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Biological Analogues. On the Nature of the Binding Sites of Copper-Containing Proteins

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Abstract: An extended series of ligands and their copper(II) complexes have been prepared as spectroscopic models for determining the geometries and ligand coordinations of copper proteins. The electronic properties of thioether, imidazole, amide anion, amine, phenolate anion, and thiolate anion coordination to copper(II) have been determined. In addition, the electronic spectra of some of these ligands in square-planar, square-pyramidal, and tetrahedral geometries about copper(II) have been obtained by appropriate ligand design. On the basis of these results and an analysis of the protein spectra, structures for the copper coordination and geometry in (blue) type I copper, copper in galactose oxidase, type III copper, and copper in oxyhemocyanin are proposed.

Copper, in its various roles in biological systems, displays differing spectroscopic and chemical properties presumably because of the differing ligand environments and coordination numbers. Included among this variety of centers are "blue" or type I copper, "nonblue" or type II copper, ESR "nondetectable" or type III copper, and the copper present in hemocyanin.[1,2]

Type I copper occurs in the blue electron-carrying proteins stellacyanin, plastocyanin, and azurin where it is the only type of copper present. It also occurs, accompanied with types II and III, in the blue oxidases, laccase, ceruloplasmin, and ascorbate oxidase. By itself, blue copper is spectroscopically spectacular; it is characterized by an exceedingly intense electronic absorption at around 600 nm ($\epsilon \sim 5000$) as well as a succession of weaker bands which start at ~450 nm and carry on to the near-infrared; a total of 9 bands are seen in the visible and near-infrared regions for plastocyanin.[3]

The geometry and ligand environment of the type I site have not been determined directly, but on the basis of spectroscopic evidence, nearly all the conceivable permutations have been proposed.[3-8] Resonance Raman studies have been interpreted both in terms of distorted tetrahedral[4] and trigonal-bipyramidal[5] coordination geometries. Cobalt(II) reconstituted azurin and plastocyanin show electronic spectra typical of distorted tetrahedral coordination,[6] and more recent, additional data, in the near-infrared region, provide persuasive evidence for tetrahedrally surrounded copper(II) in plastocyanin and stellacyanin.[3]

The presence of RS^-, from cysteine, in the coordination sphere of blue copper has been proposed on the basis of binding by *p*-mercuribenzoate,[1,2] x-ray photoelectron spectroscopy,[7] electronic and circular dichroism spectra,[3] resonance Raman data,[4,5] and inferred from the Co(II) reconstituted materials. Ligands proposed, other than RS^-, include imidazole,[3,4] deprotonated amide,[3] and unspecified oxygen donors.[5] The coordination of thioether sulfur (from methionine) instead of RS^- has also been claimed;[8] this is unlikely for various reasons, not least of which is that no methionine has been found in stellacyanin.[9] Amino acid compositions of plastocyanin,[10] azurin,[11] ceruloplasmin,[12] ascorbate oxidase,[13] and laccase[14] indicate sufficient cysteine, methionine, histidine, and tyrosine to account for any of the proposed ligand environments.

Type II or nonblue copper is usually found in combination with types I and III but it occurs alone in galactose oxidase.[15] There has been little speculation as to the geometry and ligand environment of the copper in this enzyme although RS^- (cysteine) coordination is suggested by chemical studies on the enzyme and the apoenzyme.[16] Amino acid analyses disagree on the number of cysteines present in galactose oxidase[16,17] but considerable amounts of methionine and histidine are present.

So far, type III copper has not been found alone; it occurs along with types I and II in the blue oxidases, where selective titration studies with reducing agents show that the 330-nm ($\epsilon \sim 3000$) band of these proteins is associated with the type III copper and that two electrons are required to reduce this site. Type III copper is believed to consist of magnetically coupled pairs of copper ions.[19]

Superficially, the spectra and constitution of the copper in oxyhemocyanin appear to be remarkably similar to that of type III copper.[1,2,20,21] The coppers occur as spin-coupled copper(II) pairs which bind a peroxide ion.[22] The electronic absorption spectrum of oxyhemocyanin consists of two major visible bands, one at 345 nm ($\epsilon \sim 9000$ per Cu), the other at 570 nm ($\epsilon \sim 500$ per Cu).[23] Amino acid analyses of a variety of hemocyanins[20,24] indicate a large amount of histidine and of methionine per copper pair to be present as well as cysteine, although the number involved in disulfide bridges was not determined. However, it is significant that hemocyanin can be reconstituted after the RS^- groups have been blocked in the apoenzyme.[20,21] Thus, RS^- coordination appears to be excluded and the most widely assumed coordination ligand is imidazole.

This paper describes an attempt at determining the struc-

tures and ligand environments of the copper sites in these enzymes on the basis of their electronic spectra. Although this method alone is somewhat limiting, we were optimistic because these copper-containing enzymes show such distinct and unusual spectra and the potential donor atoms are limited.

1. Models

Copper(II) can adopt square-planar, square-pyramidal, trigonal-bipyramidal, octahedral, and tetrahedral geometries,[25] which, except for the first, are generally distorted from the idealized structures. The d-d spectra shown by these coordination geometries are clearly distinctive only in the case of the tetrahedral environment[26] where the absorptions occur to much lower energies and generally show, in the distorted forms, well-separated absorption peaks; all the other geometries show closely spaced absorption manifolds. With "hard" donor ligands the molar extinction coefficients of the d-d bands are of the order of 100. Thus the spectra of the metalloproteins under discussion cannot be due only to unusual stereochemistries and must be partly connected with nature of the donor atoms. Three types of donor atoms are likely to be involved in these protein complexes, namely oxygen (carboxylate, phenolate, and water), nitrogen (amine, amide anion, and imidazole), and sulfur (thioether and thiolate). Experience suggests that of these ligands only the phenolate anion, thioether, and the thiolate anion, together with particular coordination geometries, are likely to produce the unusual features of these spectra.

Given the above constraints, we have prepared a series of monomeric copper(II) complexes with varying geometries and with various permutations of the key donor atoms. These are shown in Figure 1.

The strategy in the design of these ligands took the following into account. Thioethers coordinate very weakly to copper(II) in dipolar solvents; thus, in nearly all cases, the thioether donor atoms were placed at the inner coordination positions of the multidentate chelates, so that, upon coordination, the sulfur atoms would be constrained to bond to the copper(II) ions by virtue of the strong coordinating abilities of the terminal tertiary nitrogen atoms and the operation of the chelate effect. Even so, all the thioether complexes display concentration-dependent spectra up to about 0.1 M solutions, whereafter constant extinction coefficients are observed as the concentration is increased.

The positioning of the thioether groups in the ligands also provided a method for controlling the stereochemistries of the complexes. Extensive studies with cobalt(III) complexes of these and similar ligands have shown that thioether groups which link two five-membered aliphatic chelate rings force these rings to adopt a nonplanar arrangement about the metal.[27] This constraint is relaxed if the thioether group links a five- and a six-membered aliphatic chelate ring. In this case the rings may adopt either a planar or nonplanar relationship to each other.[27] Thus the systems II and IV shown in Figure 1, which have consecutive five-membered chelate rings, are assumed to chelate in the manner shown. It is perhaps significant that the ligand of system II forms an intractable, presumably polymeric, material when reacted with $Cu(ClO_4)_2$ in water solution; addition of *N*-methylimidazole (N-MeIm) produces a highly crystalline deep blue complex which retains its constitution in water solutions. This may suggest that this ligand cannot adopt a square-planar arrangement about copper(II) and is stabilized into a five-coordinate geometry by N-MeIm. Consistently, the ligand of system III presented no such problems because its alternating ring system can adopt the "natural" square-planar geometry of copper(II). Similar observations have been made on related systems.[28] The system I is probably slightly distorted from the square-planar geometry by virtue of interaction between the two pyridine rings.[29,30]

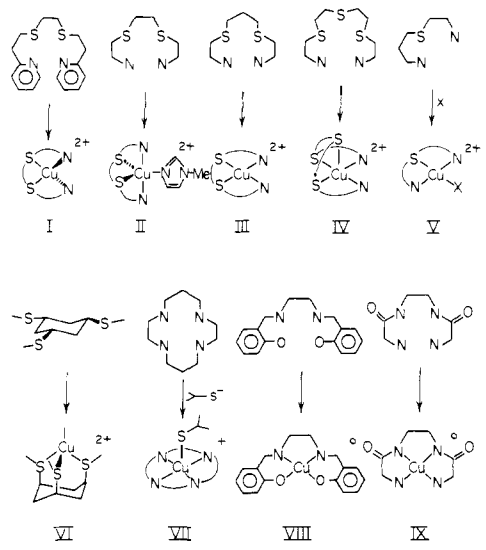

Figure 1. Structures of the various ligand and complex systems.

