Further perspectives on the charge transfer transitions of blue copper proteins and the ligand moieties in stellacyanin

(visible absorption/methionine/cysteine/cystine)

DAVID R. MCMILLIN AND MARGARET C. MORRIS

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

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ABSTRACT The charge transfer spectra of plastocyanin and azurin are analyzed in detail, taking into account the structural information that is available. The number and relative energies of the low-lying charge transfer bands attributable to cysteine and methionine sulfur are considered with reference to the energy levels associated with the donor centers and to experimental work on relevant complexes. Two cysteine $\rightarrow$ Cu(II) charge transfer transitions are assigned to bands that occur near 620 nm and near 770 nm. Energy considerations suggest that the methionine $\rightarrow$ Cu(II) transition occurs in the vicinity of 550 nm for both plastocyanin and azurin. The existence of a disulfide linkage in stellacyanin is established by thiol titrations, and the possibility exists that the fourth ligand in stellacyanin may involve disulfide sulfur. If so, the cystine $\rightarrow$ Cu(II) transition is also expected to appear around 550 nm.

The unusual absorption spectra of blue copper proteins have been the subject of considerable interest for 20 years or more (1); however, the band assignments are not yet fully established. This article has two aims. The first is to clarify, insofar as possible, the charge transfer (CT) band assignments for the two blue copper proteins azurin and plastocyanin, at the same time reconsidering some interpretations that have appeared in the literature. The second is to narrow the field of possible structures for the blue copper site in stellacyanin and to examine critically the utility of electronic spectra in identifying the ligands.

The visible absorption spectrum of plastocyanin is presented in Fig. 1 where it has been resolved into a number of gaussian components. Both d-d bands and CT bands are shown, and the CT bands are labeled A-E, the bands to lower energy having been assigned to d-d transitions (2). The first convincing proof of the CT nature of bands A and B came as a result of metal replacement studies of stellacyanin, plastocyanin, and azurin (3, 4). Bands A and B were tentatively assigned as π S(Cys) $\rightarrow$ Cu(II) and σ S(Cys) $\rightarrow$ Cu(II), respectively, from the cysteine ligand which had been previously implicated by mercurial titrations (5, 6). [The σ and π designations are carryovers from studies of octahedral complexes of halide ligands in which one of the p orbitals of the halide is σ -bonded to the metal center and the other two p orbitals are π bonded (7).] By now, there is considerable supporting evidence for these assignments (1).

Assigning the remaining CT bands proved more difficult, and little progress was made until x-ray structural analysis had established that the copper centers in plastocyanin and azurin have S_2N_2 donor environments involving a cysteine, a methionine, and two histidine side chains. However, band D has recently been convincingly assigned as π (Im) $\rightarrow$ Cu(II) in which Im denotes a coordinated imidazole group (1, 2, 8-10). The assignments of bands C and E remain controversial. Tennent

and McMillin (11) and Gray and coworkers (12) favor defining band C as S(Met) $\rightarrow$ Cu(II) CT but Solomon and coworkers (10) dispute this assignment based on polarized absorption measurements on crystalline plastocyanin.

This disagreement is unfortunate because the CT bands could be useful in deducing the ligand environment in other blue copper proteins whose structures are unknown. Stellacyanin is of particular interest in this regard because its peptide contains no methionine residues (13). Evidently, the metal binding site in stellacyanin is somewhat different from that of azurin or plastocyanin, although the site appears to remain tetrahedral (2) and to involve a cysteine sulfur (11, 14) and two histidines (15). Based on an analysis of ligand optical electronegativities, Tennent and McMillin (11) suggested that the fourth ligand may be a disulfide sulfur. A similar suggestion had been proposed by Woodruff and coworkers (16) who carried out resonance Raman studies. On the other hand, Hill and Lee (15) suggested that two cysteine ligands may be involved. This hypothesis probably can be reconciled with CT spectra because the absorption spectrum of the copper derivative of liver alcohol dehydrogenase is similar to that of a blue copper moiety (17), and one of the binding sites of alcohol dehydrogenase is known to be tetrahedral and to involve a histidine and two cysteines as ligands (18).

The model suggested by Hill and Lee (15) has been tested by carrying out thiol titrations, and the results are described below after the CT assignments for plastocyanin and azurin are reexamined.

MATERIALS AND METHODS

Materials. The acetone powder containing stellacyanin was obtained from Saito and Co. (Osaka, Japan). The NaDodSO₄ was electrophoresis purity grade and was purchased from Bio-Rad. The 5,5'-dithiobis(2-nitrobenzoic acid) was purchased from Aldrich. The phosphate buffers were passed through a Chelex-100 column to remove adventitious transition metal ions. The 2-mercaptoethanol was purchased from Eastman and was determined to be 92% active by dithiobisnitrobenzoic acid titration.

