

# The Taming of the Blue

## Progress in Understanding a Novel Copper Center in Proteins

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As is evident from this and several of the companion articles, metal ions are essential cofactors for many biological macromolecules. It has been estimated that one-third of all known enzymes depend upon at least one metal ion for catalytic function (1). Accordingly, inorganic chemists and biochemists alike are intrigued by such questions as: How is the metal ion bound to a particular protein or enzyme? What is the function of the metal? How has the particular metal ion been selected? This article discusses how the first of these questions has been answered for a well-known copper center.

Ascertaining structure, even just the local structure within the so-called active site, is a difficult task when such complex molecules as proteins are involved. X-ray methods can be used to define the structure; however, suitable single crystals are not always easily obtained, and when they are available, interpretation of the diffraction pattern is a difficult and time-consuming process. For example, Freeman and co-workers spent several years searching for a source of plastocyanin which would permit the growth of the single crystals suitable for X-ray structure determination (2, 3). In principle, the local structure about the metal can be inferred from spectroscopic measurements, but the task is a formidable one. One of the major difficulties is that so many different types of ligands can be involved. Figure 1 lists some of the amino acid side chains which contain donor atoms capable of coordinating to metal ions. Water or other exogenous ligands such as the sulfide ion may also be present. The nuclearity (bimetallic, trimetallic, etc.) of the binding site, as well as the coordination number and the coordination geometry of each metal center, are variable as well. The problem is compounded if the chromophore is novel and small molecule analogues are unavailable.

A further complication, viewed from the point of view of the coordination chemist, is that the biological structures are often of rather low symmetry. The donor set is usually mixed, and the geometry is irregular. While this complicates the process of structural elucidation, such exquisitely tailored structures are highly effective from the point of view of function. For example, the coordination geometry may be shaped to promote the selection of a particular metal ion (4) or to minimize the barrier to electron transfer in an electron carrier (5). In addition the protein structure may serve to orient the pertinent molecular orbitals involved in electron transfer (6, 7) and/or to fine tune the reduction potential (8).

In spite of the complexities, spectroscopic methods are important aids to the study of metalloproteins, even if the

crystal structure is known. The reason is that spectral methods can be applied in solution or in vivo, and in some cases structural differences may occur between solution and the solid state (9, 10). They may also occur as a result of a change in temperature (11, 12). The various approaches which have been taken in order to characterize the structure of the so-called blue copper site are outlined below. Other interesting aspects of the proteins involved, e.g., the electron-transfer reactivities, sequence homologies etc., as well as further structural details, are described in various reviews (13-19).

### The Blue Copper Site: A Case Study

Blue copper centers are found in many different organisms, from bacteria to humans. The only known substrate is the electron (and in some cases, perhaps the proton (20)), and the primary function appears to be electron transfer (3, 15). Undoubtedly, the most distinctive characteristic of a blue copper site—and the basis of the term "blue copper"—is a strong absorbance in the region of 600 nm ( $\epsilon \approx 5000 M^{-1} cm^{-1}$ ). In Figure 2 the absorption spectrum of azurin from the bacterium *Pseudomonas aeruginosa* is presented. Of course a host of copper(II) complexes absorb in the red; it is

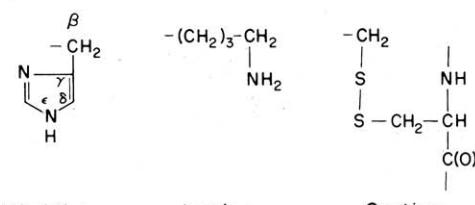
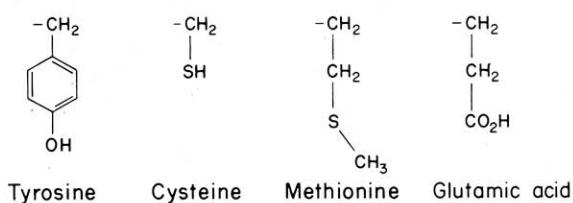


Figure 1. Side chains of selected amino acid residues. The building block of the protein backbone can be written as -NH-CHR-C(O)-, where R is the side chain.

