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LETTERS

A Structural Basis for Long-Range Coupling in Azurins from Resonance Raman Spectroscopy

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Resonance Raman spectra have been measured within the S → Cu charge-transfer band for azurins from *Alcaligenes denitrificans*, *Pseudomonas aeruginosa*, and *Alcaligenes xylosoxidans*. The spectra have similar frequencies but show significantly different intensity patterns. Comparison of the differences in structure and environment from the known X-ray crystal structures with resonance Raman spectral changes demonstrates a rough correlation with the overall protein structure. However, a much better correlation exists between the observed resonance Raman spectral changes and specific amino acid differences. Comparison of the resonance Raman spectra and amino acids within 12 Å of the copper site suggests that the protein−copper site coupling extends to at least 10 Å from the copper. This experimentally observable, long-range coupling with specific amino acids may suggest a new mechanism for the efficient long-range electron transfer seen in these proteins.

Introduction

Previous resonance Raman studies of plastocyanins,[1−3] blue copper proteins involved in photosynthesis in plants, have indicated that the spectra are sensitive to amino acid changes up to ∼12 Å from the copper metal ion and suggest that resonance Raman spectroscopy may be a powerful probe of coupling in these electron transport proteins. The frequencies of the resonance Raman modes are expected to be sensitive to the structure of the coordination sphere of the copper metal ion chromophore, while the resonance Raman intensities are influenced primarily by the excited-state dynamics. Little to no change in frequency was observed in all of the plastocyanins studied, suggesting the copper site geometry is very similar. However, the intensities appear to be much more sensitive to long-range interactions in the blue copper proteins. Two mechanisms have been suggested by which the resonance Raman intensities are influenced by the amino acid composition within 12 Å of the copper site.[1−3] The first is a short-range (<8 Å) through-bond kinetic coupling of internal coordinates of nearby amino acids. The second is electrostatic interactions over distances of up to 13 Å from the copper site. Changes of amino acids >15 Å from the copper site did not appear to affect the resonance Raman spectra.

Although these previous studies indicated two possible long-range coupling mechanisms, they suffered from a lack of known plastocyanin crystal structures; only the X-ray crystal structure of poplar *a*,[4] and recently, spinach plastocyanin[5] are known. Instead, the interpretation of these results has rested on the crystal structure of poplar *a* plastocyanin alone, coupled with molecular modeling. Although the crystal structure of a spinach plastocyanin mutant, G8D, has recently become available[5] and supports this approach, the resolution is low. In an effort to understand the structural determinants of electron and charge transfer in protein environments from a more well-defined structural basis, we report here the resonance Raman spectra of four structurally characterized azurins, an analogous blue copper protein, from three species of bacteria, *Alcaligenes denitrificans*

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(AD), *Pseudomonas aeruginosa* (PA), and *Alcaligenes xylosoxidans* (AXI and AXII). The results presented here provide a strong structural basis for the interpretation of observed resonance Raman intensity differences and frequency shifts as a result of coupling between the active site and the surrounding protein matrix. This long-range coupling may explain the efficient electron transfer seen in these proteins.

Experimental Section

Azurin from *A. denitrificans* (AD) and *P. aeruginosa* (PA) was isolated and purified by methods described previously.[6] Azurin from *A. xylosoxidans* (AXI and AXII) was isolated and purified by literature methods with slight modifications in the cell rupture and column chromatography steps.[7−9] Column chromatography was performed using Whatman CM-52 and Sephadex G-50 columns until the purity ratio (A_{280}/A_{620}) for azurin was ≤3.8 for AD, ≤2.1 for PA, 2.0−4.9 for AXI, and 3.1−4.7 for AXII. Typical yields were 13−56 mg for AD, 46−58 mg for PA, 4−24 mg for AXI, and 4−8 mg for AXII per 100 g of cell paste.