Procedure. Native stellacyanin was extracted from the acetone powder according to the procedure of Reinhammar (19). Apostellacyanin was prepared by using the method of Morpurgo (20). Protein was denatured by dialysis against 0.1 M phosphate, pH 7.0/1% NaDodSO₄. Reduced stellacyanin was prepared anaerobically by adding a 150-fold excess of 2-mercaptoethanol to denatured protein at 40°C. Reaction was complete after 1½ hr, and the protein was dialyzed against 0.1 M phosphate, pH 7.0/1% NaDodSO₄. Sulfhydryl titrations were carried out by the method of Ellman (21).

Instrumentation. The UV-visible absorption spectra were obtained with a McPherson EU-700 spectrophotometer. The

FIG. 1. Visible spectrum of plastocyanin resolved into gaussian components. Bands A through E are assigned as CT transitions. The figure has been constructed by using the data reported in ref. 2.

pH was measured at room temperature with an Orion model 601 A digital Ionalyzer.

RESULTS AND DISCUSSION

Theoretical Considerations. When assigning CT transitions, it is often useful to take into account the reduction potentials or the ionization energies and electron affinities of the centers involved. For example, if all else is the same, a relatively easily ionized ligand will give rise to a lower-energy ligand-to-metal CT transition than will a ligand more difficult to ionize. Granting that the ionization energies and electron affinities are related to orbital energies, it is important to remember that the transition energy is not simply a difference in orbital energies (22–24). A better, although still inexact, expression for the energy of a ligand-to-metal CT transition is

$$h\nu = \epsilon_M - \epsilon_L - J_{ML} + 2K_{ML}$$

in which ϵ_M is the energy of the metal-centered orbital, ϵ_L is the energy of the ligand-centered orbital, and J_{ML} and K_{ML} are the coulomb and exchange integrals, respectively, which account for electron–electron repulsive effects. J_{ML} represents the mutual repulsion between the charge densities defined by the metal-centered and the ligand-centered orbitals involved, and K_{ML} denotes the self-repulsion of the charge density defined by the overlap of the metal- and ligand-centered orbitals.

The coulomb and exchange integrals can be very significant in determining the magnitude of the transition energy (22, 24), although the exchange integral is likely to be small in the case of a CT transition.* Unfortunately, the various terms in this equation are difficult to quantify. However, some insight can be obtained from ligand ionization energies which are related to the ϵ_L values and which are available from photoelectron spectroscopy. Obviously, caution is needed with such an approach, especially when a number of close-lying transitions are being considered. Of course, strong “relaxation” can be associated with photoelectron energies, and this could confuse relative energies; however, for the ligands under consideration the photoelectron energies agree well with theoretical calculations of orbital energies.

Photoelectron Spectra of Relevant Sulfur Moieties. Literature data for the photoionization energies of relevant sulfur donors are presented in Table 1. With the exception of the di-

Table 1. Photoionization energies of sulfur ligands

Compound	Experimental energies,* eV	Calculated energies, eV
H ₂ S	10.48	10.68 [†]
	13.4	13.61 [†]
CH ₃ SH	9.41	9.97 [‡]
	11.9	12.16 [‡]
(CH ₃) ₂ S	8.68	8.03 [†]
	11.2	10.85 [†]
CH ₃ SSCH ₃	8.97	9.01 [§]
	9.21	9.28 [§]

* Ref. 25.

[†] Ref. 26.

[‡] Ref. 27.

[§] Ref. 28.

sulfide species, the first two ionization energies are found to be well separated. In each case the lowest ionization energy is associated with a nonbonding sulfur 3p orbital. The second ionization energy—which is generally >2 eV more positive—reflects an orbital with appreciable sulfur 3s character and hence the greater ionization energy. In the disulfide case, both ionization energies correspond to orbitals that are predominantly sulfur 3p in character but are split by interacting with each other (29).

