Proteins as Solvents: Blue Copper Proteins as a Molecular Ruler for Solvent Effects on Resonance Raman Intensities

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Resonance Raman spectra, excited within the S→Cu charge-transfer transition, are presented for poplar *a*, spinach, zucchini, and cucumber plastocyanin, type I "blue" copper proteins (cupredoxins) found in the photosynthetic electron transport apparatus of plants. The degree of similarity of the resulting spectra increases as the amino acid homology of the proteins increases. For example, the resonance Raman spectrum of spinach plastocyanin exhibits frequencies and relative intensities significantly different from those of poplar *a* plastocyanin, with which it is 78% homologous, while the resonance Raman spectra of zucchini and cucumber plastocyanin, which are 91% homologous, are identical. The spectra exhibit good signal/noise ratios, are reproducible from different protein preparations, and are unaffected by solvent ionic strength, indicating that the observed spectral differences reflect actual differences in ground-state or excited-state (or both) structures as a result of protein composition. The relative intensities appear to be more sensitive to amino acid composition than the frequencies are, although frequency shifts are observed in the spectra that also exhibit the largest changes in relative intensity. These spectral differences are correlated with molecular structure by performing molecular modeling of the structure and electrostatic environment of the copper site in the four plastocyanins based on the experimentally determined crystal structure of poplar *a* plastocyanin. These correlations indicate that the resonance Raman intensities are sensitive to structural or electrostatic differences (or both) in the protein environment as far as 12 Å from the copper site. This long-range sensitivity dies off with amino acid composition changes at distances >15 Å. Possible mechanisms for this long-range sensitivity are discussed.

Introduction

Spectroscopic frequencies are typically sensitive to very short-range molecular characteristics and interactions. The frequencies of condensed-phase electronic and vibrational transitions are usually interpreted in terms of the solute molecular structure (nuclear and electronic) and direct solvent−solute interactions, such as hydrogen bonding. However, the *intensities* of spectroscopic transitions can be much more sensitive to long-range structure and interactions. Examples include exciton coupling seen in the absorption spectra of proteins,[1,2] and hypochromism observed in the absorption spectrum of DNA,[3] both as a result of long-range ordering in these systems.

Raman and resonance Raman intensities are also known to be sensitive to environmental influences. Raman and resonance Raman spectra of proteins[4,5] and nucleic acids[6,7] show hypochromism as a result of the ordering of amino acids and nucleic acid bases in an alpha or double helix, respectively. Raman hypochromism arises directly from hypochromism in the absorption spectrum, since the Raman cross-section depends on the oscillator strength of the absorption band from which it derives its intensity. The Raman noncoincidence effect, in which the isotropic and anisotropic Raman bands do not have the same frequency, is thought to arise from resonant transfer of vibrational excitation via transition dipole−transition dipole interactions.[8−10] Any short-range order in the system would significantly increase the magnitude of such an interaction, although it may or may not be necessary.[9] Finally, changes in relative resonance Raman intensities can also arise from strong interactions with solvent, which may lower the symmetry of the system, allowing some strictly forbidden Raman bands to become partially allowed and acquire intensity.[11] However, no studies have been performed to determine the distance sensitivity of Raman intensities to such environmental influences. In this paper, the combination of a structurally well-characterized system with a structurally specific probe, namely, resonance Raman spectroscopy of plastocyanins, demonstrates that the resonance Raman intensities are sensitive to amino acid changes in this protein over a 12 Å distance.

Blue copper proteins provide an ideal model system for examining the effect of environment on excited-state properties. The crystal structures of many blue copper proteins have been determined.[12−16] Plastocyanins, 10-kDa type I "blue" copper proteins (cupredoxins) found in the photosynthetic electron-transport chain in plants,[17] are readily available, exhibit high homology among different species (78%), and have a well-characterized structure. In the oxidized form, they contain a Cu^{2+} ion ligated to His37, Cys84, His87, and Met92 in a distorted tetrahedral geometry (Figure 1).[12,13,17] The blue color of plastocyanin arises from a S(Cys-p)→Cu charge-transfer transition localized at the copper site. In plastocyanin, the copper ion is buried among hydrophobic amino acid residues in one end of the protein and does not show any solvent accessibility.[13,18] Because of the lack of solvent accessibility in plastocyanin, the protein plays the role of the solvent. Previously,[19] we determined that the resonance Raman-derived excited-state charge-transfer dynamics in plastocyanin are sensitive to changes in amino acid composition. These results were interpreted in terms of a model in which amino acid