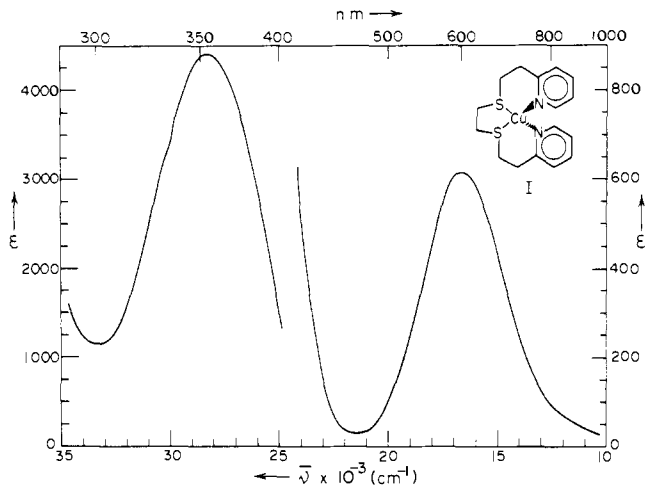

Figure 2. The absorption spectrum of system I in water solution.

Molecular models show that the adamantane-like system VI will dispose the sulfur atoms tetrahedrally about copper.

The preparations of these ligands presented few difficulties except for the case of *cis.cis*-1,3,5-tris(methylthia)cyclohexane, the tris-*p*-tosyl precursor of which was found to be highly prone to elimination by basic sulfur nucleophiles. Reaction with KSCN in diethylene glycol, however, gave a satisfactory displacement of the tosyl groups. Subsequent hydride reduction of the thiocyanate groups followed by alkylation gave the tristhioether as a low melting solid.

2. Spectra. Thioether Ligands

Figures 2, 3, and 4 show the absorption spectra of systems I, III, and IV. The spectra of systems III and IV are characterized by three absorption regions; two bands of moderate to strong intensity occur at around 40 000 and 30 000 cm^{-1} and a weak band occurs at around 17 000 cm^{-1}. The 40 000-cm^{-1} region of system I is dominated by transitions associated with the pyridine moieties, but the 30 000- and 17 000-cm^{-1} bands are clearly resolved. When these spectra are compared with those shown by the corresponding aliphatic tetraamine-copper(II) complexes, the 40 000- and 17 000-cm^{-1} bands are common to both but the 30 000-cm^{-1} band (hereinafter called the blue band) appears only in the sulfur analogues. Thus we assign the 40 000-cm^{-1} band to a $N(\sigma) \rightarrow d_{x^2-y^2}$ (Cu(II)) charge-transfer transition, the blue band to a $S \rightarrow d_{x^2-y^2}$

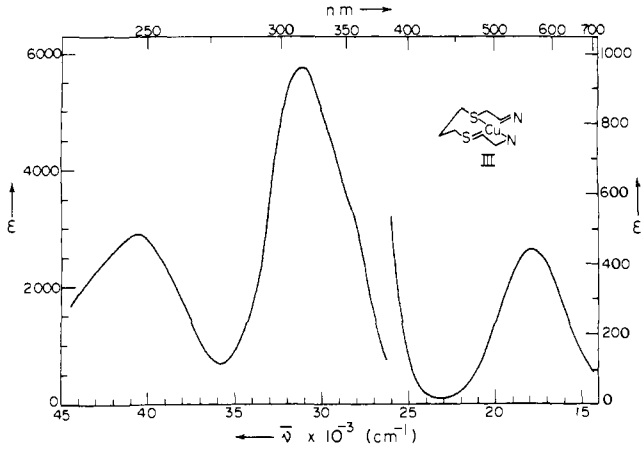

Figure 3. The absorption spectrum of system III in water solution.

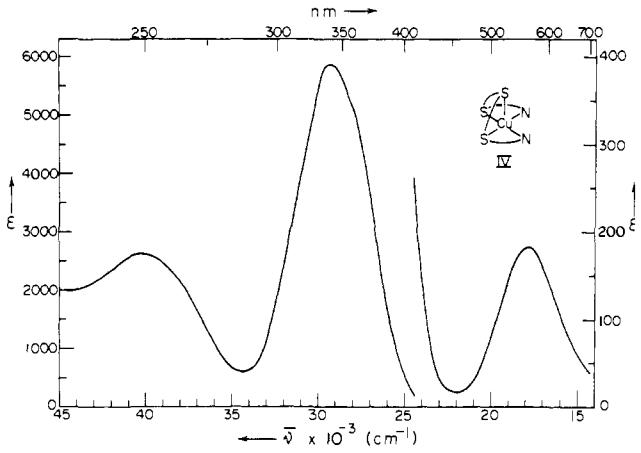

Figure 4. The absorption spectrum of system IV in water solution.

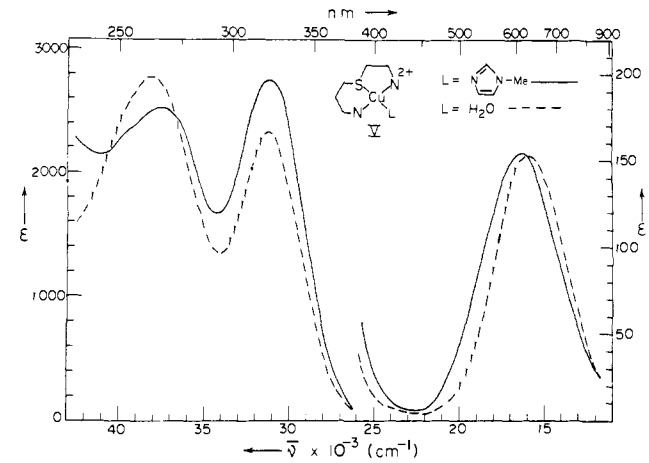

Figure 5. The absorption spectra of system V in water solution.

charge-transfer excitation, and the 17 000-cm^{-1} band, which occurs to slightly lower energies compared to the planar $[Cu(N)_4]^{2+}$ chromophore by virtue of the weaker crystal field of sulfur, is assigned to d-d transitions essentially localized at the Cu(II) ion. The d-d bands in these sulfur-containing complexes, however, are unusually intense and it seems highly probable that this enhancement arises from borrowing from the higher energy bands, principally the intense blue band. It is perhaps significant that system I shows the strongest d-d band which may be due, in part, to the ligand's tendency to adopt a distorted geometry.

The spectrum of the intensely blue complex of system I is remarkably similar to that shown by oxyhemocyanin; the lowest energy transition has nearly an identical energy and intensity and the same is true for the position of the blue band, although the intensity is somewhat less in the present case. The electronic provenance of this 30 000-cm^{-1} transition almost certainly involves a promotion from the electron pairs of sulfur to the partially filled $d_{x^2-y^2}$ level of Cu(II), but since the intensity of such transitions is related to the degree of overlap between the appropriate ligand and metal orbitals, the high intensity of the blue band suggests that a $R_2S(\sigma) \rightarrow d_{x^2-y^2}$ transition is involved. Consistently, the analogous systems, III and IV, show about the same intensity for the blue band as might be expected because of the poor overlap between the axial sulfur ligand of IV and the (in-plane) $d_{x^2-y^2}$ orbital which bonds strongly to the two in-plane sulfur atoms in both cases. We expect that the $R_2S(\pi) \rightarrow d_{x^2-y^2}$ transition, by the overlap criterion, would be weaker in intensity and, because it is not as strongly involved in bonding as the $S(\sigma)$ levels, to occur at lower energies. Inspection of the blue band for each of these complexes reveals that, probably in all three cases, this band is split into two components of different intensities. This is particularly evident in system III where the weaker component is at lower energies; the splitting is less evident in system I but the weaker component now occurs to higher energies. This reversal suggests that these weaker components may not represent the $R_2S(\pi) \rightarrow d_{x^2-y^2}$ transitions although they are likely to occur in the regions of the $R_2S(\sigma) \rightarrow d_{x^2-y^2}$ excitation. We believe the splitting arises as a consequence of the two cis-disposed sulfur ligands and that the two observed transitions both represent $R_2S(\sigma) \rightarrow d_{x^2-y^2}$ excitations. If this assumption is correct, then it follows (vide infra) that an analogous system containing only a single sulfur donor atom would give a symmetrical blue band which is half as intense as, for example, this same band in system III.

Figure 5 shows two spectra of such a system (V) and, as can be seen, the blue bands (at ~31 000 cm^{-1}) are indeed half as intense as the blue band of system III. This observation supports the assumption that blue bands involve sulfur-Cu(II) charge-transfer transitions provided the geometries are similar. Moreover, the blue band of system V appears to be symmetrical for both species. Although this last observation is, of course, not conclusive, it does lend support to our assumption about the origin of the splitting of the blue band in the previous complexes.