CT Transitions from Thiolate Sulfur. The Lewis structure of a thiolate sulfur involves three lone pairs.[†] On this basis one might naively presume that three CT transitions of roughly similar energies can be associated with a thiolate ligand. However, a more detailed analysis reveals that this model is inadequate. In fact, a free thiolate group exhibits two types of lone pairs at quite different energies (Fig. 2). Thus, the photoelectron spectrum of CH₃Cl (isoelectronic with CH₃S[−]) reveals a degenerate set of lone pairs having an ionization energy of ≈11.2 eV and a next-lowest ionization energy at 14.1 eV (30). To understand this, recall that in the free atom the sulfur 3s and 3p orbitals are affected differently by the presence of the core electrons about the sulfur nucleus and that the 3s orbital is appreciably more stable than the 3p orbitals. Vestiges of this effect are to be expected in a molecular environment, and the empirical finding is that the 3s orbital tends to concentrate in a relatively low-lying lone pair level of the sulfur moiety (see the calculations referenced in Table 1 and ref. 31). The energy level scheme alters somewhat upon coordination, but the results in Table 1 indicate that the lone pair with more 3s character is expected to occur ≈2 eV below the others. Because the coulomb integrals will be roughly comparable, a spread of 2 eV or more would then be expected to occur among the three CT transitions from the thiolate group, suggesting that only two low-energy CT bands will be observed. A schematic energy level diagram illustrating this case is also depicted in Fig. 2 where a “bent” geometry at sulfur is assumed.

CT Transition(s) from a Coordinated Thioether. A thioether moiety exhibits two lone pairs; however, the two levels have quite different energies and involve different amounts of sulfur 3s character (Table 1). The lone pair orbital which is associated with the lowest ionization energy will be involved in bond formation with the metal. A transition from this level should be readily observed and can be labeled $\sigma S(\text{Met}) \rightarrow \text{Cu(II)}$. A second transition involving the other lone pair level would occur at much higher energy. In accord with this analysis, the spectral studies by Stein and Taube (32, 33) involving pentaamine ruthen-

* Basch, H., Hollister, C. & Moskowitz, J. (1967) in *Abstracts of Papers* 154th National Meeting of the American Chemical Society, Chicago, September 1967, Inorganic Section, No. 81.

[†] For our purposes, the term “lone pair” designates a filled molecular orbital that is principally localized on the donor atom of a ligand moiety. The level involved need not be strictly nonbonding.

FIG. 2. Qualitative energy-level diagram for the interaction of a thiolate ligand with a metal acceptor orbital. The lone pair levels of the thiolate group are presented at the left; the metal orbital is presented at the right. The thiolate lone pairs rich in $3s$ and $3p$ character are labeled with appropriate subscripts.

mium(III) complexes of various sulfur donors revealed only one low-lying CT transition from a thioether sulfur. Literature data show that the $\sigma S(\text{Met}) \rightarrow \text{metal}$ transitions generally occur with about the same energy as, or at slightly higher energy than, the $\sigma S(\text{Cys}) \rightarrow \text{metal}$ transitions in a related series of complexes (33, 34). On energy grounds one therefore concludes that the transition involving methionine sulfur should fall in the region of band C in Fig. 1. Although the copper-to-S(Met) bond is long (35), this energy argument is probably still valid in view of the compensating effects that can be expected to occur upon bond lengthening—e.g., a less-negative ϵ_L term and a less-positive J_{ML} term. If all else is the same, of course, the absorption intensity of the $\sigma S(\text{Met}) \rightarrow \text{Cu(II)}$ transition would decrease as the bond length increases (36).

However, based on polarized absorption studies, Solomon and coworkers (10) rejected a $\sigma S(\text{Met}) \rightarrow \text{Cu(II)}$ assignment for band C. Relative to one of the crystal faces of plastocyanin, they found that band C appears to be allowed in both parallel and perpendicular polarizations with respect to the crystallographic a axis, and the copper-methionine bond is roughly perpendicular to this axis. On this basis, these workers preferred to assign band C to a third CT transition originating at the cysteine sulfur. However, on the basis of the energy analysis above, it seems doubtful that bands A, B, and C all could reflect lone pair levels of the thiolate sulfur. Moreover, the polarization data need not be subject to a simple geometric analysis; Tennent and McMillin (11) and others (37) have already pointed out that the CT transitions in general do not originate from or terminate at a single center. In principle, the methionine and cysteine sulfurs can interact directly in the ground state (38, 39) or in an excited state of the complex (40); however, the sulfur-sulfur distance in the protein is probably too great for direct orbital overlap (J. M. Guss and H. C. Freeman, personal communication). Alternatively, the CT chromophores could couple through space (41) or via metal orbitals (42). In this way the (predominantly) $\sigma S(\text{Met}) \rightarrow \text{Cu(II)}$ transition could "borrow" intensity—say from the (predominantly) $\sigma S(\text{Cys}) \rightarrow \text{Cu(II)}$ transition—and, given the similarity in transition energies, such an interaction looks entirely plausible. The methionine transition could thereby assume the polarization characteristics of the cysteine transition.

