Resonance Raman Spectra of "Blue" Copper Proteins and the Nature of Their Copper Sites[1a]

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Abstract: Resonance Raman spectra of five "blue" copper proteins (*Rhus vernicifera* stellacyanin and laccase, spinach plastocyanin, *Cucurbita pepo medullosa* ascorbate oxidase, and human ceruloplasmin) were measured in the region 150 to 1700 cm^{-1}, using laser excitation into their ~600-nm electronic absorption bands. From the resonance Raman and other spectroscopic and chemical evidence, it is proposed that the "blue" copper site has a distorted four-coordinate structure arising from the binding of copper to one cysteine sulfur and three nitrogen atoms, at least one of which is an amide nitrogen. A qualitative molecular orbital description relating the resonance Raman bands to the proposed structure is presented. The distinctive resonance Raman spectrum of stellacyanin suggested that its "blue" copper site differs markedly from those in the other proteins examined.

Introduction

"Blue" copper proteins[2] occur widely in nature as electron transfer agents. The structure of the "blue" copper site, essential to an understanding of the mechanism of these proteins, has not been determined. The group of proteins derives its name from the intense electronic absorption band (ϵ 1300–5000 M^{-1} cm^{-1}) centered in each protein near 600 nm. They are further characterized by electronic absorption bands in the near-infrared, low copper hyperfine coupling constants in their EPR spectra, high-positive redox potentials, and the presence of tightly bound copper with no readily exchangeable ligands. The presence of cysteine sulfur and at least one nitrogen ligand at the copper site has been proposed on the basis of chemical[3] and ENDOR[4] studies, respectively. Difficulties in conceiving a unique stereochemical structure on the basis of these properties has forestalled any attempts at synthesizing a model of the "blue" copper site.

Resonance Raman spectroscopic studies of chromophoric centers in metalloproteins have met with considerable success in such proteins as hemoglobin,[5] cytochrome *c*,[5] rubredoxin,[6] hemerythrin,[7] hemocyanin,[8] adrenodoxin,[9] and transferrin.[10] In a preliminary report,[11] we presented the resonance Raman spectra of the "blue" copper proteins, stellacyanin, laccase, and ceruloplasmin, and suggested that several nitrogen or oxygen ligands and one sulfur or imidazole nitrogen ligand were coordinated to the "blue" copper atom in a tetrahedral or five-coordinate environment. Using improved data for these proteins and results for two additional "blue" copper proteins, spinach plastocyanin and ascorbate oxidase from zucchini squash, our preliminary conclusions are confirmed and refined. The resonance Raman data together with EPR and electronic absorption evidence enable a model for the "blue" copper site to be proposed. After the original submission of this manuscript, resonance Raman spectra of ceruloplasmin, azurin, and plastocyanin in the low-frequency region were reported by Miskowski et al.[12]

Experimental Section

Laccase and stellacyanin were purified from an acetone powder of latex from *Rhus vernicifera*, the Japanese lacquer tree (obtained from Saito and Co., Tokyo, Japan), by chromatography[13] on DEAE-Sephadex A-50 and CM-Sephadex C-50. Observed absorbance ratios, A_{280}/A_{615} = 16.6 and A_{280}/A_{604} = 5.5, respectively, compare favorably with previously reported values.[13] Plastocyanin was purified from fresh spinach leaves by the procedure of Plesničar and Bendall.[14] The purified protein had an absorbance ratio, A_{278}/A_{597} = 1.6. The procedure of Lee and Dawson[15a] for isolation of ascorbate oxidase from green zucchini squash, *Cucurbita pepo medullosa*, was followed up to the stage of acetone precipitation. The protein was then further purified by chromatography[15b] on DEAE-Sephadex A-50 and had an absorbance ratio A_{280}/A_{610} = 22.8. Commercial ceruloplasmin (Schwartz-Mann) was further purified by DEAE-cellulose chromatography[16] and had an absorbance ratio A_{280}/A_{610} = 21.3.

Raman spectra were recorded with a Jarrell-Ash 25-100 double monochromator incorporating a RCA 31034 photomultiplier tube for detection and using Spectra-Physics Model 164 argon and krypton ion laser excitation. The laser beam entered along the long axis of a sealed quartz capillary tube containing freshly prepared protein solution and was focused inside the tube. The scattered light was collected at 90° to the incident beam. Spectral conditions are reported in the caption to Figure 2.