Table 1. Representative Physical Data<sup>a</sup>

Protein or Complex	Species	$M_r$	$\lambda_{\max}$ (nm)	$\epsilon_{\max}$ (M <sup>-1</sup> , cm <sup>-1</sup> )	$A_{11}$ (cm <sup>-1</sup> × 10 <sup>4</sup> )	$E_{1/2}$ (V vs SHE)
Cu(OH) <sub>2</sub> <sub>6</sub> <sup>2+</sup>			790 <sup>b</sup>	13 <sup>b</sup>	134 <sup>c</sup>	0.15
Cu(NH <sub>3</sub> ) <sub>4</sub> <sup>2+</sup>			590 <sup>b</sup>	53 <sup>b</sup>	211 <sup>d</sup>	0.00 <sup>e</sup>
Azurin	<i>Pseudomonas aeruginosa</i>	14,600	626	4800 <sup>f</sup>	60	0.32
Plastocyanin	<i>Spinacea oleracea</i>	10,800	597	4500	63	0.38
Stellacyanin	<i>Rhus vernicifera</i>	20,000	605	4080	35	0.18
Rusticyanin <sup>g</sup>	<i>Thiobacillus ferrooxidans</i>	16,500	597	2240	45	0.67 (pH = 2)
Laccase	<i>Polyporus versicolor</i>	62,000	614	4900	90	0.79

<sup>a</sup> Data from refs. (13), (14), (16), and (19), except as noted.<sup>b</sup> Bjerrum, J., Ballhausen, C. J., and Jorgensen, C. K., *Acta Chem. Scand.*, **8**, 1275 (1954).<sup>c</sup> Walker, F. A., Sigel, H., and McCormick, D. B., *Inorg. Chem.*, **11**, 2756 (1972).<sup>d</sup> [Pt(NH<sub>3</sub>)<sub>4</sub>][PtCl<sub>4</sub>] host. Fritz, H. P., and Keller, H. J., *J. Naturforschg. B*, **20**, 1145 (1965).<sup>e</sup> Cu(NH<sub>3</sub>)<sub>4</sub><sup>2+</sup>/Cu(NH<sub>3</sub>)<sub>2</sub><sup>+</sup> couple. Phillips, C.S.G., and Williams, R. J. P., "Inorganic Chemistry," Vol. 2, Oxford University Press, New York, 1966, p. 316.<sup>f</sup> Ref. (36).<sup>g</sup> Lappin, A. G., Lewis, C. A., and Ingeldew, W. J., *Inorg. Chem.*, **24**, 1446 (1985), and reference therein.

the intensity of absorbance that is so unusual in the blue proteins. In fact the absorbance of a blue copper protein is about 400 times as intense as that of Cu(OH)<sub>2</sub><sub>6</sub><sup>2+</sup> (Table 1). The second spectroscopic signature of a blue copper center, first identified by Malmström and Vånnård in 1960, is a narrow hyperfine splitting in the low-field, or  $A_{11}$ , region of the EPR spectrum (21).

#### Coordination Geometry

A tetrahedral coordination geometry for the blue copper site was initially proposed in order to rationalize the intense visible absorption and the narrow hyperfine splitting (22, 23). Since there is no center of symmetry in a tetrahedron, the ground state wavefunction of the copper center could have mixed 3d-4p orbital character. This would intensify the ligand-field or "d-d" transitions because they assume d → p character.

More recently, it has become clear that the intense visible absorption is due to low-lying charge-transfer transitions, *vide infra*. In consonance with this interpretation, a series of less intense bands in the near infrared region of the absorption spectrum have been assigned as ligand-field bands (24). The near infrared bands are prominent in the magnetic circular dichroism spectrum, consistent with their assignment as magnetic dipole-allowed, d-d transitions. It should be noted that the d-d bands of cobalt(II)-substituted derivatives of azurin, plastocyanin, and stellacyanin also indicated that the site was distorted tetrahedral (25-27). Since a single d-d transition is expected from Cu(II) in pure tetrahedral symmetry, the fact that several d-d bands are observed indicates that the site has lower symmetry. If the distortion is modeled in terms of single angular deformation, the transition energies can best be explained in terms of a  $D_2$ -type flattening along one of the twofold axes of the original tetrahedron (14, 24). As becomes apparent below, however, the observed structure has no symmetry (apart from the identity operation), and it cannot be described in terms of a particular angular deformation of a tetrahedron. Although the ligand field analyses helped to characterize the gross geometry, they failed to provide very detailed structural information.

#### Donor Set

As a result of a great deal of experimental work, the donor make up of the blue copper site was, on the other hand, rather well anticipated. In some of the first definitive experiments, NMR relaxation studies demonstrated that the site lacked an exchangeable water ligand (28, 29). This is consis-

tent with the outer-sphere electron-transfer function of this center, as there is no need for the association and dissociation of substrates.