Two further geometries about the copper ion require consideration, the trigonal-bipyramidal and tetrahedral geometries. The spectrum of the first of these, system II, is shown in Figure 6.

A single-crystal x-ray diffraction structural determination of this complex[31] shows that the molecule essentially possesses the structures shown in Figure 1 although the N-MeIm ligand roughly occupies a square plane with *one* sulfur atom and the two amino groups, apparently because a perchlorate ion resides in the sixth (quasiocatahedral) site. The precise structure in solution is unknown but there seems little doubt that the ligand will retain its twisted structure to give a distorted five-coordinate geometry, the spectroscopy of which is our principal concern.

The blue band at 31 000 cm^{-1} (Figure 6) is assigned to the $R_2S(\sigma) \rightarrow$ Cu(II) charge-transfer transition and it will be noted that its energy position is similar to that observed for the previous systems. Moreover, the intensity of this band is about the same as that of system V, and roughly half that of the systems with two in-plane sulfur atoms. The significance of this observation depends on the precise geometry of the complex and the resultant overlap between the sulfur donor atoms and the half-filled d orbital of Cu(II), but it does suggest that the geometry of this complex is different from that of system III.

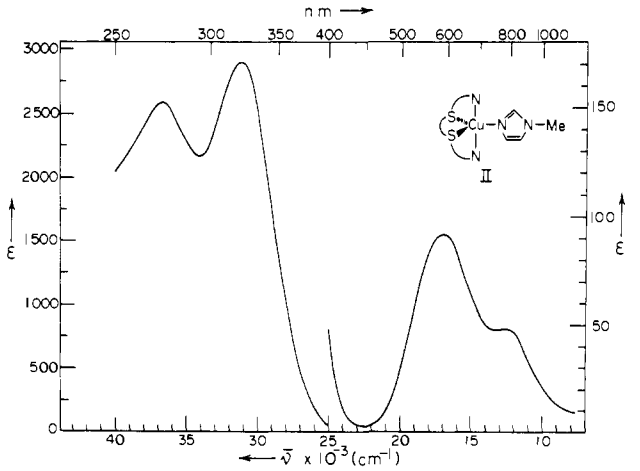

Figure 6. The absorption spectrum of system II in water solution.

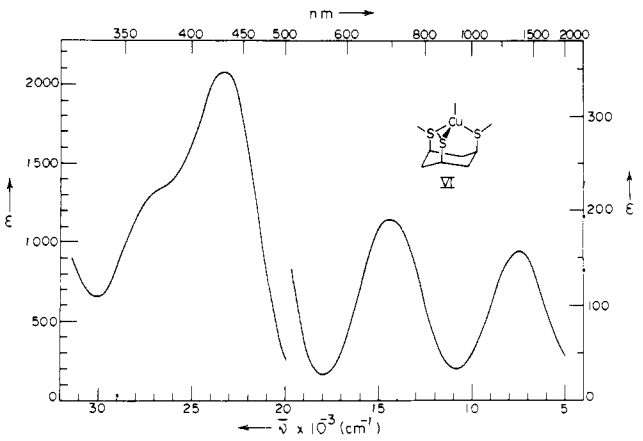

Figure 7. The absorption spectrum of system VI in acetic anhydride solution.

This conclusion is supported by the nature of the d-d transition manifold which shows a clear splitting of the components. An observed splitting of the d-d bands is commonly found in distorted five-coordinate copper(II) complexes.[25]

The spectrum of the tetrahedral species, system VI, is shown in Figure 7. The ligand, as expected, forms exceedingly weak complexes with copper(II) and the only medium we found that gave complete complexation was acetic anhydride. Even in this solvent the spectra were concentration dependent, although the intensities reached constant, maximum values at accessible concentrations. The spectrum shown refers to a maximized concentration and, moreover, the addition of excess ligand did not alter the spectrum of the original 1:1 (ligand-$Cu(ClO_4)_2$) solutions. Hence we infer that no bis-ligand complexes are interfering with the spectrum. Presumably the fourth coordination position is occupied by an oxygen donor atom, probably from the acetic anhydride. The spectrum shown by this system leaves little doubt that a tetrahedral species is formed. Two clearly resolved d-d bands are observed at low energies, one at 7500 cm^{-1} and the other at 14 500 cm^{-1}. The $R_2S(\sigma) \rightarrow$ Cu(II) charge-transfer band is displaced to lower energies compared to the previous complexes and is split into two components, one at 23 500 cm^{-1} and the other at around 26 000 cm^{-1}. The displacement of the charge-transfer band makes the complex appear bright apple green in contradistinction to the previous complexes which are an intense "royal" blue color.

3. Spectra. Thiolate Anion Coordination

We have succeeded in coordinating a RS^- ligand to copper(II) to give a relatively stable species (system VII). The brick red complex $[Cu(cyclam)](ClO_4)_2$ is insoluble in ethanol but the solid dissolves in this solvent in the presence of excess sodium thiolate to produce a deep blue solution which is conveniently stable at 10 °C. The intensities of the blue solutions depend on the concentration of the sodium thiolate but a maximum value is reached at about 15:1 thiolate to complex concentrations. The stability of the blue color depends on the temperature and the concentration of the solution; the lower the temperature and more dilute the solution, the more stable is the system. The greater stability of the dilute solutions supports the idea that RS· coupling is the driving force of the reaction. We have manipulated the conditions so that the system is stable for about 5 min before the solution intensity begins to decrease; eventually a colorless solution is obtained.

In Figure 8 we show the spectrum of the $[Cu(cyclam)SR]^+$ system in ethanol solution together with that of $[Cu(cylam)]^{2+}$ in water solution. The choice of isopropyl mercaptan is a matter

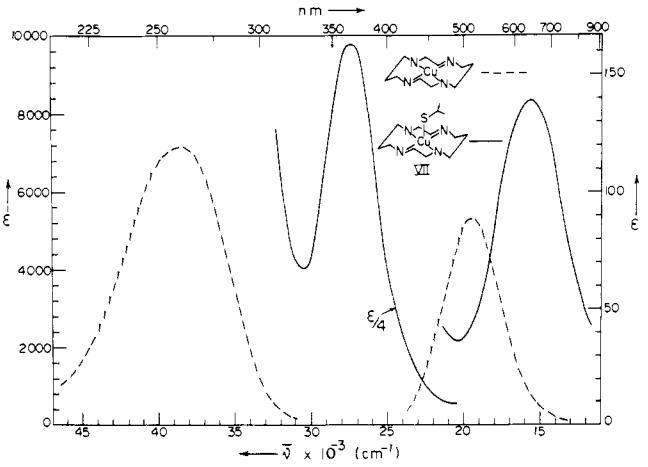

Figure 8. The absorption spectrum of system VII (—) in ethanol solution and that of $[Cu(cyclam)](ClO_4)_2$ (- - -) in water solution. The $\epsilon/4$ portion refers to the right-hand scale.

of convenience; other aliphatic homologues serve equally well. A comparison of the two spectra in Figure 8 shows that a new band at 27 500 cm^{-1} appears upon coordination of RS^-. This we ascribe to the $RS^-(\sigma) \rightarrow d_{x^2-y^2}$ charge-transfer transition. The intensity of this band is lower than that observed for the thioether system V and we believe that this is related to the poor overlap of the axially disposed RS^- ligand and the $d_{x^2-y^2}$ orbital of the copper(II) ion. In addition to the new transition, thiolate coordination causes a shift to lower energies of the d-d bands, from 19 500 to 16 000 cm^{-1}. We ascribe this shift to the raising of the energies of the d_{z^2} and d_{xz}, d_{yz} orbitals by thiolate coordination. A similar red shift is observed when $[Cu(cyclam)](ClO_4)_2$ is dissolved in dimethylformamide (DMF) containing a large excess of N-MeIm. The intense band at around 40 000 cm^{-1}, seen in $[Cu(cyclam)]^{2+}$, is observed in all the copper(II) complexes (vide supra) having primary amine coordination and is ascribed to $N(\sigma) \rightarrow d_{x^2-y^2}$ charge-transfer transitions.

4. Spectra. Phenolate Anion Coordination

Figure 9 shows the spectrum of the bright green system VIII. The visible spectrum shows three bands that can be associated with metal-ligand chelation, at 17 000, 25 500, and 30 500 cm^{-1}. The absorption at 35 500 cm^{-1} is seen in the free ligand, its sodium salt (Figure 9), and its zinc(II) complex. The weak band at 17 500 cm^{-1} occurs in the expected d-d transition region and is assigned accordingly, but the electronic provenances of the 25 500- and 30 500-cm^{-1} bands are assigned on the following arguments.

