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Resonance Raman Spectra of Plastocyanin and Pseudoazurin: Evidence for Conserved Cysteine Ligand Conformations in Cupredoxins (Blue Copper Proteins)†

Jane Han,‡ Elinor T. Adman,§ Teruhiko Beppu,∥ Rachel Codd,⊥ Hans C. Freeman,⊥ Laila Huq,⊥ Thomas M. Loehr,‡ and Joann Sanders-Loehr*,‡

Department of Chemical and Biological Sciences, Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006-1999, Department of Biological Structure, University of Washington, Seattle, Washington 98195, Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan, and Department of Chemistry, University of Sydney, Sydney 2006, New South Wales, Australia

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ABSTRACT: New resonance Raman (RR) spectra at 15 K are reported for poplar (*Populus nigra*) and oleander (*Oleander nerium*) plastocyanins and for *Alcaligenes faecalis* pseudoazurin. The spectra are compared with those of other blue copper proteins (cupredoxins). In all cases, nine or more vibrational modes between 330 and 460 cm^{-1} can be assigned to a coupling of the Cu–S(Cys) stretch with Cys ligand deformations. The fact that these vibrations occur at a relatively constant set of *frequencies* is testimony to the highly conserved ground-state structure of the Cu–Cys moiety. Shifts of the vibrational modes by 1–3 cm^{-1} upon deuterium exchange can be correlated with N–H···S hydrogen bonds from the protein backbone to the sulfur of the Cys ligand. There is marked variability in the *intensities* of these Cys-related vibrations, such that each class of cupredoxin has its own pattern of RR intensities. For example, plastocyanins from poplar, oleander, French bean, and spinach have their most intense feature at ~425 cm^{-1}; azurins show greatest intensity at ~410 cm^{-1}, stellacyanin and ascorbate oxidase at ~385 cm^{-1}, and nitrite reductase at ~360 cm^{-1}. These variable intensity patterns are related to differences in the electronic excited-state structures. We propose that they have a basis in the protein environment of the copper–cysteinate chromophore. A further insight into the vibrational spectra is provided by the structures of the six cupredoxins for which crystallographic refinements at high resolution are available (plastocyanins from *P. nigra*, *O. nerium*, and *Enteromorpha prolifera*, pseudoazurin from *A. faecalis*, azurin from *Alcaligenes denitrificans*, and cucumber basic blue protein). The average of the Cu–S(Cys) bond lengths is 2.12 ± 0.05 Å. Since the observed range of bond lengths falls within the precision of the determinations, this variation is considered insignificant. The Cys ligand dihedral angles are also highly conserved. Cu–S_γ–C_β–C_α is always near −170° and S_γ–C_β–C_α–N near 170°. As a result, the Cu–S_γ bond is coplanar with the Cys side-chain atoms and part of the polypeptide backbone. The coplanarity accounts for the extensive coupling of Cu–S stretching and Cys deformation modes as seen in the RR spectrum. The conservation of this copper–cysteinate conformation in cupredoxins may indicate a favored pathway for electron transfer.

The blue copper proteins have been the subject of intensive spectroscopic and structural studies (Adman, 1985, 1991; Solomon et al., 1986). Particularly noteworthy are their very intense (ϵ = 3000–5000 M^{-1} cm^{-1}) absorption bands in the visible spectrum (590–625 nm) arising from cysteinate S → Cu(II) charge transfer. In addition, they exhibit distinctive resonance Raman (RR) spectra, axial or rhombic EPR spectra with abnormally low copper hyperfine splitting constants, and relatively high redox potentials of 200–700 mV (compared to $E'_0 \simeq 160$ mV for the Cu^{2+}/Cu^{1+} pair). Blue copper centers are found in both mononuclear and multinuclear copper proteins. Where the biological function has been established,

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* To whom correspondence should be addressed.
‡ Oregon Graduate Institute of Science and Technology.
§ University of Washington.
∥ University of Tokyo.
⊥ University of Sydney.

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it is invariably electron transfer [see, e.g., Farver and Pecht (1984) and Ryden (1984)]. For this reason, Adman (1985) has proposed that the mononuclear copper proteins be described collectively as cupredoxins.

The cupredoxins for which high-resolution X-ray crystal structures are available comprise poplar (*Populus nigra*) plastocyanin (Guss & Freeman, 1983), *Oleander nerium* plastocyanin (Tong, 1991), algal (*Enteromorpha prolifera*) plastocyanin (Collyer et al., 1990), *Alcaligenes denitrificans* azurin (Baker, 1988), cucumber basic blue protein (Guss et al., 1988), and *Alcaligenes faecalis* pseudoazurin (Petratos et al., 1988; Adman et al., 1989). There is also a cupredoxin-type domain in the multinuclear copper protein, zucchini ascorbate oxidase (Messerschmidt et al., 1989), and the existence of similar domains in two other multinuclear copper proteins, laccase and ceruloplasmin, has been inferred from the primary structures (Messerschmidt & Huber, 1990). Despite their different plant and bacterial origins, all six proteins have similar β-sandwich structures and blue copper sites (Adman, 1991). In all cases, the copper is coordinated to one cysteine and two histidines in a distorted trigonal geometry with a weakly ligated axial methionine at a longer distance. In the azurins from *A. denitrificans* (Baker, 1988) and *Pseudomonas aeruginosa* (Adman, 1985), there is also a weakly coordinated peptide carbonyl group serving as a fifth ligand. Both the intensity of the ~600-nm absorption band ($\epsilon > 3000$ M^{-1} cm^{-1}) and the narrowness of the EPR copper hyperfine splitting ($A_{\parallel} < 65 \times 10^{-4}$ cm^{-1}) have been attributed to a strong trigonal ligand set having a symmetry lower than C_{3v} (Gewirth et al., 1987; Ainscough et al., 1987).

Despite the close similarities in the structures of the blue copper sites, there are some surprising differences. For some of the proteins, the X-band EPR spectrum is axial, whereas for others it is rhombic. Resonance Raman (RR) spectra show even greater variability. In all cases, excitation within the S(Cys) → Cu(II) CT band produces strong enhancement of four or more fundamental vibrational modes in the 330–460-cm^{-1} region. The multiplicity of these modes has been attributed to coupling between the Cu–S stretch and internal modes of the cysteine ligand (Nestor et al., 1984; Woodruff et al., 1988). However, the most intense RR mode can be located anywhere between 360 and 445 cm^{-1}.

In order to gain a better understanding of the structural factors responsible for the complexity and variability of the RR spectra, we have chosen to investigate poplar plastocyanin and *A. faecalis* pseudoazurin and to compare their spectroscopic behaviors with those of other blue copper proteins of known structure. We find that the vibrational *frequencies* among the blue copper proteins are actually fairly constant, which is consistent with the strong conservation of structure observed crystallographically. This would argue against the previous suggestion (Blair et al., 1985) that the frequency of the most intense vibrational mode can be used as an indicator of Cu–S(Cys) bond length. Rather, the uniqueness of the spectral pattern for each protein appears to be due mainly to variations in the vibrational *intensities*. Thus, the differences in the blue copper sites which are detected by RR spectroscopy are reflecting differences in the electronic excited-state structure of the Cu–cysteinate chromophore.