Over time, experimental evidence mounted for the presence of a highly polarizable ligand, ultimately identified as a thiolate sulfur from a cysteine residue. Initially, Malström and Vånnård suggested that the narrow hyperfine splitting in the EPR might be explained by a strongly covalent interaction involving the copper center (21). A clue to the nature of the ligand came from studies of plastocyanin (30). Katoh and co-workers found that the addition of mercurials bleached plastocyanin. Since mercurial groups combine strongly—although not exclusively—with thiolate groups, Katoh suggested that a cysteine sulfur was involved in binding copper. Then studies in Rome showed that the copper-depleted forms of azurin and stellacyanin also reacted with mercurials while the reaction was blocked in the native proteins (31, 32). About this time, Williams suggested that the intense visible absorption of the blue copper center could be due to a charge-transfer transition from cysteine sulfur to copper(II) (33).

Soon thereafter, metal-replacement studies showed that the energy of the intense absorption varied inversely with the electron affinity of the metal ion and established that the intense visible absorbance of the blue copper center was a charge-transfer phenomenon (25, 34, 35). Figure 2 reveals that the bands shift steadily to higher energy as the atomic

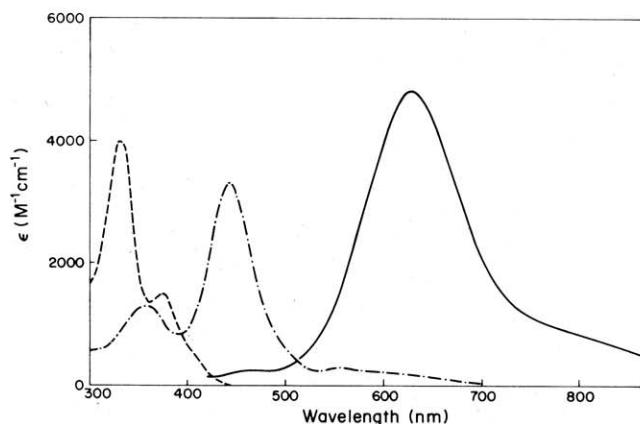


Figure 2. Charge transfer absorption spectra of native azurin (—) and the nickel(II) (- · -) and cobalt(II) (- - -) derivatives of azurin.

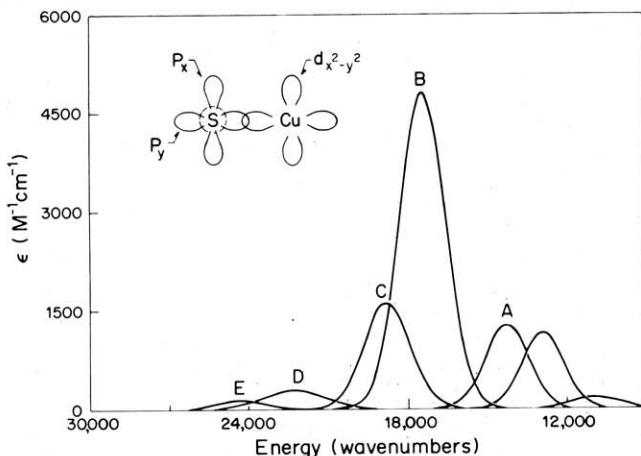


Figure 3. Visible spectrum of plastocyanin resolved into Gaussian components. The figure is based on the data in ref. (14), and bands A-E have been assigned to charge-transfer transitions. The insert depicts the relative orientations of the sulfur 3p orbitals and the 3d<sub>x<sup>2</sup>-y<sup>2</sup></sub> orbital of copper. The dashed circle denotes the 3p<sub>z</sub> orbital which is principally involved in the bond to the  $\beta$ -CH<sub>2</sub> group.

number of the first row ion decreases. When the band energies were analyzed in terms of the optical electronegativities of Jørgensen, a cysteine thiolate donor was strongly indicated (25, 35, 36).