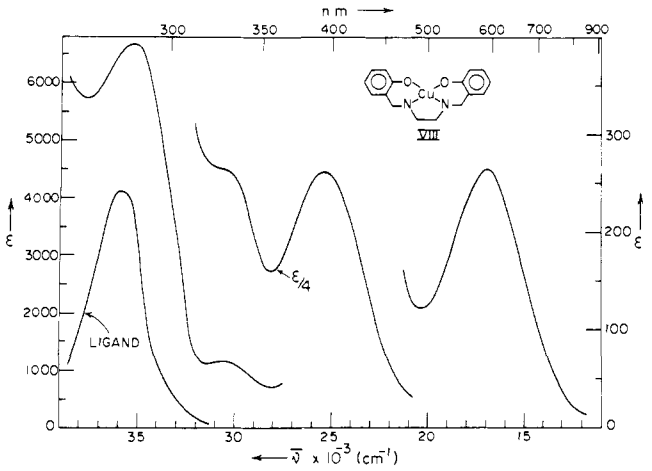

Figure 9. The absorption spectrum of system VIII in methanol solution and that of the free ligand in methanol containing 2 equiv of NaOH. The $\epsilon/4$ portion of the spectrum refers to the right-hand scale.

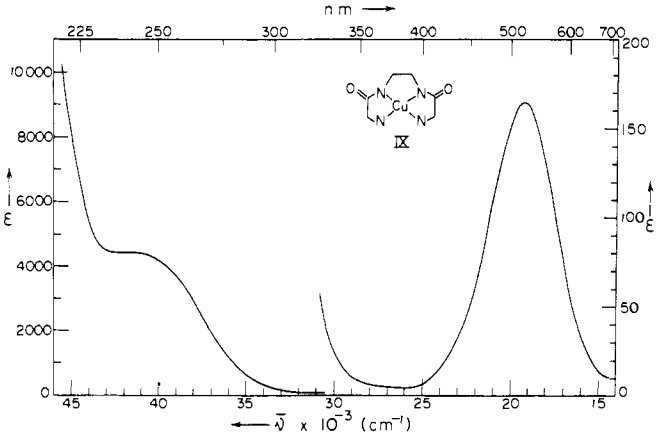

Figure 10. The absorption spectrum of system IX in water solution.

Both of these transitions are relatively weak having molar extinction coefficients of the order of 500 per phenolic residue and if either of these excitations were a $O(\sigma) \rightarrow d_{x^2-y^2}$ transition we would expect an intensity similar to that observed for the $S(\sigma) \rightarrow d_{x^2-y^2}$ and $N(\sigma) \rightarrow d_{x^2-y^2}$ transitions of square-planar complexes. Moreover, the first charge-transfer band of $[Cu(gly)_2]$ (gly = glycinate anion) occurs at around 40 000 cm^{-1} and, if the $O(\sigma) \rightarrow d_{x^2-y^2}$ transition of this complex occurs in this region, it does not seem likely that the energy of the electron pairs of the phenolate anion would be raised 15 000 cm^{-1} so that the $O(\sigma) \rightarrow d_{x^2-y^2}$ would occur at 25 500 cm^{-1}. Hence, we assign the 25 500-cm^{-1} transition to a charge-transfer band from the filled d orbitals of copper(II) to antibonding orbitals of the phenolic residue. Overlap considerations suggest that the major source of intensity of this transition is the promotion of an electron from the d_{xz}, d_{yz} orbitals of the copper ion, although this argument is modified by the precise conformation of the complex. Depending on the extent of distortion, it is conceivable that the $d_{x^2-y^2} \rightarrow \pi^*$-phenolate transition could be observed as a weak transition at low energies, ~1300 nm. Our attempts at detecting this excitation in this system were unsuccessful. Support for the origins of the 25 500-cm^{-1} band is provided by the spectrum of the bis-8-hydroxyquinoline–copper(II) complex which has this band displaced about 2000 cm^{-1} to the red which is expected because of the more extended conjugation of the quinoline residue.

We assign the 30 500-cm^{-1} band also to a Cu(II) → phenolate (π^*) transition, that is to the next unoccupied phenolate

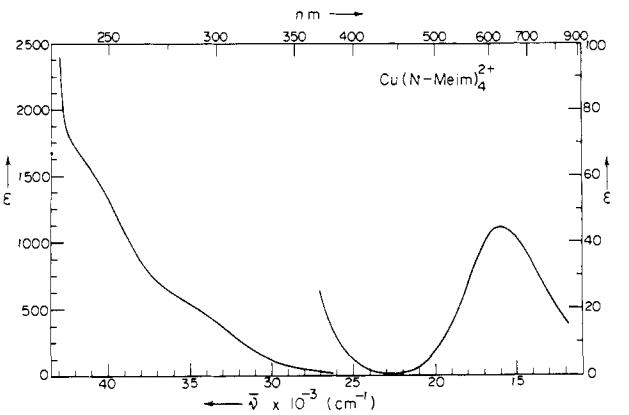

Figure 11. The absorption spectrum of $[Cu(N\text{-MeIm})_4](ClO_4)_2$ in water solution.

π^* level above the one associated with the 25 500-cm^{-1} transition. This band also occurs in the 8-hydroxyquinoline complex but is red shifted by about 2000 cm^{-1}.

5. Spectra. Imidazole and Amide Anion Coordination

Two recent reports have suggested that amide anion to copper(II)[3] and imidazole–copper(II)[22] charge-transfer bands may occur in the visible regions of the spectrum under certain circumstances. The spectra of system IX and that of $[Cu(N\text{-MeIm})_4]^{2+}$ shown in Figures 10 and 11, respectively, suggest that this is unlikely. It will be noted that the ligand field strength of the N-MeIm is considerably weaker than that observed for the amide anion-amine system. The first charge-transfer band of system IX occurs in the same region as that observed for the $N(\sigma) \rightarrow d_{x^2-y^2}$ excitation of primary amines coordinated to copper(II) so that the, presumably weaker, amide anion $N(\pi) \rightarrow$ Cu(II) charge-transfer transition occurs at or above 40 000 cm^{-1} in square planar systems.

When the spectrum in Figure 11 is compared with that of uncomplexed N-MeIm or the hydrochloride salt, the only new band in the ultraviolet region is the shoulder at 34 000 cm^{-1} which is assigned to an N-MeIm–copper(II) charge-transfer transition. We have no evidence as to its exact electronic provenance, although we suspect that both the $N(\sigma) \rightarrow$ Cu(II) and Cu(II) → N-MeIm (π) transitions occur in the featureless region 32 000 to 42 000 cm^{-1}.

6. Proposed Structures of the Metalloenzyme Sites

(a) Structure of Type I Copper. We begin by attempting to find the origins of the intense ~600 nm (16 700 cm^{-1}) band of the blue copper proteins. The present results and other observations[3] leave little doubt that this is not a d-d band, the intensity of which has been enhanced via borrowing mechanisms in a noncentric structure. It is therefore a charge-transfer band but it cannot arise because of nitrogen and oxygen coordination. The only conceivable oxygen donor is the phenolate anion but the lowest energy phenolate charge-transfer bands are much too weak to account for the intensity; the 600-nm enzyme band is ten times stronger than the charge-transfer bands in Figure 9. We are then left with either a thioether or thiolate to copper charge-transfer transition as an assignment. It cannot, however, be a thioether to copper transition since the tetrahedral system VI for which this transition, assuming the most generous value, occurs at 23 500 cm^{-1}, some 7000 cm^{-1} short of the 600-nm band. Even a tetrahedral system in the weakest possible field derived from protein ligands could not displace a thioether to copper band to 600 nm. This leaves thiolate coordination which, as we have seen, causes an expected red shift of the $RS^-(\sigma) \rightarrow$ Cu(II)

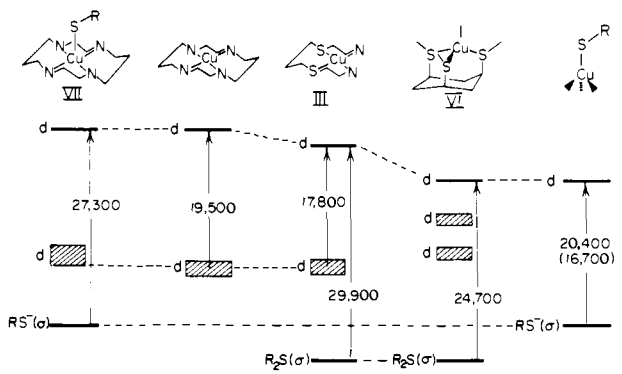

Figure 12. A concatenating energy level diagram showing how the position of the $RS^-(\sigma) \rightarrow Cu(II)$ charge-transfer transition will shift in energy from a square-planar system to the hypothetical tetrahedral complex. All the values are in cm^{-1} and 16 700 cm^{-1} is the energy position of the presumed $RS^-(\sigma) \rightarrow Cu(II)$ transition in type 1 copper. The "nonbonding" copper d orbitals are shown as "boxes" since the individual transitions are not resolved in the spectra.

