Variations in the Type I Copper Protein Coordination Group: Resonance Raman Spectrum of ^{34}S-, ^{65}Cu-, and ^{15}N-Labeled Plastocyanin

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Abstract: Resonance Raman spectra are reported for bacterially expressed poplar plastocyanin labeled with ^{34}S, ^{15}N, and ^{65}Cu isotopes. The strongly enhanced bands near 400 cm^{-1} are sensitive to all three isotopes. The strongest bands also exhibit the largest ^{65}Cu and ^{34}S shifts, confirming the involvement of Cu−S[cysteine] stretching in the enhancement mechanism. The isotope shift and intensity pattern is similar to that reported recently for *P. aeroginosa* azurin, but the modes with the largest Cu−S contribution are higher in plastocyanin, 429/420 vs 409 cm^{-1}. This frequency order is counter to a recently proposed correlation between Cu−S[cysteine] bond strength in Type 1 Cu proteins and the displacement of the Cu from the trigonal $[His]_2[Cys]$ coordination plane toward the distant fourth ligand. The out-of-plane displacement is greater for plastocyanin than for azurin, 0.34 vs 0.04 Å. The small displacement and the weaker Cu−S[cysteine] bond in azurin are attributed to (1) an additional hydrogen bond to the cysteine S atom and (2) an additional donor interaction to the Cu from a backbone carbonyl group. Observed ^{15}N shifts implicate the N atom of the cysteine peptide, and possibly of other residues, as part of the dynamical unit responsible for the 400-cm^{-1} region RR bands. The isotope shifts will provide important constraints on normal mode calculations of Cu protein RR spectra.

Introduction

The resonance Raman [RR] spectra of Type 1 copper proteins are complex and vary significantly from one protein to another[1] in ways that reflect structural alteration at the active site.[2] The potential variability of the structure is highlighted by the recent finding that in *P. aeruginosa* azurin, replacement of a ligating residue, His 117, with glycine creates an aperture in the surface of the protein through which the Cu is accessible to exogenous ligands.[3] Different ligands produce substantially different RR spectra, from which appreciable variability in the coordination geometry has been inferred.[4] Because of the sensitivity of the RR spectra to structural variation, there is much current interest in assigning and interpreting the spectral bands. To this end we report the RR spectrum for bacterially expressed poplar plastocyanin, into which ^{34}S, ^{65}Cu, and ^{15}N isotopes have been incorporated.

The RR spectra of blue Cu proteins are enhanced via excitation in their strong ~600 nm absorption bands, which arises from S→Cu charge transfer.[6] The large dipole strength and low energy of these transitions are associated with the unusual Cu^{2+} coordination environment:[2] a trigonal array of two histidine and one cysteinate ligands, with weak donor interactions from a fourth (methionine) and sometimes a fifth (backbone carbonyl) ligand along the trigonal axis (Figure 1). Since S→Cu charge transfer weakens the Cu−S bond in the excited state, the RR spectrum is expected to be dominated by Cu−S bond stretching. The natural frequency of a Cu−S oscillator at the short bond distance observed in blue Cu protein structures, ~2.1 Å, is ~400 cm^{-1}.

The expected strong RR band near 400 cm^{-1} is indeed seen[7] for model compounds in which thiolate ligands are bound to Cu^{2+} adducts of sterically hindered trispyrazolylborates.[8] These adducts have coordination geometries and electronic absorption bands that resemble the blue Cu active sites closely. Yet the blue Cu protein RR spectra are complex, with several bands near 400 cm^{-1}. Insight into the source of this complexity has been provided by a model compound in which the thiolate ligand has a *sec*-butyl substituent.[7] Its RR spectrum has three prominent bands near 400 cm^{-1}, instead of one. A normal coordinate analysis revealed the multiple bands to arise from mixing of C−C−C and C−C−S bending coordinates with the Cu−S stretching coordinate; this mixing is enhanced via torsional coordinates of the C−H bond adjacent to the S atom. Multiple bands near 400 cm^{-1} in blue Cu protein RR spectra may likewise arise from mixing of Cu−S stretching with multiple heavy atom bending modes involving the cysteinate ligand and adjacent residues.[7,9]

