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Chemistry

Spectroscopic studies and a structural model for blue copper centers in proteins

(blue copper/tetrahedral geometry/near-infrared spectra/*d–d* transitions/charge transfer transitions)

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ABSTRACT **Low temperature absorption, circular dichroism, and magnetic circular dichroism spectral studies of the blue copper proteins *Rhus vernicifera* stellacyanin, bean plastocyanin, and *Pseudomonas aeruginosa* azurin have been made. Low energy bands attributable to the *d–d* transitions $^2B_2 \rightarrow {}^2E$ and $^2B_2 \rightarrow {}^2B_1$ in a flattened tetrahedral (D_{2d}) copper(II) center are observed in these proteins at about 5000 and 10,000 cm^{-1}, respectively. The band positions accord well with ligand field calculations based on a tetrahedral structure that is distorted approximately 6° toward a square plane. The ligands in this flattened tetrahedral coordination unit in bean plastocyanin are identified from various spectroscopic experiments as His-38, Cys-85, His-88, and a deprotonated peptide nitrogen (N*) a few residues above His-38. Based on a comparison of absorption and CD intensities, the characteristic bands at about 13,000, 16,000, and 22,000 cm^{-1} in the blue proteins are assigned to the ligand to metal charge transfer transitions $\pi S \rightarrow d_{x^2-y^2}$, $\sigma S \rightarrow d_{x^2-y^2}$, and $\pi N^* \rightarrow d_{x^2-y^2}$, respectively, in the flattened tetrahedral CuN_2N^*S unit.**

X-ray crystal structure analysis has not been completed to date for any blue (or type 1) copper protein. The probability that the copper coordination environment is highly unusual, however, has long been recognized, as a result of various spectroscopic studies (1–13). A typical blue protein exhibits an intense absorption peak in the region 600–630 nm ($\epsilon >$ 3000), which is flanked by weaker bands at 450 and 800 nm. The natural circular dichroism (CD) spectra of blue copper proteins are also distinctive and similar to one another. Negative rotations are observed at 450 and about 800 nm, corresponding to the weaker absorptions, whereas a smaller positive band with a shoulder toward high energy is present in the 600 nm region (8–10). These spectral features have been assigned variously to *d–d* excitations or to charge transfer from a strongly reducing ligand to the copper(II).

Recent spectroscopic studies of the cobalt(II) derivatives of *Rhus vernicifera* stellacyanin, bean plastocyanin, and *Pseudomonas aeruginosa* azurin have indicated that at least some of the bands in the native proteins are attributable to charge transfer transitions. An intense band ($\epsilon > 2000$) that appears to be analogous to the 600 nm feature of the blue proteins is observed between 300 and 350 nm in each of the cobalt(II) derivatives (14). This shift in band position of about 16,000 cm^{-1} to higher energy [Co(II) > Cu(II)] accords well with that anticipated for a ligand to metal charge transfer transition. Assignments of specific charge transfer transitions in the native blue proteins, however, remain to be determined.

The pattern of *d–d* bands observed in the Co(II) derivatives is consistent with a distorted tetrahedral geometry for the blue site (14). Whether the site forces Cu(II) to adopt the same geometrical structure is an important question, and one that has not been answered satisfactorily prior to the present investigation. Resonance Raman spectral data on single-copper blue proteins have been reported by two groups, with somewhat different interpretations. Siiman *et al.* (15) have argued that a distorted tetrahedral geometry is to be preferred for blue copper, whereas Miskowski *et al.* (16) have suggested a five-coordinate site structure. In a recent paper, Morpurgo *et al.* (17) have also adopted a five-coordinate model for the blue copper site.

Distorted tetrahedral Cu(II) should exhibit *d–d* transitions well below 10,000 cm^{-1}, assuming that the ligand field is not unusually strong. We have undertaken, therefore, a detailed investigation of the near-infrared absorption, CD, and magnetic circular dichroism (MCD) spectra of stellacyanin, plastocyanin, and azurin. Detailed analysis of these and analogous visible spectral data has allowed assignments to be made of both the *d–d* and charge transfer transitions. Interpretation of the charge transfer spectra has been aided further by the identification of the probable ligands of Cu(II) in bean plastocyanin.