Experimental Procedures

Protein Samples. Pseudoazurin from *Alcaligenes faecalis* S-6 was purified as described previously (Kakutani et al., 1981). Samples for Raman spectroscopy were prepared from the same material which had been used for X-ray crystallography and had an A_{280}/A_{593} of 2.14. Concentrated samples ~1.5 mM in pseudoazurin were diluted 20-fold in 0.02 M KH_2PO_4 and 0.04 M KCl (pH meter reading 6.3) in either H_2O or D_2O. The samples were then concentrated by centrifugation in a Centricon 10 (Amicon) ultrafiltration device. Two more dilution and reconcentration steps were performed. Before the last reconcentration step, the dilute solutions were incubated overnight at 5 °C. The final protein concentrations were ~1.5 mM.

Poplar (*Populus nigra* var. *italica*) and oleander (*Oleander nerium*) plastocyanins were purified as described previously (Chapman et al., 1977). During the purification of poplar plastocyanin, the second ion-exchange chromatographic separation step yielded two blue components, Pc I and Pc II. These components were treated separately in subsequent steps of the procedure. For RR spectroscopy, lyophilized samples of Pc I were dissolved in 0.1 M sodium phosphate in H_2O or D_2O (pH meter reading 6.0) yielding a concentration ~0.5 mM in Cu(II) and were incubated overnight at 5 °C. Samples of Pc II (not lyophilized) were prepared in 0.1 M sodium phosphate (pH 7.0) or 0.1 M sodium acetate (pH 4.2) and concentrated to 2.5 mM in Cu(II) by ultrafiltration, as described above.

Resonance Raman Spectroscopy. RR spectra were obtained with a computer-interfaced Jarrell-Ash spectrophotometer using a Spectra Physics 2025-11 (Kr) laser for 647.1-nm excitation and a Coherent Innova 90-6 (Ar)/599-01 (dye) laser system for 570-nm excitation. The detector was an RCA C31034 photomultiplier tube with an ORTEC model 9302 amplifier/discriminator. The Raman spectra were collected in an ~150° backscattering geometry with samples at a temperature of ~15 K by using a closed-cycle helium refrigerator (Air Products Displex). For isotopic comparisons, samples were run consecutively under identical instrumental conditions. Peak positions were determined by abscissa expansion and curve fitting (typical values: 40% Gaussian, 60% Lorentzian, full width at half-height of 7–9 cm^{-1}). Although absolute frequencies are accurate to only ±1 cm^{-1}, isotope shifts are reproducible to within ±0.3 cm^{-1}.

Results and Discussion

Copper Site Structures in Pseudoazurin and Plastocyanin. Pseudoazurin from *A. faecalis* is a protein with a molecular weight of 14000 that serves as an electron donor to nitrite reductase under anaerobic conditions (Kakutani et al., 1981). Its crystal structure has been determined at high resolution (Petratos et al., 1988; Adman et al., 1989). The cupric ion in pseudoazurin has three strongly coordinated ligands (His-40, Cys-78, His-81) forming an approximately trigonal planar array with a fourth ligand (Met-86) at a longer distance, resulting in a distorted tetrahedral structure (Figure 1). Despite its name, pseudoazurin has a structure, as well as EPR and RR properties (see below), which are distinctly different from those of the bacterial azurins.

Bacterial pseudoazurin is structurally more closely related to plant plastocyanin, an electron carrier in chloroplasts. Much of the polypeptide backbone of pseudoazurin (70 of the 123 residues) can be superposed upon the structure of plastocyanin (Guss & Freeman, 1983) with an rms deviation of only 0.7 Å (Petratos et al., 1987). The copper sites of the two proteins, including the four ligating amino acids (His-37, Cys-84, His-87, and Met-92 in plastocyanin) and the connecting polypeptide backbone segments, are identical within the limits of precision of the determinations (Figure 1A). Similar ligand arrangements are observed at the blue copper sites of the cucumber basic protein (Guss et al., 1988) and ascorbate oxidase (Messerschmidt et al., 1989) despite insertions and

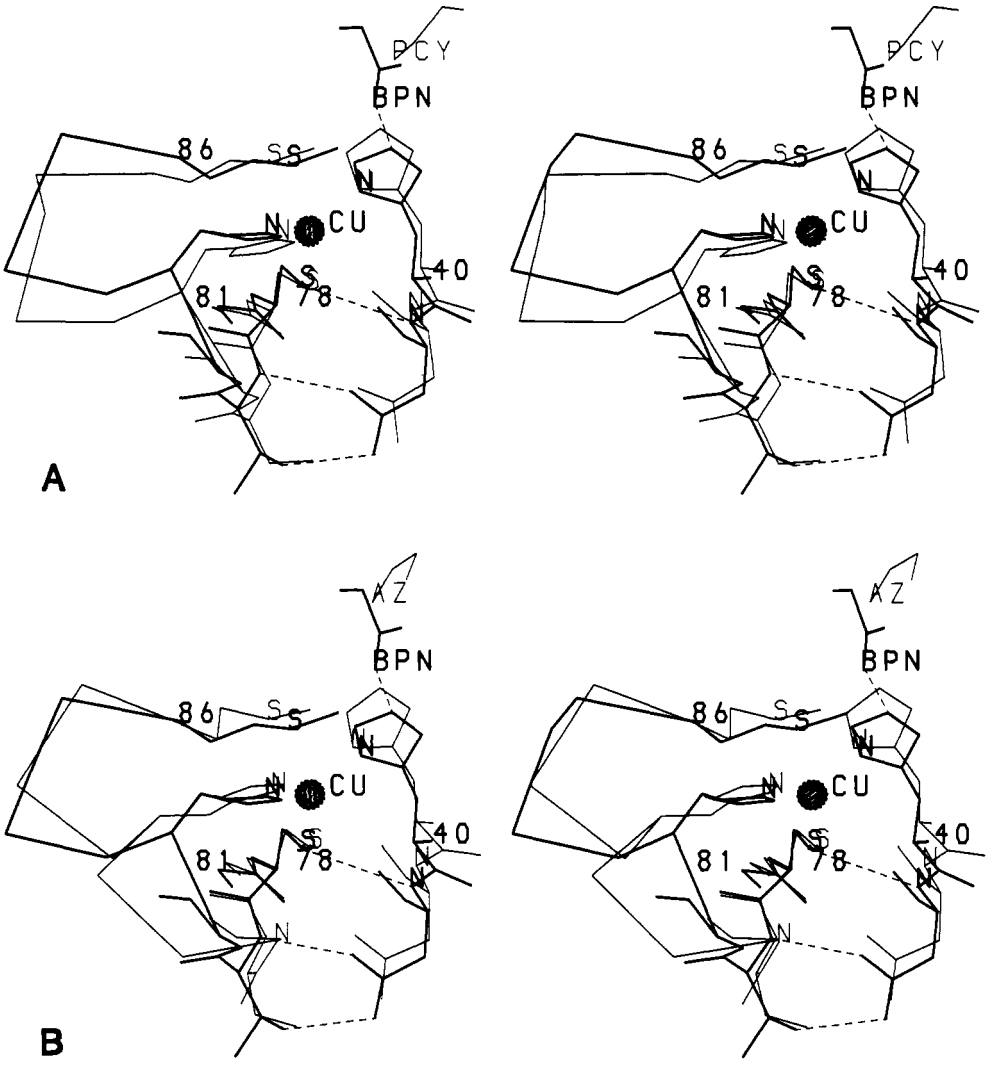

FIGURE 1: (A) Superposition of the copper sites in pseudoazurin (BPN) from *A. faecalis* (thick lines) and poplar plastocyanin (PCY) (thin lines). (B) Superposition of the copper sites in pseudoazurin (thick lines) and *A. denitrificans* azurin (AZ) (thin lines). The drawings were made from X-ray coordinates in the Brookhaven Protein Data Bank for pseudoazurin at 2.0-Å resolution (2PAZ), plastocyanin at 1.6-Å resolution (1PCY), and azurin at 1.8-Å resolution (2AZA). Also shown for pseudoazurin are the hydrogen bonds (···) between copper ligands and protein moieties and between the side chain of the conserved Asn-41 and the backbone NH and side chain of Thr-79. Analogous hydrogen bonds (not shown) are found in plastocyanin and azurin.

deletions in the loop regions of the polypeptide backbones. The azurins also maintain a similar orientation of these four ligating amino acids (Figure 1B) despite their having a weakly coordinated carbonyl as a fifth ligand (Adman, 1985; Baker, 1988).