Samples were prepared for resonance Raman experiments by quantitative dilution with a cacodylate buffer solution (0.5−1.0 M cacodylate, 0.01 M Tris-HCl, pH 8.7). Room-temperature resonance Raman spectra were taken with 300 μL aqueous solutions (0.01 M Tris-HCl, 0.38−0.45 M cacodylate, pH 8.7) having an absorbance of 4−5 OD/cm at 620 nm. The addition of cacodylate did not have a noticeable effect on the absorption or resonance Raman spectra. Resonance Raman spectra were collected using a single monochromator with a CCD detector.[3,6] Laser excitation at 568.2 nm was obtained with a Kr ion laser (Coherent, Santa Clara, CA). The laser was spherically focused onto a 5-mm (o.d.) NMR tube containing the sample in a 135° backscattering geometry and with a typical power of 100−130 mW. Only the frequencies have been calibrated in the spectra presented, by measuring solvents of known frequencies (benzene, carbon tetrachloride, and chloroform). Reported frequencies are accurate to ±2 cm^{-1}.

Results and Discussion

The azurin X-ray crystal structure from each species has been previously determined,[10−13] and the structure for PA azurin with selected amino acids in the vicinity of the copper active site is shown in Figure 1. The copper has five coordinating atoms in a distorted trigonal bipyramidal geometry. Two nitrogens from His46 and His117 and a sulfur from Cys112 form a trigonal plane around the copper and are strongly bound. A sulfur from Met121 and an oxygen from Gly45 are weakly bound axial ligands.

Figure 2 shows the resonance Raman spectra of the four species of azurin. Vibrational bands less than 300 cm^{-1} and bands near 800 cm^{-1} are not shown. The vibrational frequencies are generally similar for the four azurins. Strong modes occur between 330 and 500 cm^{-1} which have been interpreted to arise from the Cu−S(Cys) stretch mixed with other internal coordinates.[14] Weak Cu−N stretches are present at ∼212, 250, and 274 cm^{-1}, and a weak C−S stretch is present at 748 cm^{-1} (data not shown).[14] There is also an unassigned weak mode at 654 cm^{-1} (not shown). Although the spectra in Figure 2 are similar and exhibit similar frequencies, the relative intensities of the modes vary significantly between the species; these relative intensity differences were observed reproducibly with different excitation wavelengths within the S → Cu charge-transfer transition and from different preparations of each protein.

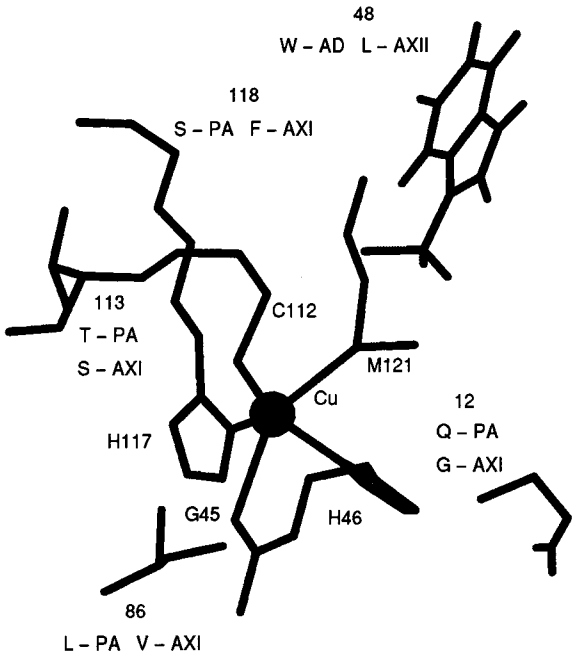

Figure 1. Partial structure of *Pseudomonas aeruginosa* azurin near the copper site.[13] Amino acid differences among the four azurins within 10 Å of the copper site are shown. One-letter codes are given for the amino acids for each of the species *Alcaligenes denitrificans* (AD[10]), *Alcaligenes xylosoxidans* (AXII[11] and AXI[12]), and *Pseudomonas aeruginosa* (PA[13]). Structures are taken from the Brookhaven Data Bank.[15]

To interpret the observed resonance Raman spectral intensity differences from a sound structural basis, a number of correlations of the known protein structures[10−13] and sequences with the resonance Raman spectra were attempted. Each pair of spectra can be quantitatively compared by calculating the χ^2 values between the spectra. The χ^2 value between the two resonance Raman spectra is calculated from $\chi^2 = (1/f)\sum(I_1 - I_2)^2$ where f is the number of degrees of freedom, I_1 and I_2 are resonance Raman spectral intensities for two species being compared, and the sum is carried out over the frequencies from 300 to 500 cm^{-1}. These χ^2 values reflect both the intensity differences and the slight frequency shifts observed in Figure 2. As expected from Figure 2, the spectra for AD and AXII are the most similar and exhibit the smallest χ^2 ($\chi^2 = 3.3 \times 10^{-2}$). Also, the spectrum of PA is most dissimilar with AD and AXII (χ^2 of 12.2×10^{-2} and 19.8×10^{-2}, respectively), and is closest to AXI ($\chi^2 = 5.4 \times 10^{-2}$).