MATERIALS AND METHODS

French bean plastocyanin (*Phaseolus vulgaris*) (18), azurin (*Pseudomonas aeruginosa*) (19), and stellacyanin (*Rhus vernicifera*) (20) were purified by standard methods. Near-infrared spectra were obtained on protein films to minimize interference by water absorption and permit a wide variation of temperature. The protein solutions were dialyzed against deionized distilled water and concentrated, first by pressurized membrane ultrafiltration and then, by placing drops on a plexiglas disk in a metal dessicator over Drierite. After three of four drops had been successively concentrated (ten to fifteen for the thick films), the film was prepared by transferring the disk to a dessicator containing a saturated potassium acetate solution. This controlled humidity allowed the film to form slowly, thereby preventing most of the cracking caused by rapid removal of excess water. The drying process was halted by transfer of the film to a dessicator charged with a saturated sodium hydrogen phosphate solution.

The near-infrared CD and MCD spectra were obtained with samples run in deuterated phosphate buffer in order to extend the spectral range to about 1850 nm. Although the infrared overtones of water do not give rise to a measureable CD spectrum, an absorbance of 1.0 or greater diminished the light level to the limit of detector response. The deuterated protein solutions were prepared by evaporating unbuffered, concentrated aqueous solutions in a Drierite dessicator as described above. The films were then transferred to a

Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; XPS, x-ray photoelectron spectra; NMR, nuclear magnetic resonance.

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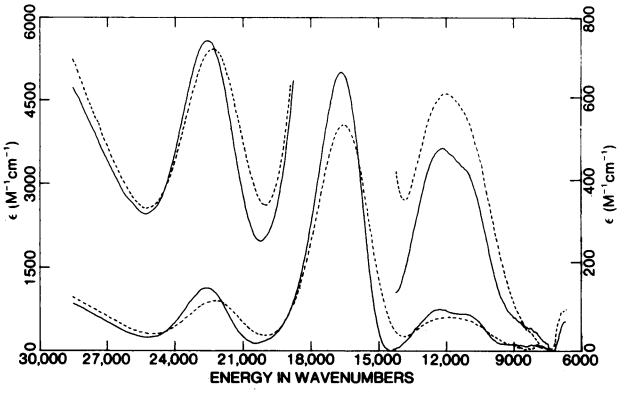

FIG. 1. The near-infrared and visible absorption spectra of stellacyanin films at 270 (——) and 35 K (– – –). Lower curves, left-hand ϵ scale; upper curves (thick film), right-hand ϵ scale (ϵ values are in M^{-1} cm^{-1}).

dessicator charged with D_2O. The films were allowed to equilibrate overnight, dried again to a film, and placed a second night in the D_2O humidifier. After the second equilibration with D_2O, the solutions were evaporated to a film and the film dissolved in deuterated phosphate buffer at pD 6. The deuterated buffer was prepared by dissolving vacuum-dried Na_3PO_4 in D_2O to form a 0.05 M solution. The pD was adjusted to 6 by adding small volumes of phosphoryl chloride ($POCl_3$), which reacted with D_2O to form D_2PO_4 and DCl. The final concentration of stellacyanin was 1.06 mM. Azurin concentrations were 0.69 and 1.50 mM for the near-infrared MCD and CD spectra, respectively. The corresponding plastocyanin concentrations were 0.41 and 2.21 mM. Solutions for the visible CD spectra were prepared by dilution with deuterated phosphate buffer until an absorbance less than 1.0 in a 1 cm cell was recorded for the 600 nm band. The final concentrations were 0.206, 0.210, and 0.197 mM for stellacyanin, azurin, and plastocyanin, respectively. The proteins were reduced by adding a small amount of concentrated sodium dithionite dissolved in nitrogen-purged D_2O.

Absorption spectra were measured on a Cary 17I spectrometer. Samples were cooled by a Cryogenics Technology, Inc., model 20 cryocooler equipped with quartz windows and a temperature controller, which allowed adjustment of the temperature to ±1 K over most of the range used. Visible CD spectra were recorded on a Cary 61 spectropolarimeter. Near-infrared CD and MCD spectra were recorded on a specially-built instrument in the laboratory of Prof. P. J. Stephens at the University of Southern California (21). Baselines were determined using deuterated phosphate buffer as a blank.