To sort out these mode mixing effects, one needs isotope labels at the atoms undergoing significant displacement in the enhanced vibrations. Isotope labeling of the proteins has

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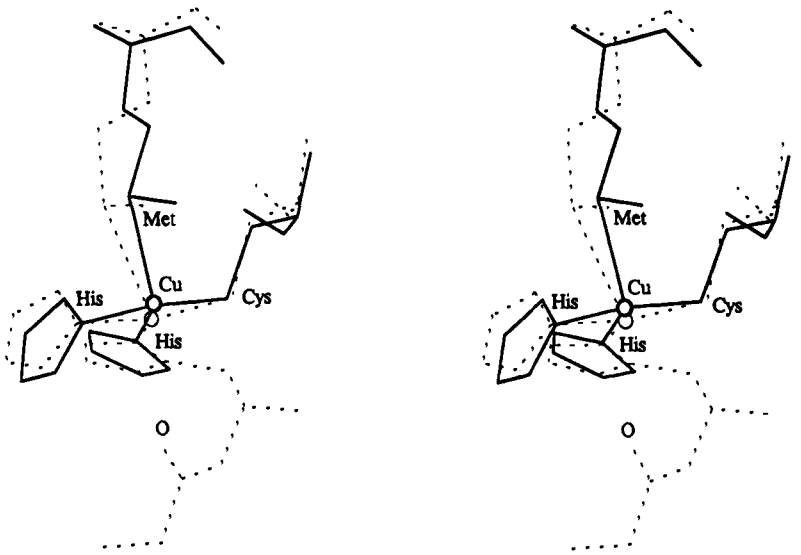

Figure 1. Stereoview of the active site structures of *poplar* plastocyanin (solid line) and *Pseudomonas aeruginosa* azurin (dotted line), drawn from the coordinates in the Brookhaven protein data bank (*poplar* plastocyanin: pdb1plc.ent; *P.a.* azurin: pdb4azu.ent). Note the displacement of the Cu atom from the N,N,S_{Cys} plane in plastocyanin relative to azurin.

become possible with the cloning of the genes and the expression of the proteins in bacteria. Recently Dave et al.[5] reported ^{34}S isotopic effects on the RR spectrum of *P. aeruginasa* azurin, while Sanders-Loehr[1b] reported the effect of ^{15}N incorporation. Labeling with ^{65}Cu had previously been accomplished by chemical removal of Cu and reconstitution with its isotope.[10] The present data permit the first detailed comparison of the mode compositions of two Type 1 Cu proteins (see Figure 1 for a comparison of the active site structures) having different RR spectra. They provide insight into the factors connecting the spectra with bonding features.

Experimental Section

Poplar plastocyanin was expressed in *E. coli* strain X90 (DE3) (*ara*$^-$, Δ*lac-pro*, *nalA*, *argEam*, *rif*^{R}, *thil*$^-$, [F′, *lacI*^{q}, *pro*$^+$])[11] using the pET-3a vector carrying the bacteriophage T7 gene promoter.[12] The ^{34}S-labeled protein was expressed in M9 minimal medium supplemented with ^{34}S-enriched (94%) magnesium sulfate (ICON, Summit, NJ) following the method of Ybe and Hecht (1994). ^{15}N-labeled protein was also produced in M9 medium supplemented with $^{15}NH_4Cl$ (97–99%) (Cambridge Isotope Laboratory), but using a revised expression protocol (Ybe and Hecht, in preparation). ^{63}Cu- and ^{65}Cu-substituted plastocyanins were expressed in LB medium containing 1 mM copper citrate (^{63}CuO (99.9%) and ^{65}CuO (99.7%) from Oak Ridge National Laboratories). After reaching logarithmic phase [OD_{600} = 0.6–1.0], isopropyl-β-D-thiogalactopyranoside (final concentration of 100 μg/mL) was added to induce expression. The cells were incubated with vigorous shaking at either 28 or 30 °C for an additional 4 h. The bacterial cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C. Plastocyanin was released by the freeze–thaw method[13,14] and dialyzed exhaustively against 0.5 mM $MgCl_2$ (water in the case of ^{34}S-labeled protein) and then exchanged into 20 mM bis-tris buffer at pH 6.0. The ^{15}N-, ^{63}Cu-, and ^{65}Cu-substituted proteins were simply concentrated in 0.5 mM $MgCl_2$ using an Amicon centriprep. Protein was isolated by anion-exchange chromatography using a 20 HQ Poros column (Perceptive Biosystems) with a 3%/mL of NaCl gradient at a flow rate of 7 mL/min. The purity index $A_{280/597}$ was ~1.2, consistent with literature values.