The resonance Raman spectra are expected to be influenced by the structure of the coordination sphere and the protein environment around the copper metal ion. These two factors can be separated and examined independently to a certain extent. The X-ray structures of these four azurins show nearly identical secondary and tertiary structures, and the protein backbones can be very closely superimposed. The root-mean-square (RMS) deviation between different pairs of azurins provides a convenient, quantitative means of comparing the azurin structures. The RMS deviation is calculated from the x, y, and z coordinates over N atoms using $\text{RMS} = (\sum[(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2]/N)^{1/2}$ and the average X-ray crystal structures of each protein.[10−13] Two RMS deviations were calculated: one using only the copper atom and the five coordinating atoms (N, N, S, S, O), and the second using all of the backbone and copper atoms (513 atoms per structure). The small RMS deviations using the entire protein backbone (RMS = 0.42−0.75 Å) indicate that the global structures of the four azurins are very

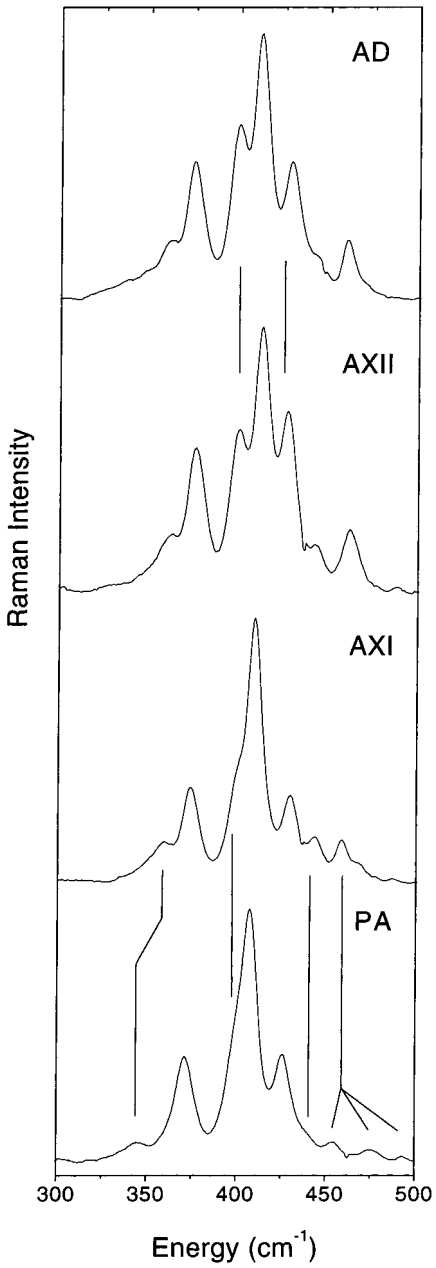

Figure 2. Resonance Raman spectra of azurins using an excitation wavelength of 568.2 nm. The spectra are labeled as in Figure 1. Vertical lines indicate species-dependent spectral changes. The spectra have been normalized to the highest intensity peak and have been displaced along the *y*-axis for clarity.

