contained 0.05 M imidazole, 0.0052 M GSH, 0.026 M methylglyoxal, and for this specific rate enhancement 0.052 M $MgCl_2$ and 0.185 M KCl (ionic strength of 0.4).
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Resonance Raman Spectra of Copper–Sulfur Complexes and the Blue Copper Protein Question

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Abstract: Resonance Raman spectra are reported for copper(II) complexes of thiaether and mercaptide ligands. The copper–sulfur stretching frequencies in the complexes are compared to those reported by previous workers for the blue copper proteins. The vibrational results strongly suggest that methionine sulfur is bound to copper in the proteins. In the case of stellacyanin, which contains no methionine, a Cu–S (disulfide) bond is suggested. The resonance Raman intensity pattern for one of the thiaether complexes is interpreted using a single-state resonance expression and an expression involving vibronic coupling and two-state resonance. The results, together with the observed intensity of the assigned copper–(methionine) sulfur vibration in the proteins, suggest possible resonance mechanisms for this low-intensity vibration, and details of the electronic assignments for the copper–(methionine) sulfur charge-transfer transitions.

Blue copper proteins, in addition to their essential biological functions, are characterized by a series of curious spectroscopic and chemical properties.[2] The question of the nature of the copper coordination environment in the proteins, which is responsible for these unique properties, is a matter of considerable interest. A central feature of many suggested structures of the blue copper environment is the presence, in addition to the more usual nitrogen and/or oxygen ligands, of a copper–sulfur bond.[3-8] Copper–sulfur bonding in these proteins is consistent with a large body of spectroscopic and chemical evidence.[4-7,9-11] Until very recently,[16] however, the identity of the sulfur ligand had not been established.[9,12-15]

Based upon electronic spectra and chemical evidence on copper thiaether complexes,[17] methionine has been suggested as a possible ligand for copper in the blue proteins. Later, the same suggestion was advanced[8] on the basis of the amino acid sequences of plastocyanin and azurin. The possibility of methionine binding has, however, generally been dismissed (see ref 18 and references therein), in favor of the more commonly accepted cysteine sulfur coordination.[3-7] It was thought by most workers, with the exception of one,[8] that *either* cysteine *or* methionine, but not both, was bound to copper in the proteins.[3-7,9-15,17]

The resonance Raman technique[19] has been applied to blue copper proteins,[5,6] and a copper–sulfur stretching frequency has been assigned to several of these systems. However, no vibrational data concerning copper bound to sp^3 sulfur (of the thiaether or mercaptide types found in proteins) was available to aid in evaluating the protein vibrational results. (Recently, the resonance Raman spectrum of a complex wherein Cu(II) is bound to mercaptide sulfur has been reported.[20]) We have applied the resonance Raman technique to copper mercaptide[21] and thiaether[17] complexes which have previously been suggested on the basis of ESR,[21] electronic absorption,[17] and redox[17] evidence as possible analogues of the copper–sulfur binding in the blue copper proteins. Our results constitute the first vibrational study of complexes in which Cu(II) is bound to thiaether and mercaptide sulfur. The resonance Raman evidence suggests that methionine or a similar neutral sulfur ligand such as disulfide is coordinated to copper in all of the blue proteins. Stellacyanin alone contains no methionine,[9,29] and therefore is unique in that it must employ one of the alternate "methionine-like" ligands.

The resonance Raman evidence for methionine binding was presented in the original and subsequent revisions of this paper. The copper ligands in plastocyanin have since been determined by X-ray structural analysis.[16] This structural determination shows that two histidine nitrogens, a cysteine sulfur, and a methionine sulfur are bound to copper in plastocyanin. Thus, unexpectedly, both previously suggested modes of copper–sulfur binding at the blue copper site actually occur, at least in plastocyanin. Despite the structural determination on plastocyanin and other crystallographic work in progress, spectroscopic evidence will continue to be important in inferring the nature of the copper environment in the remaining blue proteins, some of which may never be crystallized.