(*a*) *Bond Distances.* One of the most unusual and apparently persistent characteristics of the blue copper site is the shortness of the Cu–S(Cys) bond. The average value in the six high-resolution structures (Table I) is 2.12 Å. Although the values appear to range from 2.07 to 2.16 Å, they are subject to estimated uncertainties of at least 0.05 Å (Table I) so that they are effectively indistinguishable from one another. The estimated uncertainty of ±0.05 Å is in keeping with the structure analysis of poplar plastocyanin at 1.6-Å resolution where two independent refinements were made with two independently recorded data sets (Guss & Freeman, 1983). It is supported by the recent finding that the Cu–S(Cys) bond length in poplar plastocyanin changed from 2.16 to 2.07 Å when the structure were further refined using new data at 1.3-Å resolution (J. M. Guss, H. D. Bartunik, and H. C. Freeman, unpublished results). In the case of *A. faecalis* pseudoazurin, two separate structure determinations using different methods of data collection have yielded Cu–S(Cys) bond lengths of 2.07 and 2.16 Å for data sets at 2.0- and 1.55-Å resolution, respectively (Adman et al., 1989; Petratos et al., 1988). Thus, it is likely that the 2.12 ± 0.05 Å value for Cu–S(Cys) from Table I is applicable to the other cupredoxin structures. In contrast with the cupredoxins, low molecular weight cupric thiolate complexes more typically exhibit Cu–S bond distances of 2.2–2.4 Å (Guss et al., 1986; Anderson et al., 1986; Bharadwaj et al., 1986).

The bonds to axial ligands such as Met in blue copper proteins are much weaker than the bonds to the trigonal Cys, His_2 ligand set. The Cu–S(Met) values range from 2.6 to 3.1 Å (Table I), the difference of 0.5 Å being well beyond experimental error. The unusually long value of 3.13 Å in azurin may be a reflection of the presence of a second axial ligand. Plastocyanin and pseudoazurin have intermediate Cu–S(Met) distances near 2.80 Å, whereas the cucumber basic protein has a significantly shorter distance of 2.62 Å.

The irregular geometry of the blue copper site appears to be imposed by the protein and the framework in which the ligands are located. Thus, the ligand positions in plastocyanin are essentially unaffected by replacement of Cu(II) with Hg(II) or by removal of Cu(II) to form the apoprotein (Church et al., 1986; Garrett et al., 1984). In plastocyanin, pseudoazurin, and azurin, a high degree of ordering is also

Table I: Structural Characteristics of Blue Copper Sites

protein	M–S distance (Å) Cys	M–S distance (Å) Met	dihedral angle (deg) S_δ–M–S_γ–C_β	dihedral angle (deg) M–S_γ–C_β–C_α	dihedral angle (deg) S_γ–C_β–C_α–N
poplar plastocyanin[a]	2.07	2.82	−3	−168	169
algal plastocyanin[b]	2.12	2.92	−1	−166	166
oleander plastocyanin[c]	2.11	2.82	−4	−172	173
pseudoazurin[d]	2.16	2.76	−3	−178	166
cucumber basic protein[e]	2.15	2.62	−6	−171	166
azurin[f]	2.13	3.13	−1	−169	173
ascorbate oxidase[g]	2.1	2.9		−168	
alcohol dehydrogenase[h]	2.1			−174	190

[a] Bond lengths (esd = 0.04 Å) and dihedral angles for *P. nigra* var. *italica* plastocyanin at 1.33-Å resolution (J. M. Guss, H. D. Bartunik, and H. C. Freeman, unpublished results). [b] Bond lengths (esd = 0.05 Å) and dihedral angles for *E. prolifera* plastocyanin at 1.85-Å resolution (Collyer et al., 1990). [c] *O. nerium* plastocyanin at 1.8-Å resolution (Tong, 1991). [d] Bond lengths for *A. faecalis* S-6 pseudoazurin at 1.55-Å resolution, R = 18% (Petratos et al., 1988); dihedral angles from 2PAZ in The Brookhaven Protein Data Bank. [e] Bond lengths (esd = 0.05 Å) and dihedral angles for cucumber basic protein at 1.8-Å resolution (J. M. Guss and H. C. Freeman, unpublished results). [f] Bond lengths ±0.05 Å for *A. denitrificans* azurin at 1.8-Å resolution, R = 16.4% (Baker, 1988); bond angles from 2AZA in The Brookhaven Protein Data Bank. [g] Bond lengths ±0.2 Å and bond angles for zucchini ascorbate oxidase at 1.9-Å resolution, R = 20.5% (A. Messerschmidt, personal communication). [h] Data for the catalytic *zinc* site in horse liver alcohol dehydrogenase at 2.4-Å resolution (Chakrabarti, 1989).

evident from the lower thermal parameters in the vicinity of the copper site (Guss et al., 1986; Adman et al., 1989; Ainscough et al., 1987).

An important factor in maintaining the structural integrity of the blue copper site is the hydrogen-bond network involving the ligands and adjacent residues (Guss & Freeman, 1983; Adman et al., 1989). In both pseudoazurin and plastocyanin (Figure 1A), the sulfur atom of the ligating Cys (residue 78 in PAz/84 in Pc) is the acceptor of an NH···S hydrogen bond from the peptide-backbone N (residue 41/38). The orientation of the imidazole ring of the more buried His ligand (residue 40/37) is stabilized by a hydrogen bond to either a side chain or a backbone carbonyl (residue 9/13). The geometry of the Cu site is further stabilized by two interactions involving the residues immediately following the ligand His and the ligand Cys. The residue adjacent to the ligand His is a conserved Asn (residue 41/38) whose side-chain amide group forms hydrogen bonds with the N(peptide) atom and side-chain O_γ atom of a Thr/Ser (residue 79/85) adjacent to the ligand Cys. All these interactions occur with minor variations in all the known cupredoxin structures.

(*b*) *Dihedral Angles.* Copper–ligand dihedral angles are a sensitive indicator of the extent to which the Cys ligand conformation is conserved in blue copper proteins. In keeping with the near superimposability of copper-site structures, these dihedral angles have approximately constant values (Table I). Thus, the S_δ(Met)–Cu–S_γ–C_β(Cys) angle, the Cu–S_γ–C_β–C_α(Cys) angle, and the S_γ–C_β–C_α–N(Cys) angle have values near 0°, −170°, and +170°, respectively. A dihedral angle of 180° or 0° means that the four contributing atoms must be coplanar. In the case of the blue copper proteins, this near coplanarity extends over *six* atoms from the S_δ of methionine to the N(amide) of cysteine, as can be seen in Figure 1. The apparent conservation of atomic positions at the blue copper site is all the more remarkable in view of the extensive differences among the primary and tertiary structures of other parts of the protein molecules. For example, the sequence identity between pseudoazurin and plastocyanin is only 20% (Petratos et al., 1987) and that between consensus sequences of azurin and plastocyanin is only 7% (Ryden, 1984).