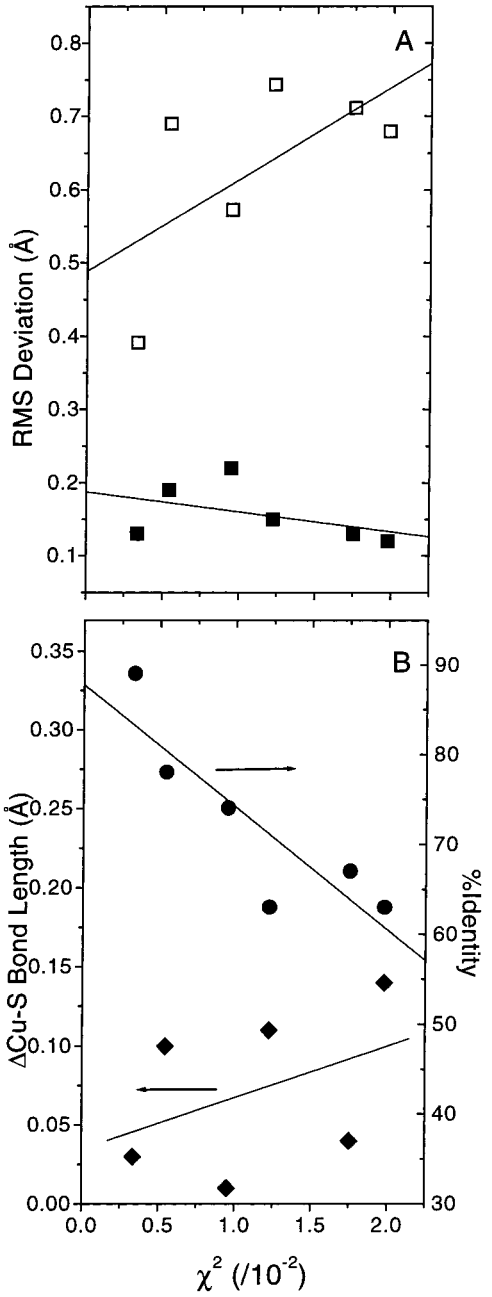

Figure 3. Correlations of the azurin structures and sequences with resonance Raman spectral differences. Graph A shows the correlation of the spectral differences with the RMS deviation of the core atoms (R = ■) and the protein backbone (R = □). Graph B shows the correlation of the spectral differences with changes in Cu−S bond length (R = ◆), and % identity (R = ●). Note that the Raman spectral difference parameter χ^2 is expected to increase as the structural difference parameters, RMS deviation and ΔCu−S bond length, increase. However, χ^2 is expected to *decrease* as the % identity increases, as the % identity is a measure of environmental similarity.

similar to one another. The correlation coefficient of the resonance Raman difference parameter χ^2 vs RMS deviation is 0.63 (Figure 3A), indicating a very rough relationship between overall protein backbone structure and the resonance Raman spectrum.

A similar examination of the copper center also shows few structural differences. The RMS deviation values are small (RMS = 0.12−0.22 Å), indicating that the azurin structures are also very similar at the copper center. Correlation of the resonance Raman spectral difference parameter χ^2 with the RMS deviation of the copper site is poor (Figure 3A), yielding a correlation coefficient of −0.45. Counterintuitively, this suggests that the spectra tend to be more *similar* as the structural *differences* increase. A more detailed structural comparison of the copper center is given in Table 1. Because the most intense peaks near 400 cm^{-1} have been assigned to modes involving the Cu−S(Cys) bond,[14] it may be expected that the resonance Raman spectra would correlate with the Cu−S bond length. However, this is not what is observed, as the correlation coefficient of the resonance Raman spectral difference parameter χ^2 vs $\Delta Cu-S = |d_{Cu-S,species1} - d_{Cu-S,species2}|$, where *d*, the bond length, is 0.41 (Figure 3B). To determine if other copper site structural factors influence the resonance Raman spectrum, we compared the spectra of AD and AXII azurin, which have only two significant structural differences at the copper site. First, the Cu−O bond in AD azurin is 3.13 Å, similar to the 2.93 Å in PA azurin, and significantly different from the 2.75 Å in

TABLE 1: Distances around Copper Atom in Azurins[a]

bond	AD	AXII	AXI	PA
Cu−N (H46)	2.08 Å	2.02 Å	1.97 Å	2.06 Å
Cu−N (H117)	2.00 Å	2.02 Å	1.92 Å	2.03 Å
Cu−S (C112)	2.15 Å	2.12 Å	2.16 Å	2.26 Å
Cu−S (M121)	3.11 Å	3.26 Å	3.18 Å	3.13 Å
Cu−O (G45)	3.13 Å	2.75 Å	2.51 Å	2.93 Å
Cu−NNS plane	0.13 Å	0.00 Å	0.13 Å	0.11 Å
ref	10	11	12	13

[a] X-ray crystal structures of each species were obtained from the Brookhaven Databank.[15] The Cu−NNS plane is the distance from the copper metal to the plane defined by the strongly ligated N, N, and S atoms.