Further visible absorption studies (14), in conjunction with circular dichroic spectra (37, 38), revealed that the visible absorbance of the blue copper site was actually comprised of several transitions. The component transitions which have been resolved in the case of plastocyanin are indicated in Figure 3, and the most recent assignments are compiled in Table 2. The most intense component is assigned as  $\sigma$ S(Cys)  $\rightarrow$  Cu(II). The notation signifies that the transition originates from a molecular orbital which can be construed as predominantly a thiolate "lone-pair" orbital which exhibits sigma-type overlap with the half-filled d orbital of copper(II) (Fig. 3). This transition occurs at somewhat higher energy than the  $\pi$ S(Cys)  $\rightarrow$  Cu(II) analogue because the sigma lone-pair is preferentially stabilized by interaction with the metal orbital.

The existence of a Cu(II)-thiolate chromophore was a bit of a surprise because the following reaction:



occurs quite rapidly when the two components are combined in solution (39). Evidently, the protein structure impedes this reaction, presumably by blocking the formation of the disulfide bond. Via some clever synthetic work, several examples of reasonably stable, small molecule copper(II)-thiolate linkages are now known (40-42).

Methionine sulfur is an alternative donor that could have explained the low-lying charge-transfer transition, since polythiaether complexes of Cu(II) also exhibit intense visible absorbance (43). The debate over an S(Cys) versus an S(Met) ligand was not finally resolved (it turned out not to be a question of either/or) until the crystal structures became available. However, the results of the mercurial titrations and the fact that  $\sigma$ S  $\rightarrow$  Cu(II) and  $\pi$ S  $\rightarrow$  Cu(II) transitions were resolved argued convincingly for the presence of thiolate sulfur. (In the region of interest, only a  $\sigma$ S(Met)  $\rightarrow$  Cu(II) transition is expected from methionine sulfur (44).)

Simultaneously, progress was being made in the identification of the other donors. In 1975 Markley and co-workers produced good evidence that there were two histidine ligands in the binding site of plastocyanin (45). This was possible because the  $\delta$ -C and the  $\epsilon$ -C hydrogens of the imidazole group (Fig. 1) appear as singlets in the aromatic region

Table 2. Band Assignments for Plastocyanin

Band <sup>a</sup>	$\lambda_{\max}^b$ (nm)	Orbital Assignment <sup>c</sup>	
		Ref. (44)	Ref. (62)
A	752	$\pi$ S(Cys) $\rightarrow$ Cu(II)	$p_x$ (Cys) $\rightarrow$ Cu(II)
B	606	$\sigma$ S(Cys) $\rightarrow$ Cu(II)	$p_y$ (Cys) $\rightarrow$ Cu(II)
C	552	$\sigma$ S(Met) $\rightarrow$ Cu(II)	$s, p_z$ (Cys) $\rightarrow$ Cu(II) or $\pi$ (His) $\rightarrow$ Cu(II)
D	450	$\pi$ (His) $\rightarrow$ Cu(II)	$\pi$ (His) $\rightarrow$ Cu(II)
F	428		$\pi$ (His) $\rightarrow$ Cu(II) or $\sigma$ S(Met) $\rightarrow$ Cu(II)

<sup>a</sup> See Figure 3 for labels.

<sup>b</sup> From Ref. (24).

<sup>c</sup> Recent studies establish that  $\pi$ (Im)  $\rightarrow$  Ru(III) and  $\sigma$ (RS<sup>-</sup>)  $\rightarrow$  Ru(III) transitions can occur at similar energies. This is in accord with the  $\pi$ (His)  $\rightarrow$  Cu(II) assignment of Band C. (Krogh-Jespersen, K., and Schugar, H. J., *Inorg. Chem.*, **23**, 4390 (1984)).

of the <sup>1</sup>H-NMR spectrum where individual resonances can be reasonably well resolved. In addition, because the imine nitrogen of the imidazole ring is subject to protonation, the  $\delta$  and  $\epsilon$  resonances exhibit a characteristic pH dependence (46). However, the apparent pK is much lower when the imine lone pair is coordinated to the metal ion. Finally, in the oxidized form of the protein, the resonances of the coordinated imidazoles are severely broadened by the paramagnetic Cu(II) center. By monitoring these several effects, various workers have found that two histidine ligands are usually bound at blue copper centers (47, 48). It can be noted that ENDOR and spin-echo EPR techniques methods have been used to detect the nitrogen donors as well (49-51). Subsequently, Schugar and co-workers identified  $\pi$ N(His)  $\rightarrow$  Cu(II) charge-transfer transitions in model compounds (52), and this has led to assignments of analogous transitions in blue copper spectra (14). The suggested assignments are included in Table 2.

