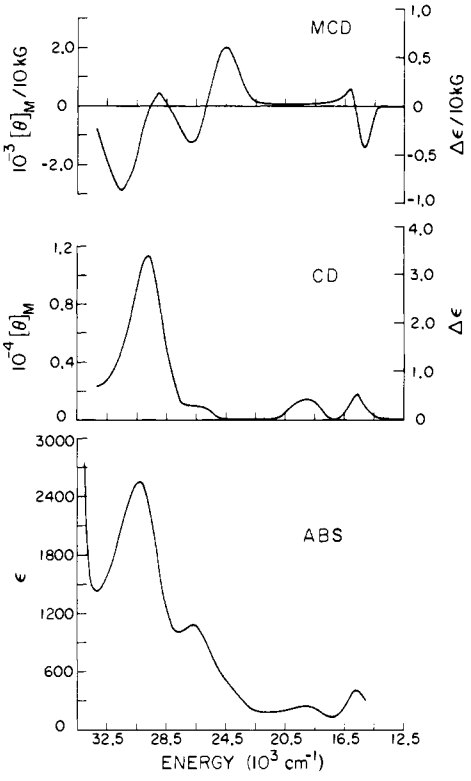

Figure 3. Electronic absorption (below), CD, and MCD (above) spectra of cobalt(II) azurin in the uv-visible region. Spectra were taken at 25 °C in Tris buffer (pH 8).

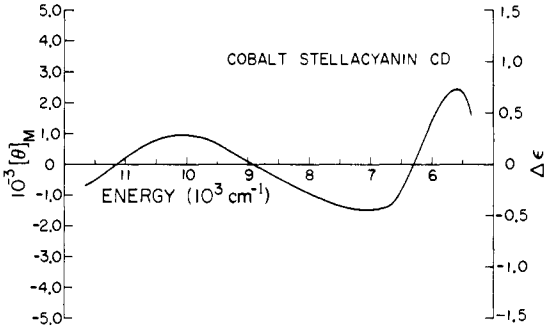

Figure 4. Near-infrared CD spectrum of cobalt(II) stellacyanin in D_2O-Tris buffer (pH 8.1) at 25 °C: pathlength, 1 cm; protein concentration, 0.1 mM.

with that shown by pseudo-tetrahedral compounds of Co(II).[18,20]

Further structural insight may be gained from an analysis of the d-d transitions in the near-infrared region. The latter transitions are difficult to observe in absorption spectral experiments, owing to their very low intensities and overlap with vibrational overtone bands. Vibrational bands, however, exhibit very small CD[16] and MCD and electronic transitions can be more sensitively observed with these techniques. Further, magnetic dipole allowed transitions give rise to large natural CD and this phenomenon is particularly well suited to the identification of the lowest d-d transitions of Co(II). The CD spectrum for cobalt(II) stellacyanin down to ~5400 cm^{-1} is shown in Figure 4. Transitions are clearly observable down to the lowest measured energy. This fact combined with the high intensities of the ligand field absorptions observed in the visible region eliminate an octahedral (six-coordinate) site possibility.[9-11] Indeed, it is evident that only a distorted tetrahedral geometry can accommodate satisfactorily all the available spectroscopic results on the Co(II) derivatives of the blue proteins. A further indication of a distorted tetrahedral site