AXII azurin (Table 1). Second, in AD azurin the copper sits 0.13 Å above the NNS plane, as in AXI and PA azurin, while it is coplanar in AXII azurin (Table 1). These structural differences in the axial ligands do not qualitatively correlate well with the resonance Raman spectra, suggesting that differences in the copper site structure of this magnitude do not significantly influence the resonance Raman intensities. All of these results argue that the azurin resonance Raman spectra of these species do not correlate well with the structure of the copper site and suggest that it is the protein environment, constructed by the amino acid side chains, which may be the determining factor in the resonance Raman spectrum and excited-state dynamics.

The protein forms the environment around the copper metal site via the amino acid side chains. The microscopic local solvent properties, such as dielectric constant, polarity, or hydrophobicity, are determined by the amino acid side chains, while charged and dipolar amino acid residues create an electrostatic potential around the copper metal ion. To understand the effect of amino acid composition on the resonance Raman spectra, the % identity was calculated for each pair of azurins. The % identity is calculated from % identity $= [1 - (\Delta AA/129)] \times 100\%$, where ΔAA is the number of amino acid changes. Only identical amino acids were used and sequence length differences were treated as amino acid changes. This method probably underestimates the role of the amino acid environment, as the homology between these proteins is much higher than the identity. The % identity correlates well with the spectral difference parameter χ^2, with a correlation coefficient of -0.88 (Figure 3B). The negative sign indicates that the spectra become less different as the amino acid content becomes more similar, as expected. This good correlation supports the hypothesis that the protein environment is the primary influence on the resonance Raman intensities in these proteins.

The ultimate goal of this work is to determine if specific resonance Raman spectral changes can be related to specific amino acid changes. In this way, it would be possible to look at specific interactions with distance to understand the long-range coupling in these electron-transfer proteins. AD and AXII azurins have the highest number of identical amino acids and the most similar resonance Raman spectra. Within 16 Å of the copper metal ion there are only four amino acid changes (Table 2). The only difference within 10 Å of the copper site is a change in residue 48 from Trp in AD azurin to Leu in AXII azurin (Figure 1). This residue is also only two amino acids along the polypeptide strand from His46 and Gly45 which coordinate directly to the copper metal. In the plastocyanins,[1−3] changes in amino acids that are within a few amino acids of the copper center ligands along the same polypeptide strand were suggested to rotate the normal modes, resulting in a redistribution of intensity between two vibrational modes. This model is supported here by the observed changes in relative intensities of the modes at ∼400 and 428 cm^{-1}; in the spectrum of AD azurin the 428 cm^{-1} mode is less intense than the 400 cm^{-1} mode, while it is more intense than the 400 cm^{-1} mode in the spectrum of AXII azurin. A second amino acid change is residue 16 from an Asp in AD azurin to an Asn in AXII azurin. This amino acid change removes a negative charge 13.4 Å from the copper site, but leaves a dipole. A third amino acid change is residue 42 which changes from Ser in AD to Val in AXII and results in a loss of a dipole 12.8 Å from the copper site. Note that the distance to the charges was measured from the copper ion to the atoms of the side chain which nominally carry the charge. In plastocyanin, dipole changes up to 12−13 Å from the copper site were seen to slightly affect the absolute intensities in resonance-enhanced modes.[1] In azurin, it appears that these two changes have a minimal effect on the resonance Raman spectrum. The fourth amino acid change is residue 7 changing from Ile in AD to Val in AXII. This is a minor, homologous change 10.5 Å from the copper and is unlikely to affect the spectrum. In plastocyanin it was found that homologous changes near the copper site on β-strands that are not directly covalently linked to the copper site have no effect on the resonance Raman spectra.[1]

TABLE 2: Amino Acid Differences in Azurin

residue change[a]			distance[b], Å	electrostatic[c]
		AD/AXII		
7	Ile	Val	10.5	0/0
16	Asp	Asn	12.6	−/polar
42	Ser	Val	12.0	polar/0
48	**Trp**	**Leu**	**8.9**	**ϕ/0**
		AXI/PA		
8	Ala	Gln	11.1	0/polar
12	**Gly**	**Gln**	**10.0**	**0/polar**
16	Asp	Asn	12.1	−/polar
34	Lys	Ser	12.6	+/polar
40	Ala	Pro	10.7	0/0
71	Asn	Asp	11.0	polar/−
73	Val	Leu	11.1	0/0
86	**Val**	**Leu**	**6.3**	**0/0**
89	Gly	Ser	11.6	0/polar
110	Tyr	Phe	10.6	polar,ϕ/ϕ
113	**Ser**	**Thr**	**7.6**	**polar/polar**
118	**Phe**	**Ser**	**8.2**	**ϕ/polar**