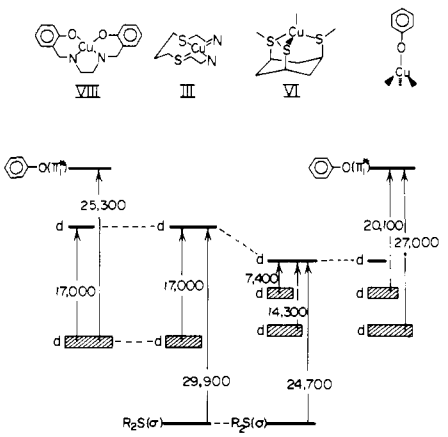

Figure 13. A concatenating energy level diagram interconnecting the lowest energy "nonbonding" Cu(II) (d) → phenolate (π_1^*) transition of the square-planar system with that of the hypothetical tetrahedral molecule. π_1^* is the lowest unoccupied phenolate orbital.

transition compared to thioether coordination. That the 600-nm band is a $RS^-(\sigma) \rightarrow Cu(II)$ charge-transfer band is supported by the following argument.

Figure 12 presents the argument diagrammatically and the energy values shown therein are taken from the present spectra. We assume the nonbonding d levels of system III and [Cu(cyclam)]$^{2+}$ in water solution are approximately the same and that the different absorption energies of the d-d transitions in the two systems arise from an energy displacement of the $d_{x^2-y^2}$ orbital. With this assumption, we can compare energies of the $RS^-(\sigma) \rightarrow Cu(II)$ and $R_2S(\sigma) \rightarrow Cu(II)$ transition to a common reference. The $RS^-(\sigma) \rightarrow Cu(II)$ absorption appears symmetrical (Figure 8), but the $R_2S(\sigma) \rightarrow Cu(II)$ absorptions are split when two or more (cis disposed) thioether units are coordinated. We have fitted the components of these charge-transfer bands by Gaussian analysis and have taken the mean of the two absorption maxima as the energy for the $R_2S(\sigma) \rightarrow Cu(II)$ transition. By including the value for the energy of the $R_2S(\sigma) \rightarrow Cu(II)$ absorption of the tetrahedral system, the shift in the d levels in going from a tetragonal to a tetrahedral field is obtained (Figure 12). Hence we obtain a value of 20 400 cm^{-1} for a $RS^-(\sigma) \rightarrow Cu(II)$ transition of the hypothetical tetrahedral system. This is 3700 cm^{-1} short of the intense blue band observed in the enzymes. Given the approximations, however, we believe that the above argument provides persuasive support for the view that blue copper is tetrahedrally surrounded and has thiolate coordination.

We now turn to the assignment of a number of weaker bands clustered about the strong blue band. A single band at 22 570 cm^{-1} is observed for stellacyanin[3] whereas plastocyanin shows two bands, at 21 540 and 23 640 cm^{-1} (mean = 22 590 cm^{-1}), in this region.[3] Azurin has a comparable band at 20 840 cm^{-1}. The molar extinction coefficients of these transitions vary between 200 and 1500. A recent report[3] has ascribed these absorptions to amide anion $N(\pi) \rightarrow Cu(II)$ charge-transfer transitions, but we consider this unlikely in view of the energy of the first charge-transfer band of system IX (Figure 10). We assign these bands, and others, to a progression arising from a transition of an electron from the various copper(II) d levels to the unoccupied phenolate π^* orbitals of tyrosine. This hypothesis is supported by the intensities which are similar to those observed for system VIII (Figure 9) and by the following argument.

Figure 13 shows a concatenating energy level diagram similar to the one in Figure 12 and is subject to similar assumptions. It will be noted that the energies of the $R_2S(\sigma) \rightarrow Cu(II)$ charge-transfer transitions have been used to fix the relative energies of the d orbitals of the tetrahedral relative to the square-planar systems. Thus the highest energy of the (lowest energy) $Cu(II) \rightarrow$ phenolate(π^*) transition of the hypothetical tetrahedral system should occur at around 27 000 cm^{-1}. This value, like the value obtained in the calculation for the $RS^-(\sigma) \rightarrow Cu(II)$ excitation, is about 4000 cm^{-1} too high and probably reflects in part the weaker ligand field experienced by copper in the enzymes compared to the hypothetical model. The calculation, however, does lend support to the hypothesis of phenolate coordination to a tetrahedrally coordinated copper(II) ion in these enzymes. If this is true, then by fixing the energy of any one of these bands and knowing the energies of the d-d transitions in the actual enzymes, we can predict where the individual transitions of the $Cu(II) \rightarrow$ phenolate(π^*) progression should occur in the absence of configuration interaction.

Figure 14 summarizes the results of such a calculation. It will be seen that the correlation between prediction and experiment is remarkably good, particularly in view of the fact that most of the absorption maxima of these bands in the enzymes are heavily overlapped.[3] There is, however, a complication to this analysis which arises from the observation, in the model system, that a second $Cu(II) \rightarrow$ phenolate(π^*) transition lies about 5500 cm^{-1} above the first and, given the energy spread of the d levels in the enzymes, these are expected to lie in the range 18 000 to 28 000 cm^{-1} (Figure 14). Thus the first and second progressions will tend to overlap although the model systems suggest, as may have been predicted, that the higher energy progression will be somewhat weaker in intensity than the first and therefore may have only a small effect on the absorption peak positions where overlap occurs. In any case, the presence of a second $Cu(II) \rightarrow$ phenolate(π^*) transition predicts that a band should occur at around 28 000 cm^{-1} in the enzyme spectra. Indeed, the spectrum of stellacyanin does show absorption in this region;[3] a very weak absorption is observed at 28 000 cm^{-1} in plastocyanin.[3]

The two remaining ligands are not identifiable by electronic spectroscopy and therefore do not include thioether ligands. There is evidence[32] which suggests that two imidazole ligands may be involved and our models do not preclude this. On this assumption, our proposal for the structure of the copper in blue proteins is shown in Figure 15. The d-d bands show clearly that a distorted tetrahedron is involved. This has been discussed in detail elsewhere.[3]

(b) Structure of Galactose Oxidase. The visible absorption spectrum of galactose oxidase is characterized by three absorption bands having molar extinction coefficients of the order of 1000[15]. These occur at 22 500, 15 870, and 12 900 cm^{-1},

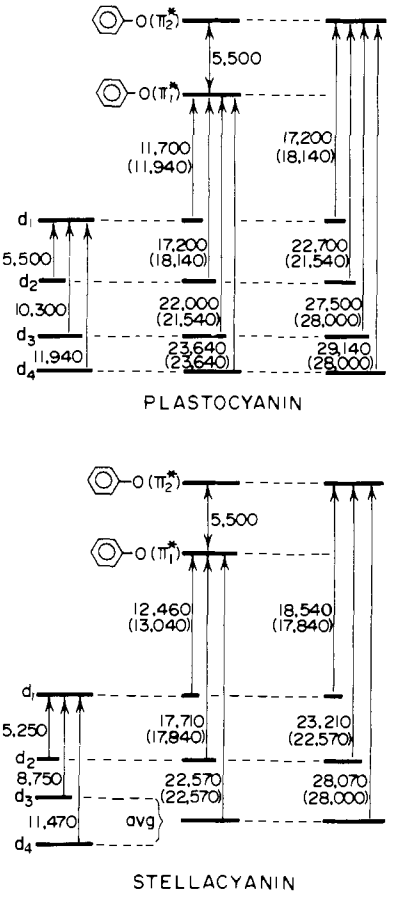

Figure 14. The calculated and predicted Cu(II) (d) → phenolate(π^*) transition energies (cm^{-1}) in plastocyanin and stellacyanin. The values of the d-level spacings are taken from ref 3. Observed transitions in the enzymes are shown in parentheses just below the calculated values. All the calculated values depend on fixing one energy level in each protein. For this purpose, the 23 640-cm^{-1} transition was taken for plastocyanin and the average (avg) value of 22 570 cm^{-1} was used as a "fix" for stellacyanin. In the latter, unlike the former, a broad unresolved band is observed at 22 570 cm^{-1}.

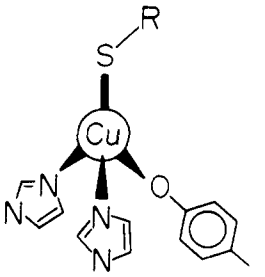

Figure 15. The proposed structure and ligand coordination in the blue type 1 copper proteins.

