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The Copper Coordination Group in "Blue" Copper Proteins: Evidence from Resonance Raman Spectra†

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ABSTRACT: Tunable dye laser excitation in the intense ~600-nm absorption band of azurin, plastocyanin, and ceruloplasmin provides resonance enhanced Raman spectra. They consist of a complex set of bands, at least three or four in number, between 350 and 470 cm^{-1}, which are assignable to Cu-N or Cu-O bond stretching, and a weak band near 270 cm^{-1}, which probably arises from Cu-S stretching. A weak band at 765 cm^{-1} found in plastocyanin may arise from C-S stretching. Analysis of the Raman intensity pattern, as well as of the nature of the resonant electronic transition, leads to a model of the "blue" copper site involving approximately trigonal-bipyramidal coordination, with a sulfur and two nitrogen ligands in the equatorial plane, and less strongly bound nitrogen or oxygen ligands at axial positions. This arrangement would be well poised for stabilization of Cu(I) upon reduction.

Among copper containing proteins, those with "blue" copper centers have attracted particular attention because of their intense light absorption ($\epsilon \sim 5 \times 10^3\ M^{-1}\ cm^{-1}$) near 600 nm, which gives rise to their deep blue color (Malkin and Malmstrom, 1970). While the energy of this absorption band, along with those of neighboring, less intense bands, is in line with expected "d-d" electronic transitions centered on Cu^{2+} ions, its intensity is some 50 times greater than is observed for simple complexes.

"Blue" copper centers are found in the copper oxidases which contain several copper atoms per molecule and it was once thought that the anomalous absorption arose from copper-copper interactions. Similar absorption was subsequently found for proteins containing isolated copper atoms, e.g., azurin, stellacyanin, and plastocyanin (the last of which contains two noninteracting copper atoms, one each in two identical subunits), demonstrating that the "blue" copper centers are a unique kind of mononuclear copper complex. The intense visible absorption is associated with copper(II) as shown by electron paramagnetic resonance

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(EPR) spectroscopy. Reduction to copper(I) bleaches the color completely.

The biological function of "blue" copper appears to be electron transfer exclusively. The "blue" copper ions are inaccessible to solvent or exogenous ligands. Plastocyanin is an essential component of the electron transport chain of plant photosynthesis. In the multicopper oxidases, the role of the "blue" copper center appears to be intramolecular electron transfer (Holwerda and Gray, 1974).

Resonance Raman spectroscopy, which involves laser excitation within an absorption band of the molecule under study, can be used to probe the vibrational modes of biological chromophores in their usual high dilution (Spiro, 1974). Here we report resonance Raman spectra of three proteins with "blue" copper centers: azurin, plastocyanin, and ceruloplasmin. They provide evidence on the constitution of the "blue" copper coordination group, and on the nature of the electronic transition. Since submission of this manuscript Raman spectra have been reported for ceruloplasmin and two other "blue" proteins, stellacyanin and laccase, by Siiman et al. (1974). Their data are in good agreement with ours.

Experimental Section

Plastocyanin was prepared from spinach leaves by a combination of published methods. The essential features were: isolation of intact chloroplasts (Katoh and Takamiya, 1964), disruption of these by freezing and detergent treatment (Borchert and Wessels, 1970), and purification of the protein from the chloroplast extract by ammonium sulfate precipitation combined with DEAE-cellulose and Sephadex G-75 chromatography (Katoh et al., 1962). The Raman sample was in 0.01 *M* Tris buffer at pH 7.4 and had a purity index (absorbance at 280 nm/absorbance of 600 nm) of 1.4. Azurin was isolated from *Pseudomonas aeruginosa* by the method of Horio et al., (1963) and then subjected to further chromatography on DEAE-cellulose and Sephadex G-75 columns. The Raman sample had a purity index of 4.1 in 0.01 *M* Tris buffer at pH 7.4. Ceruloplasmin was purchased from Miles Laboratory and was dissolved in pH 5.8 acetate buffer (0.1 *M*). Protein concentrations for the Raman samples were 0.5–0.7 m*M* for azurin, 1.4 m*M* for plastocyanin, and 0.9 m*M* for ceruloplasmin. In experiments to determine Raman intensities, $NaClO_4$ was added to the solutions (0.2 *M*) and protein peak heights were compared to the height of the 928-cm^{-1} (ν_1) peak of ClO_4^-.