By 1977-17 years after its discovery—the blue copper site could be described as a distorted tetrahedral center with two imidazole donors (from histidines) and a thiolate sulfur from a cysteine residue. The identity of the fourth donor was uncertain, although plenty of predictions were available (14), including methionine sulfur. The latter came from an analysis of the protein sequence and the observation that a particular methionine was conserved in plastocyanins from several different species (53).

#### Direct Structural Studies

The first detailed 3-dimensional structural information about a blue copper protein appeared in 1978 when Freeman and co-workers reported the crystal and molecular structure of plastocyanin from poplar leaves (54). The plastocyanin molecule can be described as a barrel-like structure consisting of eight "strands" (staves) comprised of short runs of a single polypeptide chain which undergoes a series of loops and hairpin turns (Fig. 4). Copper is found at one end of the barrel, usually termed the northern end, and is coordinated to the sulfur atoms of Cys 84 and Met 92 and to the  $\delta$ -nitrogens of His 37 and His 87. Recently, the structure has been refined to 1.6 Å resolution; the bond angles and bond distances are reported in Figure 5 (3).

It was satisfying to find that the X-ray structure confirmed the presence of cysteine sulfur and the histidine nitrogen donors as predicted by the spectroscopic analyses. The long Cu-S(Met) distance indicates that the interaction is weak in comparison with the other donors, and this may be the reason why this group was so hard to identify by spectroscopic means. In the first approximation the donor arrangement can be described in terms of deformation along a threefold axis (the Cu-S(Met) bond) of an idealized tetrahedral structure, but the real symmetry is much lower than axial.

Soon after the plastocyanin structure was reported, the

structure of azurin from the bacterium *Pseudomonas aeruginosa* appeared (55). More recently, the *Pseudomonas* structure has been further refined (56), and the structure of azurin from *Alcaligenes denitrificans* has also appeared (57). So far, the azurin structures are known at considerably lower resolution than the plastocyanin structure. Although there are many differences in the detailed structures (16), the coordination geometry of copper is similar in azurin and plastocyanin. However, in both azurins there is the suggestion that a peptide oxygen may act as a distant fifth ligand. If so, the coordination geometry might be better described as a distorted trigonal bipyramidal with axial S(Met) and O(peptide).

In 1978 the EXAFS of *Pseudomonas* azurin was also analyzed and interpreted in terms of a short Cu-S interaction and 2-3 Cu-N interactions at 1.97 Å (58). A second sulfur ligand was tentatively identified at a longer distance, but with considerably less precision. These basic conclusions were confirmed in subsequent analyses (59-61).

#### Single-Crystal Spectroscopy

The polarizations of the charge-transfer transitions of a single crystal of plastocyanin have been investigated in an

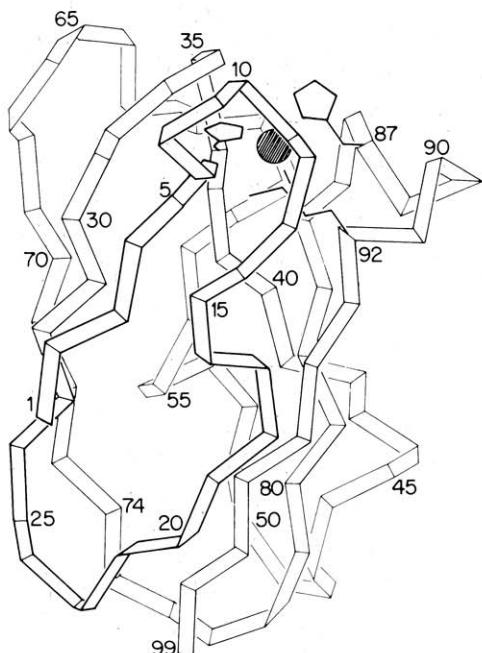


Figure 4. A ribbon drawing of plastocyanin which illustrates the polypeptide folding. The copper atom and the copper ligands are also indicated. The figure is an adaptation of one that appears in ref. (16).