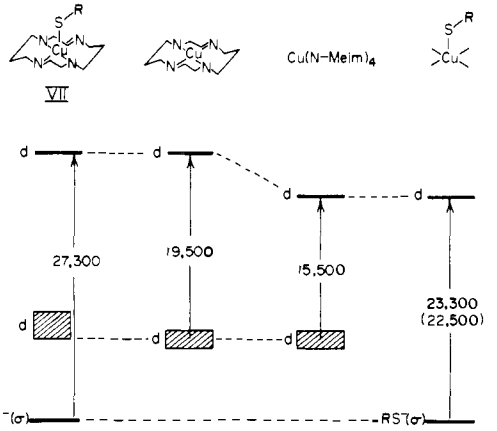

Figure 16. An interconnecting energy level diagram which attempts to predict the energy of the $RS^-(\sigma) \rightarrow Cu(II)$ transition in the hypothetical square-pyramidal ion, $[Cu(N\text{-}MeIm)_4RS]^+$. The calculated value is 23 300 cm^{-1} and the observed value of the assumed $RS^-(\sigma) \rightarrow Cu(II)$ in galactose oxidase is 22 500 cm^{-1}.

the last two bands being broad and overlapped. In fact, the two low-energy absorptions are remarkably similar in relative intensity and energy position to those observed for system II (Figure 6). The d-d bands of system II occur at around 17 000 and 12 400 cm^{-1} and hence we assign the 15 870- and 12 900-cm^{-1} absorptions of galactose oxidase to d-d transitions whose intensities have been enhanced by borrowing from higher energy transitions.[33] Moreover, the general profile of the d-d manifold suggests that the copper(II) ion in the enzyme is in a somewhat distorted five-coordinate environment.

On the basis of the present models, the energy and intensity of the 22 500-cm^{-1} enzyme band could arise as a result of either phenolate coordination (Figure 9) or an axially coordinated thiolate anion (Figure 8). We conclude that phenolate coordination is unlikely because the chemical evidence on the enzyme suggests thiolate coordination[16,34] but, more significantly, because we would expect a Cu(II) → phenolate(π^*) transition to be split in the same way as the d-d band (cf. arguments in Figure 14).

The positions of the d-d bands in the enzyme indicate a crystal-field strength of about that observed for $[Cu(N\text{-}MeIm)_4]^{2+}$ (Figure 11). Assuming this, together with similar assumptions to those used before, we arrive at an energy of 23 300 cm^{-1} for the $RS^-(\sigma) \rightarrow Cu(II)$ transition in the enzyme (Figure 16) which is close to the actual value of 22 500 cm^{-1}. In order to account for the low intensity of this band, we assume that axial coordination of the thiolate ligand is involved in a distorted square-pyramidal geometry.

The above considerations assumed that the recorded spectrum of galactose oxidase refers only to the divalent copper complex. The argument would require modification if, as kinetic results suggest,[19] the resting enzyme contains a variable amount of copper(III) or its equivalent. The possibility that Cu(III) is accessible in this protein has led to the suggestion that amide anion coordination may be involved.[35]

(c) Type II Copper. No spectroscopic transitions uniquely attributable to type II copper have been observed because it occurs in conjunction with type I and type III copper, both of which have a very strong contiguous absorptions in the visible region.

(d) Type III Copper. Titration studies[18] show the intense 330-nm absorption of fungal laccase is due to type III copper. The energy position strongly suggests that this band is due to a $R_2S(\sigma) \rightarrow Cu(II)$ charge-transfer transition of a square-planar or quasi-square-pyramidal copper(II) system (Figures 2, 3, 4, and 6). Moreover, the high intensity suggests that the sulfur is bonded in the square plane. No other definite absorption bands appear for type III copper after type I copper has been reduced. It would appear, however, that thiolate coordination in the square plane is excluded. The extinction coefficients based on the molar concentration of type III copper for the ~330-nm bands are 4100 for ceruloplasmin (human serum),[36] 3200 for laccase (*Rhus succedenea*),[37] and 5300 for ascorbate oxidase (cucumber)[38] which are in excellent agreement with the extinction coefficients obtained for the $R_2S(\sigma) \rightarrow Cu(II)$ transition of the present model systems.

The mushroom protein tyrosinase (*Agaricus bisporus*) is believed to have two pairs of copper atoms in the active sites[39] and, in the resting state, is nearly colorless. Oxytyrosinase can be generated from the resting enzyme by the controlled addition of hydrogen peroxide. The now colored and diamagnetic

enzyme exhibits a strong absorption at 345 nm.[40] A similar result has been obtained with *Neurospora* tyrosinase.[41] These results suggest that these tyrosinases also have thioether coordination to square-planar or square-pyramidal copper(II) of the "oxy" forms and to this extent, at least, they resemble type III copper.

(e) **Copper in Hemocyanin.** To the extent that type III copper and oxyhemocyanin have intense bands at 330 and 345 nm,[23] respectively, and each contains magnetically coupled copper pairs, the structures seem to be similar. Moreover, the ESR spectra of the nitrosyl derivatives of hemocyanin and mushroom tyrosinase are remarkably similar.[42] That type III copper and the hemocyanins form a class with similar functional and physiochemical properties has been noted before.[43] The extinction coefficients for the ~345-nm band of hemocyanins, however, are about 9000 which is approximately twice that observed for type III copper and for the present two-sulfur models. Monomeric copper(II) complexes with four thioether donor atoms generate extinction coefficients of about 9000 for the $R_2S(\sigma) \rightarrow$ Cu(II) transition.[8] The high intensity of the 345-nm band in hemocyanin may indicate that more than one thioether ligand is bound to the copper or that other transitions lie beneath this absorption. None of the other biological ligands has strong absorption in this region except the "tail" of the imidazole-Cu(II) charge-transfer bands and the second charge-transfer transition associated with phenolate-Cu(II) coordination. The other possibility is that the $O_2^{2-}(\sigma) \rightarrow$ Cu(II) charge-transfer absorption may intrude into this region but since, as yet, such a transition in a well-characterized model system has not been observed, the matter is not settled. The poorly characterized brown precipitate obtained from mixing H_2O_2 and cupric acetate does have a band at around 370 nm.[44]

Octopus oxyhemocyanin has an absorption at 440 nm with an extinction coefficient of about 500 per copper.[23] The resonance intensity profile of the $O\text{-}O^{2-}$ stretching frequency of the Raman spectrum shows that the 440-nm band does not contribute to the intensity of the peroxide stretch.[22] Thus it is highly probable that the 440-nm band is not associated with an O_2^{2-}-Cu(II) charge-transfer transition. A transition at about 440 nm and with about the same intensity is observed in system VIII and we ascribed it to the first Cu(II) $\rightarrow$ phenolate(π^*) charge-transfer band. It thus seems plausible to assign a similar electronic provenance to this band in hemocyanin. Consistent with this hypothesis of phenolate binding are the observations that, either by ethoxyformylation or spectrophotometric tyrosine titration or by pH titration, tyrosine becomes accessible upon the removal of the copper ions.[45,46]

To lower energies oxyhemocyanin shows an envelope of transitions with a maximum extinction at 570 nm and a shoulder at about 680 nm.[23] These two components are resolved in the circular dichroism spectra.[23] The maximum extinction coefficient of this envelope is about 500 per copper and, in this respect as well as energy position and the general band profile, has a remarkable resemblance to the corresponding band observed in system I. Thus, we are inclined to assign these bands in oxyhemocyanin to d–d transitions whose intensity has been enhanced through mixing with intense higher energy transitions. This intensity borrowing may account for the Raman intensity profile of the $O\text{-}O^{2-}$ stretch.[22] If the 570-nm envelope is due to d–d transitions and since no absorption is observed to lower energies,[23] then a square-planar or square-pyramidal geometry about each copper in oxyhemocyanin is suggested.

The above observations suggest that copper in oxyhemocyanin is bound to thioether and the phenolate anion in a tetragonal geometry. There is evidence that at least two imidazoles are associated at the active site[45,46] and of course the peroxide ion is coordinated. From ESR data, it would appear that the copper pairs are about 6 Å apart in the nitrosyl derivative and that in methemocyanin the spin is quenched.[43] It is therefore attractive to postulate that the phenolate oxygen atom acts as a bridge linking the two copper atoms. Such oxygen bridging is ubiquitous in copper(II) chemistry.[47,48]

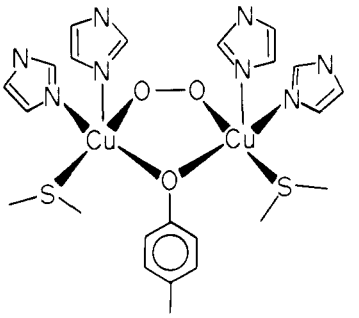

Figure 17. Proposed structure and ligand environment of the copper site in oxyhemocyanin. The diagram is meant to show that the phenolate oxygen atom, the peroxide ligand, the thioether ligands, and an imidazole occupy an approximate square-planar array. The particular ligand isomerism in the plane is drawn as a matter of convenience. Two imidazoles are in quasi-axial positions. Type III copper may have a similar environment although there is no evidence for the peroxide ligand in its resting state.