Raman spectra were obtained in 1-mm capillary tubes by transverse excitation with the beam of an argon (Coherent Radiation CR-5) or tunable dye (Spectra Physics Rhodamine 6G) laser. The 90° scattered light was analyzed with a Spex 1401 double monochromator equipped with a cooled ITT FW-130 photomultiplier and dc amplification.

Results

Figure 1 compares Raman spectra for the three proteins under study, obtained by excitation in the intense absorption band near 600 nm. While there are clearly differences in detail, the same pattern is observed in each case: a complex set of strong bands between 350 and 470 cm^{-1}, and an isolated weak band near 270 cm^{-1}. The ~270-cm^{-1} band was near the limit of signal/noise resolution for azurin and ceruloplasmin, but it was easily reproducible for plastocyanin, which gave the highest quality spectra. Above 500 cm^{-1} the spectra were featureless, except for a weak band at 765 cm^{-1} found for plastocyanin. The positions of the observed bands are listed in Table I.

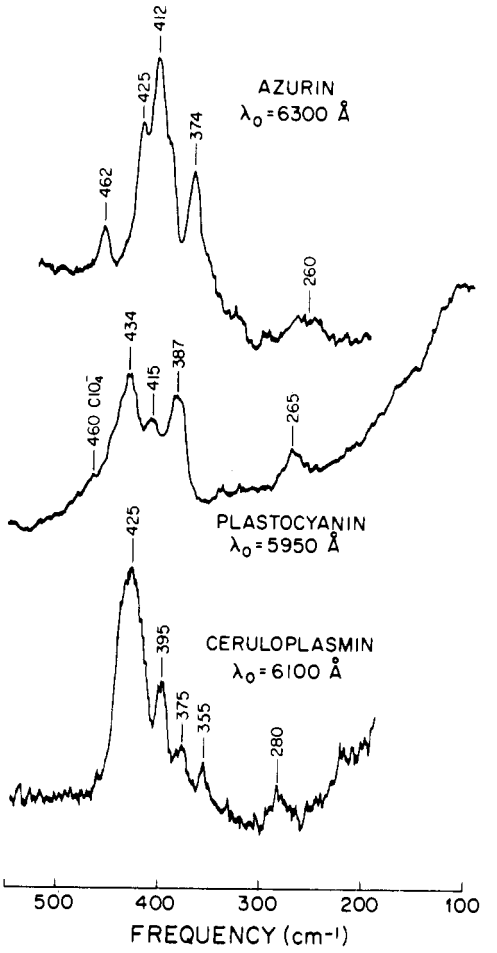

FIGURE 1: Resonance Raman spectra of "blue" copper proteins, obtained with tunable dye (Rhodamine 6G) excitation at the indicated wavelengths; spectrometer parameters: slit width, 10 cm^{-1}; scan rate, 50 cm^{-1}/min; sensitivity, 10^{-9} A; laser power, ~60 mW.

Table I: Vibrational Frequencies (cm^{-1}) Observed in Resonance Raman Spectra of "Blue" Copper Proteins.

Azurin	Plastocyanin	Ceruloplasmin
250	265	280
		355
374	382	375
412	415	395
422	434	425
462		
	765	

The Raman spectra are strongly enhanced via resonance with the ~600-nm electronic transition. Figure 2 shows that, within the limited tuning range of the tunable dye laser, the observed Raman intensities roughly track the strong absorption band for ceruloplasmin and plastocyanin. More definitely, excitation with green (5145 Å) or blue (4880 Å) Ar^+ laser lines, in a region of low absorption, gave negative results, although nonresonance Raman scattering should have been much stronger, both because scattering intensity increases with the fourth power of the incident energy, and because of the greater power of the Ar^+ lines (~1 W vs. ~0.1 W for the dye laser output). The observed Raman bands are all polarized, with the depolarization