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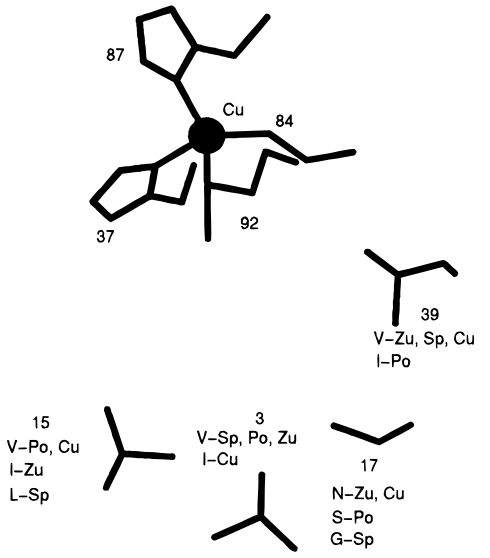

Figure 1. Partial structure of poplar *a* plastocyanin near the copper site, after Guss et al.[12,13] One-letter codes are shown for the amino acid residues that differ between poplar *a* (Po), spinach (Sp), zucchini (Zu) and cucumber (Cu) plastocyanins (see Table 1 footnote for amino acid abbreviations). Note that amino acid residue 17 is the closest amino acid difference between zucchini and spinach plastocyanin.

differences 8 Å away from the copper site change the mixing of internal coordinates, resulting in different normal-mode descriptions and intensities of the resonance-enhanced vibrations in plastocyanin.

In this paper we further explore the relationship between protein composition and the resonance Raman spectrum. Resonance Raman spectra, excited within the S→Cu charge-transfer transition, are presented for poplar *a*, spinach, zucchini, and cucumber plastocyanin. The degree of spectral similarity, both in frequency and intensity, is directly correlated with the amino acid homology. Comparison of the resonance Raman spectra of zucchini and cucumber plastocyanin lends further support to the idea that the normal modes of the resonance-enhanced vibrations are delocalized over at least 8 Å from the copper site along the polypeptide strands directly connected to the coordinating copper ligands. Surprisingly, the resonance Raman intensities in plastocyanin also appear to be sensitive to the dipolar properties of amino acids up to 12 Å distant from the copper site, which are not directly coupled through a covalent linkage. Possible physical origins of this long-range sensitivity are discussed.

Experimental Section

Protein Purification. Poplar leaves were obtained from local trees. Spinach leaves were obtained from local supermarkets. Zucchini and cucumber leaves were obtained from plants grown at a local farm. Plastocyanin was isolated from poplar, spinach, cucumber, and zucchini leaves according to literature procedures, with slight modifications.[20,21] The isolation and purification of poplar *a*, zucchini, and cucumber plastocyanin were identical to that of parsley, described earlier.[17] The isolation of spinach plastocyanin was also described previously.[19] Typical yields were 29−40 mg for poplar, 3−10 mg for spinach, 9−28 mg for zucchini, and 6−17 mg for cucumber plastocyanin per kilogram of leaves. The purity of plastocyanin, determined by the A_{278}/A_{597} ratio, was 1.1 for poplar *a*, 1.4 for spinach, 1.2 for zucchini, and 1.3 for cucumber.

Resonance Raman Spectroscopy. Plastocyanin samples for the resonance Raman experiments were prepared by quantitative dilution of plastocyanin (50 mM Tris−HCl, pH 7.6) with a cacodylate/Tris buffer solution (0.5 M cacodylic acid, 50 mM Tris−HCl, pH 7.6). The cacodylate/Tris buffer solution was made fresh every week because of slight decomposition on this time scale. The addition of cacodylate provided an internal intensity standard and did not have a noticeable effect on the absorption or resonance Raman spectra of plastocyanin. Room-temperature resonance Raman spectra of plastocyanin were obtained with 300-μL sample solutions having an absorbance of 5−7 *A*/cm at 597 nm ($\epsilon = 4500\ M^{-1}\ cm^{-1}$).[22] Resonance Raman scattering was excited by spherically focusing the laser onto a spinning 5-mm (o.d.) NMR tube containing the sample solution in a 135° backscattering geometry. Laser excitation was obtained with Kr^+, Ar^+, and dye lasers (Coherent, Santa Clara, CA). The wavelengths used were 514.5, 530.9, 568.2, 594, 612, and 647.1 nm. The laser power was 60−100 mW. Multichannel detection of the resonance Raman scattering was obtained with a liquid nitrogen-cooled charge-coupled device (CCD) detector (Princeton Instruments, Trenton, NJ) connected to the first half of a double monochromator (Spex Industries, Metuchen, NJ). Spectral slit widths were 5−7 cm^{-1}. Frequency calibration was done by measuring Raman scattering of solvents of known frequencies (benzene, chloroform, and carbon tetrachloride). Reported frequencies are accurate to ±2 cm^{-1}.