Results

Resonance Raman spectra, in the region 150–1700 cm^{-1}, were obtained by laser excitation into the electronic absorption band centered in each protein near 600 nm. The positions of the excitation wavelengths relative to the absorption band are shown for stellacyanin in Figure 1. Since, for each protein, excitation at 568.2 and 647.1 nm (and 514.5 nm for stellacyanin) gave resonance Raman spectra in which the relative intensities of the bands were the same, it appears that only one allowed electronic transition in this region is responsible for the resonance Raman spectra. Resonance Raman spectra (Figure 2) of stellacyanin, laccase, plastocyanin, ceruloplasmin, and ascorbate oxidase between 600 and 150 cm^{-1} show at least two intense bands in the 330–470-cm^{-1} region. Even stellacyanin, plastocyanin, and laccase, which contain only one "blue" copper atom, thus avoiding the problem in the other proteins of multiple and possibly nonequivalent "blue" copper sites, exhibit two or three intense resonance Raman bands in this region. We have previously[11] assigned these bands to Cu–N (from a peptide bond or an amide side chain) or possibly Cu–O stretching vibrations. Support for the former and preferred assignment was gained from the position and intensities of ν(Cu–N(peptide)) in Cu(II)–peptide complexes and from the observation of three weak bands, in the proteins, between 600 and 1700 cm^{-1}, which were assigned to amide group vibrations.[11] Spectra of plastocyanin and ascorbate oxidase also exhibit these characteristic weak bands (Table 1). In addition, the medium-intensity band near 260 cm^{-1} was assigned[11] to ν(Cu–S) (cysteine) or ν(Cu–N) (imidazole). Chemical evidence[3] for cysteine sulfur as a ligand in the "blue" copper sites (vide infra) favors assignment of

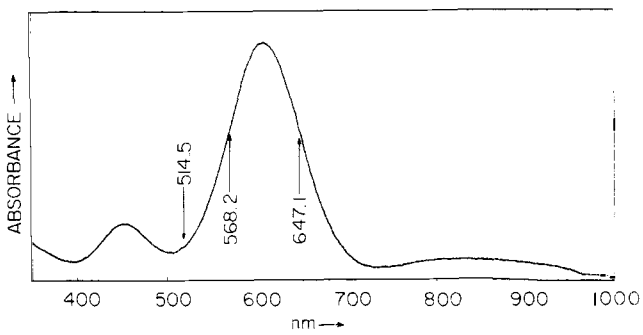

Figure 1. Electronic absorption spectrum of stellacyanin (~3.3 mg/ml, 0.05 M phosphate buffer pH 5.5), showing positions of laser excitation lines.

ν(Cu-S) to the 260-cm^{-1} feature. Improved resolution in the low-frequency region has allowed this band to be observed in its entirety for laccase and plastocyanin (compare Figure 2 with Figure 1 in ref 11). Vibrational assignments are summarized in Table I.

Discussion

Assignment of the resonance Raman bands establishes the identity of the Cu ligands as nitrogen (including N from amide) and sulfur. Further interpretation of the resonance Raman spectra, in structural terms, is based upon a "blue" copper model, derived from electronic absorption and EPR data, together with some qualitative orbital overlap criteria.