Experimental Section

The copper mercaptide and polythiaether complexes were prepared using previously described procedures.[17,21] The BF_4^- salt of the Cu(II) complex of 2,6-bis(methylthiomethyl)pyridine[22] (SNS) was obtained from Dr. P. S. Bryan and the Cu(II) complex of 2,2′-bis(2-benzim-

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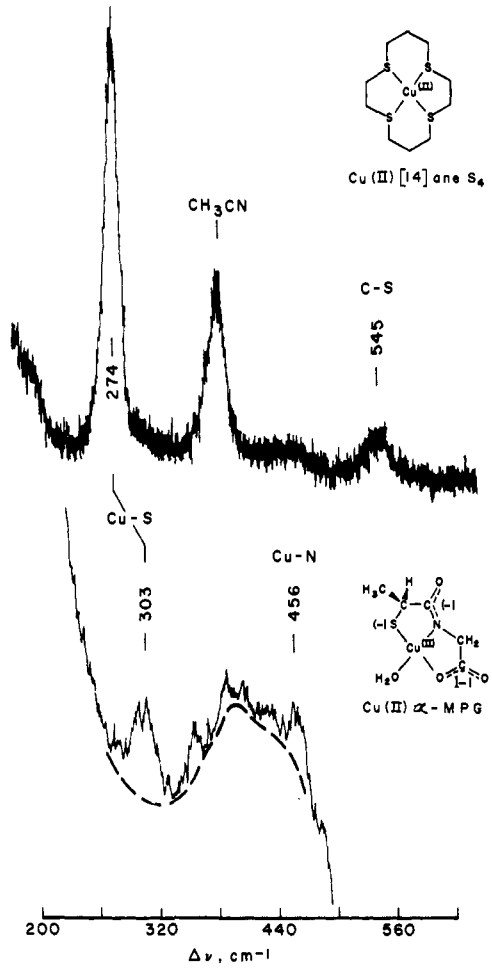

Figure 1. Resonance Raman spectra in the metal-ligand stretching frequency regions of $Cu^{II}[14]aneS_4$ (top) and Cu^{II}-α-MPG (bottom). The excitation wavelengths were 4579 Å for $Cu^{II}[14]aneS_4$ and 4880 Å for Cu^{II}-α-MPG. The broad peak "baseline" (dashed line) in the Cu^{II}-α-MPG spectrum is due to the cell material of the Spex rotating sample accessory.

idazole) ethyl thioether (NSN) was obtained from Dr. G. R. Dukes. Raman spectra were obtained using the apparatus described elsewhere[23] or on a Cary 82 spectrometer. To obtain the resonance Raman spectra of the thiaether complexes, solutions were prepared in spectroscopic grade acetonitrile and/or nitromethane. The acetonitrile was stored over decolorizing carbon and filtered through a 0.45-μm Millipore filter. The concentrations of the polythiaether complexes were approximately 5×10^{-3} M, those of the SNS complex were $(5-7) \times 10^{-3}$ M, and those of Cu^{II}NSN were approximately 1×10^{-2} M. The samples were contained in 1 mm o.d. capillary tubes to record the resonance Raman spectra. Copper(II) α-mercaptopropionylglycine [Cu^{II}-α-MPG] was approximately 1×10^{-2} M in water and was contained in a Spex rotating sample accessory for the Raman experiment. The visible spectra were recorded using a Cary 118 or Cary 14 UV-visible spectrophotometer. The resonance Raman intensities of the 274-cm^{-1} peak in $Cu^{II}[14]aneS_4$ were measured by the cut-and-weigh peak integration method. The 380-cm^{-1} peak of acetonitrile was used as an internal intensity reference at each excitation wavelength. It was assumed that the Raman scattering of acetonitrile did not undergo appreciable resonance enhancement. All experimental intensities were corrected for concentration differences and normalized to the intensity of the 274-cm^{-1} peak at $\nu_0 = 21468$ cm^{-1}.