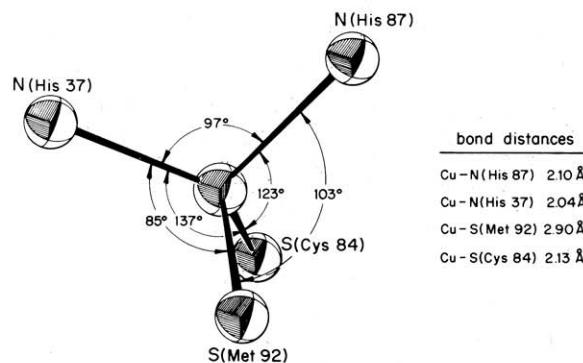


Figure 5. An ORTEP drawing of the copper atom and its donor set in plastocyanin. The orientation is approximately the same as that of Figure 4. The S(Cys84)-Cu-S(Met92) angle is 108°.

attempt to place the assignments on a more secure experimental basis (62). Although the analysis is complicated by the low site symmetry, the  $\sigma$ S(Cys)  $\rightarrow$  Cu(II) transition is expected to be polarized parallel to the Cu-S(Cys) bond. (However, the polarization of the  $\pi$ -type transition is not necessarily the same (63).) Solomon and co-workers have used the polarized absorption data to arrive at the assignments listed in Table 2 (62). They find no evidence for the  $\sigma$ S(Met)  $\rightarrow$  Cu(II) transition in the energy regime where it is expected (near that of  $\sigma$ S(Cys)  $\rightarrow$  Cu(II) (44)). This may be due to the long Cu-S(Met) distance and consequently weak orbital overlap. Single crystal EPR studies were also described (62), and they indicate that the  $d_{x^2-y^2}$  orbital, the orbital which contains the unpaired electron, is in a plane more or less perpendicular to the Cu-S(Met) axis.

Single-crystal EXAFS studies of plastocyanin have also been reported, and they revealed no hint of S(Met) even when the Cu-S(Met) bond was oriented for maximum interaction with the departing electron (64). This may have resulted from a dynamic disorder in the Cu-S(Met) bond due to the weak binding of methionine (64).

#### Vista

Blue copper proteins emerged more than 2 billion years ago (17). Nevertheless, the core of ligands is conserved in proteins from a wide variety of species, and it seems safe to infer that this site is highly evolved for the electron transfer function. While the details of the copper binding are now well understood, we cannot claim the same degree of understanding as regards the functional aspects of this unique structure. Before the proteins were characterized, who would have imagined that a distorted tetrahedral ligand environment containing a thiol group would be a favorable environment for Cu(II)? Yet even the simplest of organisms can specifically synthesize these proteins with a copper cofactor in spite of the low cellular abundance of copper. Of interest in this regard, recent calorimetry studies of azurin reveal that the binding energy of Cu(II) exceeds those of other first-row transition ions (65). This suggests the binding of copper may be favored thermodynamically. Is this a consequence of the particular donors involved or their geometrical arrangement? More specifically, does the remote location of the S(Met) donor bias the site toward copper? Another possibility is that the fourth ligand (and perhaps its identity) varies from protein to protein and that this permits a fine tuning of the potential, etc., for particular systems (14). The problem of metal selection aside, how does the protein architecture influence the electron transfer function? Do charged residues on the surface direct the binding of redox partners (66)? Is the redox orbital of copper oriented specifically to promote the physiological function (16)? These questions and many others are just beginning to be answered. The last chapter in our understanding of the novel blue copper center has not been written.

#### Acknowledgment

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#### Literature Cited

- (1) Malmström, B. G. *Chem. Scr.*, **21**, 7 (1983).
- (2) Chapman, G. V., Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., and Venkatappa, M. P., *J. Mol. Biol.* **110**, 187 (1977).
- (3) Guss, J. M., and Freeman, H. C., *J. Mol. Biol.*, **169**, 521 (1983).
- (4) Frausto da Silva, J. J. R., and Williams, R. J. P., *Struct. Bonding (Berlin)*, **29**, 67 (1976).
- (5) Gray, H. B., and Malmström, B. G., *Comments Inorg. Chem.*, **2**, 203 (1983).
- (6) Salemme, F. R., *Ann. Rev. Biochem.*, **46**, 299 (1977).
- (7) Poulos, T. L., and Mauk, A. G., *J. Biol. Chem.*, **258**, 7369.
- (8) Sweeney, W. V., and Rabinowitz, J. C., *Ann. Rev. Biochem.*, **49**, 139 (1980).
- (9) Bertini, I., Lanini, G., Luchinat, C., Monnanni, R., *Inorg. Chim. Acta*, **107**, 153 (1985).
- (10) Mims, W. B., Davis, J. L., and Peisach, J., *Bioophys. J.*, **45**, 755 (1984).
- (11) Musci, G., Desideri, A., Morpurgo, L., Garnier-Suillerot, A., and Tosi, L., *Biochem. J.*,