The conservation of cysteine ligand dihedral angles is also observed in other classes of proteins that contain metal thiolate bonds. The crystal structures for the $Fe(Cys)_4$ sites in rubredoxins (Adman et al., 1977; Frey et al., 1987) and the $FeS_4(Cys)_4$ sites in ferredoxins (Backes et al., 1991) reveal that the geometry of a particular cysteine ligand is maintained in homologous proteins. The distribution of Fe–S_γ–C_β–C_α angles in rubredoxins is two at ∼180° and two at ∼270°, whereas in ferredoxins it is two at ∼70° and two at ∼270° (Chakrabarti, 1989). A similar range of values is observed for the equally conserved S_γ–C_β–C_α–N dihedral angles in rubredoxins and ferredoxins. Chakrabarti (1989) has observed that the S_γ–C_β–C_α–N angle tends to be near 180° when the M–S_γ–C_β–C_α angle is also close to 180°, as in the blue copper sites (Table I).

In the crystal structure of liver alcohol dehydrogenase, the catalytic Zn(II) is coordinated to two cysteines, one histidine, and a water (Eklund & Bränden, 1983). One of the cysteine ligands appears to have a short metal–sulfur bond and Zn–S_γ–C_β–C_α–N coplanarity (Table I). This is the probable reason why Cu(II)-substituted alcohol dehydrogenase exhibits the optical, EPR, and RR spectroscopic properties of a blue copper site (Maret et al., 1986). Although the structure of the Cu(II) derivative has not been determined crystallographically, it is likely to be close to that of the native zinc enzyme as the ligand geometry is known to be preserved in the Co(II) and Cd(II) analogues as well as in the apoprotein (Schneider et al., 1983, 1985).

Resonance Raman Spectra of Plastocyanin and Pseudoazurin. Raman spectra of blue copper proteins are maximally enhanced by excitation within the intense S(Cys) → Cu(II) CT band near 600 nm (Woodruff et al., 1988). All exhibit a large set of cupric–cysteinate-related vibrational modes between 350 and 450 cm^{-1}. The pattern of relative intensities of these modes appears to be characteristic of the particular type of blue copper protein. For the plastocyanins, the strongest peak is near 425 cm^{-1} and the next strongest is near 375 cm^{-1}, with an additional four to five smaller peaks in this region (Figure 2). There is also an isolated ν(Cu–His) mode near 265 cm^{-1}. This pattern is observed for plastocyanins from oleander and poplar (Figure 2), as well as from spinach and bean (Blair et al., 1985).

The RR spectra now reported are for oleander plastocyanin (Figure 2A) and two closely related forms of poplar plastocyanin, Pc I and Pc II (Figure 2B,C). Many poplar trees from which Pc I is isolated also yield Pc II. The X-ray structural data for poplar plastocyanin have all been derived from Pc I since no suitable crystals of Pc II have yet been prepared. The amino acid sequence for poplar Pc II was determined in 1980 (R. P. Ambler, personal communication to H.C.F.). It is in agreement with that recently published by Dimitrov et al. (1987) for poplar Pc *b*, with the exception of residues 39–40 where no difference from Pc I was noted. The 11 amino acid differences between Pc I and Pc II (at residues 1, 21, 22, 23, 39, 40, 45, 46, 76, 81, and 97) are conservative. None of the differences involves a residue close to the copper site in Pc I. Nevertheless, the RR spectra (Figure 2B,C) exhibit small differences in peak frequencies and relative intensities, suggesting that even remote amino acid changes do have a small effect on the blue copper site. As expected from the refinements of the poplar plastocyanin structures at pH 6.0 and 4.2 (Guss & Freeman, 1983), there are no significant differences between the RR spectra at pH 7.0 and 4.2 (Figure 2 legend).

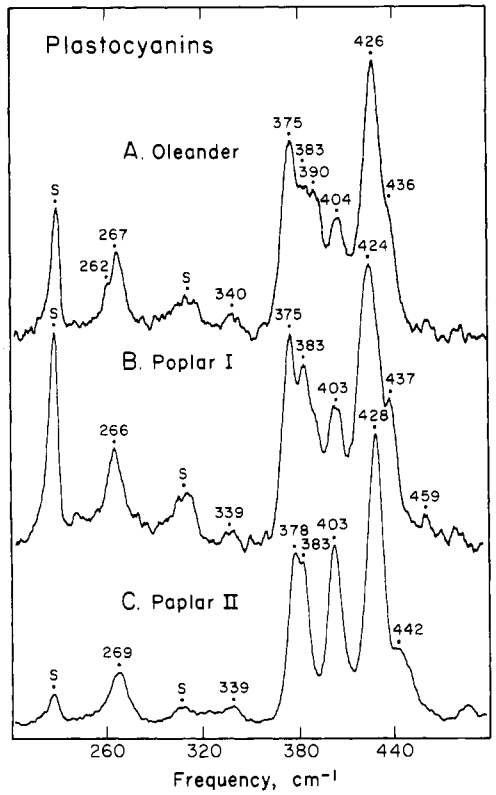

FIGURE 2: Resonance Raman spectra of plastocyanins from oleander (A), poplar component I (B), and poplar component II (C). Spectra A and B were obtained on samples ~0.5 mM in Cu(II), pH 6.0, at 15 K with 570-nm excitation (65 mW) with a resolution of 4 cm^{-1} and a scan rate of 0.5 cm^{-1}/s, and are accumulations of 20 scans each. Spectrum C was obtained on a sample 2.5 mM in Cu(II) in 0.1 M phosphate (pH 7.0) plus 3-fold excess $K_3Fe(CN)_6$ under similar conditions except for a resolution of 5 cm^{-1} for 10 scans. Omission of the $K_3Fe(CN)_6$ or changing the buffer to 0.1 M acetate (pH 4.2) caused no significant changes in the RR spectrum of poplar Pc II. S denotes peaks from frozen solvent (translational modes of ice).

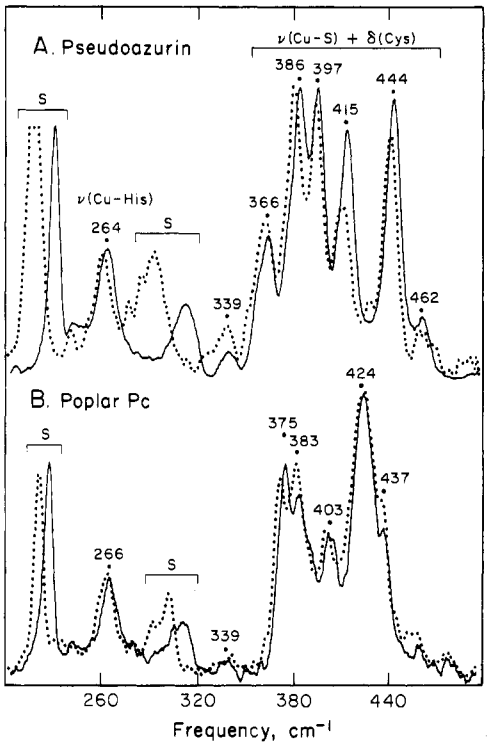

FIGURE 3: Resonance Raman spectra of pseudoazurin and plastocyanin in H_2O (—) and D_2O (···). (A) Pseudoazurin from *A. faecalis* (1.5 mM in H_2O, 1.2 mM in D_2O). Spectra were obtained at 15 K with 647.1-nm excitation (70 mW) with a resolution of 5 cm^{-1}, a scan rate of 0.5 cm^{-1}/s, and 20 scans each. (B) Poplar plastocyanin I (0.5 mM in H_2O, 0.6 mM in D_2O). Spectral conditions are as given in the legend to Figure 2. The indicated peak frequencies are for protein in H_2O.