Results and Discussion

Table I summarizes the copper-sulfur stretching frequencies of the copper(II) mercaptide and thiaether complexes of the present study, the copper-sulfur frequencies of copper thiaether complexes with mixed donor groups (SNS and NSN), and the assigned copper-sulfur stretching frequencies

Table I. Copper-Sulfur Stretching Frequencies in Mercaptide and Thiaether Complexes and in Blue Copper Proteins

Cu(II) complex[a]	type	ν(Cu-S), cm^{-1}	references
α-MPG	mercaptide	303	present work
$[12]aneS_4$	thiaether	280	present work
$[13]aneS_4$	thiaether	272	present work
$[14]aneS_4$	thiaether	274	present work
$[15]aneS_4$	thiaether	258	present work
$[16]aneS_4$	thiaether	247	present work
$[12]aneS_3$	thiaether	247	present work
$[15]aneS_5$	thiaether	282	present work
$[20]aneS_6$	thiaether	280	present work
$(Et)_2TTU$	thiaether	276	present work
$(SNS)_2$	thiaether	260	34
NSN	thiaether	274	35
azurin (*Pseudomonas aeruginosa*)	protein	260	5
plastocyanin (spinach)	protein	265, 262	5, 6
ceruloplasmin (human)	protein	280, 250	5, 6
stellacyanin (*Rhus vernicifera*)	protein	267	6
laccase (*Rhus vernicifera*)	protein	259	6
ascorbate oxidase (zucchini squash)	protein	262	6

[a] Representative structures of mercaptide and tetrathiaether complexes are given in Figure 1. Systematic nomenclature of polythiaether complexes may be found in ref 17. Solvents for the proteins were aqueous phosphate buffer systems.[5,6]

in the blue copper proteins. Figure 1 shows the structures of $Cu^{II}[14]aneS_4$ and Cu^{II}-α-MPG and their respective resonance Raman spectra. The copper-sulfur stretching vibrations of the complexes are assigned on the basis of their strong enhancement in resonance with the S → Cu(II) charge-transfer transition which occurs at approximately 400 nm in each complex. Also, the depolarization ratio of 0.1 at 274 cm^{-1} for $Cu^{II}[14]aneS_4$ is expected of the symmetric copper-sulfur vibration in effectively fourfold symmetry.[24]

The data for the copper thiaether complexes show that, despite an enormous range of geometric constraints about the copper center (trigonal, square planar, square pyrimadal, C_{2v}, pseudooctahedral, flattened tetrahedral), changes in the number of sulfur donors present from one to six, and variations in the number and types of nonsulfur donor groups, the frequency of the copper-thiaether sulfur stretching vibration varies only between 247 and 282 cm^{-1}. The copper ion lies well above the plane of the four sulfur atoms in the $[12]aneS_4$, $[13]aneS_4$, and presumably $[12]aneS_3$ complexes[25] compared to its in-plane position in the $[14]aneS_4$ complex[26] and, presumably, the larger macrocycles. The $(Et)_2TTU$ complex is nonmacrocyclic and the sulfur donors are arranged in a very flat tetrahedral (D_{2d}) geometry.[25] The SNS and NSN complexes incorporate nitrogen ligands (pyridine and benzimidazole) into the primary coordination sphere of the copper. Therefore, it appears that large effects upon a Cu(II)-S (thiaether) vibrational frequency, due to constraints imposed by ligand or protein structure, are not to be expected. The resonance Raman spectra of the macrocyclic complexes will be discussed in greater detail elsewhere.[27]

The Cu-S (mercaptide) stretching frequency in Cu^{II}-α-MPG is observed at 303 cm^{-1}. Inasmuch as Cu^{II}-α-MPG contains two stronger, negatively charged donors (peptide nitrogen and carboxylate oxygen) in addition to mercaptide, this 303-cm^{-1} frequency is probably at the low end of the range expected for Cu(II)-S (mercaptide) bonds. The Cu-N (pep-

tide) stretching mode in Cu^{II}-α-MPG is observed at 456 cm^{-1}.