RESULTS

The 270 K absorption spectrum of a stellacyanin film from 28,000 to 7000 cm^{-1} is consistent with solution data reported by Peisach *et al.* (4). A new band at about 9000 cm^{-1} is partially resolved when the film is cooled to 35 K (Fig. 1). Our visible CD curve of stellacyanin accords well with the spectrum reported by Falk and Reinhammar (9). Near-infrared CD spectra of stellacyanin are shown in Fig. 2. Bands are prominent in the expanded spectrum (B) at 8100 and approximately 5000 cm^{-1}. The latter band abruptly ends owing to the onset of D_2O vibrational absorption. The MCD curve in the near-infrared shows weak positive and negative features at about 10,800 and 8800 cm^{-1}, respectively.

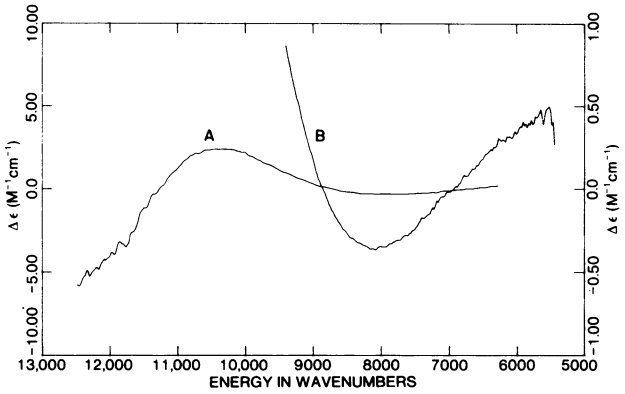

FIG. 2. The near-infrared CD spectra of 1.06 mM stellacyanin in a pD 6 deuterated phosphate buffer at 290 K. Curve A, left-hand $\Delta\epsilon$ scale; curve B, right-hand $\Delta\epsilon$ ($\Delta\epsilon$ values are in M^{-1} cm^{-1}).

The 270 K absorption spectrum of bean plastocyanin is similar to that reported for the *Chenopodium album* protein (3). The major absorption band has a maximum at 16,700 cm^{-1} and is assigned the solution ϵ of 4500 (7). Peaks are also observed at 12,900 and 21,800 cm^{-1}. The high energy band shows considerable asymmetry, and a shoulder is resolved (about 24,000 cm^{-1}) at 35 K (Fig. 3). A band at about 10,000 cm^{-1} is also observed in the 35 K spectrum.

The plastocyanin visible CD spectrum has the same general shape as the stellacyanin CD curve. The negative band at 12,800 cm^{-1} and the positive feature at 16,500 cm^{-1} are more intense in plastocyanin, whereas the positive shoulder at about 19,000 cm^{-1} is less prominent. The main difference in the absorption and CD spectra of stellacyanin and plastocyanin occurs in the activity around 22,500 cm^{-1}. In plastocyanin, two absorption peaks are observed, at 21,800 and 24,000 cm^{-1}; the CD curve shows a negative feature at 21,200 cm^{-1} and a positive band at 24,100 cm^{-1}. Stellacyanin exhibits only one absorption band and one CD feature, both at 22,500 cm^{-1}.

A negative band at 9200 cm^{-1} and positive activity at lower energy are observed in the near-infrared CD spectrum of plastocyanin. The CD curve also shows positive activity toward higher energy, as in stellacyanin, and presumably turns negative to match the minimum at 12,500 cm^{-1} in the visible spectrum. The MCD spectrum has a minimum at about 11,000 cm^{-1} and shows slight positive activity at lower energy. All near-infrared CD activity is lost when the sample of plastocyanin is reduced with dithionite.