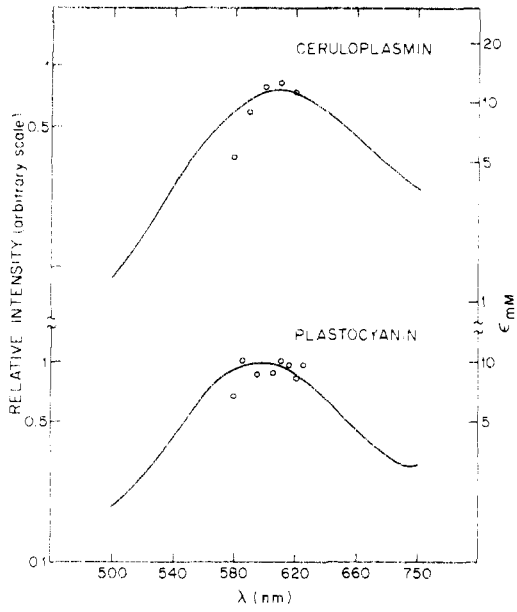

FIGURE 2: Excitation profiles for the 425-cm^{-1} band of ceruloplasmin and the 434-cm^{-1} band of plastocyanin. The solid curves are the absorption spectra and the circles are Raman intensities (peak heights) relative to the ν_1 band of ClO_4^-.

ratio near one-third, as expected for resonance-enhanced totally symmetric vibrations (Albrecht and Hutley, 1971).

No Raman spectra could be obtained on reduced azurin or plastocyanin, using any of the laser frequencies available to us. This negative result is not surprising, since upon reduction the "blue" copper centers lose all absorption in the visible region and therefore, presumably, have no mechanism available for resonance Raman enhancement.

Discussion

Raman Spectral Assignments. Inasmuch as the observed Raman bands are clearly enhanced by resonance with the intense absorption band near 600 nm, they must arise from vibrations which couple to the electronic transition. The chromophore is the "blue" copper center, and the vibrations can confidently be assigned to the copper coordination group. Indeed, the observed frequency shifts are in the range 250–500 cm^{-1}, where copper–ligand stretching vibrations are expected. There are no doubt low-frequency deformation modes of the polypeptide chain which also occur in this range, but it is unlikely that any of them undergo resonance enhancement, especially as higher frequency skeletal polypeptide modes, whose coupling to the chromophore might be more probable, are unobserved.

Consequently, we attribute the observed Raman bands to "blue" copper–ligand stretching modes. In view of the coupling to the ~600-nm absorption band, the Raman spectra contain no information about the non-"blue" copper centers (types II and III) in ceruloplasmin, which do not have intense absorption near 600 nm (Malkin, 1973).

The constitutions of the "blue" copper coordination groups are not known with certainty. Possible ligand atoms are nitrogen, oxygen, and sulfur. A recent ENDOR study of stellacyanin has verified nitrogen coordination (Rist et al., 1970), at a site inaccessible to solvent. Sulfhydryl coordination has been suggested by *p*-mercuribenzoate binding studies on azurin (Finazzi-Agro et al., 1970), plastocyanin (Katoh and Takamiya, 1964), and stellacyanin (Morpurgo et al., 1972; McMillin et al., 1974), and confirmed for stellacyanin by spectroscopic characterization of a cobalt(II) derivative (McMillin et al., 1974). Mercaptide sulfur ordinarily reduces copper(II), with production of disulfide. Presumably this reaction is prevented in the protein by steric inaccessibility of a second mecaptide group and/or a second copper(II) ion. On the other hand Briving and Deinum (1975) report evidence that tree laccase contains two disulfide links and only one free sulfhydryl, and that the latter can be bound by mercurial agents without loss of the "blue" copper ESR signal. Fluorescence studies implicate a tryptophan residue near the "blue" copper site of azurin (Finazzi-Agro et al., 1970).

The complex of strong Raman bands observed for azurin, plastocyanin, and ceruloplasmin fall in the frequency range 350–470 cm^{-1}, which is characteristic for the stretching of Cu–N or Cu–O (Nakamoto, 1970) when the ligand atoms are not part of, or attached to, an aromatic ring. The ligand atoms responsible for these vibrations could therefore be nitrogen from lysine or arginine side chains, oxygen from glutamate, aspartate, or serine side chains, or they could be nitrogen and/or oxygen from peptide units. Studies of complex formation by peptides have documented the avidity of Cu^{2+} for peptide nitrogen (Freeman, 1973).

When the ligand atom is part of, or attached to an aromatic ring, the metal–ligand stretching frequency decreases appreciably because of strong kinematic coupling with ring deformation modes. In copper(II)–imidazole complexes the Cu–N stretching vibrations have been assigned near 250 cm^{-1} (Goodgame et al., 1969, 1971). The isolated weak band observed near 270 cm^{-1} in the present Raman spectra could be assigned to a Cu–imidazole, or possibly a Cu–tyrosine or Cu–tryptophan vibration.