Assignment of the Electronic Absorption Spectra. In the visible and near-infrared regions, the electronic absorption and circular dichroic spectra[17] of stellacyanin, laccase, and ceruloplasmin are comprised of five or six bands attributable to "blue" copper. The high intensity of the ~600-nm absorption band (ϵ 1300-5000 M^{-1} cm^{-1}) was originally attributed[18] to the presence of a bond between the copper and a sulfur from a cysteine residue. Coordination of Cu^{2+} (readily reducible) to RS^- (readily oxidizable) should result in a small energy separation between the lowest empty metal orbital and the highest filled ligand orbital. A charge-transfer transition between these two orbitals would then lead to the type of low-energy feature seen in the absorption spectra of the proteins. Support for the assignment of the 600-nm band to a charge-transfer transition comes from the electronic spectra of Co(II) stellacyanin,[3c] in which the 604- and 450-nm bands of native Cu(II) stellacyanin shift to 355 and 300 nm. Assignment of the 600-nm as well as the 450-nm (ϵ 250-800 M^{-1} cm^{-1}) band to S → d(Cu) excitations appear most appropriate. The argument for a S → Cu charge-transfer band is strengthened by chemical evidence for the presence of cysteine at the "blue" copper site. Mercurial titrations[3b,19] of several "blue" copper proteins and the respective apoproteins show that one additional titratable cysteine residue per "blue" copper atom is present in the apoprotein. Complete primary amino acid sequences of azurin[20] and various plastocyanins[21] have been reported; all have at least one cysteine residue per copper atom, available for Cu-S(cys) coordination. Other "blue" copper proteins—stellacyanin, laccase, and ceruloplasmin—for which amino acid analyses[22] are known, have at least one cysteine residue per mole of protein. The two absorption bands in the near-infrared region,[17] 700-900 nm (ϵ 300-2000 M^{-1} cm^{-1}), can be assigned to copper ligand field transitions. The high intensities of these d-d bands probably arise from spin-orbit coupling of doublet ligand field states to nearby higher energy doublet S → Cu charge-transfer states. Similar high d-d band intensities have been observed in some copper-halide complexes. In the series of compounds, $[Cu(bpy)_2X]ClO_4$, upon varying X^- from Cl^- to I^- a four-

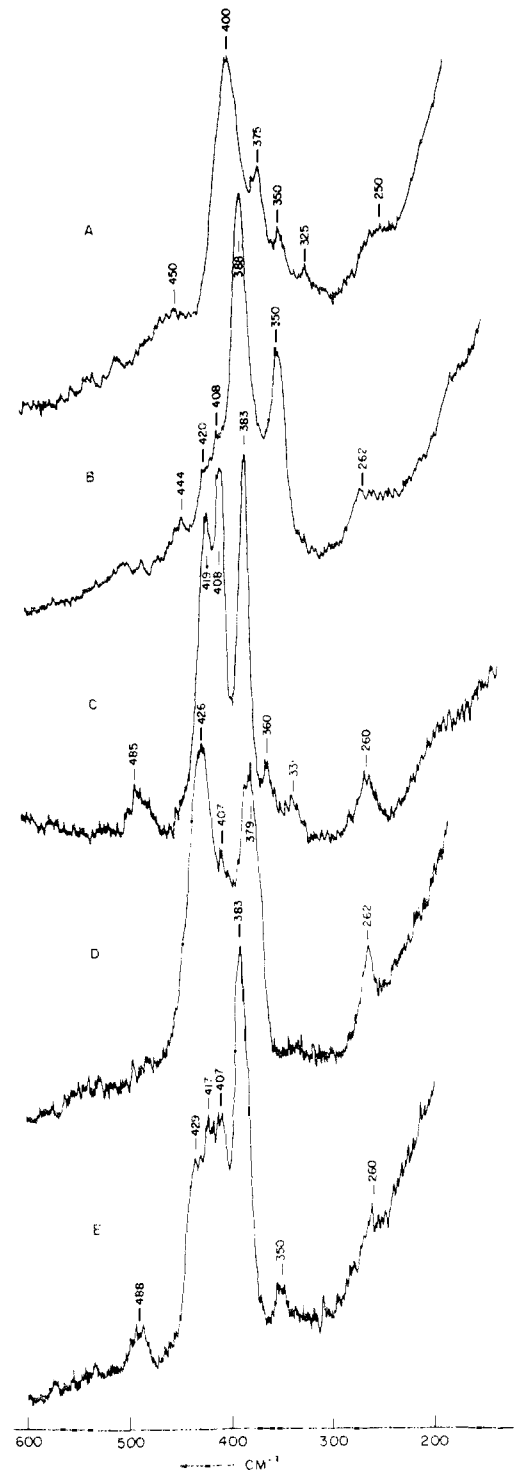

Figure 2. Resonance Raman spectra of ceruloplasmin, 11.6 mg/ml, in 0.05 M acetate buffer, pH 5.5 (A); stellacyanin, 8.4 mg/ml, in 0.05 M phosphate buffer, pH 5.5 (B); laccase, 42.5 mg/ml, in 0.05 M phosphate buffer, pH 5.5 (C); plastocyanin, 1.5 mg/ml, in 0.05 M phosphate buffer, pH 6.9 (D); ascorbate oxidase, 42.4 mg/ml, in 0.05 M phosphate buffer, pH 7.0 (E). Experimental condition: time constant, 5 s; scan rate, 30 cm^{-1}/min; excitation, 647.1 nm Kr^+.