Molecular Modeling. All structural and electrostatic modeling calculations were performed on an Indigo^2 workstation (Silicon Graphics, Inc., Palo Alto, CA) by using the InsightII molecular modeling environment software containing the Biopolymer, DelPhi, and Discover modules (Molecular Simulations, Inc., Palo Alto, CA) as described previously.[19] Briefly, the atomic coordinates for poplar *a* plastocyanin[12] were obtained from the Brookhaven Protein Data Bank.[23] We used the following procedure to generate the structure of spinach, zucchini, and cucumber plastocyanin. Because of the high degree of homology between the proteins, the structure of poplar *a* plastocyanin was used as the starting point.[17] The amino acids of poplar *a* plastocyanin were "mutated" to those of spinach, zucchini, or cucumber plastocyanin by using the Biopolymer module of InsightII. The new amino acids were constrained to lie along the coordinates of the old amino acids as much as was feasible. The poplar *a* plastocyanin structure from the X-ray coordinates and the resulting spinach, zucchini, or cucumber plastocyanin structures were then minimized by using the AMBER[24,25] force field with the Discover software as described previously.[19] This approach permits a comparison between the two structures while minimizing the systematic errors introduced by the choice of energy-minimization force field and parameters.

Calculation of the electrostatic potential in the protein was performed by using the finite difference solutions to the linear Poisson−Boltzmann equation[26] as implemented in the DelPhi[27,28] module of InsightII and as described previously for plastocyanin.[19] Briefly, the solvent and protein coordinates are mapped on a three-dimensional grid, and each grid point is assigned a value for the charge, dielectric constant, and ionic strength. Boundary conditions are set by the Coulomb equation. For these calculations, the ionic strength was assumed to be 0, a low dielectric (2) was assigned to the protein, a high dielectric (80) was assigned to the region outside the protein, and the standard DelPhi protein formal charges, in which 1−2 atoms of each amino acid residue carry the total formal charge at physiological pH for that residue, were used for plastocyanin. The calculations were set up with 65 grid points for each dimension and a minimum 15 Å border space between the edge of the protein and the edge of the box.

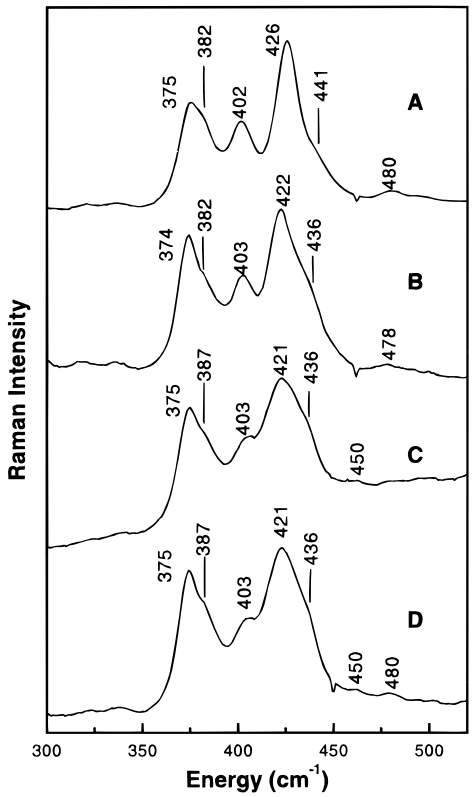

Figure 2. Resonance Raman spectra of poplar *a* (A), spinach (B), zucchini (C), and cucumber (D) plastocyanin excited with 150 mW of 568-nm light between 300 and 520 cm^{-1}. The spectra are the sum of three scans and have only been frequency-calibrated. Vibrational bands at lower (265 cm^{-1}) and higher frequency (~820 cm^{-1}) are not shown.

