The First Direct Evidence for Copper(II)-Cysteine Vibrations in Blue Copper Proteins: Resonance Raman Spectra of ^{34}S-Cys-Labeled Azurins Reveal Correlation of Copper-Sulfur Stretching Frequency with Metal Site Geometry

Bakul C. Dave, Juris P. Germanas,* and
Roman S. Czernuszewicz*

Department of Chemistry
University of Houston
Houston, Texas 77204

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Proteins containing blue (or type 1) copper centers (cupredoxins) participate in electron transfer in a number of important biological processes, including photosynthesis and respiration.[1] The Cu center in these proteins possesses an array of unusual properties, including an intense absorption centered around 600 nm, a small EPR hyperfine coupling constant, and a relatively high reduction potential.[2] Crystal structure analyses of several cupredoxins[3] have revealed that the Cu atom is coordinated by a highly conserved set of residues (two histidines and a cysteine) in a nearly trigonal geometry, with weaker axial interactions with a methionine sulfur atom, and, in the case of azurin, a peptide carbonyl group.[3,4]

Despite the conserved nature of the core residues, type 1 Cu centers display substantial variation in their electrochemical and spectroscopic properties.[2] Their redox potentials vary from 170 to 680 mV, and the value of the redox enthalpy has been postulated to be proportional to the strength of the Cu-S bond.[5] Blue copper EPR spectra are classified as axial or rhombic; additionally, UV-vis absorption spectra display an absorbance centered near 450 nm of variable intensity. Han *et al.*[6] have suggested that these spectral characteristics are related to the degree of the displacement of the Cu atom out of the N_2S plane determined by the three strong ligands.

Resonance Raman (RR) spectroscopy has provided substantial insights into type 1 Cu centers.[7] The RR spectra of cupredoxins, however, are unusually complicated and exhibit a complex set of dominant bands (up to nine) of variable frequencies and relative intensities in the 300-450-cm^{-1} region which derive their RR activity from excitation into the $S(\pi) \rightarrow Cu(d_{x^2-y^2})$ charge-transfer (CT) transition near 600 nm.[6-8] In order to determine the importance of the strength of the Cu-S bond in controlling the spectroscopic and electrochemical features of blue Cu sites, we have measured the RR spectra of wild type (WT) and two Cu center mutants, M121G[9] and H46D,[10] of *Pseudomonas aeruginosa* azurin with natural isotope abundance or labeled with ^{34}S in the cysteine sulfur atom.[11] The RR spectra of these proteins allow us to report the first direct identification of the (predominantly) Cu-S stretching vibration in cupredoxins and to correlate Cu-S bond strength with metal site structure in azurin and its site-directed mutants.

Figure 1 displays the high-resolution RR spectra for the natural abundance WT azurin and its M121G and H46D mutants, and their corresponding ^{34}S-Cys-substituted proteins, obtained in the 360-435-cm^{-1} region with 647.1- (WT) and 568.2-nm (M121G, H46D) excitation wavelengths.[12] Each spectrum shows four strong RR peaks in this region that are characteristic for the type 1 structure of the Cu(II)-Cys chromophores.[7] The most intense one in WT azurin occurs at 408 cm^{-1} with a shoulder at $\sim$400 cm^{-1}. The other peaks are found at 373 and 428 cm^{-1} and remain largely unaltered by replacing methionine 121 with glycine (M121G) or histidine 46 with aspartate (H46D); the 373-cm^{-1} peak shifts slightly to lower (371 cm^{-1}) and higher (375 cm^{-1}) frequencies in M121G and H46D mutants, respectively, whereas the 428-cm^{-1} peak loses less than 1 cm^{-1} in M121G and $\sim$2 cm^{-1} in H46D azurins.