	Power, mW	Slit width, cm^{-1}	Sensitivity, cps
A	80	10.0	2000
B	80	7.0	1000
C	60	7.0	2000
D	40	7.0	2000
E	30	7.0	1000

to fivefold increase in Cu(II) ligand field band intensities was observed[23] owing to successively lower energy halide → metal charge-transfer bands.

Table I. Vibrational Assignments for Resonance Raman Bands (cm^{-1}) of "Blue" Copper Proteins

Stellacyanin	Laccase	Plastocyanin	Ceruloplasmin	Ascorbate oxidase	Tentative assignment
267	259	262	250	262	ν(Cu–S)
	331		325		δ(CNC) or δ(CCN)
350	360		350	350	ν_3(Cu–N)[a]
388	383	379	375	383	ν_2(Cu–N)[a]
410	408	407	400	407	
	419			417	ν_1(Cu–N)[a]
424		426		429	
444			450		
	485			488	δ(C=O)
748	750	750	750		δ(C=O)
1240			1240	1240	ν(C–N(peptide)) or amide III
1650	1650	1660	1650	1660	ν(C=O)

[a] Cu–O could contribute in this region and an oxygen ligand has not been excluded.

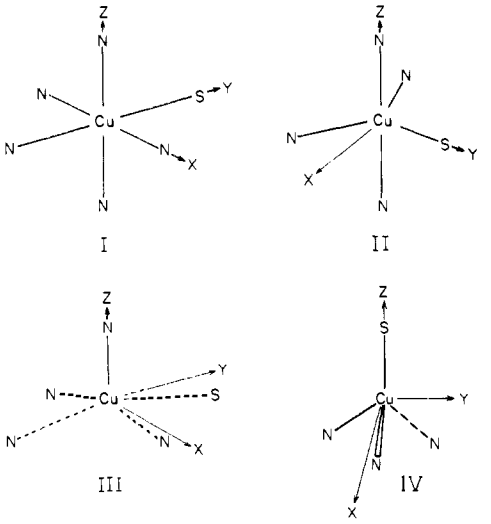

Figure 3. Possible stereochemical arrangements at the "blue" copper site; structure IV is the preferred model. One of the nitrogen ligands may be replaced by oxygen.

Previously, the 600-nm band was assigned to a ligand field transition,[22a] with its intensity accounted for in terms of low symmetry fields and d–p mixing.[24] However, in well-characterized Cu(II) complexes of any geometry, the molar extinction coefficients of the Cu(II) ligand field bands rarely exceed 300 M^{-1} cm^{-1}, an order of magnitude below that of the 600-nm band in the "blue" copper proteins. Furthermore, only four d–d transitions are possible for a Cu(II), d^9 configuration, while five or six bands occur in the protein spectra.

Model for the "Blue" Copper Site. In our initial communication[11] we recognized that the most likely stereochemistry for "blue" copper was either tetrahedral or five-coordinate. Miskowski et al.[12] agree with our conclusion[11] that the "blue" copper site consists of a nitrogen–sulfur core, but while they interpret the data on the basis of a trigonal-bipyramidal structure, we favor a tetrahedral model on the following basis.

Usually, the number and position of ligand field bands[25] of transition metal complexes are helpful in predicting metal–ligand coordination and geometry. However, in the case of the "blue" copper site, the very subtle differences between the electronic absorption spectra of five-coordinate, distorted tetrahedral, and octahedral structures prevent a clear-cut identification from the spectra alone. However, when EPR data and certain criteria for the metal–ligand orbital overlap required to produce a low-energy charge-transfer band are also considered, a model for the Cu(II) stereochemistry in "blue" copper proteins can be proposed. This general model, and the orbital overlap criteria, satisfactorily explain intensity patterns in the resonance Raman spectra of the proteins. (While we consider only CuN_xS models, oxygen cannot be excluded by the Raman data. This does not affect the following EPR argument since local site symmetry would be little perturbed by replacement of a N by an O.)