A preferred assignment of the band near 270 cm^{-1}, however, is to a Cu–S stretching mode, in view of the chemical and spectroscopic evidence for involvement of cysteine sulfur at the binding site. Such modes have not been recorded in small molecule work, presumably because of the instability of Cu(II)–mercaptide complexes. Indeed, metal–sulfur stretching vibrations are poorly documented in general, but what evidence exists suggests that for divalent ions they may be expected in the range 250–300 cm^{-1} (Nakamoto, 1970). Symmetric stretching vibrations of cysteine sulfur to Fe(III) bonds have been assigned at 314 cm^{-1} in rubredoxin (Long et al., 1971) and 350 cm^{-1} in adrenodoxin (Tang et al., 1973) from resonance Raman studies. Stretching vibrations for a given ligand are expected at lower frequencies for dipositive than for tripositive cations, and a frequency near 270 cm^{-1} for Cu(II)–S(cysteine) stretching therefore seems reasonable. In any event sulfur is twice as massive as oxygen or nitrogen, and if the Raman bands between 350 and 470 cm^{-1} arise from Cu–N or Cu–O stretching, then this region cannot contain a Cu–S stretching mode, unless the Cu–S stretching force constant is about twice as high as the Cu–N or Cu–O stretching force constants. This seems unlikely, since metal–ligand force constants generally decrease with increasing ligand size (Hershbach and Laurie, 1961). While the protein no doubt imposes an unusual coordination geometry on the copper ion, it is unlikely to force a factor of two increase in the strength of a given copper–ligand bond. The inference is that a Cu–S stretching mode, if present in the molecule, either gives rise to the ~270-cm^{-1} band, or is too weak to be detected.

The Raman spectra show at least four bands between 350 and 470 cm^{-1} for azurin and ceruloplasmin. Only three peaks are observed for plastocyanin, but the complex enve-

lope may well hide additional components. It seems likely that all the Raman bands arise from fundamental vibrations. Other mechanisms for explaining extra bands, e.g., combination bands or Fermi resonance, are possible, but seem unlikely, since the required lower frequency fundamentals are not themselves observed in the Raman spectra. If fundamental vibrations are indeed responsible for the complex Raman emission between 350 and 470 cm^{-1}, then there appear to be at least four N or O ligands in the "blue" copper coordination sphere in addition to the probable S ligand. Moreover, the symmetry of the coordination group must be rather low. Otherwise the vibrational frequencies would be expected to span a narrower range. In the limit of a tetragonal complex with four equivalent N or O ligands, only one polarized band in the ~400-cm^{-1} region would be observed.

While the coordination groups of the "blue" copper centers are evidently similar in the three proteins, they do display appreciable differences among corresponding Raman bands, both in relative intensity and in frequency. Especially noteworthy is the ~30-cm^{-1} separation between the 462-cm^{-1} azurin band and the highest frequency bands in plastocyanin and ceruloplasmin, and the ~20-cm^{-1} separation between the 355-cm^{-1} ceruloplasmin band and the lowest frequency bands in plastocyanin and azurin. These differences could arise either from changes in force constants or from altered kinematic coupling produced by changes in geometry.

The weak band at 765 cm^{-1} found in plastocyanin, which gave the best quality spectra, may arise from a carbon–sulfur stretching mode of the bound cysteine group, which could experience some resonance enhancement. Alternatively it may represent an overtone of the strong band at 387 cm^{-1}, although in that event one might have expected another overtone for the even stronger band at 434 cm^{-1}, which is not observed. Siiman et al. (1974) observed weak (but resonance enhanced) bands near 1650 and 1240 cm^{-1}, as well as near 750 cm^{-1}, and suggested assignments to C=O stretching, C—N stretching, and C=O bending vibrations of peptide groups bound to the copper atom.

The Nature of the Resonant Electronic Transition. Because the energy of the ~600-nm absorption band is normal for a Cu^{2+} "d–d" transition, efforts have been made to rationalize its high intensity within the framework of crystal field theory, by invoking distortion of the copper coordination sphere from its normal tetragonal symmetry (Blumberg, 1966; Brill and Bryce, 1968). Due to the very large intensification needed, about a factor of 50, the needed distortions are somewhat arbitrary in a chemical sense; a number of nontetragonal cupric complexes are known, but none of their d–d bands approach those of the "blue" copper sites in intensity. Intensity borrowing from nearby allowed transitions cannot be invoked to ease this problem, since the first intense absorption above the ~600-nm band is that of the aromatic amino acid residues at ~280 nm, a factor of two higher in energy. An additional difficulty is that circular dichroic spectra reveal too many transitions in the visible region for all of them to be assigned to "d–d" transitions (Tang et al., 1968; Falk and Reinhammer, 1972).