- 213, 503 (1983).
- (12) Woodruff, W. H., Norton, K. A., Swanson, B. I., and Fry, H. A., *Proc. Natl. Acad. Sci., U.S.A.*, **81**, 1263 (1984).
  - (13) Fee, J. A., *Struct. Bonding (Berlin)*, **23**, 1 (1975).
  - (14) Gray, H. B., Solomon, E. I., *Metal Ions. Biol.*, **3**, 1 (1981).
  - (15) Farver, O., Pecht, I., in "Copper Proteins and Copper Enzymes," Vol. I, (*Editor: Lontie, R.*), CRC Press, Boca Raton, FL, 1984, pp. 183-214.
  - (16) Adam, E. T., *Top. Mol. Struct. Biol.*, **6**, 1 (1985).
  - (17) Ryden, L., in "Copper Proteins and Copper Enzymes," Vol. I, (*Editor: Lontie, R.*), CRC Press, Boca Raton, FL, 1984, pp. 157-182.
  - (18) Holwerda, R. A., Wherland, S., and Gray, H. B., *Ann. Rev. Biophys. Bioeng.*, **5**, 363 (1976).
  - (19) Lappin, A. G., *Met. Ions. Biol. Syst.*, **13**, 15 (1981).
  - (20) Freeman, H. C., *Coord. Chem.*, **21**, 29 (1981).
  - (21) Malmström, B. G., and Vännågård, T. J., *J. Mol. Biol.*, **2**, 118 (1960).
  - (22) Blumberg, W. E., in "The Biochemistry of Copper," (*Editors: Peisach, J., Aisen, P., and Blumberg, W. E.*), Academic Press, New York, 1966, pp. 49-64.
  - (23) Brill, A. S., and Bryce, G. F., *J. Chem. Phys.*, **48**, 4398 (1968).
  - (24) Solomon, E. I., Hare, J. W., Dooley, D. M., Dawson, J. H., Stephens, P. J., and Gray, H. B., *J. Amer. Chem. Soc.*, **102**, 168 (1980).
  - (25) McMillin, D. R., Rosenburg, R. C., and Gray, H. B., *Proc. Natl. Acad. Sci., U.S.A.*, **71**, 4760 (1974).
  - (26) Solomon, E. I., Rawlings, J., McMillin, D. R., Stephens, P. J., and Gray, H. B., *J. Amer. Chem. Soc.*, **98**, 8046 (1976).
  - (27) Hauenstein, B. L., Jr., and McMillin, D. R., *Met. Ions. Biol. Syst.*, **13**, 319 (1981).
  - (28) Blumberg, W., and Peisach, J., *Biochim. Biophys. Acta*, **126**, 269 (1966).
  - (29) Koenig, S. H., and Brown, R. D., *Ann. N.Y. Acad. Sci.*, **222**, 752 (1973).
  - (30) Katoh, S., and Takamiya, A., *J. Biochem. (Tokyo)*, **55**, 378 (1964).
  - (31) Morpurgo, L., Finazzi-Agrò, A., Rotilio, G., and Mondovi, B., *Biochim. Biophys. Acta*, **271**, 292 (1972).
  - (32) Finazzi-Agrò, A., Giovagnoli, C., Avigliano, L., Rotilio, G., and Mondovi, B., *Eur. J. Biochem.*, **34**, 20 (1973).
  - (33) Williams, R. J. P., *Inorg. Chim. Acta Rev.*, **5**, 137 (1971).
  - (34) McMillin, D. R., Holwerda, R. A., and Gray, H. B., *Proc. Natl. Acad. Sci., U.S.A.*, **71**, 1339 (1974).
  - (35) Tennent, D. L., and McMillin, D. R., *J. Amer. Chem. Soc.*, **101**, 2307 (1979).
  - (36) McMillin, D. R., *J. Bioinorg. Chem.*, **8**, 179 (1978).
  - (37) Tang, S. P. W., Coleman, J. E., and Myer, Y. P., *J. Biol. Chem.*, **243**, 4286 (1968).
  - (38) Falk, K. E., Reinhammar, B., *Biochim. Biophys. Acta*, **285**, 84 (1972).
  - (39) Amundsen, A. R., Whelan, J., and Bosnich, B., *J. Amer. Chem. Soc.*, **99**, 6730 (1977).
  - (40) Thompson, J. S., Marks, T. J., and Ibers, J. A., *Proc. Natl. Acad. Sci., U.S.A.*, **74**, 3114 (1977).