Low-temperature EPR studies[2] suggest that "blue" copper occupies a site of lower than axial symmetry. Four structures—distorted octahedral (I), distorted trigonal bipyramidal (II) with the sulfur equatorial, square pyramidal (III), and flattened tetrahedral (IV)—appear acceptable at this point (Figure 3).

The ground electronic state of a d^9 Cu(II) complex is determined by the highest energy singly occupied d orbital: $d_{x^2-y^2}$ or d_{z^2} for I; d_{z^2}, $d_{x^2-y^2}$, or d_{xy} for II; $d_{x^2-y^2}$ for III; and d_{xz} (or d_{yz}) for IV. The two forms of structure II, which is the model favored by Miskowski et al.,[12] compressed or elongated, appear unlikely on the basis of EPR evidence. Compressed trigonal bypyramids such as $[Cu(NH_3)_2(NCS)_3]^-$, which possesses D_{3h} symmetry, and $[Cu(bpy)_2I]^+$, $[Cu(bpy)_2(tu)]^{2+}$ (bpy, 2,2′-bipyridyl; tu, thiourea), which have C_{2v} symmetry, have been well characterized.[26] The latter are particularly relevant because they are analogous to the model, CuN_4S, trigonal bipyramidal with cys S in the trigonal plane, proposed for "blue" copper by Miskowski et al.[12] In contrast to the EPR spectra of the "blue" copper proteins which show $g_\parallel > g_\perp$, the EPR spectra of these model trigonal bipyramids all show $g_\perp > g_\parallel$, and we eliminate a compressed trigonal-bipyramidal stereochemistry for "blue" copper on this basis. An elongated trigonal-bipyramidal structure would show $g_\parallel > g_\perp$ (as would elongated octahedral, tetrahedral, and planar structures when the singly occupied orbital is $d_{x^2-y^2}$ or d_{xy}, d_{yz}, d_{xz}), but a high degree of anisotropy would be expected in the copper hyperfine coupling constants, $A_\parallel$ and $A_\perp$. However, the $A_\parallel$ values that are found in EPR spectra of "blue" copper proteins[2] indicate a Cu(II) hyperfine interaction approaching isotropy.

Of the remaining three structures, only IV can readily yield a low-energy charge-transfer transition. It has been emphasized[12] that good π(S)–d(Cu) orbital overlap is necessary to produce an intense π(S) → d(Cu) charge-transfer absorption band. In structures I and III the relevant π(S) orbitals are π_x(S) and π_z(S), both of which have zero or minimal orbital overlap with the d_σ(Cu) orbitals, $d_{x^2-y^2}$ or d_{z^2}. Thus, in our opinion, structure IV appears to be the most likely model for the "blue" copper chromophore. Good σ overlap between σ(N) and either d_{xz} or d_{yz} is possible as well as good π overlap between π_x(S), π_y(S) and d_{xz}, d_{yz}, respectively.

Interpretation of the Resonance Raman Spectra in Terms

of the Model and Orbital Overlap Criteria. The most striking feature of the resonance Raman spectra is the high intensity of the Cu–N bands compared with that of the band arising from the Cu–S vibration. At first sight, this is puzzling in view of the involvement of the Cu and S atoms in the charge-transfer transition held responsible for the Raman intensity enhancement. However, this apparent anomaly can be readily explained on the basis of the proposed model, taken with certain orbital overlap criteria. When the Raman intensity is derived from a single excited state,[27] the intensities of the resonance Raman bands are related to the molecular orbitals that constitute the ground and excited electronic states through the vibronic transition moment. These molecular orbitals are formed in the usual manner by the mixing of symmetry-related Cu atomic orbitals and ligand atomic orbitals. Copper d orbital–ligand orbital overlap can be taken as a qualitative measure of participation of either a given ligand orbital in a molecular orbital based mainly on Cu or a given Cu d orbital in a molecular orbital based mainly on the ligand. Therefore, it is the copper–ligand bonding possessing optimum orbital overlap which gives the largest contribution to the molecular orbitals and leads to intense resonance Raman bands that are associated with vibrations of the Cu–ligand type. The molecular orbital that describes the ground electronic state of "blue" copper mainly contains the singly occupied antibonding $3d_{xz}$ (or $3d_{yz}$) orbital. This Cu d orbital forms the strongest σ bonds with the ligand donor atom σ orbitals, and therefore a molecular orbital based mainly on $3d_{xz}$ (Cu) contains contributions from these ligand orbitals. Since the low-energy absorption band, giving rise to intensity enhancement, is ascribed to a $\pi(S) \rightarrow d_{xz}$ (Cu) excitation and the molecular orbital based on d_{xz} (Cu) participates in both ground and excited state functions, *we expect intense resonance Raman bands from Cu–ligand bonds possessing good* $3d_\sigma$ *(Cu)–*σ *(ligand) overlap.*