An alternative assignment of the ~600-nm band, suggested by Williams (1971), is to a charge transfer transition from a reducing ligand to Cu^{2+}. The obvious choice for the ligand is mercaptide sulfur, in view of the evidence from mercurial binding studies, with the exception of the study by Briving and Deinum (1975), that a sulfhydryl group is involved in copper binding. Recently Gray and coworkers (1974) have succeeded in replacing Cu^{2+} with Co^{2+} in stellacyanin, and have definitely characterized a sulfur → cobalt charge transfer transition.

The resonance Raman spectrum of the "blue" copper centers contains a band which can be assigned to Cu–S stretching. If a sulfhydryl group is in fact bound to Cu^{2+} at these centers, then sulfur → copper charge-transfer absorption is *definitely* expected in the visible region of the optical spectrum, Cu^{2+} being a better oxidant than Co^{2+}.

The resonance Raman spectra are dominated by bands near 400 cm^{-1}, where Cu–N or Cu–O stretching vibrations are expected. They are all polarized, and must arise from A terms in the Raman tensor expansion, since B terms involve vibronic coupling between nearby allowed electronic transitions (Tang and Albrecht, 1970), and there are no nearby allowed transitions with which the resonant ~600-nm transition can couple. A terms involve coupling to a single resonant electronic transition via the Frank–Condon overlaps (Tang and Albrecht, 1970). These are largest for vibrations whose normal coordinates most nearly reproduce the structural distortions found in the excited state.

The electronic configuration of copper(II) is d^9, and four of the five orbitals are doubly occupied, while the fifth is singly occupied. The singly occupied (highest energy) orbital is expected to be oriented in the direction of (that is, be antibonding with respect to) any nitrogen ligands in the coordination sphere, as nitrogen ligands are considerably higher than oxygen or sulfur ligands in the spectrochemical series (Jorgensen, 1961). This orbital must also be the terminal orbital in the ~600-nm electronic transition, whether the latter is a "d–d" or a ligand-to-metal charge transfer transition. Consequently the bonds to nitrogen ligands are expected to be appreciably weakened in the excited state, and high intensity is therefore expected for Cu–N stretching Raman modes.

The form of the excited state distortion depends on the properties of the donor orbital as well as the terminal orbital. If the transition is "d–d" in character, then it is difficult to specify the orientation of the donor orbital with respect to the ligands, in the absence of detailed structural information. A charge transfer assignment, however, requires a specific orientation. The donor orbital in a S → Cu charge transfer transition is either a σ or π orbital on the sulfur atom. If it is the σ orbital, then the transition should alter the Cu–S bond strength appreciably, while if is the π orbital, the effect on the Cu–S bond should be much smaller. Since the only Raman band assignable to Cu–S stretching, ~275 cm^{-1}, is much weaker than the Cu–N stretching modes, a S(σ) → Cu charge transfer assignment of the resonant electronic transition appears to be excluded, but a S(π) → Cu assignment is possible.

By analogy with the well-studied charge transfer spectra of metal halide complexes (Jorgensen, 1970) the S(σ) → Cu transition should lie 4–8000 cm^{-1} higher than the S(π) → Cu transition, and may be assigned to part or all of the absorption band near 450 nm (Malkin and Malmstrom, 1970). (The low-energy absorption band near 800 nm can then be assigned to d–d transitions, in agreement with McMillin et al.'s (1974) assignment for stellacyanin.) This set of assignments would imply a reversal of the usual ($\sigma > \pi$) charge transfer intensity pattern (Jorgensen, 1970). The weakness of the σ charge transfer transition would be consistent with the view that the terminal orbital does not over-

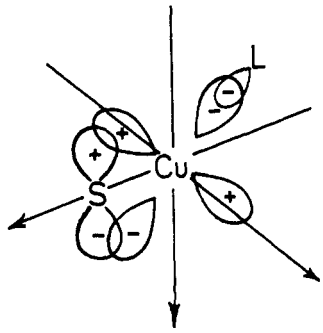

FIGURE 3: "Optimal" interaction of a copper d orbital with S(π) and L(σ) orbitals. Only one L ligand is shown.

lap significantly with the sulfur σ orbital. High intensity for the π charge transfer transition would, however, imply substantial overlap of the terminal orbital with the donor π orbital on the sulfur atom.