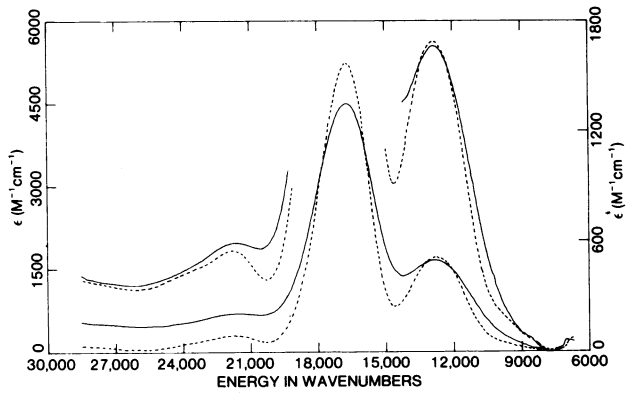

FIG. 3. The near-infrared and visible absorption spectra of plastocyanin films at 270 (——) and 35 K (– – –). Lower curves, left-hand ϵ scale; upper curves (thick film), right-hand ϵ scale.

Table 1. Absorption* and CD spectral data for blue copper proteins

Protein	$\bar{\nu}$ (cm^{-1})	$\Delta\epsilon$ (M^{-1} cm^{-1})	ϵ (M^{-1} cm^{-1})	γ	Assignment
Stellacyanin	5,250	0.45	~100	~0.018	$^2B_2 \rightarrow {}^2E$
	8,750	−0.35	~100	~0.014	$^2B_2 \rightarrow {}^2B_1$
	11,470	2.4	565	0.017	$^2B_2 \rightarrow {}^2A_1$
	13,040	−5.0	341	0.059	$\pi S \rightarrow d_{x^2-y^2}$
	16,580	3.6	3,549	0.0041	$\sigma S \rightarrow d_{x^2-y^2}$
	17,840	0.75	1,542	0.0019	†
	22,570	−7.35	942	0.031	$\pi N^* \rightarrow d_{x^2-y^2}$
Plastocyanin	5,500	0.125	~100	0.005	$^2B_2 \rightarrow {}^2E$
	10,300	−0.165	200	0.0033	$^2B_2 \rightarrow {}^2B_1$
	11,940	~1.5	1,162	0.0052	$^2B_2 \rightarrow {}^2A_1$
	13,540	−3.78	1,289	0.012	$\pi S \rightarrow d_{x^2-y^2}$
	16,600	4.08	4,364	0.0037	$\sigma S \rightarrow d_{x^2-y^2}$
	18,140	0.4	1,163	0.0014	†
	21,540	−1.32	300	0.018	$\pi N^* \rightarrow d_{x^2-y^2}$
	23,640	1.26	~100	~0.050	†
Azurin	10,300	−0.475	82	0.023	$^2B_2 \rightarrow {}^2B_1$
	12,940	−3.6	686	0.021	$\pi S \rightarrow d_{x^2-y^2}$
	15,940	3.96	3,798	0.0042	$\sigma S \rightarrow d_{x^2-y^2}$
	17,770	0.72	504	0.0057	†
	20,840	−1.11	185	0.024	$\pi N^* \rightarrow d_{x^2-y^2}$

* Data for each blue protein are taken from a gaussian resolution of the 35 K near-infrared and visible absorption spectrum.
† Not assigned.

The 270 K absorption spectrum of a film of azurin is in agreement with earlier solution results (8). Narrowing of the 12,400 cm^{-1} band at 35 K reveals a weak absorption at approximately 10,000 cm^{-1} (Table 1). Our CD spectrum of azurin in the visible region is quite similar to that reported by Tang *et al.* (8). The near-infrared CD spectrum shows a minimum at 9250 cm^{-1} and increasing positive activity at lower energy. The corresponding MCD curve exhibits a negative band at about 10,500 cm^{-1}. It should be noted that reduced azurin shows no CD activity at all in the near-infrared region.

DISCUSSION

New absorptions and CD spectral features attributable to *d-d* transitions have been observed in the near-infrared region for stellacyanin, plastocyanin, and azurin (Table 1). The low energies of these transitions (about 5000 and 10,000 cm^{-1}) rule out structures based on six, five, and square-planar-four coordinate geometries for the blue copper center (22, 23). It also has been shown that the ground state of either type of trigonally-distorted, tetrahedral Cu(II) geometry is inconsistent with the electron paramagnetic resonance (EPR) g values for blue proteins (E. I. Solomon, J. W. Hare, and H. B. Gray, manuscript in preparation). Thus, the choice is either a flattened tetrahedral (D_{2d}) structure or a geometry based on a very unusual coordination number for Cu(II). We strongly prefer the flattened tetrahedral structure, as the ligand field treatment below, based on this structure, accommodates the spectroscopic data in a most satisfactory manner.