In the low-frequency regions of the resonance Raman spectra (Figure 1), the intense ν(Cu–N) bands are distributed around three frequencies, viz. close to 415 and 380 cm^{-1} for laccase, plastocyanin, ascorbate oxidase (and also azurin)[12] and close to 380 and 350 cm^{-1} for stellacyanin. The broadness of the feature around 400 cm^{-1} in ceruloplasmin suggests that it is composed of more than one band. This may be due to the superposition of individual ν(Cu–N) stretching vibrations or the presence of more than one kind of "blue" copper site in this protein. Using the foregoing rationale we ascribe the intense ν(Cu–N) resonance Raman features to Cu–N bonds in which the σ(N) orbitals are directed toward d_σ(Cu)(d_{xz}). The intensity of the ν(Cu–S) band near 260 cm^{-1} is weak. This may be explained by the very small σ(S)–d_σ(Cu) overlap that is expected in our model IV. Thus the model allows the Cu–S bond to show a strong electronic absorption feature, through a charge-transfer transition resulting from a $3p_\pi(S) \rightarrow d_{xz}$(Cu) excitation but weak resonance Raman enhancement due to poor d_σ(Cu)–σ(S) overlap.

No carbon–sulfur vibrations were observed in the resonance Raman spectra since there is no multiple bonding (i.e., $p_\pi(S)$–$p_\pi(C)$ overlap) through the S–C bond of the cysteine residue. However, weakly enhanced vibrational modes due to amide (in which multiple bonding can occur) were observed, providing supporting evidence for amide nitrogen ligand(s) binding to the copper atom. The amide modes will derive their intensity enhancement from mixing of $3p_\pi(S)$ and $2p_\pi(N)$ orbitals (and therefore $2p_\pi(C)$ and $2p_\pi(O)$ orbitals) by overlap of $3d_\pi$ and $4p_\pi$ copper orbitals with $\pi(S)$ and $\pi(N)$ orbitals.

The stellacyanin spectrum is clearly distinct from those of ascorbate oxidase, laccase, and plastocyanin, particularly in the intensity of the 350-cm^{-1} ν(Cu–N) band. The intensity of this feature in stellacyanin may arise from a nitrogen ligand that is closer, in this protein, to the *xz* plane. This would give better σ overlap between σ(N) and d_{xz}(Cu) orbitals. The same N ligand may give rise to the weak 350-cm^{-1} features in ceruloplasmin, laccase, and ascorbate oxidase. A flattened tetrahedral configuration for the "blue" copper site in stellacyanin is also consistent with its standard redox potential,[2] E_0, which is +0.18 V. This value is near the redox potential of the aqueous Cu(II)|Cu(I) couple. The redox potentials[2] of plastocyanin (E_0 = +0.37 V), laccase (E_0 = +0.39 to +0.43 V), and azurin (E_0 = +0.33 V) are all considerably higher. For these proteins, a "blue" copper site geometry between planar (the usual structure for four-coordinate Cu(II) complexes)[28] and tetrahedral (the usual structure for four-coordinate Cu(I) complexes)[28] is appropriate. Such a model accords with the "entatic state" hypothesis[29] which suggests that an effective copper electron transfer site will have a geometry intermediate to those of Cu(II) and Cu(I) sites.