If the ~600-nm absorption band does indeed arise from S → Cu charge transfer, then the intensity patterns of the resonance Raman spectrum and the absorption spectrum imply strong interaction of the terminal d orbital *both* with σ orbitals on the nitrogen ligands and a π orbital on the sulfur ligand. This requirement would exclude any structure close to the normal tetragonal stereochemistry of copper(II) complexes (Freeman, 1973). The optimum L-Cu-S angle for simultaneous overlap of a copper d orbital with L(σ) and S(π) orbitals is 135°, as shown in Figure 3.

The Raman data are consistent with assignment of the ~600-nm resonant absorption band to either an enhanced "d-d" or S → Cu charge transfer transition. If the latter assignment is correct then the absence of a strongly enhanced Cu-S stretching mode implies a nontetragonal coordination geometry, with a L-Cu-S angle not far from 135°. Since strong distortion from tetragonality is also a minimum requirement for adequate intensification of the absorption band for a "d-d" assignment, a nontetragonal coordination geometry for the "blue" copper site seems very likely.

A third alternative assignment for the ~600-nm absorption band would be charge transfer to Cu(II) from a nitrogen or oxygen containing ligand. Strong Raman enhancement would then be expected only for bands in the ~400-cm^{-1} region, as is observed. The existence of N or O ligands sufficiently reducible to allow charge transfer at such a low energy seems chemically implausible, however.

Models Based on S → Cu Charge Transfer. Since, in our opinion, the weight of the chemical and spectroscopic evidence currently favors a S → Cu charge-transfer assignemnt for the ~600-nm resonant electronic transition, it seems worth pursuing the geometric constraints suggested for such an assignment by the resonance enhancement data. Two idealized geometries permit the L-Cu-S angles to approach the value, 135°, for optimum simultaneous overlap of L(σ), Cu(d), and S(π) orbitals. The first of these is monosubstituted tetrahedral. Three strongly bound L groups would establish a doubly degenerate σ-antibonding orbital (d_{xz}, d_{yz}, if the Cu-S axis is taken as the z axis) as the highest energy d orbital, and this orbital would be poorly oriented for σ interactions with the sulfur ligand, but fairly well oriented for π interactions. The second possibility is based on trigonal-planar symmetry. Taking the z axis perpendicular to the plane, the σ-antibonding d orbitals are then derived primarily from d_{xy} and $d_{x^2-y^2}$ which would be degenerate in D_{3h} symmetry. In the reduced (C_{2v}) symmetry of a MSL_2 complex, these orbitals divide into one involved in σ bonding with the sulfur ligand ($d_{x^2-y^2}$, if the Cu-S axis is taken as the x or y axis) and one involved in σ bonding with the two L ligands (d_{xy}). The orbital d_{xy} is well oriented for π interactions with a p-π sulfur orbital, and if it is the highest energy d orbital, our requirement is again satisfied. Less regular structures could maintain the overlap requirement to some degree, but large distortions would decrease S(π) interactions and/or increase S(σ) interactions with the highest energy d orbital, and substantially alter the special spectroscopic properties of the site.

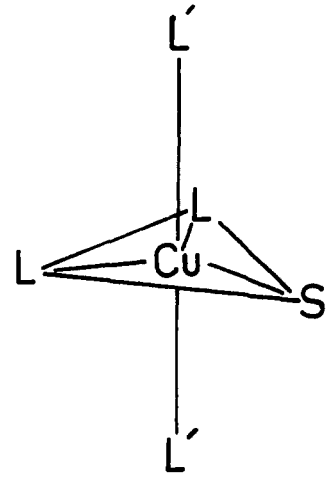

FIGURE 4: Proposed structure for the "blue" copper site. The L groups are presumably nitrogen ligands, and more strongly bound than the L′ ligands.