All of the blue copper proteins for which amino acid analyses have been performed, with the exception of stellacyanin, contain both cysteine and methionine residues. Stellacyanin alone contains no methionine but, as potential sulfur donors, one cysteine residue and one disulfide linkage. In general, four possibilities exist as to the nature of copper coordination to sulfur in proteins. These are the following (nonsulfur ligands omitted).

1. Deprotonated cysteine (mercaptide) coordination:

$$\cdot\text{protein-S-Cu}^{2+}\quad (-)$$

2. Methionine (thiaether) coordination:

$$\begin{array}{l}\text{protein-}\ \text{S-Cu}^{2+}\\ \quad\ |\\ \quad\ \text{CH}_3\end{array}$$

3. Protonated cysteine (thiol) coordination:

$$\begin{array}{l}\text{protein-}\ \text{S-Cu}^{2+}\\ \quad\ |\\ \quad\ \text{H}\end{array}$$

4. Cystine (disulfide) coordination:

$$\begin{array}{l}\text{protein-S-Cu}^{2+}\\ \quad\ |\\ \text{protein-S}\end{array}$$

From the electrostatics of mercaptide vs. thiaether bonding to Cu(II) and the probable force constants of the two types of bonds, it is expected that the Cu-S (mercaptide) stretching frequency should be higher than that of Cu-S (thiaether). The data in Table I confirm this expectation. The Cu-S stretching frequencies in the proteins are expected to be generally consistent with those of the copper-sulfur complexes. Thus, a Cu-S (deprotonated cysteine) vibration in the proteins will have a similar frequency to that of a Cu-S (mercaptide) bond in a complex, and a Cu-S (methionine) frequency will resemble that of a Cu-S (thiaether) bond. It is evident from Table I that the assigned Cu-S vibrations of the proteins are more consistent with Cu-S (thiaether) frequencies than with the Cu-S (mercaptide) frequency. The observed Cu-S (mercaptide) frequency in Cu^{II}-α-MPG appears inconsistent with both the low-frequency (~260 cm^{-1}) protein vibrations and the next higher frequency, intense peaks which occur between 350 and 383 cm^{-1} in the proteins. However, as we have pointed out, the 303-cm^{-1} Cu-S vibration in Cu^{II}-α-MPG probably represents the low end of the range expected of Cu-S (mercaptide) stretching frequencies. Indeed, in view of the now-known plastocyanin structure,[16] one of the higher frequency peaks in the resonance Raman spectra of the proteins must be assigned to the Cu-S (cysteine) stretch. Specifically, in the case of *Pseudomonas aeruginosa* azurin the Cu-S (cysteine) and Cu-N (histidine) stretching vibrations occur at 372, 408, and 426 cm^{-1}. The weaker peaks in this region are assigned to ligand deformations or ligand-Cu(II)-ligand deformations mixed with ligand modes.[28]

Inspection of the amino acid sequence of stellacyanin[29] reveals no obvious homology between it and the proposed binding sites in other blue copper proteins.[7,8] Stellacyanin contain two types of sulfur residues, including one cysteine and one disulfide link. We propose that the 267-cm^{-1} peak in stellacyanin is due to a Cu-S (disulfide) bond. There is evidence for such a bond in the Cu(II) complex of oxidized glutathione.[30] The vibrational frequency of a Cu-S (disulfide) bond may be expected to be close to that of Cu-S (thiaether). The remaining possibility for sulfur coordination in stellacyanin which might result in a 267-cm^{-1} Cu-S stretch is coordination of the sole cysteine residue as protonated cysteine (thiol). We consider this possibility to be remote, inasmuch as coordination of thiols to Cu(II) is, at present, unknown. We note, however, that certain complexes of iron(II)[31] and ruthenium(II)[32] contain coordinated thiol, and that the spectroscopic and bonding properties of coordinated thiols resemble those of thiaethers rather than mercaptides.