A structure consistent with the above considerations is the one shown in Figure 17. Type III copper may also have a similar structure.

7. Spectroscopic Assignments

We now briefly outline what we believe to be the origins of the splitting of the $R_2S(\sigma) \rightarrow$ Cu(II) charge-transfer bands. When two cis disposed thioether ligands are present, two wave functions associated with the sulfur σ orbitals are generated, namely $(1/\sqrt{2})(\sigma_1 + \sigma_2)$ and $(1/\sqrt{2})(\sigma_1 - \sigma_2)$. In the case of the array of three sulfur ligands, system VI, three functions are generated of which a pair of degenerate. The functions are $(1/\sqrt{3})(\sigma_1 + \sigma_2 + \sigma_3)$ and $(1/\sqrt{6})(2\sigma_1 - \sigma_2 - \sigma_3)$, $(1/\sqrt{2})(\sigma_2 - \sigma_3)$ of which the last two functions are degenerate. Taking the simpler two-sulfur case, it is readily shown or seen by oribital inspection that the overlap between the symmetric and antisymmetric functions, $(1/\sqrt{2})(\sigma_1 + \sigma_2)$ and $(1/\sqrt{2})(\sigma_1 - \sigma_2)$, respectively, and the orbitals on the central metal ion will be different. Hence the resultant molecular orbitals will be of different energies. In addition, the electrostatic (exciton) interaction of the individual transition dipole moments associated with each $R_2S(\sigma) \rightarrow$ Cu(II) transition will be different for the two coupling modes. Thus, both the electrostatic and exchange interactions will contribute to the splitting of the $R_2S(\sigma) \rightarrow$ Cu(II) excitation. An identical argument can be advanced for the three-sulfur case.

A summary of the assignments for the absorption bands of the copper proteins is given in Tables I and II. Most of the details in these tables have been covered previously except the $RS^-(\pi) \rightarrow$ Cu(II) assignments in the blue proteins. These transitions are not resolved in our model systems and the assignments shown in Table I are based on the assumption that the $RS^-(\pi) \rightarrow$ Cu(II) transition will be weaker and displaced to lower energies compared to the $RS^-(\sigma) \rightarrow$ Cu(II) transition. In the thiolate anion, two transitions are possible since there are two $RS^-(\pi)$ orbitals but we expect that the splitting of the two $RS^-(\pi)$ levels will be small.

8. Experimental Section

Preparations. System I. 1,8-Bis(2′-pyridyl)-3,6-dithiaoctane[30] (3.04 g) dissolved in ethanol (25 mL) was added to $Cu(ClO_4)_2 \cdot 6H_2O$ (4.1 g) in water (5 mL). An immediate intensely blue precipitate was formed. The mixture was heated on a steam bath for a few minutes

Table I. Type I Copper

Protein	Absorption band, cm^{-1} [a]	Assignment [b]
Stellacyanin	5 250	$d_2 \rightarrow d_1$ [c]
	8 750	$d_3 \rightarrow d_1$
	11 470	$d_4 \rightarrow d_1$
	13 040	$(1/\sqrt{2})(S(\pi a) + S(\pi b)) \rightarrow d_1$
		$(1/\sqrt{2})(S(\pi a) - S(\pi b)) \rightarrow d_1$
		$Cu(d_1) \rightarrow Ph(\pi_1^*)$ [d]
	16 580	$RS^-(\sigma) \rightarrow Cu(d_1)$
	17 840	$Cu(d_2) \rightarrow Ph(\pi_1^*)$
		$Cu(d_1) \rightarrow Ph(\pi_2^*)$
	22 570	$Cu(d_{3,4}) \rightarrow Ph(\pi_1^*)$
		$Cu(d_2) \rightarrow Ph(\pi_2^*)$
	~28 000	$Cu(d_{3,4}) \rightarrow Ph(\pi_2^*)$
Plasto-	5 500	$d_2 \rightarrow d_1$
cyanin	10 300	$d_3 \rightarrow d_1$
	11 940	$d_4 \rightarrow d_1$
		$Cu(d_1) \rightarrow Ph(\pi_1^*)$
	13 540	$(1/\sqrt{2})(S(\pi a) + S(\pi b)) \rightarrow d_1$
		$(1/\sqrt{2})(S(\pi a) - S(\pi b)) \rightarrow d_1$
	16 600	$RS^-(\sigma) \rightarrow d_1$
	18 140	$Cu(d_2) \rightarrow Ph(\pi_1^*)$
		$Cu(d_1) \rightarrow Ph(\pi_2^*)$
	21 540	$Cu(d_3) \rightarrow Ph(\pi_1^*)$
		$Cu(d_2) \rightarrow Ph(\pi_2^*)$
	23 640	$Cu(d_4) \rightarrow Ph(\pi_1^*)$
	~28 000	$Cu(d_4) \rightarrow Ph(\pi_2^*)$

[a] Data taken from ref 3. [b] See Figure 15. [c] The energies of the d orbitals are $d_1 > d_2 > d_3 > d_4$ of which one is quasi-degenerate.[3] [d] Ph refers to the phenolate anion for which $\pi_2^* > \pi_1^*$ in energy.

Table II. Type II and Type III Copper and Oxyhemocyanin

Protein	Absorption band, cm^{-1}	Assignment [d]
Galactose oxidase [a]	12 900	$d_2 \rightarrow d_1$
	15 870	$d_3 \rightarrow d_1$
	22 500	$RS^-(\sigma) \rightarrow d_1$
Type III copper	~30 000	$R_2S(\sigma) \rightarrow d$
Oxytyrosinase [b]	~16 500	$d \rightarrow d$
	~29 000	$R_2S(\sigma) \rightarrow d$
Oxyhemocyanin [c]	14 300	$d_2 \rightarrow d_1$
	17 500	$d_3 \rightarrow d_1$
	22 700	$Cu(d_{2,3}) \rightarrow Ph(\pi_1^*)$
	28 800	$R_2S(\sigma) \rightarrow d_1$
		$O_2^{2-} \rightarrow d_1$ [e]

[a] Data taken from ref 15. [b] Data taken from ref 40. [c] Data taken from ref 23. [d] Symbolism is the same as in Table I. [e] See difference spectrum (Figure 1) in O. Farver, M. Goldberg, D. Lancet, and I. Pecht, *Biochem. Biophys. Res. Commun.*, **73**, 494 (1976).

and then was cooled to 0 °C. The precipitate was collected and washed with cold water, then with ethanol, and finally with ether. It was recrystallized from hot water (250 mL) to give 4.2 g of dark blue crystals. Anal. Calcd for $[Cu(C_{16}H_{20}N_2S_2)](ClO_4)_2$: C, 33.9; H, 3.6; N, 4.9; Cl, 12.5; S, 11.3. Found: C, 33.8; H, 3.7; N, 4.9; Cl, 12.5; S, 11.4.

System II. 1,8-Diamino-3,6-dithiaoctane[49] (1.8 g) was dissolved in warm ethanol (20 mL) and added to $Cu(ClO_4)_2 \cdot 6H_2O$ (3.7 g) in warm water (20 mL). To the resulting blue solution was added 1-methylimidazole (0.84 g) in water (5 mL) whereupon the color of the reaction mixture intensified. The solution was filtered and excess $NaClO_4$ was added to the filtrate which was then cooled to 0 °C. The product slowly crystallized and after 1 h at 0 °C the blue crystals were collected and were washed first with ethanol and then with ether. It was dissolved in hot water and was filtered. Excess $NaClO_4$ was added to the hot filtrate and on cooling large deep blue crystals formed (2.0 g). Anal. Calcd for $[Cu(C_6H_{16}N_2S_2)(C_4H_6N_2)](ClO_4)_2$: C, 22.9; H, 4.2; N, 10.7; S, 12.2; Cl, 13.5. Found: C, 23.1; H, 4.3; N, 10.7; S, 12.2; Cl, 13.6.

System III. 1,9-Diamino-3,7-dithianonane[49] (1.94 g) was dissolved in ethanol (10 mL) and added to $Cu(ClO_4)_2 \cdot 6H_2O$ (3.7 g) in water (15 mL). A deep blue solution resulted. It was filtered and the filtrate was taken to near dryness under vacuum. Ethanol (50 mL) was added to the residue and the mixture was allowed to stand at 25 °C for 15 min. The solid was collected and washed with ethanol and ether. It was recrystallized from hot water by the addition of excess $NaClO_4$ to the hot solution and then by cooling the solution at 0 °C. The product deposited as maroon plates (2.8 g). Anal. Calcd. for $[Cu(C_7H_{18}N_2S_2)](ClO_4)_2$: C, 18.4; H, 4.0; N, 6.1; Cl, 15.5; S, 14.0. Found: C, 18.6; H, 4.0; N, 6.2; Cl, 15.5; S, 14.0.

