Cryoresonance Raman Spectroscopy of Plastocyanin, Azurin, and Stellacyanin. A Reevaluation of the Identity of the Resonance-Enhanced Modes

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Low-temperature techniques have been enormously important in infrared and nonresonant Raman studies of relatively simple molecular systems. However, reports of resonance Raman studies of biomolecules under cryogenic conditions are rare. Among the few studies of this type are those by Loehr et al.[1a] and by Ondrias et al.[1b] We report the resonance Raman spectra of the blue copper proteins plastocyanin (from spinach), azurin (from *Pseudomonas aeruginosa*), and stellacyanin (from *Rhus vernicifera*) obtained at nominal cryostat temperatures as low as 12 K.

In this communication we establish that cryoresonance Raman (CRR) leads to dramatic improvements in signal to noise and spectral resolution in the blue copper proteins. We argue that a number of the resonance-enhanced modes near 400 cm^{-1} in these proteins are internal motions of the coordinated ligands, including the histidine imidazole rings. This suggests a reinterpretation of the resonance Raman spectra.

Figure 1 compares the resonance Raman spectrum of azurin in water at ca. 300 K to that of the same solution frozen at 12 K. The improved signal to noise (due to base line suppression and greater peak height) and resolution (due to line narrowing) of the CRR spectrum are obvious. Figure 2 compares the CRR spectra of azurin (AZ), plastocyanin (PL), and stellacyanin (ST) in the 200–500-cm^{-1} frequency region. The observed frequencies of the Raman peaks between 300 and 500 cm^{-1} and their relative intensities are presented in Table I.

Raman spectra as a function of temperature within the solid phase (ca. 30–273 K) show line narrowing as temperature is reduced. We suggest that at low temperatures the line widths are determined by inhomogeneous broadening arising from subtle variations in long-range environments of the various oscillators. As temperature is raised, the line widths are eventually dominated by homogeneous broadening arising, most likely, from vibrational dephasing involving low-energy modes of the protein. Similar effects have been suggested[1b] to explain line narrowing and frequency shifts observed in heme proteins. Although we achieved indicated cryotip temperatures as low as 12 K, we emphasize that this is merely the nominal temperature; laser irradiation of the sample results in local heating and a temperature at the scattering point that we estimate to be 20 °C higher.

The intense features in the resonance Raman spectra of blue copper proteins are centered around 400 cm^{-1} (somewhat lower in stellacyanin) with overtones, combinations, and the C–S(cys) stretch near 800 cm^{-1} and weak features elsewhere.[2-5] Because the resonant electronic transition near 600 nm is assigned as RS^-(cys) → Cu^{2+} ligand-to-metal charge transfer,[6] Franck–Condon arguments have led to the assignment of the strongest features in the resonance Raman spectra as essentially pure metal–ligand stretches.

This vibrational assignment presents difficulties because, for azurin and plastocyanin, the four ligands bound to copper have