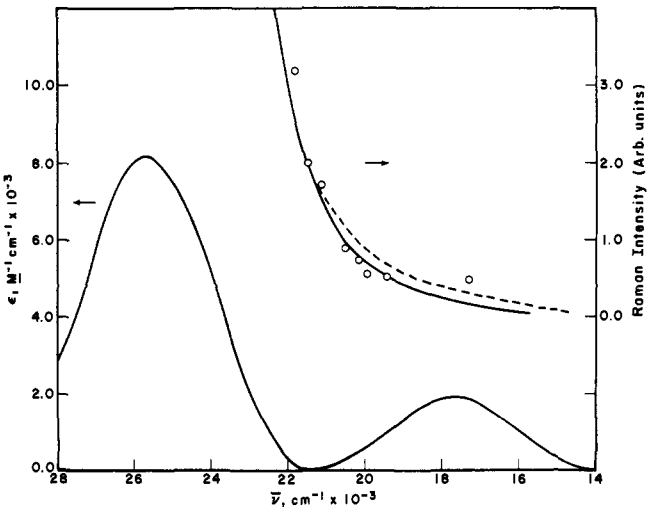

Figure 2. Electronic absorption spectrum and Raman excitation profile for the 274-cm^{-1} vibration of Cu^{II}[14]aneS$_4$. Theoretical excitation profiles were calculated using equations described in the text; solid line was calculated by F_A expression, dashed line by F_{AB} expression. The estimated errors in the experimental Raman intensities are 10%.

It has been previously noted that the intensity of the ~260-cm^{-1} peak in the resonance Raman spectra of the proteins is unexpectedly weak for a Cu-S vibration in resonance with its own intense (~600 nm, $\epsilon \simeq 5000$ M^{-1} cm^{-1}) S(σ) → Cu(II) charge-transfer transition.[7] In order to study the intensity behavior of a Cu-S vibration resonance enhanced by direct S(σ) → Cu(II) charge transfer, we have measured the Raman excitation profile of the 274-cm^{-1} vibration of Cu^{II}[14]aneS$_4$. Figure 2 shows the electronic absorption spectrum as well as the calculated[33] and experimental Raman excitation profiles for this complex. The electronic absorption bands at 390 nm (ϵ 8200 M^{-1} cm^{-1}) and 563 nm (ϵ 1900 M^{-1} cm^{-1}) are assigned to the expected (ref 18 and references therein) S(σ) → Cu(II) and S(π) → Cu(II) charge-transfer transitions, respectively.

The theoretical excitation profiles were calculated using equations previously derived[33] for single-state resonance (with the 390-nm electronic transition):

$$F_A = \frac{\nu_r^4 a_1^2}{[(\nu_{1g}^0 - \nu_0)^2 + \Gamma_1^2]^2}$$

and simultaneous enhancement by the two electronic transitions including vibronic coupling (of the 390- and 563-nm transitions):

$$F_{AB} = \left[\frac{a_1^2}{[(\nu_{1g}^0 - \nu_0)^2 + \Gamma_1^2]^2} + \frac{b_{21}^2}{[(\nu_{1g}^0 - \nu_0)^2 + \Gamma_1^2][(\nu_{2g}^0 - \nu_0)^2 + \Gamma_2^2]} + \frac{2a_1 b_{21}[(\nu_{1g}^0 - \nu_0)(\nu_{2g}^0 - \nu_0) + \Gamma_1\Gamma_2]}{[(\nu_{1g}^0 - \nu_0)^2 + \Gamma_1^2]^2[(\nu_{2g}^0 - \nu_0)^2 + \Gamma_2^2]}\right]$$

where ν_{1g}^0 and ν_{2g}^0 are the peak frequencies of the 390- and 563-nm transitions and ν_0 is the Raman excitation frequency. Damping factors (Γ_i) were taken as the half-widths at half-height of the entire absorption envelopes. The numerator term