Table II: Resonance Raman Vibrational Frequencies and Deuterium Isotope Shifts for Cupredoxins[a]

plastocyanin[b]		pseudoazurin[b]		azurin[c]		Cu(II)–ADH[d]	
H_2O	ΔD_2O	H_2O	ΔD_2O	H_2O	ΔD_2O	H_2O	ΔD_2O
266	−1	264	−1	266		264	−2
				287	−2	316	−2
339		339		333		342	
		359		348		*358	↓
375	−3	366	−1	373	−1	372	↓
383	−1	*386	−3			380	+2
403	−2	*397		401	−2	401	−3
		415	−3↓	*409	−1	*419	+2
*424				428	−2		
437	−1	*444	−2	441	−2		
459		462	−1	455	−1		

[a] Frequencies and isotope shifts in D_2O (in cm^{-1}) are rounded to the nearest whole number. An asterisk (*) denotes peaks of highest intensity. An arrow (↓) denotes a decrease in intensity. [b] Poplar plastocyanin I and *A. faecalis* pseudoazurin (this work). [c] *P. aeruginosa* azurin (T. den Blaauwen, G. W. Canters, J. Han, T. M. Loehr, and J. Sanders-Loehr, unpublished results). [d] Cu(II)-substituted alcohol dehydrogenase containing NADH (Maret et al., 1986).

Pseudoazurin from *A. faecalis* has its own unique pattern of RR intensities in the Cu–Cys region with three equally strong peaks at 386, 397, and 444 cm^{-1} (Figure 3A). In addition, a moderately strong peak at 415 cm^{-1} and five peaks of lower intensity between 339 and 462 cm^{-1} are observed. Yet another intensity pattern is seen for azurin where proteins from *seven* bacterial species all have their strongest RR peak at ~410 cm^{-1} (Blair et al., 1985; Ainscough et al., 1987).

Deuterium Isotope Shifts in RR Spectra. Another common attribute of the RR spectra of blue copper proteins is that many of the vibrational modes show small shifts to lower energy when the proteins are equilibrated in D_2O (Nestor et al., 1984; Ainscough et al., 1987; Sharma et al., 1988). The RR spectra of pseudoazurin and plastocyanin show the expected deuterium sensitivity as documented in Figure 3 and Table II. Both proteins exhibit a −1-cm^{-1} shift in the ~265-cm^{-1} peak as well as shifts of −1 to −3 cm^{-1} for most of the peaks in the 330–460-cm^{-1} region.

There is general agreement that the deuterium dependence of the ν(Cu–His) mode at ~265 cm^{-1} is due to exchange of one or two protons on the imidazole ring (Nestor et al., 1984; Blair et al., 1985). The deuterium effects in the 330–460-cm^{-1} region have also been ascribed to histidine contributions (Woodruff et al., 1988), but that interpretation may not be correct. We have observed similar deuterium-dependent shifts in the RR spectra of dinuclear and tetranuclear iron–sulfur proteins which lack histidine ligands (Mino et al., 1987; Backes et al., 1991). These observations indicate that deuterium isotope effects on metal–sulfur vibrational modes are due to the hydrogen-bonding interactions of sulfur ligands with exchangeable amide NH groups of the polypeptide backbone. Thus, the deuterium shifts in the RR spectra of plastocyanin, pseudoazurin, azurin, and Cu-substituted alcohol dehydrogenase (Table II) can be attributed to hydrogen bonding of a cysteine–thiolate ligand (Figure 1; Mino et al., 1987; Maret et al., 1986).

The deuterium dependencies of the RR spectra of plastocyanin and pseudoazurin are similar in the number of affected peaks and in the extent of the isotope shifts (Table II). A

similar pattern of deuterium sensitivity is observed for azurins from *P. aeruginosa* (Table III) and *A. denitrificans* (Ainscough et al., 1987). Although the azurins have two hydrogen bonds to the cysteine–sulfur ligand compared to only one in plastocyanin and pseudoazurin, the azurins do not show a correspondingly greater deuterium isotope sensitivity. It is possible that the hydrogen-bonding amides in *P. aeruginosa* azurin do not exchange well with deuterium (M. van de Kamp and G. W. Canters, personal communication). In native Zn(II)-containing alcohol dehydrogenase, the Cys-46 ligand is also hydrogen bonded to the amide NH of residue 48 (H. Eklund, personal communication). This feature is likely to be conserved in the Cu(II)-substituted protein where the deuterium-isotope effects (Table II) are even more remarkable than for native blue copper proteins. The spectral changes in Cu(II)–alcohol dehydrogenase involve large frequency shifts to both higher and lower energy as well as marked decreases in various peak intensities, all of which can be attributed to hydrogen-bonding effects (Maret et al., 1986). Similarly, the 415-cm^{-1} peak of pseudoazurin undergoes a significant lowering of intensity in D_2O (Figure 3A).

Resonance Raman Frequencies. The origin of the complex set of vibrational modes between 330 and 460 cm^{-1} in the RR spectra of blue copper proteins has been the subject of much discussion (Woodruff et al., 1988). Enhancement profiles (RR intensities versus excitation wavelength) have shown that the vibrational intensities all track the ~600-nm S(Cys) → Cu(II) CT absorption band (Ainscough et al., 1987; Maret et al., 1986; Musci et al., 1985). Therefore, each of these RR frequencies is expected to have a Cu–S stretching component contributing to its normal mode. Involvement of the axial methionine ligand can be ruled out because the substitution of selenomethionine for methionine in azurin has no detectable effect on the RR spectrum (Thamann et al., 1982). Similarly, contributions from the histidine ligands are likely to be minimal because ^{15}N isotopic substitution in an exogenous imidazole ligand of Cu(II)–alcohol dehydrogenase has no effect on the RR spectrum of the Cu–cysteinate chromophore (Maret et al., 1986). These considerations, as well as the smaller than expected Cu-isotope dependence, have led to the conclusion that the vibrational modes between 330 and 460 cm^{-1} must represent admixtures of the Cu–S(Cys) stretch and cysteine ligand deformation modes (Nestor et al., 1984).

Potential candidates for cysteine ligand deformation modes in the 300–500-cm^{-1} range include the S_γ–C_β–C_α bend involving the S_γ ligating group, the C_β–C_α–CO bend, the C_β–C_α–N bend, the N–C_α–CO bend, and a Cu–S_γ–C_β–C_α torsional mode (Nestor et al., 1984). Coupling of ν(M–S) with δ(S–C–C) is expected to be strongly dependent on the M–S_γ–C_β–C_α dihedral angle, being maximal at 180° and minimal at 90° (Han et al., 1989). This type of coupling has been detected in the RR spectra of iron–sulfur proteins such as $Fe(Cys)_4$ rubredoxin (Czernuszewicz et al., 1986), $Fe_2S_2(Cys)_4$ ferredoxin (Han et al., 1989), and $Fe_4S_4(Cys)_4$ high-potential iron protein (Backes et al., 1991), all of which contain one or more Fe–S_γ–C_β–C_α dihedral angle near 180°. As can be seen in Table I, all of the blue copper proteins which have been structurally characterized have Cu–S_γ–C_β–C_α dihedral angles close to 180°. The S_γ–C_β–C_α–N dihedral angle is also close to 180°, making the amide nitrogen a part of the coplanar grouping (Figure 1). Thus, it is likely that the ν(Cu–S) mode in blue copper sites is coupled to both the δ(S–C_β–C_α) and δ(C_β–C_α–N) modes, as well as to several other cysteine-related deformations. In contrast, the vibrational spectra of Cu(II)–imidazole complexes exhibit ν(Cu–Im) modes below 300 cm^{-1} and imidazole ring motions above 800 cm^{-1} (Larrabee & Spiro, 1980).