Neither the present work nor previous theories of the "blue" copper site satisfactorily explain the occurrence of an S → Cu charge-transfer band at such a low energy as 16 670 cm^{-1}. One possible rationale is that another amino acid residue is interacting with the cysteine sulfur ligated to Cu(II). An analogy can be drawn with the S (cysteine)–imidazole (histidine)–asparagine charge-relay system proposed in papain[30] in which the histidine and asparagine residues acting in concert are said to increase the nucleophilicity of the cysteine sulfur. A similar charge-relay system, not necessarily involving the same amino acid residues (through Cu(II)–Cu(I) plastocyanin difference NMR spectra[31] have implicated a histidine residue in the vicinity of the copper atom), may operate in "blue" copper proteins to increase the basicity of the cysteine sulfur atom so that an unusually low-energy S → Cu charge-transfer transition can arise. In addition, such a charge-relay system could provide a pathway for the transfer of electrons from substrate to copper.

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Kinetics and Mechanism of the Copper(I)-Induced Homogeneous Ullmann Coupling of *o*-Bromonitrobenzene

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Abstract: A kinetic investigation of the coupling of *o*-bromonitrobenzene to form 2,2′-dinitrobiphenyl in a homogeneous acetone solution containing copper(I) trifluoromethanesulfonate, aqueous ammonia, and acetonitrile has revealed that the reaction is second order in aryl halide and first order in copper(I). The formation of the very minor product, nitrobenzene, is first order in each of these two reactants. Free *o*-nitrophenyl radicals have been excluded as intermediates by the finding that the product distribution is insensitive to the presence of tetrahydrofuran, whereas this additive has the effect of greatly increasing the yield of nitrobenzene when *o*-nitrophenyl radicals are generated in the same media by decomposition of the *o*-nitrobenzenediazonium ion. An organocopper intermediate in the coupling process is indicated by the effect of added ammonium ion which greatly increases the ratio of nitrobenzene to biaryl; the ammonium salt increases the second-order rate constant for production of nitrobenzene but has no appreciable effect on the rate constant for biaryl production. The suggested mechanism, which is completely consistent with these data, involves a reversible oxidative addition of the CBr bond to copper(I) to form an organocopper(III) intermediate which may either displace a bromide ion from a second aryl bromide molecule or become protonated by the medium.

The widely used Ullmann coupling reaction[1-3] produces a biaryl when an aryl halide and copper powder are heated at various temperatures, but frequently in the vicinity of 200°. The mechanism has been extensively reviewed in recent years.[1-4] It now appears agreed[1c,2,4] that the central mechanistic feature is the formation of an organocopper intermediate of unknown oxidation state.[5] However, the exact nature of this intermediate and the processes by which it is formed and consumed remain obscure. From time to time reported experimental results have been interpreted as supporting the presence of radicals during Ullmann coupling,[2,6-8] but none of the evidence is compelling with regard to the actual participation of radicals in the coupling mechanism itself; in fact, the recent finding that iodomaleate and iodofumarate esters couple essentially stereospecifically renders vinyl radicals very unlikely intermediates,[9] and evidence against radical intermediates in the heterogeneous Ullmann coupling of an aryl halide has also been obtained.[10]

In other types of reactions, the mechanistic questions which thus far remain unanswered are usually explored by kinetic studies, but until now these have been impossible owing to the heterogeneous nature of the reaction mixtures. However, the recent discovery[11] of a homogeneous Ullmann reaction performed in organic solvents containing copper(I) trifluoromethanesulfonate (triflate) and aqueous ammonia has now rectified this situation, and we now present the results of a kinetic and mechanistic investigation of this reaction.

Results

Since *o*-iodonitrobenzene couples too rapidly at room temperature for convenient kinetic measurements,[11] a survey was made[12] of the effect of reaction conditions on the product distribution using *o*-bromonitrobenzene, which under the conditions used previously gave *o*-nitroaniline, nitrobenzene, and a low yield of 2,2′-dinitrobiphenyl. It was found that under the conditions described for the kinetic runs in the Experimental Section, a 90% yield of coupling product was formed along with 7.8% of nitrobenzene and about 1% each of *o*-nitrophenol and *o*-nitroaniline; the reac-