The trigonal geometry would permit two additional ligands on the z axis, as shown in Figure 4. In view of the inference from the Raman band multiplicity that there are at least four N or O ligands in addition to the S ligand, we favor the five-coordinate structure over the alternate four-coordinate structure.[1] In the trigonal-bipyramidal structure, the two axial ligands, L′, which may be N or O, must be more weakly bound than the equatorial ligands, L, in order to maintain d_{xy} as the highest energy d orbital. Vibrational mixing between Cu-L and Cu-L′ stretching modes would allow for some resonance enhancement of the latter.

If the coordination group were strictly C_{2v}, as shown in the idealized structure in Figure 4, it would display one symmetric Cu-L and one symmetric Cu-L′ stretching mode. The two corresponding antisymmetric stretching modes would be at different frequencies, depending on the size of the interaction force constants, but would have little Raman intensity, since nontotally symmetric modes can experience resonance enhancement only through vibronic mixing of the resonant electronic transition with some other allowed electronic transition (Tang and Albrecht, 1970).

If the C_{2v} molecular symmetry is lowered somewhat, through inequivalent ligands or distortions of the bond angles, the antisymmetric modes gain symmetric character and corresponding resonance enhancement. The observed resonance Raman intensity patterns, one or two strong bands between 350 and 470 cm^{-1}, with two or three weaker bands, are consistent with modest reductions from C_{2v} symmetry. The form of the distortion is evidently different for

[1] The ligand field spectrum of cobalt(II)-stellacyanin (McMillin et al., 1974) is completely consistent with the spectrum of trigonal-planar cobalt(II) in zeolite sites (Klier, 1969).

the different proteins since the intensity patterns differ considerably in detail.

The trigonal-bipyramidal model is consistent with the suggestion of Williams (1971) that the peculiar spectroscopic properties of the "blue" copper centers reflect a coordination group which compromises the normal coordination geometry of Cu^{2+} and Cu^{+}, thereby promoting efficient electron transfer. The normal preference of Cu^{+} is to *avoid* tetragonal coordination, but it is otherwise quite variable. Tetrahedral coordination has frequently been emphasized for Cu^{+}, but trigonal coordination is also known (Eller and Corfield, 1971; Lewis et al., 1970; Lewin et al., 1972; Weininger et al., 1972). Cu^{+}, being a "soft" metal center (Pearson, 1963), binds preferentially to sulfur, and it is reasonable to suppose that if the "blue" Cu^{2+} centers have a Cu–S bond, it is maintained upon reduction. In the trigonal-bipyramidal model, all that is required to generate a stable trigonal $Cu(I)SL_2$ complex is further weakening of the Cu–L′ bonds. Thus, the model plausibly moves the cupric ion some distance along the reaction coordinate for electron transfer, with respect to its "normal" position in small molecules, in line with the "entatic state" concept of Vallee and Williams (1968).

An additional point which bears on the structure of the "blue" copper centers is the surprising lack of correlation between their optical properties and their reduction potentials. The latter are highly variable, whereas the wavelength of the intense visible absorption, which might be expected to provide a measure of the Cu^{2+} acceptor orbital energy, is relatively constant. Thus, the reduction potential of plastocyanin (E_0' = 0.34–0.37 V, λ_{max} 597 nm; Katoh et al., 1962) is nearly 0.2 V higher than that of stellacyanin (E_0' = 0.184 V, λ_{max} 605 nm; Reinhammer, 1972) while among the multi-copper oxidases (Reinhammer, 1972), the "blue" copper site of *Polyporus versicolor* laccase (E_0' = 0.785 V, λ_{max} 610 nm) is nearly 0.4 V higher than that of *Rhus vernicifera* laccase ($E_0' \simeq$ 0.4 V, λ_{max} 614 nm). In the trigonal-bipyramidal model, this lack of correlation can be explained by postualting marked variability among different proteins with respect to the axial ligands, L′, but not with respect to the equatorial ligands, L. Since, to a first approximation, the axial ligands do not interact with the half-filled orbital, d_{xy}, they should have little influence on the in-plane $S(\pi) \rightarrow Cu(d_{xy})$ charge-transfer energy. The reduction potential, however, depends on the *total* energy of the Cu^{2+} ion, which *would* be affected by the axial ligands. The observed variation in the Raman intensity patterns, within the same basic scattering mechanism, is consistent with appreciable variation in the axial ligands from one protein to another.

The reduction potential is also a function, of course, of the energy of the reduced form of the "blue" copper site. Consequently some or all of the variation in reduction potential might conceivably arise from variation in the Cu^{+} coordination geometry. It is unfortunate that the Cu^{+} coordination group is not amenable to study through current spectroscopic techniques.