The coordinate system used for the flattened tetrahedral model is illustrated in Fig. 4. A rotation of 45° about the z-axis transforms this system into the standard tetrahedral one. A distortion from tetrahedral toward square planar geometry may be described by a change of the angle (β) between the z-axis and the metal-ligand bonds. The electronic energies (W) of the ligand field states of Cu(II) as a function of β are as follows (23):

$$W(^2A_1) = W(a_1 = d_{z^2}) = -12(3\cos^2\beta - 1)Ds + 3\delta Dt$$

$$W(^2B_1) = W(b_1 = d_{xy}) = 12(3\cos^2\beta - 1)Ds + 1/2(35\sin^4\beta - \delta)Dt$$

$$W(^2E) = W(e = d_{xz}, d_{yz}) = -6(3\cos^2\beta - 1)Ds + 2\delta Dt$$

$$W(^2B_2) = W(b_2 = d_{x^2-y^2}) = 12(3\cos^2\beta - 1)Ds - 1/2(\sin^4\beta + \delta)Dt$$

where $\delta = 35\cos^4\beta - 30\cos^2\beta + 3$, $Ds = ze\langle r^2\rangle/21a^3$ and $Dt = ze\langle r^4\rangle/21a^5$.

The ground state of Cu(II) in flattened tetrahedral coordination is $^2B_2$. The value of β for stellacyanin was fixed at 60° in order to give reasonable values for $\Delta W(^2A_1 - {}^2B_2)$ in the tetrahedral and square planar limiting geometries. Taking $\Delta W(^2E - {}^2B_2) = 5250$ and $\Delta W(^2B_1 - {}^2B_2) = 8750$ cm^{-1} (Ds = 765, Dt = 444 cm^{-1}), values of $\Delta W(^2A_1 - {}^2B_2)$ are calculated to be 22,800 and 6915 cm^{-1} at the D_{4h} ($\beta = 90°$) and T_d($\beta = 54.74°$) limits, respectively. The energy separation of 22,800 cm^{-1} is correctly denoted $\Delta W(^2A_{1g} - {}^2B_{1g})$, as $^2A_1$ and $^2B_2$ become $^2A_{1g}$ and $^2B_{1g}$, respectively, in D_{4h}. The other D_{4h} transitions, $^2B_{1g} \rightarrow {}^2E_g$ and $^2B_{1g} \rightarrow {}^2B_{2g}$, are predicted at 24,890 and 15,550 cm^{-1}, respectively. All the calculated D_{4h} values should be reduced by about 20%, allowing for the slight increase (about 0.1 Å) in metal-ligand bond lengths that is expected to accompany a change from tetrahedral to square planar coordination. Such an increase has been observed for $CuCl_4^{2-}$, which exhibits both flattened tetrahedral and square planar structures (24). The ad-

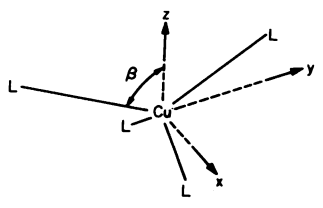

FIG. 4. Reference coordinate system for the flattened tetrahedral (D_{2d}) ligand field model.