  - (41) Hughley, J. L., IV, Fawcett, T. G., Rudich, S. M., Lalancette, R. A., Potenza, J. A., and Schugar, H. J., *J. Amer. Chem. Soc.*, **101**, 2617 (1979).
  - (42) Toftlund, H., Becker, J., Olesen, P. H., and Pedersen, J. Z., *Isr. J. Chem.*, **25**, 56 (1985).
  - (43) Ferris, N. S., Woodruff, W. H., Rorabacher, D. B., Jones, T. E., and Ochrymowycz, L. A., *J. Amer. Chem. Soc.*, **100**, 5939 (1978).
  - (44) McMillin, D. R., Morris, M. C., *Proc. Natl. Acad. Sci., U.S.A.*, **78**, 6567 (1981).
  - (45) Markley, J. L., Ulrich, E. L., Berg, S. P., and Krogmann, D. W., *Biochemistry*, **14**, 4428 (1975).
  - (46) Markley, J. L., *Acc. Chem. Res.*, **8**, 70 (1975).
  - (47) Ulrich, E. L., and Markley, J. L., *Coord. Chem. Rev.*, **27**, 109 (1978).
  - (48) Cass, A. E. G., and Hill, H. A. O., in "Copper Proteins and Copper Enzymes," Vol. I, (*Editors: Lontie, R.*), CRC Press, Boca Raton, FL, 1984, pp. 63-91.
  - (49) Rist, G. H., Hyde, J. S., and Vännågård, T., *Proc. Natl. Acad. Sci., U.S.A.*, **67**, 79 (1970).
  - (50) Roberts, J. E., Cline, J. F., Lum, V., Freeman, H., Gray, H. B., Peisach, J., Reinhammar, B., and Hoffman, B. M., *J. Amer. Chem. Soc.*, **106**, 5324 (1984).
  - (51) Mims, W. B., and Peisach, J., *Biochemistry*, **15**, 3863 (1976).
  - (52) Schugar, J. J., in "Copper Coordination Chemistry: Biochemical and Inorganic Perspectives," (*Editors: Karlin, K. D., and Zubietta, J.*), Adenine Press, Guilderland, NY, 1983, pp. 43-47.
  - (53) McLendon, G., and Martell, A. E., *J. Inorg. Nucl. Chem.*, **39**, 191 (1977).
  - (54) Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., and Venkatappa, M. P., *Nature (London)*, **272**, 319 (1978).
  - (55) Adman, E. T., Stenkamp, R. E., Sieker, L. C., and Jensen, L. H., *J. Mol. Biol.*, **123**, 35 (1978).
  - (56) Adman, E. T., and Jensen, L. H., *Isr. J. Chem.*, **21**, 8 (1981).
  - (57) Norris, G. E., Anderson, B. F., and Baker, E. N., *J. Mol. Biol.*, **165**, 501 (1983).
  - (58) Tullius, T. D., Frank, P., and Hodgson, K. O., *Proc. Natl. Acad. Sci., U.S.A.*, **75**, 4069 (1978).
  - (59) Peisach, J., Powers, L., Blumberg, W. E., and Chance, B., *Biophys. J.*, **38**, 277 (1981).
  - (60) Blumberg, W. E., and Powers, L., *Fed. Proc.*, **41**, 3462 (1983).
  - (61) Co, M. S., and Hodgson, K. O., in "Copper Proteins and Copper Enzymes," Vol. I, (*Editor: Lontie, R.*), CRC Press, Boca Raton, FL, 1984, pp. 93-113.
  - (62) Penfield, K. W., Gay, R. R., Himmelwright, R. S., Eickman, N. C., Norris, V. A., Freeman, H. C., and Solomon, E. I., *J. Amer. Chem. Soc.*, **103**, 4382 (1981).
  - (63) Murrell, J. N., *Quart. Rev.*, **15**, 191 (1961).
  - (64) Scott, R. A., Hahn, J. E., Doniach, S., Freeman, H. C., Hodgson, K. O., *J. Amer. Chem. Soc.*, **104**, 5364 (1982).
  - (65) Engeseth, H. R., and McMillin, D. R., to be published.
  - (66) Chapman, S. K., Davies, D. M., Watson, A. D., and Sykes, A. G., *ACS Symp. Ser.*, **211**, 177 (1983).