A final concern regarding the assignment of band C is the fact

Table 2. Results of thiol titrations

Sample	Concentration, mol/liter*	Conditions	$\Delta A_{412}^\dagger$	Thiol, mol/mol protein [‡]
apo	3.7×10^{-5}	pH 7	0.413	0.82
apo + 1% NaDodSO ₄	5.1×10^{-5}	pH 7, denatured	0.647	0.95
apo + 1% NaDodSO ₄ + HSCH ₂ CH ₂ OH	3.7×10^{-5}	pH 7, denatured, and reduced	1.583	3.15

apo, Apoprotein.

* Calculated from A_{804} of a native reference.

† Corrected for dilutions.

‡ Calculated by using $\epsilon_{412} = 13,600 \text{ l mol}^{-1} \text{ cm}^{-1}$.

that a similar band appears in the spectrum of stellacyanin, yet this protein is devoid of methionine residues (13). A plausible explanation for this happenstance is presented in the next section.

The Case of Stellacyanin. After considering the energies of the CT bands, Tennent and McMillin (11) suggested that cystine sulfur may replace methionine sulfur as a ligand in stellacyanin, in which case band C might be assigned as $\sigma S(\text{cystine}) \rightarrow \text{Cu(II)}$. Subsequently, Gray and coworkers (14) arrived at the same conclusion. Comparison of the $\sigma S \rightarrow \text{Co(III)}$ CT energies from the thioether and disulfide sulfurs supports this conjecture (34). On the other hand, Hill and Lee (15) suggested that the ligand set of stellacyanin may involve two cysteine and two histidine residues. They argued that the peptide actually contains three cysteine residues as opposed to the one cysteine and one cystine reported in the sequence determination and that the oxidation of a pair of cysteines may have occurred during the sequence analysis. However, our thiol titrations (Table 2) strongly support the presence of cystine in the apoprotein because the apoprotein is prepared by dialysis against cyanide, which is reducing (43), and because the apoprotein binds Cu(II) to give native protein. Therefore, the presence of cystine in the peptide is indicated.

CONCLUSIONS

Our analysis suggests that only three low-lying CT bands are likely to originate from the methionine and cysteine sulfurs of azurin and plastocyanin. In each case, bands A and B can be convincingly assigned to π and σ components associated with

Table 3. Band assignments

Protein	Band	cm^{-1}	nm*	Assignment
Plastocyanin	A	13,330	752	$\pi S(\text{Cys}) \rightarrow \text{Cu(II)}$
	B	16,500	606	$\sigma S(\text{Cys}) \rightarrow \text{Cu(II)}$
	C	18,100	552	$\sigma S(\text{Met}) \rightarrow \text{Cu(II)}$
	D	22,200	450	$\pi(\text{Im}) \rightarrow \text{Cu(II)}$
	E	23,340	428	Unassigned
Azurin	A	12,830	779	$\pi S(\text{Cys}) \rightarrow \text{Cu(II)}$
	B	15,850	631	$\sigma S(\text{Cys}) \rightarrow \text{Cu(II)}$
	C	17,650	567	$\sigma S(\text{Met}) \rightarrow \text{Cu(II)}$
	X [†]	20,790	481	$\pi(\text{Im}) \rightarrow \text{Cu(II)}$
Stellacyanin	A	12,680	789	$\pi S(\text{Cys}) \rightarrow \text{Cu(II)}$
	B	16,220	617	$\sigma S(\text{Cys}) \rightarrow \text{Cu(II)}$
	C	17,490	572	$\sigma S(\text{cystine}) \rightarrow \text{Cu(II)}$
	X [†]	22,210	450	$\pi(\text{Im}) \rightarrow \text{Cu(II)}$

* From ref. 14.

† Bands D and E are not resolved.

S(Cys) $\rightarrow$ Cu(II) CT. The assignment of band C is problematic. Although the energy appears to be correct for σ S(Met) $\rightarrow$ Cu(II), the oscillator strength and polarization characteristics of this CT are difficult to anticipate. On the other hand, the possibility of assigning a third CT to S(Cys) seems doubtful from energy considerations. Moreover, any contribution to the intensity of band C from CT from a π orbital of imidazole would also be expected to be modest because the metal orbital probably only overlaps significantly with the $2p$ orbital of the coordinated nitrogen atom which in turn forms only part of the π orbital of the coordinated imidazole. All points considered, it seems likely that CT from methionine sulfur makes some contribution to band C. Consistent with our view, Gray and co-workers (12) recently made a similar assignment in the spectrum of ascorbate oxidase. By assuming this assignment, the analogous band in stellacyanin might then be interpreted in terms of a cystine ligand, but this inference must be considered tenuous until the identity of the fourth ligand is established by independent means. For convenience our suggested assignments are presented in Table 3.

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