Results

Resonance Raman spectra of poplar *a*, spinach, zucchini, and cucumber plastocyanin excited within the S→Cu charge-transfer transition are shown in Figure 2. Note that the spectra reported are simply the sum of three scans and have been frequency-calibrated; no additional manipulations of the data have been performed. The spectra of all four plastocyanins appear similar; five intense bands, assigned to the Cu−S stretch coupled to other vibrations of the copper active site,[29−37] are observed at ~375, 382, 402, 422, and 436 cm^{-1}. Another small band is observed in some of the spectra at 450 cm^{-1}. Additional bands at 265 and ~820 cm^{-1} (not shown in Figure 2) have been attributed to the Cu−N stretches, and to overtone and combination bands of the 300−500 cm^{-1} vibrations,[29−37] respectively. Although many of the frequencies are similar among the four plastocyanins, particularly between spinach, zucchini, and cucumber plastocyanin, the relative resonance Raman intensities appear to vary with species. Figure 2 shows that spinach and poplar *a* plastocyanin exhibit very different relative intensities and slightly different frequencies, whereas the resonance Raman spectra of zucchini and cucumber plastocyanin exhibit identical frequencies and relative intensities. Close examination and comparison of the resonance Raman spectra for spinach and zucchini plastocyanin demonstrate a slight difference in the two spectra (Figure 2B and 2C). Figure 2 shows a slight intensity increase at ~412 cm^{-1} on going from spinach to zucchini plastocyanin, the result of a band gaining intensity or an increase in bandwidth of the 403 or 421 cm^{-1} (or both) bands. This intensity difference was reproducible in the resonance Raman spectra recorded on different days with different preparations of the plastocyanin and at all excitation wavelengths within the S(Cys-π)→Cu charge-transfer absorption band. These differences are surprising because spinach and zucchini plastocyanin are 87% homologous, having 86 identical amino acid residues out of 99.

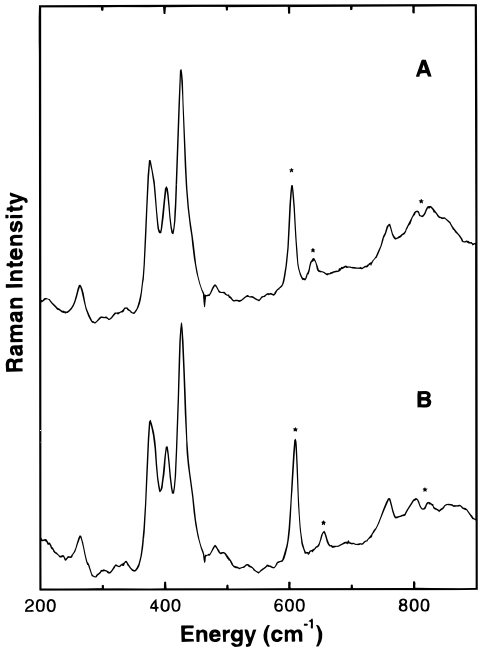

Figure 3. Resonance Raman spectra of poplar *a* plastocyanin at (A) pH 7.6 and (B) pH 5.0. The spectra are the sum of three scans and have only been frequency-calibrated. Asterisks indicate bands from the cacodylate internal standard used in the experiments.

Figure 3 shows the effect of bulk solvent pH on the poplar *a* plastocyanin resonance Raman spectrum. The resonance Raman spectrum at pH 5.0, the minimum pH at which poplar *a* plastocyanin is stable, is identical to the spectrum at pH 7.6. The resonance Raman spectrum for each plastocyanin at pH 5.0 was unchanged from that at pH 7.6. The insensitivity of the resonance Raman spectrum to changes in bulk solvent pH is somewhat surprising, given that the two copper-coordinating histidines have nominal pK_a values of 6.3. Thus, the lack of a pH effect in the resonance Raman spectrum between pH 7.6 and 5.0 nicely illustrates two key points: The copper site is inaccessible to solvent, and the resonance Raman spectrum is insensitive to changes in the pH of the external solvent. Furthermore, since the ionic strength is higher in the pH 5.0 solution, these results indicate that the resonance Raman spectrum is insensitive to changes in bulk ionic strength. *Therefore, the different relative intensities of the resonance Raman spectra in Figure 1 arise from changes in the protein itself, not from any changes in bulk solvent properties.*

Discussion

To understand the differences in the resonance Raman spectra as a function of amino acid composition, we attempted a comparison of the structural and electrostatic differences in the proteins as a result of these amino acid changes, similar to the approach in our previous paper.[19] To facilitate the discussion of these mechanisms, we consider pairs of plastocyanins.