A necessary constraint on any model for the blue copper center is that it explains the electron spin resonance data for these systems, especially the axial nature of the *g*-tensor and the uniquely small hyperfine splitting. Only a few trigonal models have been studied in detail, but these do yield essentially axial EPR results (Brill and Venable, 1966). There is no simple explanation for the hyperfine coupling magnitude; however, a combination of covalency (that is, the effect of low-lying charge-transfer states) and low symmetry may prove to be adequate. A comparison of detailed magnetic calculations based on the trigonal-bipyramidal model with solution and/or single-crystal EPR data would be necessary to establish the validity of this point.

Conclusions

The interpretation of the Raman characteristics of the "blue" copper sites depends on the assignment of the ~600-nm resonant electronic transition. The Raman data are not in themselves inconsistent with a "d–d" assignment provided a "d–d" transition can in fact take on appreciable allowed character. At a minimum this would require a severe distortion from the normal tetragonal Cu(II) coordination geometry, as Blumberg (1966) and Brill and Bryce (1968) have pointed out. If, on the other hand, the ~600-nm transition is $S \rightarrow Cu(II)$ charge transfer in character, then the orbital overlap requirements imposed by the observed Raman and absorption spectral intensity patterns again imply nontetragonal geometry. Both assignments therefore exclude a tetragonal Cu(II) coordination site. A third assignment, charge transfer from a reducible N or O ligand, is also consistent with the Raman data, but is chemically implausible.

While a "d–d" assignment does not lend itself to further analysis of the Raman data, a specific model does emerge, if the assignment is $S \rightarrow Cu$ charge transfer. This model is basically a trigonal bipyramid with a cysteine mercaptide ligand sharing the equatorial plane with two strong field ligands, L, which are presumably nitrogen ligands (but not imidazole). The axial positions are occupied by less strongly bound ligands, L′, which might be nitrogen or oxygen ligands. The idealized C_{2v} symmetry is lowered by inequivalency of the L or L′ ligand or by small distortions of the bond angles, and the details of the symmetry lowering differ from one protein to another. This model provides a ready path for stabilization of Cu^{+} in the reduced sites, and also provides a plausible rationale for the lack of correlation between the optical properties and reduction potentials of the "blue" sites.

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Isolation and Identification of 1,25-Dihydroxyvitamin D_2†

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ABSTRACT: The chemical synthesis of [3α-^{3}H]vitamin D_2 of high specific activity has been described. With the use of this radioactive material, the existence of a polar metabolite believed to be the active form of vitamin D_2 in the rat and chick has been demonstrated. It has been isolated in pure form from an in vitro chick kidney mitochondrial system and identified as 1,25-dihydroxyvitamin D_2 by means of mass spectrometry, ultraviolet absorption spectrophotometry, and specific derivative synthesis. Its antirachitic activity equals that of 1,25-dihydroxyvitamin D_3 in the rat.

The recent extensive study of the metabolism of vitamin D_3 has provided overwhelming evidence to support the theory that it must be converted in the liver to 25-hydroxyvitamin D_3 (25-OH-D_3)[1] (Blunt et al., 1968; Ponchon et al., 1969; Horsting and DeLuca, 1969) and then in the kidney to 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (Fraser and Kodicek, 1970; Gray et al., 1971; Holick et al., 1971a,b; Lawson et al., 1971) before it can stimulate either intestinal calcium transport (Boyle et al., 1972) or bone calcium mobilization (Holick et al., 1972a).

More complete elucidation of the metabolism of vitamin D_2 on the other hand has awaited the synthesis of a radioactive vitamin D_2 of sufficiently high specific activity in order to detect low levels of metabolites at physiological dose levels. However, Suda et al. (1969) were able to isolate and identify 25-hydroxyvitamin D_2 (25-OH-D_2) from mammalian blood using a vitamin D_2 of low specific activity. Drescher et al. (1969) demonstrated the formation of this metabolite in the rat and chick and pointed out the inability of the chick to raise the blood concentration of 25-OH-D_2 to the steady-state level seen for 25-OH-D_3. Beyond these observations little is known concerning the functional metabolism of vitamin D_2.

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[1] Abbreviations used are: 25-OH-D_3, 25-hydroxyvitamin D_3; 25-OH-D_2, 25-hydroxyvitamin D_2; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3.