In contrast, the dominant 408-cm^{-1} RR band of WT azurin and its weak shoulder at 400 cm^{-1} are significantly affected by both mutations at the Cu site, and a splitting into two distinct bands at 400 and 410 cm^{-1} becomes especially apparent when the Cu-bound His46 is replaced with Asp in H46D. Not only are the two peak maxima further apart in the H46D azurin spectrum, but their intensity pattern changes dramatically; while in the WT protein the higher energy peak at 408 cm^{-1} is predominantly resonance enhanced with either the 647.1- or 568.2-nm (not shown) excitations, the lower energy peak at 400 cm^{-1} becomes as intense as the 410-cm^{-1} line in the H46D mutant when excited at 568.2 nm and becomes the strongest feature when excited at 647.1 nm (not shown). Likewise, the 400-cm^{-1} shoulder of M121G mutant intensifies markedly relative to the 408-cm^{-1} peak in the 568.2-nm excited spectrum and becomes the dominant peak in the 647.1-nm excited spectrum (not shown).

Identification of bands is greatly aided by ^{34}S-Cys substitution, and we observe changes in the RR frequencies which provide persuasive evidence for Cu-S stretching in azurin and its mutants. Thus, the strongest band in the WT azurin spectrum at 408 cm^{-1} downshifts the most when the protein is enriched in ^{34}S, -3.8 cm^{-1} (Figure 1), whereas a deconvolution of the two peaks in the this region showed that the shoulder at $\sim$400 cm^{-1} remains unaffected on ^{34}S substitution. Superior enhancement of this band by excitation in resonance with the S $\rightarrow$ Cu CT transition and the large ^{34}S shift unambiguously demonstrate the existence of the (predominantly) ν(Cu-S) vibration in the RR spectrum of native azurin, confirming earlier suggestions.[7a,8a] Assuming an isolated two-body oscillator model for the Cu-S(Cys) stretch, an isotope shift of -7.7 cm^{-1} is calculated for the 408-cm^{-1} frequency upon

* Authors to whom correspondence should be addressed.

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(11) The ^{34}S-enriched samples of *P. aeruginosa* azurin and its site-specific mutants were prepared by growing bacterial cultures in the minimal medium M9 using 90%-^{34}S-enriched ammonium sulfate (ICON, Summit, NJ). Protein isolation and purification were carried out according to already established procedures.[10]

(12) All the RR spectra were recorded at $\sim$77 K by collecting backscattered photons directly off the surface of a frozen protein solution ($\sim$1 mM in 10 mM pH 8 tris buffer) kept in a liquid-N_2 Dewar by using the Raman instrumentation described in the following: Czernuszewicz, R. S. In *Methods in Molecular Biology*; Jones, C., Mulloy, B., Thomas, A. H., Eds.; Humana Press: Totowa, NJ, 1993; Vol. 17, pp 345-374.

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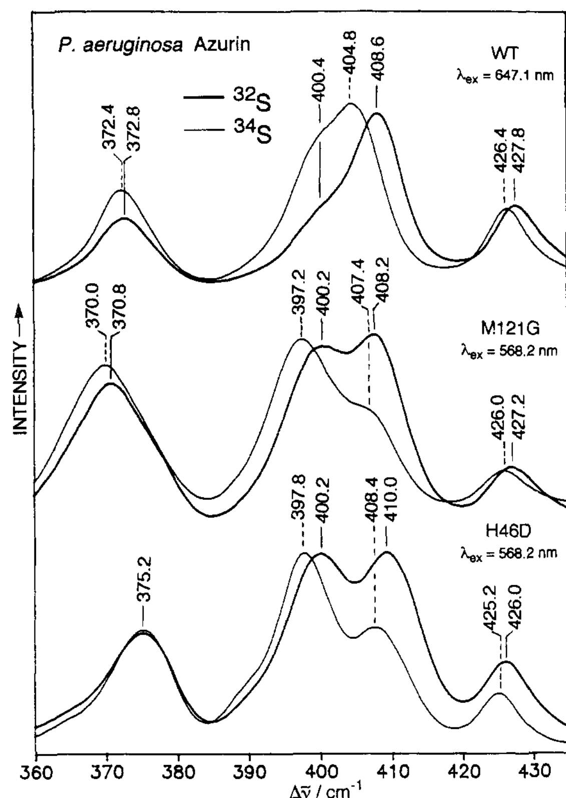

Figure 1. Low-temperature (77 K) RR spectra of *P. aeruginosa* azurins (thick-line traces) and their ^{34}S-Cys-labeled proteins (thin-line traces) in the region between 360 and 435 cm^{-1}: top, wild type (WT) excited at 647.1 nm; middle, M121G mutant, and bottom, H46D mutant, both excited at 568.2 nm. Conditions: 150-mW laser power, 2-cm^{-1} slit widths, average of three scns at 1 s/point and 0.2 cm^{-1}/s increments; no base-line correction or smoothing was applied. To ensure accurate isotope measurements, the natural abundance and ^{34}S-labeled proteins were placed side by side on the cold finger so that the RR data could be collected under the same conditions. All spectra were calibrated using the known vibrational frequencies of CCl_4 and CH_2Cl_2.