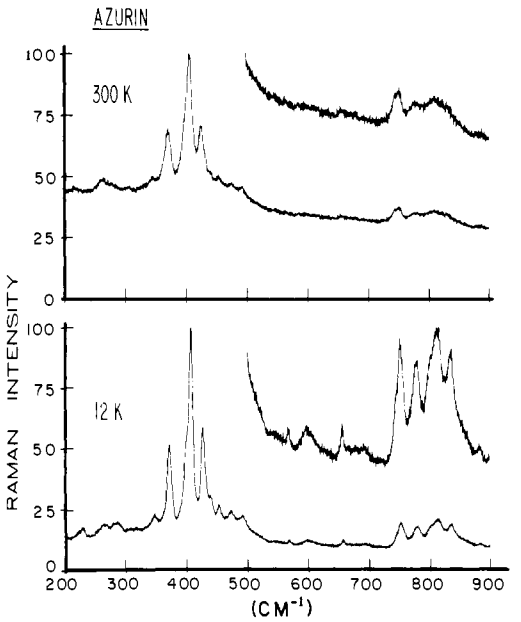

Figure 1. Resonance Raman spectra of a 7×10^{-3} M aqueous solution of azurin contained in a sealed 1-mm path length suprasil cell. Both spectra employed identical solution and illumination conditions; the sample cell was held at approximately a 30° angle to the laser beam by a copper block that was coupled to a Displex cryotip. The Raman scattering was collected at 90° to the incident beam. For the top trace, the cryotip was at ambient temperature; for the bottom trace, the tip was cooled to a nominal temperature of 12 K. The features near 800 cm^{-1} are overtones and combinations, except for the C–S stretch fundamental at 752 cm^{-1}. The peaks at 230 and 600 cm^{-1} are due to ice. Spectral conditions: laser, λ = 647.1 nm, 6 mW c.w.; spectrometer, spectral slit width 5 cm^{-1}, scan speed 1 cm^{-1}/s, 32 scans averaged together. Solution conditions: pH = 6.5, 0.01 M phosphate buffer.

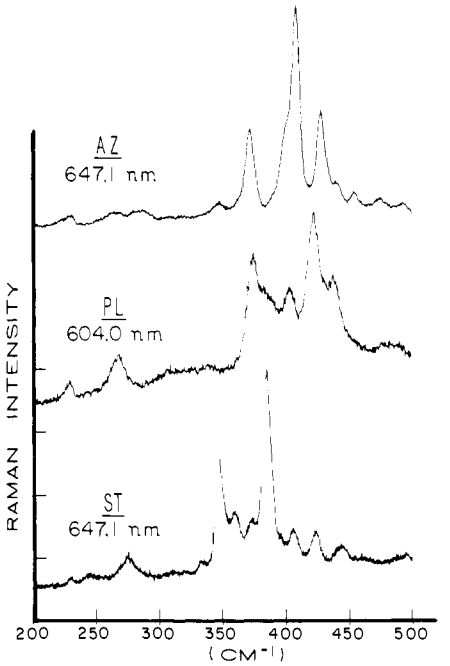

Figure 2. Comparison of the resonance Raman spectra (200–500 cm^{-1}) of azurin (AZ), plastocyanin (PL), and stellacyanin (ST) at cryotip temperatures of ca. 12 K. Conditions were the same as those stated in the caption to Figure 1. Laser wavelengths are as shown above.

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Table I. Resonance Raman Frequencies and Relative Intensities of the Blue Copper Proteins between 300 and 500 cm^{-1}

plastocyanin		azurin		stellacyanin	
ν, cm^{-1}	I_{rel}[a]	ν, cm^{-1}	I_{rel}[a]	ν, cm^{-1}	I_{rel}[a]
343	0.08	348	0.04	334	0.06
377	0.7	373	0.4	348	0.6
387	0.5	400	0.4, sh	359	0.3
393	0.4				
407	0.5	409	1.0	373	0.2
425	1.0	428	0.5	386	1.0
442	0.6	441	0.1	407	0.2
		455	0.07		
				424	0.14
480	0.1	474	0.04	444	0.08
491	0.1	492	0.03	492	0.06

[a] I_{rel} of the strongest peak in each spectrum is taken as 1.0; all other peak intensities are given as fractions of the strongest peak.

been identified crystallographically as one cysteine sulfur, one methionine sulfur, and nitrogens from two histidine imidazole residues,[7,8] While a stretching frequency near 400 cm^{-1} is reasonable for a RS^-–Cu^{2+} bond,[9] it is unprecedentedly high for a M^{2+}–N(imidazole) bond. The latter typically have stretching frequencies near 250 cm^{-1}.[10-12] The Cu–S(methionine) bond in plastocyanin appears from the crystal structure to be exceptionally long (~2.8 vs, ~2.3 Å for typical Cu^{2+}–S(thioether) bonds).[8,13] Considering that normal Cu^{2+}–S(thioether) stretching frequencies are near 270 cm^{-1},[9,14] it appears inconceivable that the freqnency due to the long Cu–S(methionine) bond in the proteins could be near 400 cm^{-1}.