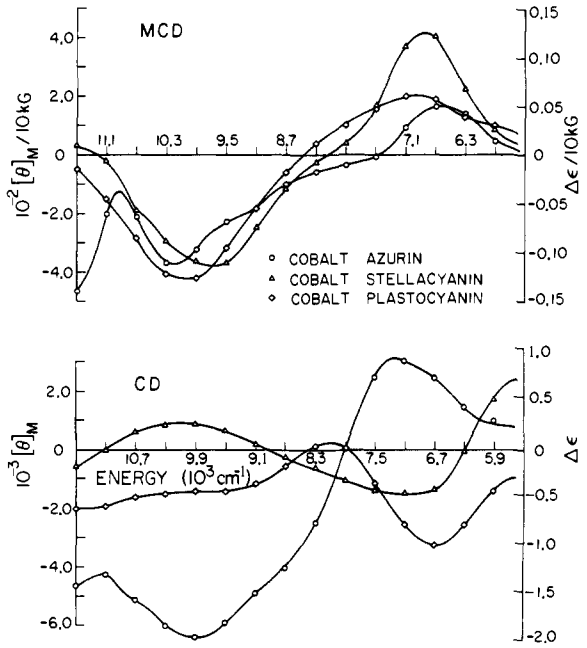

Figure 5. CD (below) and MCD (above) spectra in the near-infrared region for the Co(II) derivatives of stellacyanin (△), plastocyanin (◇), and azurin (○).

Table I. Energies of the Ligand Field States in Co(II) Protein Derivatives[a]

		$^4T_1(^4F)$			$^4T_1(^4P)$		
Protein	$^4T_2$			Av			Av
Stellacyanin	5600	6800	9 700	8250	15 500	18 500	17 000
Azurin	*b*	6600	10 150	8375	15 700	19 200	17 400
Plastocyanin	*b*	6900	10 000	8450	15 200	19 700	17 400

[a] Energies in cm^{-1}; ground state (zero) is $^4A_2$. [b] Not observed.

structure is contained in the CD and MCD spectra shown in Figure 5. The second spin-allowed transition in a tetrahedral d^7 complex is $^4A_2 \rightarrow {}^4T_1(^4F)$ and the effects of spin-orbit coupling are such that *C* terms of opposite sign (pseudo A term) result.[22] This pattern is observed for all three cobalt(II)-substituted proteins.

Viewed together, our results provide a convincing case for a distorted tetrahedral coordination geometry for Co(II) positioned in a type 1 copper site. Moreover, it appears that the metal-binding site must be fairly rigid, as a very similar geometrical structure has been deduced for Cu(II) from extensive spectroscopic studies of native stellacyanin, plastocyanin, and azurin.[3] Near-tetrahedral coordination for Cu(II) is particularly impressive, as in this case the site-structure rigidity built by the protein must overcome electronic stabilization energy associated with a Jahn-Teller distortion toward square planar geometry. Such site inflexibility contrasts with the situation observed in carboxypeptidase A, where substantial variations in coordination geometry among various dipositive metal ion derivatives have been observed.[9,10]

The d-d transitions in each of the cobalt(II) proteins may now be assigned in detail. The two visible bands must be low-symmetry-split components of the $^4A_2 \rightarrow {}^4T_1(^4P)$ transition. This splitting cannot be associated with spin-orbit coupling, as the variation is too large over the three coblat(II) proteins. The average transition energy is estimated to be 17 200 cm^{-1}. The approximate energy of the $^4T_1(^4F)$ state is obtained by averaging the positions of the two oppositely signed *C* terms associated with the splitting of $^4A_2 \rightarrow {}^4T_1(^4F)$. These peak energies are set out in Table I. A ligand field calculation using the average term energies gives $Dq = 490$ and $B = 730$ cm^{-1}, which are quite reasonable parameter values for the proposed[3] N_2N^*S donor set.[23] The larger CD intensity of the 5600-cm^{-1} band in the cobalt(II) stellacyanin spectrum (Figure 4) indicates its assignment as one component of the magnetic-dipole-allowed $^4A_2 \rightarrow {}^4T_2$ transition, which should be centered around $10Dq = 4900$ cm^{-1}.

It is of interest that the average positions of the d-d transitions of the three Co(II) protein derivatives may be described satisfactorily by quite similar *Dq* and *B* values. Only the low symmetry splitting is different in the three cases, as judged by the positions of the components of $^4A_2 \rightarrow {}^4T_1(^4P)$ and the natural CD. Thus, the ligands comprising the blue copper site must be the same, or at least extremely similar, in the three proteins studied.

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