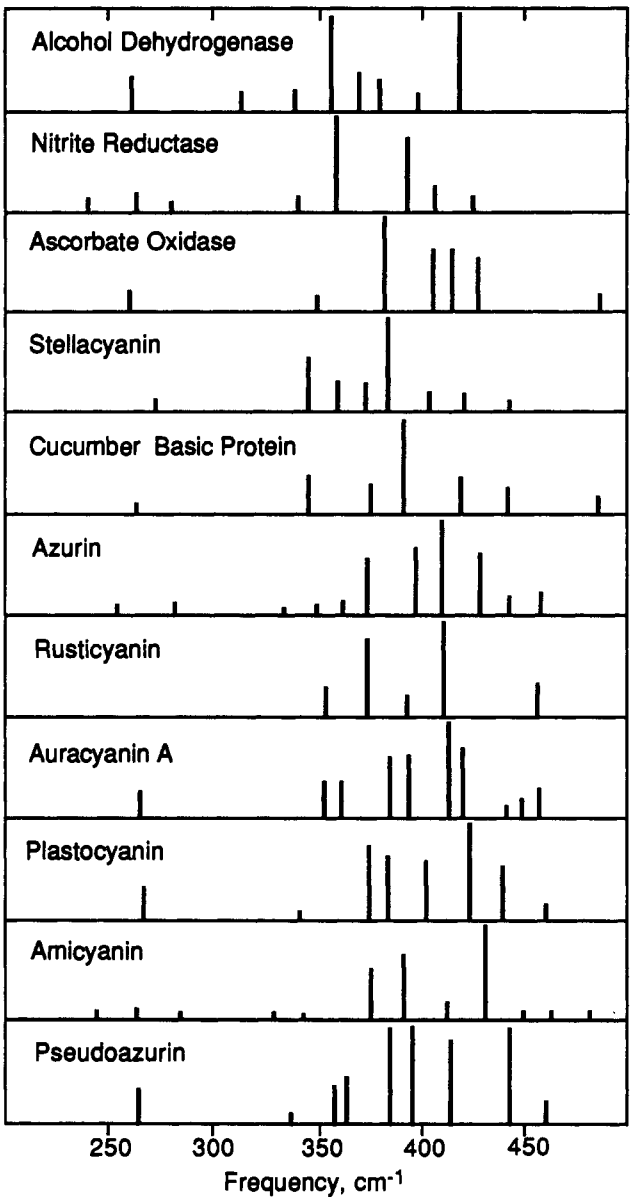

FIGURE 4: Relative peak intensities in the resonance Raman spectra of blue copper proteins. Data taken from the following sources: Cu(II)–alcohol dehydrogenase (ADH) (Maret et al., 1986), *Achromobacter cycloclastes* nitrite reductase (NiR) (J. Sanders-Loehr and B. A. Averill, unpublished results), zucchini ascorbate oxidase (AO) (Siiman et al., 1976), *Rhus vernicifera* stellacyanin (Sc) (Nestor et al., 1984), cucumber basic protein (CBP) (R. B. McInnes, R. S. Armstrong, and H. C. Freeman, unpublished results), *A. denitrificans* azurin (Az) (Ainscough et al., 1987), *Thiobacillus ferroxidans* rusticyanin (Rc) (T. A. Andary, R. S. Armstrong, and H. C. Freeman, unpublished results), *Chloroflexus aurantiacus* auracyanin A (Arc) (J. D. McManus, D. C. Brune, J. Han, J. Sanders-Loehr, and R. E. Blankenship, unpublished results), *Paracoccus denitrificans* amicyanin (Amc) (Sharma et al., 1988), poplar plastocyanin (Pc) and *A. faecalis* pseudoazurin (PAz) (this work).

Comparison of the RR spectra of blue copper proteins reveals that the [ν(Cu–S) + δ(Cys)] modes have a *fairly constant set of frequencies*, yielding the spectral alignments shown in Table II. This constancy has been overlooked in the past because of the striking differences in peak intensities in the raw spectra, but it is more obvious from the graphical depiction in Figure 4. All of the proteins have a peak near 265 cm^{-1} attributable to ν(Cu–His). In the 330–460-cm^{-1} region, as many as nine additional modes are observed. Each such mode

is confined to a narrow range of frequencies across all of the blue copper proteins. For example, most of the proteins have a peak at 440 ± 5 cm^{-1}, even though it varies from the least intense feature in stellacyanin to the most intense in pseudoazurin. Similarly there are eight other common features near 340, 360, 375, 385, 400, 415, 430, and 460 cm^{-1}. The vibrational mode which occurs at each of these frequencies probably arises from a different combination of the Cu–S(Cys) stretch with particular cysteine deformations.

Since vibrational frequencies are a reflection of the structure of the ground state (Carey, 1982), the relative constancy of these frequencies in the RR spectra of blue copper proteins is consistent with the highly conserved structure of the Cu–cysteinate moiety (Table I). The features of particular importance would be the short Cu–S(Cys) bond distance of ~2.13 Å that raises the frequencies into the 400-cm^{-1} region and the near coplanarity of the Cu–S_γ–C_β–C_α–N moiety that leads to kinematic coupling of cysteine deformation modes with ν(Cu–S). The appearance of characteristic cupredoxin-type RR spectra for the multi-copper proteins ascorbate oxidase, laccase, and ceruloplasmin (Siiman et al., 1976) indicates a similar Cys ligand coplanarity, as has also been observed in the crystal structure of ascorbate oxidase (Table I). Furthermore, a strong conservation of the H_β–C_β–S_γ–Cu dihedral angle in blue copper proteins has been implicated by ENDOR spectroscopic studies of plastocyanin, azurin, stellacyanin, and laccase (Werst et al., 1991).

Resonance Raman Intensities. There is a striking variability in the intensities of the different [ν(Cu–S) + δ(Cys)] modes in the blue copper RR spectra (Figure 4), and each class of protein has its own characteristic intensity pattern. In the plastocyanins, the feature at 424 cm^{-1} is the most strongly enhanced; in azurins, it is the feature at 409 cm^{-1}; and in pseudoazurin, it is a set of three peaks at 386, 397, and 444 cm^{-1} (Table II). It has been previously suggested (Blair et al., 1985) that the most intense peak in the spectrum corresponds to the vibration with the largest ν(Cu–S) contribution, and, thus, the frequency of this vibration should be related to the Cu–S(Cys) bond length. This suggestion seems unlikely on several grounds: (i) the strong conservation of vibrational frequencies (vide supra) suggests a common set of vibrational modes for all blue copper proteins with each vibrational mode representing a particular combination of ν(Cu–S) and δ(Cys) components and (ii) blue copper proteins such as pseudoazurin and Cu(II)-substituted alcohol dehydrogenase (Figure 4) have equally high intensities in two to three peaks which differ widely in energy. *Rather, the relative constancy of RR frequencies can be taken as an indication of a conserved short Cu–S(Cys) bond of ~2.13 Å in all blue copper sites.* On the basis of its Raman frequencies (Figure 4), stellacyanin is also likely to have a short Cu–S(Cys) bond, despite the suggestion of a longer 2.2 Å Cu–S(Cys) distance from EXAFS analyses (Peisach et al., 1982; Feiters et al., 1988).