Poplar *a*−spinach plastocyanin pair. The differences in the resonance Raman spectra of poplar *a* and spinach plastocyanin have been discussed in detail previously[19] and are attributed to changes in the normal-mode description as a result of different amino acid compositions. Specifically, the substitution of a valine in spinach for an isoleucine in poplar *a* at position 39, just 2 amino acids from the copper-coordinating

TABLE 1: Amino Acid Differences in Three Plastocyanins

poplar *a*−spinach		spinach−zucchini		zucchini−cucumber	
residue[a]	distance, Å[b]	residue	distance, Å	residue	distance, Å
1 I V	17.0	1 V I	18.1	**3 V I**	**11.4**
2 D F	16.8	2 F E	17.0	**15 I V**	**11.6**
7 A G	**10.4**	**8 G D**	**13.6**	18 D N	16.5
8 D G	**12.5**	**15 L I**	**11.7**	20 S T	22.1
15 V L	**11.7**	**17 G N**	**12.2**	23 A S	25.9
17 S G	**12.7**	23 S A	26.6	28 V T	15.5
18 E D	15.2	26 E K	20.9	52 A S	17.0
21 I V	19.9	53 A G	17.9	71 K E	16.7
22 S A	25.5	**58 S N**	**13.7**	73 N Q	21.1
23 P S	26.9	**69 T V**	**13.5**		
26 K E	19.8	73 T N	20.6		
39 I V	**8.6**	79 T S	21.2		
45 S E	19.1	81 K S	18.1		
53 S A	19.1				
66 K P	**11.8**				
70 F Y	**11.5**				
71 E K	16.7				
73 A T	20.6				
75 S T	25.2				
76 N E	24.5				
79 E T	20.0				
81 S K	15.0				

[a] The sequence number is followed by the 1-letter code of the amino acid in the first plastocyanin of the pair and the 1-letter code of the amino acid in the second plastocyanin of the pair. Values in bold are for residues within 15 Å of the copper site. A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine. [b] This is the closest distance between an amino acid side-chain atom and the copper ion. Values in bold are for residues within 15 Å of the copper site.

histidine 37, appears to distort the polypeptide backbone. This distortion does not appear to affect the ground-state structure of the copper ion and the immediately coordinating ligands, but does change the normal-mode description via a differential composition-dependent mixing of internal coordinates.[19] The different normal-mode descriptions are consistent with the slight frequency changes and much larger changes of relative intensity observed in the resonance Raman spectrum. Throughout the rest of this paper, this composition-dependent rotation of the normal modes will be referred to as a through-bond mechanism, because a covalent pathway between the chromophore and the amino acid substitution is necessary for this mechanism to be operative.

Zucchini−cucumber plastocyanin pair. The comparison of zucchini and cucumber plastocyanins provide an ideal system for testing the model presented above. For these two plastocyanins, the resonance Raman spectra are identical. The amino acid differences closest to the copper site in these two plastocyanins occur on β-strands that are not directly covalently linked to the copper site. In zucchini and cucumber plastocyanin, the only amino acid differences within 15 Å of the copper site are at positions 3 (Val-zucchini, Ile-cucumber) and 15 (Ile-zucchini, Val-cucumber); more dramatic changes in composition occur at distances further from the copper site (Table 1).[17] Both of these proximal substitutions occur at a through-space distance ~10 Å from the copper site, slightly longer than the through-space distance between the copper site and the closest amino acid differences in the poplar *a*−spinach plastocyanin pair.[19] The latter substitution is the identical amino acid change in going from poplar *a* to spinach plastocyanin but occurs at a different position in the sequence. Although the same specific amino acid residues are involved in this comparison of the two pairs of plastocyanins, these residues in the zucchini−cucumber pair now occur on β-strands that are not directly covalently linked to the copper site, and no differences are observed in the resonance Raman spectrum (Figure 2). This evidence supports our model for the resonance Raman spectral differences in the spinach−poplar *a* plastocyanin pair, i.e., that the differences in the resonance Raman spectrum are due to composition-dependent normal modes. Because the through-bond distance between the copper site and the nearest amino acid difference is >80 Å in the zucchini−cucumber pair, mixing of internal coordinates is not a physically reasonable mechanism for the changes in the resonance Raman relative intensities. *These results suggest that the through-bond mechanism for an amino acid composition-dependent rotation of the normal modes operates on through-bond distances of up to 8 Å. Furthermore, since no differences are observed in the resonance Raman spectra of zucchini and cucumber plastocyanin, effects from substantially different changes in amino acid composition at distances >15 Å from the copper site can probably be ignored.*