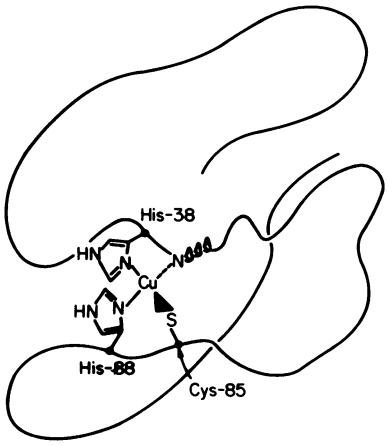

FIG. 5. Structural model for the blue copper center in bean plastocyanin. The coordination core is CuN_2N^*S (β about 60°). The short section of perturbed α-helix is believed to be a few residues above His-38.

justed values for the D_{4h} d-d transition energies are in reasonable agreement with the observed band positions in square planar Cu(II) complexes containing nitrogen-donor ligands (22, 25). In sharp contrast, ligand field parameters derived from β values a few degrees below or above 60° give absurd energy differences in the D_{4h} limit. For example, $\Delta W(^2A_{1g} - {}^2B_{1g})$ is calculated to be 58,800 cm^{-1} from $\beta = 57°$ parameters (Ds = 2240, Dt = 505 cm^{-1}), and 5050 cm^{-1} from $\beta = 70°$ ones (Ds = 77, Dt = 320 cm^{-1}).

The derived ligand field parameters ($\beta = 60°$, Ds = 765, Dt = 444 cm^{-1}) predict $^2A_1$ to be 11,540 cm^{-1} above the $^2B_2$ ground state for stellacyanin. An absorption band and CD maximum are observed near this energy. The resolved absorption band has a molar extinction coefficient of 565, which is approximately five times larger than that observed for the maximum at 8750 cm^{-1}. It is expected that the $^2B_2 \rightarrow {}^2A_1$ transition should be more intense, as it is electric dipole allowed, whereas $^2B_2 \rightarrow {}^2B_1$ is not.

Excited d-level energies for plastocyanin and azurin are very similar. Taking $\Delta W(^2E - {}^2B_2) = 5500$, $\Delta W(^2B_1 - {}^2B_2) = 10{,}300$ cm, and $\beta = 60°$ for both proteins, Ds and Dt are calculated to be 764 and 508 cm^{-1}, respectively, and the $^2B_2 \rightarrow {}^2A_1$ transition is predicted to be at 12,520 cm^{-1}. Plastocyanin exhibits a gaussian-resolved peak at 11,940 cm^{-1} (Table 1), which may be attributed to $^2B_2 \rightarrow {}^2A_1$. The CD activity is somewhat lower in plastocyanin than in stellacyanin, but the ratios between the various transitions in this energy region are similar. In azurin, the relatively intense band at about 13,000 cm^{-1} probably overlaps extensively with absorption owing to $^2B_2 \rightarrow {}^2A_1$.

Structural model for blue copper

The d-d transitions in stellacyanin, plastocyanin, and azurin have been analyzed successfully in terms of a slightly flattened ($\beta = 60°$) tetrahedral geometry for blue copper centers. Research aimed at identification of the ligands comprising such a tetrahedral center has been particularly intense in the case of plastocyanin. Direct evidence for a sulfur ligand has come from x-ray photoelectron spectral (XPS) experiments on bean plastocyanin, where a large shift of the *S2p* core energy of the single cysteine (Cys-85) residue in the protein upon metal incorporation [164.5, apo; 169.8, native; 168.8 eV, Co(II) derivative] was observed (26).

The two histidines in spinach plastocyanin have been found in nuclear magnetic resonance (NMR) titration experiments to exhibit pK values below 5. This suggests that they are coordinated to copper (27). It is reasonable to assume therefore, that the analogous two residues in the bean protein (28), His-38 and His-88, are also ligands. The fourth ligand in the proposed donor set for bean plastocyanin has been identified in extensive infrared spectral studies (29). These experiments have revealed that a short section of α-helix in apoplastocyanin is strongly perturbed upon metal [Cu(II) or Co(II)] incorporation. They thereby implicate a backbone peptide nitrogen or oxygen as a ligand. The preference of copper for nitrogen donors, as well as evidence from charge transfer spectra (*vide infra*), favor coordination by a deprotonated peptide nitrogen (N*). Consideration of the bean plastocyanin sequence places the α-helix, and therefore the backbone peptide nitrogen, a few residues above His-38 (23). Our model for the blue copper center in bean plastocyanin based on the available spectroscopic evidence is shown in Fig. 5.