Whereas vibrational frequencies in resonance Raman spectra are solely a function of the structure of the molecule in the electronic ground state, vibrational intensities are related to the change in the geometry of the chromophore in the electronic excited state (Carey, 1982). Intensities are maximized when the excited-state displacement of atoms occurs along the normal coordinate of a vibration (Nishimura et al., 1978; Shin & Zink, 1989). Thus, the variability of RR intensities in blue copper proteins may be related to the degree of displacement which occurs in the excited state for the atoms participating in a particular [ν(Cu–S) + δ(Cys)] mode. Since these excited-state effects are specific for each class of blue copper protein, it is likely that they arise from differences in amino acid composition and protein structure in the vicinity of the blue copper site (but beyond the highly conserved Cys, His_2, Met ligand set).

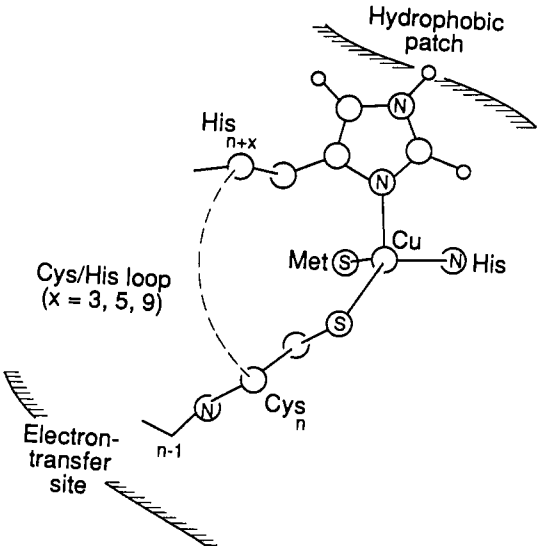

FIGURE 5: Common features of blue copper sites in cupredoxins. Electron donors or acceptors which react with cupredoxin-type domains are believed to utilize either the hydrophobic patch and/or the electron-transfer site adjacent to residue (n–1).

Table III: Correlation of Ligand Loop Size and Maximal Raman Intensity

protein	residue no.[a] Cys (n)	His (n+x)	x	max Raman intensity[b]	EPR spectral type[c]
alcohol dehydrogenase	46	67	21	358 (419)	rhombic
nitrite reductase	136	145	9	362	axial
ascorbate oxidase	508	513	5	383	axial
stellacyanin	87	92	5	385	rhombic
cucumber basic protein	79	84	5	391	rhombic
azurin	112	117	5	409	axial
rusticyanin	138	143	5	412	rhombic
auracyanin	123	128	5	415	rhombic
plastocyanin	84	87	3	424	axial
amicyanin	86	89	3	430	axial
pseudoazurin	78	81	3	444 (386)	rhombic

[a] Residue in amino acid sequence. x = the difference between Cys(n) and His (n+x). Source of sequence data: ADH, AO, CBP, Az, Pc, and PAz (references in Table I); NiR (Fenderson et al., 1991); Sc (Guss et al., 1988); Rc (M. Ronk, J. E. Shively, E. A. Shute, and R. C. Blake, personal communication); Arc (J. Van Beeumen, J. D. McManus, and R. E. Blankenship, personal communication); Amc (Sharma et al., 1988). The protein sources and abbreviations are as described in the legend to Figure 4. [b] Frequency of the most intense Raman peak in cm^{-1} (additional peak of high intensity) from references in Figure 4. [c] X-band EPR data sources: Cu-ADH (Maret & Kozlowski, 1987); NiR (B. A. Averill, personal communication); AO (Mondovi & Avigliano, 1984); Sc, CBP, Az, Rc, Pc, and Amc [references cited by Adman (1985)]; Arc (J. D. McManus and R. E. Blankenship, personal communication); PAz (Kakutani et al., 1981).

We propose that the conformation of the peptide loop connecting the Cys and His ligands (Figure 5) has an effect on the enhancement of particular RR modes in blue copper proteins. A common motif in the iron–sulfur proteins (rubredoxins, ferredoxins, and high-potential iron proteins) is that two cysteine ligands are separated by two amino acids, i.e., that the metal is coordinated by the S_γ atoms of Cys(n) and Cys(n+3) (Backes et al., 1991). A similar (n+3) spacing is observed between the Cys and His ligands in some of the blue

copper proteins, but many have an (n+5) or even larger spacing (Table III). The three proteins which have the smallest spacing (n+3) show strongest enhancement of the mode near 430 cm^{-1} (plastocyanin, amicyanin, and pseudoazurin). The proteins with (n+5) spacing have their most intense mode at either 410 cm^{-1} (azurin, rusticyanin, and auracyanin) or 385 cm^{-1} (ascorbate oxidase, stellacyanin, and cucumber basic protein). The copper sites of laccase and ceruloplasmin (as deduced from amino acid sequence alignments with ascorbate oxidase) also have an (n+5) spacing between Cys and His ligands (Messerschmidt & Huber, 1990), and these proteins exhibit maximal RR intensities at 400 and 383 cm^{-1}, respectively (Siiman et al., 1976). The two proteins with an even longer polypeptide segment between the Cys and His ligands (Cu-substituted alcohol dehydrogenase and nitrite reductase) show the strongest enhancement of the mode near 360 cm^{-1}.

Studies of aromatic amino acids have shown that their Raman intensities can vary considerably, depending on the nature of the protein environment (Hildebrandt et al., 1988). Thus, it is reasonable that the intensities of the cysteinate-related deformation modes are also sensitive to the protein environment in the vicinity of the cysteine ligand. Despite the highly conserved conformation of the cysteine ligand, the variability in the conformation of the *adjacent* residues clearly results in differences in protein structure and amino acid side-chain placement (Figure 1). Analysis of the S(Cys) $\rightarrow$ Cu(II) electronic transitions in cupredoxins as a function of temperature suggests that they are coupled with ligand deformation modes (Cupane et al., 1990). Thus, a number of atoms of the ligating residues would be expected to undergo geometric displacement in the electronic excited state. The extent of displacement which a particular atom experiences may be influenced by its environment (e.g., orientation and nature of amino acids in and around the Cys/His loop). This would then affect the Franck–Condon overlaps for a particular [ν(Cu–S) + δ(Cys)] vibrational mode. It is likely that the different types of Raman intensity patterns observed in Table III and Figure 4 correspond to different, but conserved, Cys/His loop conformations within each class of cupredoxins.

The characteristic Raman intensity pattern that is conserved within each class of cupredoxins (Figure 4) is surprisingly unaffected by metal substitution. Replacement of Cu(II) by Fe(III) or Ni(II) in alcohol dehydrogenase (Maret et al., 1986), Ni(II) in azurin (Ferris et al., 1979), or Ni(II) in stellacyanin (Musci et al., 1985) results in some differences in vibrational frequencies but the RR intensity pattern is preserved. This suggests that the coupling of the ν(M–S) vibration with cysteine deformations is the same in all of these cases. Thus, the conformation of the cysteine ligand and the Cys/His loop appears to be more important than the nature of the metal in determining the unique set of excited-state displacements which occurs in each type of blue copper protein.