Spinach−zucchini plastocyanin pair. Comparison of the spinach and zucchini resonance Raman spectra shows a very slight difference, namely, a slight increase in intensity at ~412 cm^{-1} on going from spinach to zucchini plastocyanin. Similar small changes in intensity as a result of solvent have been observed in high-resolution IR spectra[38] of clusters and in resonance Raman spectra[11] of I_3^-. Results presented here indicate that proximal amino acid substitutions may be the cause of the differences observed in the resonance Raman spectra of these two plastocyanins. Figure 1 and Table 1 indicate two differences in amino acid composition within 15 Å of the copper site, a change of a leucine to an isoleucine at position 15, and a change of a glycine to an asparagine at position 17, on going from spinach to zucchini. The first substitution is simply a change in branching of the amino acid side-chain and occurs at a through-space distance of 10 Å on a β-strand that is not directly covalently linked into the copper site. Following the arguments presented above for the zucchini−cucumber comparison, this type of substitution is not expected to have any effect on the resonance Raman spectrum. The second substitution occurs at a slightly larger through-space distance than the first substitution, 12 Å, and is also not directly covalently linked into the copper site. This substitution is a much larger change in the amino acid side-chain, a hydrogen side-chain in spinach is replaced with an acetamide side-chain in zucchini. The asparagine is expected to have very different electrostatic, hydrogen bonding, polarity, and structural properties from a glycine.

We considered several physical mechanisms in an effort to understand which of the properties of asparagine is responsible for the changes in the resonance Raman spectrum. Resonance Raman intensities are sensitive to the change in equilibrium geometry on going from the electronic ground-state to the excited-state, and are therefore sensitive to changes in both the ground-state and excited-state normal-mode descriptions and potential energy surfaces. Thus, factors that may affect one or both of these potential energy surfaces must be considered.

The ground-state structural consequences of these amino acid differences in spinach and zucchini plastocyanin can be examined by using molecular modeling techniques.[19] A comparison of the minimized geometries of spinach and zucchini plastocyanin is shown in Table 2 and demonstrates that the root-mean-square difference (RMSD) in the structures of the two plastocyanins near the copper site are very small, 0.17 Å. This difference is well within the 1.33 Å resolution of the poplar *a* plastocyanin structure.[12,13] Even at distances 15 Å from the

TABLE 2: Geometry Comparison of Three Plastocyanins[a]

parameter[b]	spinach	zucchini−cucumber
rmsd1, Å	0.1666 (70)	0.05635 (70)
rmsd2, Å	0.3999 (538)	0.1490 (538)
rmsd3, Å	0.4147 (566)	0.2406 (594)

[a] The values in the table are the minimum root-mean-square difference between the minimized geometry of the two plastocyanins in each pair; the number in parentheses represents the number of atoms used in the comparison. [b] *rmsd1*, minimum root-mean-square difference over the Cu ion and residues 37, 84, 87, and 92; *rmsd2*, minimum root-mean-square difference over the Cu ion and residues 11−14, 36−39, and 83−93; *rmsd3*, minimum root-mean-square difference over the Cu ion and residues 11−14, 16, 17 (zucchini−cucumber comparison only), 36−39, and 83−93.

copper site (row 3 in Table 2), the RMSD is very small compared with the crystallographic resolution and suggests that these amino acid substitutions have no significant effect on the structure. This conclusion is reinforced by the resonance Raman spectra themselves. Past studies[31−35] have suggested that the resonance Raman spectra of blue copper proteins are exquisitely sensitive to the coordination geometry. The identical frequencies and similar intensities observed here suggest that the perturbation is small. All of these results argue that the normal-mode descriptions in zucchini and spinach plastocyanin are the same and that a mechanism other than mixing of internal coordinates may be influencing the resonance Raman spectra of plastocyanins.

Similar results were obtained with a simple electrostatic calculation.[19] The calculated electrostatic potentials, in units of kT/e, for the copper ion and nonhydrogen atoms of the coordinating amino acid residues 37, 84, 87, and 92 are 2175, 2102, and 2115 for spinach, zucchini, and cucumber plastocyanin, respectively. These calculated electrostatic potentials are similar within a reasonable error for this calculation and indicate that the electrostatic environment of the copper site is very similar in the three plastocyanins. This result is expected from the almost identical absorption spectra of plastocyanins from all plant species.[19] Taken together, the results of the geometry minimization and electrostatic potential calculation suggest that the ground-state structure is very similar in the two plastocyanins.