A CuN_2N^*S (β about 60°) model is also reasonable for azurin. Recent XPS experiments have shown (E. I. Solomon, P. J. Clendening, H. B. Gray, and F. J. Grunthaner, unpublished results) that a sulfur is bound to copper in azurin, and the electronic spectroscopic properties of both Cu(II) and Co(II) forms of the protein are closely similar to those of analogous bean plastocyanin derivatives (14). It is probable that the near-tetrahedral binding site in each blue protein is rather rigid, as ligand-field stabilization factors strongly favor a square planar geometrical structure for four-coordinate Cu(II). The site-structure rigidity built by the protein must overcome the electronic stabilization energy associated with a Jahn-Teller distortion toward square planar Cu(II) geometry, thereby contributing to the relative instability of the oxidized state of the system. Thus the relatively high reduction potentials of blue copper proteins may be attributable, at least in part, to electronic factors associated with the rigidly-constrained, CuN_2N^*S ($\beta = 60°$) site structure.

Charge transfer transitions

We shall now present an interpretation of the intense absorption bands observed at about 13,000, 16,000, and 22,000 cm^{-1} in blue copper proteins. Our analysis is based on the assumption that the energies of the highest occupied ligand orbitals in a CuN_2N^*S unit decrease according to $\pi S > \sigma S > \pi N^* > \pi N$, which would appear to be reasonable in view of the results obtained from studies of charge transfer spectra of related, square planar copper(II) complexes (30–34). Charge transfer excited states, derived from transitions of the type $\pi \rightarrow d_{x^2-y^2}$, are both electric and magnetic dipole allowed. The transition $\sigma S \rightarrow d_{x^2-y^2}$, on the other hand, is only electric dipole allowed. The rotational strength (R) of a CD band is related to the electric dipole (μ_{el}) and the magnetic dipole (μ_{mag}) moments by

$$R = \mathrm{Im}[\int \psi_i^* \mu_{el} \psi_f d\tau \cdot \int \psi_i^* \mu_{mag} \psi_f d\tau] \quad [1]$$

where ψ_i and ψ_f are the initial and final states, respectively (35). The rotational strength may also be determined from the experimental quantity $\Delta\epsilon$, or $\epsilon_l - \epsilon_r$, according to Eq. 2:

$$R = 22.9 \times 10^{-40} \int (\Delta\epsilon/\nu) d\nu \quad [2]$$

Further, the dipole strength (D) is related to the molar extinction coefficient ϵ by

$$D = 91.8 \times 10^{-40} \int (\epsilon/\nu) d\nu \quad [3]$$

and $4R/D \approx \gamma$, the Kuhn anisotropy factor (35). Moscowitz has approximated the integral $\int (\epsilon/\nu) d\nu$ as $\ln 2 \sqrt{\pi}\, \epsilon^0 \delta^0 / \nu^0$,

where ϵ^0 is the maximum value of ϵ, δ^0 is the half-width at half-maximum, and ν^0 is the frequency of ϵ^0 (36). Assuming that δ^0 and ν^0 are the same for corresponding absorption and CD bands, γ may be calculated from Eq. 4:

$$\gamma = |\Delta\epsilon/\epsilon| \qquad [4]$$

Bands associated with π charge-transfer transitions, which are magnetic-dipole-allowed, are expected to have much larger γ values than those attributable to $\sigma S \rightarrow d_{x^2-y^2}$, as the intensity-giving mechanism in the latter case is purely electric dipole in origin. Values of γ, $\Delta\epsilon$, and ϵ for the electronic spectral features of stellacyanin, plastocyanin, and azurin are listed in Table 1. The results clearly indicate that the bands at approximately 13,000 and 22,000 cm^{-1} be assigned to π charge-transfer, as they have relatively large values of γ. Specific assignments are $\pi S \rightarrow d_{x^2-y^2}$ at 13,000 cm^{-1} and $\pi N^* \rightarrow d_{x^2-y^2}$ at 22,000 cm^{-1}. The 16,000 cm^{-1} band in each protein exhibits a γ value well below 0.005, and is attributed to a $\sigma S \rightarrow d_{x^2-y^2}$ charge transfer transition. Finally, it should be noted that theory predicts (37) that the CD associated with π and σ charge transfer transitions will have opposite signs, as is observed experimentally.

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