^{34}S labeling. This simple calculation predicts that the strongest RR band of WT azurin arises from the vibrational mode which is at least 49% ν(Cu–S) in character.

We also observe that the ~370-cm^{-1} band is virtually insensitive to the ^{34}S substitution in all three proteins. The 428-cm^{-1} band is somewhat sensitive, its ^{34}S isotope shift decreasing from −1.4 cm^{-1} in WT to −1.2 cm^{-1} in M121G to −0.8 cm^{-1} in H46D. This indicates that the 428-cm^{-1} band also arises from a vibrational mode which involves some motion of the cysteinyl sulfur atom; however, sulfur participation in this mode decreases as the coordination environment is altered at the Cu site.

Examination of the RR spectra of the M121G and H46D azurins reveals that, unlike for WT azurin, both components of the 400–410-cm^{-1} doublet are influenced by $^{32/34}$S exchange, implying a redistribution of the Cu–Cys normal coordinates which give rise to these vibrational modes. The results of these experiments are striking in that the lower energy components of the doublet are downshifted much more than the higher energy ones, −3 cm^{-1} vs −0.8 cm^{-1} in M121G and −2.4 cm^{-1} vs −1.6 cm^{-1} in H46D (Figure 1). This suggests a maximum contribution of the Cu–S stretching coordinate to the 400-cm^{-1} rather than 408/410-cm^{-1} peaks in azurin mutants and, consequently, a decreased Cu–S bond strength relative to the WT.

Previously, Han *et al.* had postulated that increased absorption at ~450 nm and rhombicity of the EPR signal of cupredoxins are related to a more tetrahedral site where the Cu atom has moved away from the plane formed by the three strong ligands.[6] In the case at hand, while the EPR spectrum of the WT protein is axial,[2] the spectra of the M121G[10] and H46D[11b] proteins are rhombic. Additionally, while the absorption spectrum of the WT displays a weak feature at 460 nm (ϵ = 580), the corresponding absorption in the spectra of M121G (453 nm, ϵ = 900) and H46D (458 nm, ϵ = 1200) azurins is significantly enhanced. Thus the EPR and optical spectra suggest significant separation between the Cu atom and the plane formed by the three strong ligands (N_2S in WT and M121G, NOS in H46D) in the mutant proteins relative to the WT.[6]

In the crystallographically characterized mutant of azurin, M121Q, that displays a rhombic EPR signal and an increased ~450-nm absorption similar to those of the M121G and H46D azurins, the Cu atom is significantly out of the N_2S plane (0.3 Å).[4c] We propose that the metal center in the M121G and H46D mutants is also displaced from the equatorial plane due to its increased interaction with axial ligands, as seen in crystals of M121Q azurin. The possible axial ligands include water and Met121. Water coordination is likely for M121G azurin since its spectroscopic properties are nearly identical to the M121(stop codon) mutant that is proposed to have a water molecule as a fourth ligand.[13] In the H46D azurin, a weakened coordination of Cu to the carboxylate side chain of Asp46 is expected to lead to increased interaction with the side chain of Met121.[10b] The RR spectra of the ^{34}S-labeled azurins clearly show a weakened Cu–S interaction in the M121G and H46D mutant proteins relative to the WT. Increases in copper–S(Cys) bond lengths would be expected in the mutant azurins if the Cu atom were moved out of the trigonal plane relative to the ligand atom positions that are fixed within the protein framework.

In conclusion, ^{34}S isotopic substitution and RR spectroscopy can provide valuable insight into the strengths of Cu–S interactions in blue copper proteins and the geometric features of their metal centers. The determination of the redox enthalpies of the mutant proteins to obtain a relationship between Cu–S bond strengths and redox thermodynamics is currently underway.

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