The resonance Raman intensities are also sensitive to changes in the excited-state structure or dynamics, and reflect the slope of the excited-state potential energy surface at the Franck−Condon geometry along each normal mode.[39−41] A plausible explanation for the relative intensity differences observed here is that the excited-state potential energy surfaces are slightly different in the two plastocyanins as a result of the different amino acid composition. The resonant electronic transition here is to a nominal charge-transfer state, which may be more sensitive to the environment than is the ground-state. However, the similarity of the absorption spectra[19] of all plastocyanins studied so far indicates that the protein environments are very similar, or that the absorption spectrum is insensitive to the protein environmental perturbations as a result of different amino acid composition. We believe the latter is true; spinach and poplar *a* plastocyanins have identical absorption spectra but very different resonance Raman spectra.[19] Furthermore, the absorption band is broad and structureless: Small changes in the Franck−Condon factors of individual modes may be hidden in the vibronic progressions of other modes and in the broad homogeneous component to the absorption spectrum,[19] producing a very similar absorption spectrum.

The question remains as to how the excited-state potential energy surface is perturbed by the protein environment. The most obvious mechanism is a direct change of the equilibrium excited-state geometry or slope, or both, via a strong solvent−chromophore interaction, such as a permanent dipole−permanent dipole or a permanent dipole−transition dipole interaction, resulting in a lowering of the symmetry at the copper site. Solvent symmetry lowering has been observed in many systems[11,42,43] and characteristically "turns on" intensity in non-totally symmetric Raman transitions, breaking the symmetry restrictions on excited-state distortions of the chromophore. In resonance Raman spectroscopy, the solvent dynamics may introduce broadening of the vibrational band in addition to increasing the intensity. Perhaps the additional permanent dipole from the asparagine at position 17 in zucchini plastocyanin is interacting with the copper site excited-state permanent dipole or the transition dipole strongly enough to perturb the vibrational spectrum of the copper site, either by turning on the resonance Raman intensity of a strictly forbidden vibrational band through symmetry lowering or by broadening the vibrational bands.

To determine whether this is a reasonable mechanism, we roughly estimated the magnitude of the Asn17 permanent dipole−copper site excited-state permanent dipole interaction by using

$$V = \frac{\mu_N \mu_{IV}}{\epsilon R^3}[\cos\theta_{N,IV} - 3\cos\theta_{N,R}\cos\theta_{IV,R}] \qquad (1)$$

where V is the interaction energy, μ_N is the permanent dipole on asparagine 17, μ_{IV} is the permanent dipole of the charge-transfer excited-state (assumed to lie along the Cu−S bond, pointing in the direction of Cu), ϵ is the dielectric constant, R is the distance between the dipole centers, and $\theta_{N,IV}$, $\theta_{N,R}$, and $\theta_{IV,R}$ are the angles between the dipoles, between μ_N and the vector connecting the dipoles, and between μ_{IV} and the vector connecting the dipoles, respectively. For this calculation, electrostatic charges of 0.675, −0.47, −0.867, 0.344, and 0.344 electrons localized on the C, O, N, H_1, and H_2 atoms of asparagine 17, respectively, and the minimized geometry were used to give $\mu_N = 3.1$ D, pointing roughly between the C═O and C−N bonds of the asparagine side-chain. The minimized geometry of zucchini plastocyanin gives R = 13 Å, $\theta_{N,IV} = 112.9°$, $\theta_{N,R} = 41°$, and $\theta_{IV,R} = 80°$. Although the excited-state permanent dipole in plastocyanin, μ_{IV}, has not been measured, a rough estimate can be obtained by assuming nominal charges of 2^+ and 1^- on the copper and cysteine sulfur, respectively, in the ground-state and a transfer of 0.25 electrons[44,45] upon going to the charge-transfer excited-state. This rough calculation yields an excited-state dipole of 13 D lying along the Cu−S bond. Assuming a dielectric constant of 2, the calculated interaction energy is −430 J/mol. This value is small and is probably an underestimate of the interaction energy magnitude; eq 1 assumes a point dipole, an assumption that may not be true in this case. At small distances, it has been shown[46] that eq 1 underestimates the magnitude of the interaction energy. Since the interaction is both distance- and direction-dependent, different vibrational modes may be affected differently. Consequently, different relative intensities may be observed. Similarly, for the permanent dipole−transition dipole interaction energy, μ_N, ϵ, $\theta_{N,IV}$, $\theta_{N,R}$, and $\theta_{IV,R}$ are the same as above and the transition dipole of plastocyanin, μ_{IV}, is 2.8 D.[19] The calculated interaction energy is −90 J/mol. As above, this value is small and probably underestimates the magnitude of the interaction energy. Since the interaction energy is greater between the permanent dipoles, this interaction is expected to dominate. Also, this rough calculation predicts a greater permanent dipole in the ground electronic state and, therefore,

a greater interaction energy. The ground-state permanent dipole−asparagine permanent dipole interaction may lead to broadening of specific Raman peaks through an inhomogeneous effect, such as that observed for the OH stretches in water. An independent measurement of the permanent dipoles in the ground- and excited-states is needed to provide a better quantitative model for dipolar effects on the resonance Raman spectrum of plastocyanin.