Comparison of EPR and RR Properties. A characteristic and common feature of the blue-copper site is the narrow copper-hyperfine splitting observed in the EPR spectrum (Gewirth et al., 1987). However, the degree of rhombicity in the X-band EPR spectrum is highly variable, ranging from strongly axial, as in plastocyanin, to strongly rhombic, as in stellacyanin (Table III). Q-band EPR spectra, on the other hand, exhibit marked rhombic character for all of these proteins (Penfield, 1985), even for those classed as axial from X-band EPR analysis. The extent of the axial or rhombic nature of the EPR spectra has been proposed by Gewirth et al. (1987) to be related to the orientations of copper–ligand bond directions with respect to the copper orbitals. Given the nearly superposable $Cu(His)_2(Cys)(Met)$ sites of poplar plastocyanin and pseudoazurin (Figure 1A) and the indistinguishable bond distances (within 0.05 Å) for all ligands, including the long Cu–Met bond, it is difficult to discern the structural origin of the different EPR character (axial and rhombic, respectively, in Table III) of these two proteins. Further, we have noted no simple correlation between the X-band EPR spectra and RR frequencies or intensities. This may be due to the fact that RR spectra reflect the geometry of the cysteine ligand and its connectivity to the protein, whereas EPR spectra are more influenced by the electronic structure of the copper and its entire ligand set.

Role of Conserved Structures in Electron Transfer. The strongly conserved conformation of the Cys ligand (Table I) is not the only feature that the Cu sites of cupredoxins have in common. Judging by the known structures of plastocyanin, pseudoazurin, and azurin, the imidazole ring of the His ligand in the Cys/His loop also has a remarkably conserved orientation (Figure 1). In all three proteins, as well as in amicyanin, the imidazole ring of this His ligand protrudes into a hydrophobic patch on the surface of the molecule (Figure 5). While the solvent accessibility of the imidazole-ring edge may vary from protein to protein, the patch is directly implicated in several interactions between proteins and their electron-transfer partners: (i) plastocyanin utilizes this His pathway with inorganic redox reagents as well as with its biological oxidant, $P700^+$ (Sykes, 1985), (ii) the hydrophobic patch in azurin is required for electron transfer from cytochrome c_{551}, as revealed by site-directed mutagenesis (van de Kamp et al., 1991), and (iii) the hydrophobic patch in amicyanin is used in the interaction with methylamine dehydrogenase (its electron donor), as shown by the crystal structure of the protein–protein complex (L. Chen, F. S. Mathews, and V. L. Davidson, personal communication).

The strong conservation of Cys ligand conformation may also be of functional significance in electron transfer. In a number of cupredoxins, one or both the residues adjacent to the Cys ligand have a side chain which interacts with an electron donor or acceptor at a second electron-transfer site (Figure 5). In plastocyanin, the conserved residue Tyr 83 adjacent to the Cu-binding residue Cys 84 has long been implicated in electron transfer between the blue copper site and both biological and inorganic redox partners [e.g., Colman et al. (1978) and Roberts et al. (1991)]. In nitrite reductase, the residue preceding the blue copper ligand, Cys 136, is His 135 which is a ligand of the type 2 Cu at the presumed catalytic site (Godden et al., 1991). In ascorbate oxidase, the Cu-binding residue Cys-508 is immediately preceded and followed by residues His-507 and His-509, each of which is a ligand of a Cu atom in the catalytically active trinuclear Cu cluster (Messerschmidt et al., 1989). The existence of similar Cu–Cys–His–Cu pathways in laccase and ceruloplasmin can be inferred from sequence homology between these proteins and ascorbate oxidase (Messerschmidt & Huber, 1990).

The strong conservation of the (n–1)–Cys(n)–Cu structural motif (Figure 5) makes it likely that electron transfer occurs through the bonds of the Cu-ligated Cys and its neighboring residue(s). The fact that the $Cu–S_\gamma–C_\beta–C_\alpha–N$ bonds are approximately coplanar in all crystallographically characterized cupredoxins (Table I) suggests that coplanarity is an important feature of through-bond electron-transfer pathways. Current discussions of electron-transfer pathways have focused on distance and bonding effects (Beratan et al., 1991). In light of the present work, it appears that the dihedral angles in a

through-bond electron-transfer pathway may also be important variables.

Conclusions

(1) The crystal structures of six cupredoxins (three plastocyanins, pseudoazurin, cucumber basic protein, and azurin) show a coplanar array of five atoms in the copper cysteinate moiety that is a highly conserved feature of blue copper sites.

(2) The coplanarity of the cupric cysteinate chromophore ($Cu-S_\gamma-C_\beta-C_\alpha$ and $S_\gamma-C_\beta-C_\alpha-N$ dihedral angles close to 180°) leads to a coupling of ν(Cu–S) with δ(Cys) modes. This coupling accounts for the presence of nine or more vibrational modes in the resonance Raman spectrum.

(3) The same set of vibrational modes in the 330–460-cm^{-1} region has been observed in the RR spectra of all cupredoxin-type proteins thus far examined. This includes ceruloplasmin, laccase, stellacyanin, rusticyanin, auracyanin, and amicyanin for which X-ray structures are not yet available. A coplanar copper–cysteinate configuration is likely for each of these proteins.

(4) The intensities of the different RR modes vary from protein to protein. However, the spectral patterns fall into four distinct categories with maximal RR intensities at ~360, 385, 410, or 430 cm^{-1}. These categories correlate with the presence of ≥8, 4, 4, and 2 amino acids, respectively, between the Cys and His ligands.

(5) The RR spectra of cupredoxins arise mainly from the Cu–cysteinate chromophore, and the variable Raman intensities are a reflection of a change in the environment of the cysteinate ligand. Thus, RR intensities are sensitive to variations in the size and conformation of the Cys/His loop or to changes more remote from the Cu site (as in the case of Pc I and Pc II). In contrast, RR intensities show little sensitivity to metal replacement [e.g., Ni(II) for Cu(II)] or changes in other ligands (e.g., exogenous imidazole in alcohol dehydrogenase) which apparently do not alter the cysteinate portion of the chromophore.

(6) The conserved conformations of the cysteinate side chain, backbone, and flanking amino acids may be important in optimizing electron-transfer pathways which utilize the Cu–S(Cys) framework.

Added in Proof

Recent experiments provide the first definitive evidence for an imidazole ligand contribution to the RR spectrum. The histidine-ligand mutant (H117G) of *P. aeruginosa* azurin can be reconstituted into a type 1 copper site by the addition of exogenous imidazole [den Blaauwen, T., van de Kamp, M., & Canters, G. W. (1991) *J. Am. Chem. Soc. 113*, 5050]. Reconstitution with [^{15}N]imidazole leads to downshifts of 0.5–1.0 cm^{-1} in the RR modes at 266, 373, 401, 409, and 428 cm^{-1}. Replacement of the imidazole by other ligands such as water or chloride causes these same vibrational modes to downshift by ~3–6 cm^{-1} (T. den Blaauwen, G. W. Canters, J. Han, T. M. Loehr, and J. Sanders-Loehr, unpublished results). However, the basic RR spectral pattern is unchanged, supporting our assignment of cysteine ligand vibrational modes as the dominant components of the RR spectrum.

Registry No. Cu, 7440-50-8; Cys, 52-90-4; NiR, 9080-03-9; His, 71-00-1; Met, 63-68-3.

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