a_1 is calculated directly from the F_A fit to the data at values of ν_0 higher than 19 436 cm^{-1}. The value for b_{21} was calculated from this value of a_1, using the relative intensities of the respective absorption envelopes, assuming that a_1 and b_{21} are directly proportional to the respective extinction coefficients. No value of a_2 produced a satisfactory F_{AA}[33] fit to the intensity data. In the arbitrary, relative units of Figure 2, the predicted Raman intensity in direct resonance with the 390-nm [$S(\sigma) \rightarrow Cu(II)$ charge transfer] transition is 110 compared to the measured value of only 0.46 in direct resonance with the 563-nm [$S(\pi) \rightarrow Cu(II)$ charge transfer] transition. Since the damping factors (Γ_i) in these calculations were the half-widths of the entire corresponding absorption envelopes, they represent the maximum values that Γ_i can attain. Therefore, the calculated value of F_A or F_{AB} in direct resonance with the 390-nm absorption ($F_A = F_{AB} = 110$ in our arbitrary units) represents a minimum value.

Considering the results of the excitation profile for the Cu-S vibration in Cu^{II}[14]$aneS_4$, the ~260-cm^{-1} Cu-S (methionine) vibration in the proteins is so weak that the resonant electronic transition at ~600 nm cannot be substantially due to *methionine* $S(\sigma) \rightarrow Cu(II)$ charge transfer. The plastocyanin structure determination,[16] considered in light of the Raman intensities, makes it amply clear that the previous assignment[4] of this transition as primarily *cysteine* $S(\sigma) \rightarrow Cu(II)$ charge transfer is correct. This assignment, together with reasonable minor contributions to the resonant electronic transition in the blue copper proteins, is consistent with the low intensity of the ~260-cm^{-1} Raman peak. One possible detailed assignment is that the ~600-nm transition contains some contribution from methionine $S(\pi) \rightarrow Cu(II)$ charge transfer. Under this condition, the methionine $S(\sigma) \rightarrow Cu(II)$ charge-transfer transition occurs at ~400 nm. The ~260-cm^{-1} peak would then have to gain most of its intensity from resonance with the cysteine $S(\sigma) \rightarrow CU(II)$ charge transfer. This indirect enhancement could be a weak effect causing the intensity of the ~260-cm^{-1} peak to be low. Alternately, the assignments of the electronic transitions in the proteins could correspond in detail to those previously proposed[4b] with the additional suggestion that the ~600-nm electronic transition in the proteins is composed of both cysteine and methionine $S(\sigma) \rightarrow Cu(II)$ charge transfer. Since the ~260-cm^{-1} Cu-S vibration is now in direct resonance with the methionine $S(\sigma) \rightarrow Cu(II)$ transition, the contribution of methionine $S(\sigma) \rightarrow Cu(II)$ charge transfer to the total transition intensity at ~600 nm must be small. In addition, the ~260-cm^{-1} peak may gain some of its intensity from indirect resonance with the more intense cysteine $S(\sigma) \rightarrow Cu(II)$ charge transfer.

If, as we suggest, the low-frequency (267 cm^{-1}) vibration in stellacyanin is a Cu-S (disulfide) stretch, the mechanism for intensity enhancement of this mode must be indirect resonance with cysteine $S(\sigma) \rightarrow Cu(II)$ charge transfer. This is dictated by the Cu-S (disulfide) chromophore which, by analogy to the Cu(II) glutathione complex, is expected to exhibit only weak transitions ($\epsilon \simeq 100$ M^{-1} cm^{-1})[30] in the 600-nm region of its electronic spectrum. As in the methionine case, an indirect resonance mechanism is consistent with the observed low intensity of the low-frequency peak.

The data presented offer strong evidence for the assignment of the low-frequency (~260 cm^{-1}) peak in the resonance Raman spectra of the blue copper proteins as a Cu-S (methionine) stretching vibration, or for coordination of another neutral sulfur ligand (viz., disulfide or thiol). We suggest that the ligand responsible for this vibration is indeed methionine in all of the methionine-containing proteins. In stellacyanin, we suggest that the ligand is disulfide, with thiol or nonsulfur ligands as less likely possibilities.

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