Resonance Raman spectra were excited with the 647.1 nm Kr^+ laser line (Coherent Innova 100-K3). The laser power was 60 mW. The scattered light was collected using a 0.85 m double monochromator (SPEX 1404) equipped with a photomultiplier (Hamamatsu R1220) photon multiplier tube detector. The slit resolution was 3 cm^{-1}. To ensure accurate isotopic measurements, the protein samples were mounted on a copper cold finger which was kept at cryogenic temperature during the RR measurement.[15]

Results and Discussion

Plastocyanin Expression. ^{34}S-labeled protein was obtained by a published method that adds $CuSO_4$ just before cell harvest.[13] However, more recently we have found that the yield of holoprotein can be improved by growing bacterial cells harboring the plastocyanin gene in the presence of copper citrate. Thus, ^{15}N-, ^{63}Cu-, and ^{65}Cu-substituted proteins were isolated by growing bacterial cells in the presence of 1 mM copper citrate. This strategy yields significantly more holoprotein because citrate chelates copper, thus keeping it soluble. The protein yield was also improved by lowering the growth temperature from 30 to 28 °C.

Resonance Enhancement and the Cu–S Displacement. Figure 2 compares RR spectra of natural abundance and isotopically-labeled plastocyanin, obtained with 647.1-nm excitation, near resonance with the Cu→S[cys] charge transfer absorption band. Several overlapping RR bands are observed in the 400-cm^{-1} region, whose frequencies and relative intensities agree with those reported previously.[16] To obtain accurate wavenumbers with which to determine the isotope shifts, the RR spectra were deconvoluted into a minimum set of 50/50 Gaussian/Lorentzian bands, as illustrated in Figure 3. The widths of the deconvoluted bands were constrained to be the same in the different isotopic spectra.

The band wavenumbers and isotope shifts are listed in Table 1 and compared with those of *P. aeruginosa* azurin. In both proteins the ^{65}Cu and ^{34}S shifts are distributed among the multiple bands near 400 cm^{-1}, showing that Cu–S stretching

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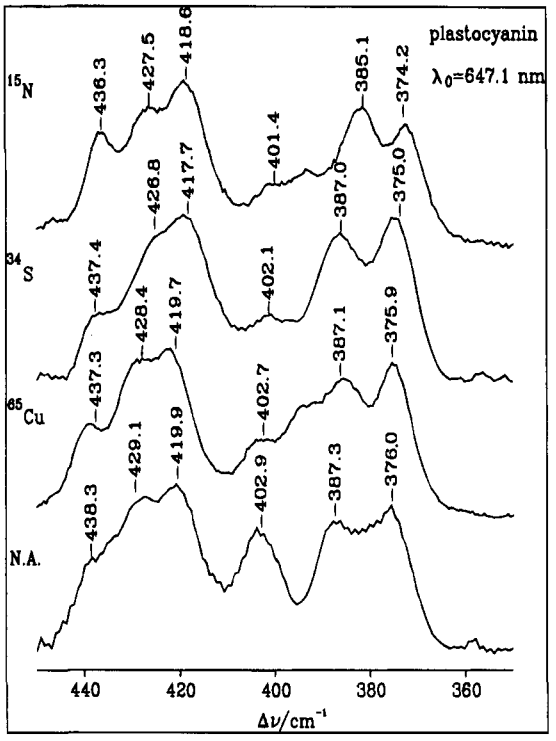

Figure 2. 647.1-nm excited resonance Raman spectra of isotopically labeled and natural abundance plastocyanin from *poplar*.