From the foregoing it appears that only one of the metal–ligand stretching modes of the blue copper chromophore is actually expected to be near 400 cm^{-1}. Yet Table I shows that nine modes in this frequency range have intensities within approximately an order of magnitude of one another. Clearly, some of these modes must be other than copper–ligand stretches. We may discard LCuL, CuSC, and CuNC deformations as reasonable possibilities at these frequencies. We are left with vibrations of the ligands thermselves. These include the C–S–C angle bend of methionine, the S–C–C bend and α-carbon deformations of cysteine, the C–S stretches of cysteine and methionine, and imdazole ring deformations as motions that might plausibly be resonance enhanced. The C–S stretches are expected[9,14] and observed[5] (for cysteine) near 750 cm^{-1}. Considering the long Cu–S(methionine) bond and the belief[6] that methionine charge transfer contributes insignificantly to the electronic absorbance of the blue copper chromophore, observation of the C–S–C bend of methionine appears unlikely. It is reasonable for one of the modes to be the S–C–C bend. Resonance enhancement of two or three cysteine α-carbon deformations, while less likely, cannot be excluded. The most reasonable assignment for the remaining modes is as histidine imidazole ring deformations. While the IR and Raman spectra of solid imidazole do not show peaks in the 300–500-cm^{-1} region,[15,16] IR spectra of the partially deuterated sample[16] exhibit frequencies that, allowing for the maximum deuterium shift, suggest that normal imidazole has internal motions near 400 cm^{-1}. Further, preliminary results[17] of low-temperature Raman studies of histidine show peaks in the 300–500-cm^{-1} region. It is noted that previous normal mode analyses of imidazole[15,16] do not take into account the existence of modes in this region and that, in any case, imidazole itself may be an inadequate vibrational model for the alkyl-substituted imidazole represented by histidine.

If imidazole ring deformations are resonance enhanced in these proteins, it is clear that these deformation motions may mix with the M–L stretches. Indeed, one explanation of the "anomalously high copper–imidazole stretching frequencies" is that none of the modes near 400 cm^{-1} need be Cu–N stretches at all, but could instead be highly mixed motions involving the Cu–S stretch and internal ligand modes including imidazole ring deformations. We suggest that the Cu–N stretches appear, as they do in model systems,[11,12] in the 200–300-cm^{-1} region (see Figures 1 and 2). In agreement with earlier work[2-5] we assign the Cu–S(cys) stretch either as one of or distributed among the strong peaks near 400 cm^{-1}.

The foregoing assignment provides a reasonable mechanism for significant variability of the frequencies of analogous RR modes from one protein to another. The extent of the interaction between the Cu–L stretches and the ligand deformations should be very dependent upon the geometry of the CuL_4 core and also the orientations of the imidazole rings and cysteine dihedral angles with respect to the Cu–L bonds. The protein structure controls these structural parameters and therefore may control the magnitude of the stretch–deformation interactions.

We note the remarkably similar appearance of the spectra of plastocyanin and stellacyanin between 300 and 500 cm^{-1} (Figure 2). Each of the peaks of stellacyanin is approximately 30 cm^{-1} lower in frequency than the corresponding feature in plastocyanin, and stellacyanin exhibits an extra feature at 424 cm^{-1}. This similarity is not evident in the room-temperature solution RR spectra.[2-4]

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Metal Electrodes Bonded on Solid Polymer Electrolytes: Platinum Bonded on Solid Polymer Electrolyte for Electrooxidation of Methanol in Perchloric Acid Solution

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The catalytic activity of a platinum electrode bonded to a solid polymer electrolyte (SPE) membrane with either cation- or anion-exchange properties was examined for electrooxidation of methanol. The stability of the catalytic activity was found to be much better than that of a bulk Pt electrode.

Recently, various SPE-coated electrodes were prepared, and their chemical properties were investigated.[1,2] We prepared platinum directly bonded onto an SPE membrane (Pt–SPE). The procedure was as follows.[3] The membrane was swelled in boiling water for 0.5–2 h and then mounted on a vessel so that one face of the membrane contacted a 0.01–0.02 M H_2PtCl_6 aqueous solution and the other face contacted a 0.1–1.5 M $NaBH_4$ aqueous alkaline solution. The reducing agent, borohydride, was allowed

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