[a] The residue number is listed first followed by the amino acids for the two species to be compared. [b] This distance is measured from the copper site to the closest amino acid side chain atom. Residues in bold are within 10 Å of the copper site and are shown in Figure 1. [c] Electrostatic changes are differences in charge or polarity of the amino acids. In the table, "0" is uncharged and nonpolar, "ϕ" is aromatic, "polar" is uncharged and polar, "−" is negatively charged, and "+" is positively charged.

The other pair of species which yield similar resonance Raman spectra is PA and AXI. The amino acid % identity is 78% and the resonance Raman spectra have a general similarity. A detailed examination of the spectra, however, reveals several significant changes (Figure 2). There are a total of 12 amino acid changes within 16 Å of the copper site, and so it would be difficult to ascribe specific spectral changes to individual amino acid changes in this case. However, it may be possible to identify amino acid changes which are likely to be more important. There are three amino acid changes which occur within two amino acids along polypeptide strands ligated to the copper. Two conservative changes are Thr113 in PA azurin changing to Ser in AXI azurin and Phe110 in PA azurin to Tyr in AXI azurin, with Tyr adding a dipole 12.8 Å from the copper site in AXI. Both of these changes are on the same polypeptide strand as Cys112, although Tyr110 is physically closer to Met121. A

significant change is Ser118 in PA azurin changing to Phe in AXI azurin. Ser has a dipole 8.8 Å from the copper site which is replaced by the aromatic ring of Phe in AXI azurin. This amino acid is adjacent to His117 and is expected to cause a significant redistribution of intensity in the spectra. There are also three charge and three dipolar residue differences between AXI and PA azurin. AXI azurin has a negative charge (Asp16) 12.9 Å from the copper and a positive charge (Lys34) 15.1 Å from the copper, while PA azurin has a negative charge (Asp71) 11.8 Å from the copper metal (distance is from Cu to side chain atoms which nominally carry the charge). In each case, the charged amino acid is replaced by a dipole at a similar distance in the respective proteins; Asp16, Lys34, and Asn71 in AXI azurin become Asn, Ser, and Asp in PA azurin, respectively. PA azurin has three additional dipoles compared to AXII. These are Gln8, Gln12, and Ser89 which replace Ala8, Gly12, and Gly89 in AXI. The distribution of charges and dipoles forms an electrostatic environment which may change the potential energy surface of the charge-transfer excited-state and/or the electronic coupling through the protein in its electron-transfer function. The remaining three changes within 16 Å of the copper site are homologous replacements. The first two are Leu86 and Leu73 in PA azurin changing to Val in AXI azurin. These are minor changes which are not likely to have much effect on the resonance Raman spectra. The third change is Pro40 in PA azurin to Ala in AXI azurin. Going from the conformationally restricted Pro to Ala is likely to decrease the structural rigidity of AXI compared to PA and may have some structural effect on the copper site.

Conclusion

The results presented here demonstrate a sound structural basis for the correlation between protein environment and excited-state properties, as reflected in the resonance Raman intensities, by systematically examining the resonance Raman spectra of a series of homologous structurally determined azurins. Confirmation of the two mechanisms by which the protein affects the excited-state dynamics, via a through-bond kinetic coupling of internal coordinates and through-space electrostatic interactions, is tentatively provided by the known structures of the four azurins presented here. Because the resonance Raman intensities and frequencies appear to be sensitive to these coupling mechanisms, resonance Raman spectroscopy could provide a uniquely powerful probe of long-range coupling in these proteins and provide a mechanistic insight in the efficient long-range electron transfer observed in these proteins. Clearly, probing the putative differential sensitivity of the resonance Raman intensities to electrostatic and kinetic coupling factors in the protein environment by using structurally characterized site-directed azurin mutants is necessary. Such studies are in progress.

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