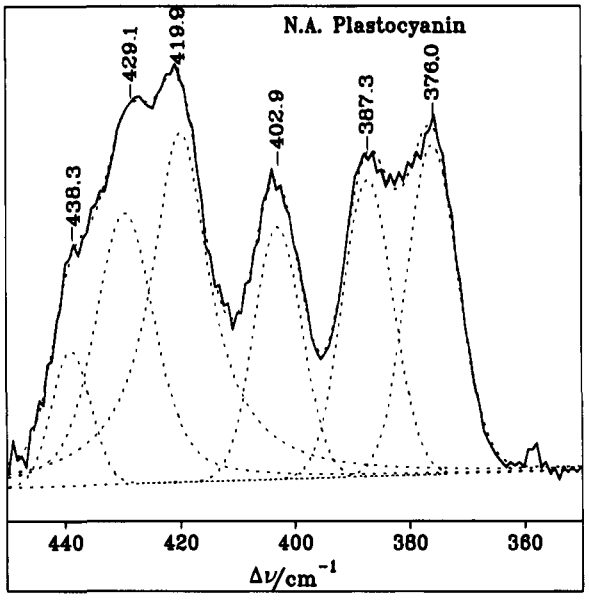

Figure 3. Peak resolution of RR bands for N.A. plastocyanin: solid line, protein spectrum; dotted line, deconvoluted peaks.

is mixed with other coordinates. For an isolated Cu−S oscillator with a 400-cm^{-1} frequency the expected ^{34}S shift is 8 cm^{-1}.[5] The total shift for the six plastocyanin bands in this region, 7.6 cm^{-1} (Table 1), is close to the Cu−S oscillator value, whereas for azurin, the sum of the reported ^{34}S shifts[5] is somewhat smaller, 5.6 cm^{-1}. For both proteins, the sum of the ^{65}Cu shifts, 2.0−2.4 cm^{-1}, is close to the Cu−S oscillator value, 2.2 cm^{-1}.

For both plastocyanin and azurin, the largest ^{34}S shifts are observed for the strongest RR bands, 429 and 420 cm^{-1} for plastocyanin and 409 cm^{-1} for azurin. This observation strongly supports the view that the Cu−S bond extension is the main geometry change in the charge transfer excited state; consequently the mode with the largest Cu−S stretching contribution acquires the greatest resonance enhancement. The higher

Table 1. Comparison of the RR Frequencies and Isotopic Shifts for *P.a. Azurin* and *poplar* Plastocyanin (cm^{-1})

P.a. azurin				*poplar* plastocyanin[d]			
freq	$\Delta^{65}Cu$[a]	$\Delta^{34}S$[b]	$\Delta^{15}N$[c]	freq	$\Delta^{65}Cu$	$\Delta^{34}S$	$\Delta^{15}N$
372.4	0.6	0.4	0.9	376	0.1	1.0	1.8
400.4	0.6	0	2.7	387.3	0.2	0.3	2.2
408.6	0.6	3.8	1.4	402.9	0.2	0.8	1.5
427.8	0.2	1.4	3.8	419.9	0.2	2.2	2.4
455	0		2.6	429.1	0.7	2.3	1.6
				438.3	1.0	0.9	2.0

[a] From reference 21. [b] From reference 5. [c] From references 1b and 27. [d] Present work.

frequencies observed for plastocyanin imply that its Cu−S bond is stronger than that of azurin. This order is in general agreement with crystallographic[17,18] and EXAFS[19,20] bond distance estimates, although reported values for azurin[18,20] are quite variable. The RR spectrum provides a sensitive guide to bond strength differences, which can be quantitated via the force constants, once a reliable force field is available for the active sites. We note that Blair et al.[21] obtained encouraging results by applying Badger's rule[22] to the "local oscillator" frequency of blue Cu proteins, calculated as the intensity-weighted average of the RR frequencies in the 400-cm^{-1} region. They also found that the local oscillator frequency scales linearly with the enthalpy of Cu[II] reduction, consistent with a dominant influence of the Cu−S bond strength on the reduction potential.