System IV. (a) Ligand. Sodium (9.2 g) was dissolved in absolute ethanol (300 mL) and the solution was cooled to 0 °C under nitrogen. First, 2-mercaptoethyl sulfide (15.4 g) was added and then, without delay, bromoethylamine hydrobromide (40.1 g) in ethanol (50 mL) was rapidly added. A white precipitate formed at once. The mixture was stirred and held at 80 °C for 1.5 h. The solid was filtered and washed several times with ethanol. The filtrate and washings were taken down to an oil under vacuum. This oil was extracted with chloroform and the remaining solid was filtered and washed with ether. The filtrate and washings were taken to dryness under vacuum and the residue was recrystallized from chloroform by the addition of ether. This material (12.5 g) is impure and we have been unable to purify it by either chromatography or by crystallization from various solvent mixtures. That the expected product is indeed present is established by the copper derivative.

(b) Complex. The crude 1,11-diamino-3,6,9-trithiadecane (2.4 g) was taken up in warm water (50 mL) and $Cu(ClO_4)_2 \cdot 6H_2O$ (3.7 g) in water (50 mL) was added. A grey-blue precipitate formed at once. It was warmed on a steam bath for 10 min and, after cooling, the precipitate was collected and washed with methanol and ether. The solid was dissolved in hot water and filtered. Sodium perchlorate was added to the hot filtrate and upon cooling the pure product deposited as grey-blue needles (2.5 g). [The ligand was removed from the pure complex with NaCN but even this material could not be obtained pure. It seems that the ligand is unstable in the free state.] Anal. Calcd for $[Cu(C_8H_{20}N_2S_3)](ClO_4)_2$: C, 19.1; H, 4.0; N, 5.6; Cl, 14.1; S, 19.1. Found: C, 19.0; H, 4.2; N, 5.5; Cl, 14.0; S, 19.0.

System V. (a) Ligand. Sodium (3.9 g) was dissolved in ethanol (75 mL) and solid 2-aminoethanethiol hydrochloride (9.7 g) was added to the solution under nitrogen. The mixture was stirred at 80 °C for 15 min and then was cooled to 5 °C. 3-Bromo-*N*-propylphthalimide[50] (22.8 g) in ethanol (30 mL) was then added, whereupon a vigorous reaction ensued. The mixture was refluxed for 1.5 h and then was cooled. The salts were collected and washed with ethanol. The ethanol was removed under vacuum to give an oil.

This oil was dissolved in ethanol (50 mL) and hydrazine hydrate (6.4 g; 99–100%) was added and the mixture was refluxed for 1.5 h. Then hydrochloric acid (19 mL; 12 M) was added carefully and the resultant mixture was refluxed for a further 30 min. Most of the ethanol was removed under vacuum and the resulting solid was collected and washed with water (50 mL). The filtrate and washings were combined and reduced, under vacuum, to ca. 15 mL. To this liquid was added solid KOH until a brown oil separated. The oil was removed and dissolved in methylene chloride which was quickly dried over $MgSO_4$ and the solvent was removed under vacuum. The product was twice distilled under high vacuum to give 2.6 g of a colorless liquid (bp 69–71 °C at 0.1 mm). Anal. Calcd for $C_5H_{14}N_2S$: C, 44.7; H, 10.5; N, 20.9; S, 23.9. Found: C, 44.7; H, 10.7; N, 20.8; S, 23.8.

(b) Complexes. The 1-methylimidazole and aquo complexes of copper containing the above ligand were prepared in aqueous solution using a 1:1:1 ratio of N-MeIm–$Cu(ClO_4)_2 \cdot 6H_2O$–ligand and a 1:1 ratio of $Cu(ClO_4)_2 \cdot 6H_2O$–ligand for the two species. The concentration was 5×10^{-3} M.

System VI. (a) Ligand. A mixture of KSCN (100 g) in diethylene glycol (100 mL) was heated and stirred at 100 °C under nitrogen to give a clear solution. To this was added *cis,cis*-1,3,5-tris(toluenesulfonyloxy)cyclohexane[51] (23.8 g) in one portion. The mixture was kept at 100 °C for 4.5 h to give a clear solution which was cooled and diluted with water (250 mL) and then extracted with CH_2Cl_2–ether (1:1). The extracts were dried over Na_2SO_4 and the solvent removed under reduced pressure to yield *cis,cis*-1,3,5-trithiocyanocyclohexane (9.0 g) as a pale yellow solid which, without purification, was taken up in dry THF (110 mL) and was added dropwise over 0.75 h to a stirred suspension of lithium aluminum hydride (10 g) in ether (220 mL). The mixture was refluxed for 19 h and then, after cooling, the

$LiAlH_4$ was destroyed and the aluminum salts dissolved by the cautious dropwise addition of hydrochloric acid (300 mL; 8 M). The layers were separated and the aqueous layer was extracted with CH_2Cl_2–ether (4:1). The combined organic layers were washed with water, then with dilute $NaHCO_3$, and with a saturated brine solution. After being dried with Na_2SO_4, the solvents were removed under reduced pressure to yield a pale yellow, slightly malodorous oil (4.8 g). This was dissolved in aqueous NaOH (180 mL; 10%) which was cooled and stirred with CH_3I (36 mL). After 2 h, the excess CH_3I was removed by evaporation and the solution was thoroughly extracted with CH_2Cl_2–ether (1:1). After drying the extracts with Na_2SO_4, the solvent was removed under reduced pressure. An orange-red oil (3.2 g) remained. This was chromatographed on Al_2O_3 (Brockman activity II). Elution with CH_2Cl_2–hexane (1:11) gave a yellow oil (1.2 g) which on molecular distillation (100 °C; 0.1 mm) yielded a colorless oil. On standing at 25 °C the oil crystallized. It was recrystallized from a small volume of methanol after the solution was allowed to stand at −5 °C for 24 h. Pure *cis,cis*-1,3,5-tris(methylthia)cyclohexane deposited as white plates (1.0 g): mp 44–46 °C; NMR ($CDCl_3$–Me_4Si) δ 0.9–1.67 (m, 1, methylene), 2.10 (s, 3, methyl) 2.10–2.87 (m, 2, methylene and methine). Anal. Calcd for $C_9H_{18}S_3$: C, 48.6; H, 8.2; S, 43.3. Found: C, 48.6; H, 8.1; S, 43.1.

(b) Complex. Copper(II) perchlorate hexahydrate (0.03824 g) was dissolved in acetic anhydride (10 mL) at 40 °C and *cis,cis*-1,3,5-tris(methylthia)cyclohexane (0.02233 g) in acetic anhydride (10 mL) was added to the copper solution. Spectra were run at various concentrations of this 1:1 copper to ligand mixture. It was found that a 5.0×10^{-3} M solution in each of the species gave a spectrum with maximum intensity. Further concentration did not increase the intensity. Addition of excess ligand up to 2:1 did not alter the spectrum.

System VII. A finely powdered sample of [Cu(cyclam)]-$(ClO_4)_2$ (0.00231 g) (prepared by mixing 1 equiv of cyclam[52] and $Cu(ClO_4)_2 \cdot 6H_2O$ in a small volume of water) was placed in a 5-mL volumetric flask. To this was added 5 mL of an ice-cold ethanol solution containing sodium ethoxide and isopropyl mercaptan so that the final solution would be 1×10^{-3} M in copper, 25×10^{-3} M in sodium ethoxide, and 30×10^{-3} M in isopropyl mercaptan. The mixture was vigorously shaken at 0 °C to give a clear blue solution, the spectrum of which was measured at 10 °C without delay. We have tried various permutations of the above ratios but have found that the concentrations and procedure just given result in the most stable system (5–10 min at 10 °C) with the maximum intensities of the absorption bands. We have checked the effect of adding the mercaptan alone and of adding sodium ethoxide alone. The blue color does not develop in either case.

System VIII. This complex was obtained as green-blue needles after slight modifications of the published procedures.[53]

System IX. (a) Ligand. Aqueous ammonia (500 mL; 27%) was added to *N,N*-bis(chloroacetyl)ethylenediamine[54] (11.7 g) and the mixture was stirred at 25 °C for 48 h. The now clear solution was taken to dryness under reduced pressure. The residue was recrystallized from water–methanol (1:1) to yield the ligand as a white solid (5.2 g). It is the dihydrochloride salt. Anal. Calcd for $C_6H_{16}N_4O_2Cl_2$: C, 29.2; H, 6.5; N, 22.7; Cl, 28.7. Found: C, 29.0; H, 6.3; N, 22.5; Cl, 28.7.

(b) Complex. Spectra were obtained on 1×10^{-2} M solutions of complex which were prepared in water by mixing equivalent amounts of $CuCl_2 \cdot 2H_2O$ and the above ligand and then adding 4 equiv of sodium hydroxide solution. A violet solution resulted.

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