Other types of interactions were considered as a possible mechanism for the spectroscopic differences observed between zucchini and spinach plastocyanin. Anharmonic mixing of the eigenfunctions of a Raman-allowed fundamental and Raman-forbidden overtone or combination band of the same symmetry species can lead to intensity in the overtone or combination band via Fermi resonance.[47,48] Typical experimental tests for the presence of Fermi resonance include varying the solvent or isotopic composition of the molecule shifting the frequency of the Raman fundamental involved in the Fermi resonance perturbs the interaction and changes the relative intensities. This has been observed in several systems, including thymine[49] and ethyl 4-(dimethylamino)benzoate.[50] Certainly, several vibrational modes from the protein or chromophore may have an overtone at the same frequency as the resonance-enhanced Raman modes near 400 cm^{-1}. However, it is unclear how a forbidden band can pick up intensity in zucchini plastocyanin when there is no change in the fundamental frequencies between spinach and zucchini plastocyanin. This lack of frequency shift indicates that Fermi resonance is probably not responsible for the different relative intensities in the resonance Raman spectrum.

Vibrational hypochromism, as has been observed in both Raman and resonance Raman scattering of nucleic acids,[4−7] is another possible mechanism for the different relative resonance Raman intensities observed here. Vibrational hypochromism is thought to arise directly from hypochromism in the absorption spectrum; a lower absorptivity results from interaction of induced dipoles in the medium with an electronic-transition dipole and decreases the intensity of Raman and resonance Raman scattering for the modes coupled to that electronic transition. While this mechanism may be operative in the blue copper proteins, we have no indication that the absorption spectrum is undergoing any alteration. The reported purity ratios (A_{278}/A_{597}) for zucchini and spinach plastocyanin are those that would be predicted if the absorptivities in these species were the same as for other species of plastocyanin which have been measured. Thus, Raman hypochromism does not appear to explain the observed intensity changes.

The accumulated evidence and arguments presented here suggest that the relative resonance Raman intensity differences between spinach and zucchini plastocyanin are due to direct differences in excited-state structure or dynamics, or both. The change in the excited-state potential energy surface appears to arise from a small permanent dipole−permanent dipole interaction over a distance of 12 Å in the blue copper protein. These results suggest that the resonance Raman intensities observed in these blue copper proteins are sensitive to environmental properties over a 12 Å distance. The generalization of these results to any condensed-phase environment is intuitive but not straightforward. Electrostatic effects and intermolecular interactions are certain to produce spectroscopic effects. However, implementing a quantitative equation to model these effects is difficult in condensed phases because protein environments are anisotropic and heterogeneous, while condensed phases are generally homogeneous in nature. Obviously, more work needs to be performed to determine the relevance of these results to specific intermolecular interactions and their effect on spectroscopic observables in condensed phases.

Conclusions

The results presented here demonstrate some of the structural and electrostatic factors affecting the resonance Raman spectrum in blue copper proteins and, by extension, resonance Raman spectra of molecules in condensed phase. Specifically, changes in the amino acid composition can have dramatic effects on the resonance Raman spectrum through a composition-dependent rotation of the normal modes. This "through-bond" mechanism appears to be operative up to distances of at least 8 Å in this protein environment. In addition, a "through-space" mechanism appears to affect the resonance Raman intensities, probably as a result of a permanent dipole−permanent dipole interaction, up to distances of 12−13 Å from the copper site. Finally, differences in the protein amino acid composition in plastocyanin at distances >15 Å have no effect on the resonance Raman spectrum. The use of proteins as structured solvents provides a unique opportunity to systematically study and perhaps control the factors that affect molecular spectroscopy and excited-states in condensed phase.

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Supporting Information Available: Two figures showing the resonance Raman spectra of zucchini and cucumber plastocyanin excited at 568, 612, and 647 nm (3 pages). See any current masthead page for ordering information and Web access instructions.

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