Cu−S Bonding Trends. The relationship of RR intensity and Cu−S bond has been traced in azurin mutants. Dave et al.[5] found that the dominant intensity and the largest ^{34}S shift switches from the 409-cm^{-1} band in wild-type protein to the 400-cm^{-1} band when the axial methionine is replaced with glycine [M121G], or a histidine ligand is replaced with aspartate [H46D]. The shift of intensity and isotope sensitivity to a lower frequency band indicated a weakening of the Cu−S bond in the mutants. This weakening was ascribed by Dave et al. to a displacement of the Cu atom from the trigonal N,N,S coordination plane, consistent with the appearance of a rhombic EPR spectrum and intensification of an absorption band at 450 nm. These spectral characteristics have been associated in other proteins with displacement of the Cu atom and a more tetrahedral coordination environment.[23]

Pursuing this line of reasoning, Andrew et al.[24] examined data for a wide range of Type 1 Cu proteins and mutants and

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found a negative correlation between the frequency of the strongest RR band and the intensity ratio of the ~450- and ~600-nm absorption bands, suggesting a steady weakening of the Cu−S[cysteine] bond as the Cu coordination geometry becomes tetrahedral. Plastocyanin is found to lie on this correlation, but wild-type azurin deviates from it. The strongest RR band is lower in frequency for azurin than for plastocyanin, and so is the 400/600-nm absorptivity ratio.[24] The lower absorptivity ratio is consistent with the crystal structures, which show the Cu atom to be almost exactly in the N,N,S plane (0.04-Å displacement) for azurin[18] but 0.34 Å out of the plane, toward the axial methionine, in plastocyanin[17] (Figure 1). There are two additional interactions in azurin which may account for structural differences: (1) The cysteine S ligand atom accepts one H-bond in plastocyanin (from the peptide nitrogen of Asn38)[17] but two in azurin (from the peptide nitrogens of Asn47 and Phe114).[18] The extra H-bond is expected to lower the thiolate negative charge and weaken the Cu−S bond, as is observed. (2) The azurin Cu ion experiences an extra donor interaction, as a result of the placement of a backbone carbonyl group on the trigonal axis opposite the methionine ligand. These balancing axial interactions may anchor the Cu ion in the trigonal plane. The carbonyl oxygen is too far away (3.13 Å) to form a bond to the Cu ion,[25] but the extra polar interaction may be sufficient to increase the Cu−S_{Met} distance substantially, 3.15 Å in azurin vs 2.83 Å in plastocyanin.

^{15}N Shifts and Mode Compositions. In the ^{15}N-labeled protein, all the RR bands in the 400-cm^{-1} region are shifted down by about 2 cm^{-1}. A similar shift pattern was reported for azurin.[1b] Interpretation of these shifts is not straightforward, since the all the N atoms of the protein are labeled, including those in the backbone and those in the side chains. The latter include the pairs of N atoms in the histidine ligands. The Cu−His stretching vibrations give rise to RR bands near 270 cm^{-1}, which are known[1b] to be ^{15}N sensitive. In the 400-cm^{-1} region, there are no in-plane modes of the imidazole ring, but there may be out-of-plane modes, involving the ring C and N atoms. These modes are not expected to couple strongly to the intensity-yielding Cu−S stretching cysteine deformation modes, and indeed, no shifts are seen in the 400-cm^{-1} region when the histidines are labeled with ^{15}N.[1b]

A stronger effect is expected from the N atom of the cysteine peptide bond. This atom is connected by two C atoms to the ligating S atom, and the CCN bending coordinate is capable of mixing with the Cu−S stretch, just as the CCC bending coordinate was found to do in the *sec*-butanethiolate adduct of the trispyrazolylborate Cu model complex.[7] Evidence for involvement of this coordinate is provided by the significant shifts reported for the 400-cm^{-1}-region bands when the Cu proteins are equilibrated with D_2O.[26] Exchange of the amide protons is expected to lower the CCN frequency. Additional involvement of other amide groups along the polypeptide chain cannot be excluded. Indeed they have been implicated by the normal mode calculation of Urushiyama et al.[9] on a 169-atom fragment of plastocyanin, which produced modes having contributions from a large number of residues. Whether the calculation provides an accurate description of the modes giving rise to the RR bnads or not is uncertain. The additional isotope data now available for plastocyanin and azurin will provide important constraints on further modeling efforts, which are currently in progress.

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