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Native Azurin and its Ni(II) Derivative: A Resonance Raman Study

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Summary: Resonance Raman spectra are reported for native Cu(II) Pseudomonas aeruginosa azurin and its Ni(II) substituted derivative. The spectrum of the native azurin includes a low frequency feature and bands in the first overtone region not previously reported. The spectrum of the Ni(II) derivative exhibits three major peaks in the metal-ligand stretching region shifted to lower frequency relative to the M-L peaks in the spectrum of native azurin. Resonance enhanced ligand modes are observed which indicate that at least two of the ligands in Ni(II) azurin (cysteine and at least one histidine) are the same as in native azurin. The data also suggest that the disposition of ligands about the metal may be more nearly tetrahedral in the Ni(II) derivative than in native azurin.

Introduction: The identity of the ligands bound to copper and their protein-constrained geometric arrangement are thought to be responsible for many of the unusual properties of blue copper proteins including azurin and plastocyanin (1). For plastocyanin (2a) and azurin (2b) the ligands have been identified by x-ray crystallography to be a cysteine sulfur, a methionine sulfur, and two imidazole nitrogens from histidine residues. Qualitative similarities among the resonance Raman spectra of the other blue copper proteins (with the exception of stellacyanin) which have been investigated (3,4) suggest that the copper-bound residues are the same in each case. Stellacyanin contains no methionine (5) and exhibits a unique resonance Raman spectrum (3,4). Possible copper ligands in stellacyanin are discussed

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elsewhere (6). In the present study we report detailed resonance Raman investigations of native Cu(II) azurin and a laboratory-modified derivative wherein copper(II) has been replaced by nickel(II). In the native protein, we observe overtones and low frequency features which have not been reported previously and which allow refined vibrational assignments. The criteria for these assignments, and perhaps the assignments themselves, are expected to be applicable to the other blue copper proteins. The resonance Raman spectrum of Ni(II) azurin is consistent with coordination of Ni(II) to the same protein residues which are coordinated to copper(II) in native azurin. If this is the case, our results suggest that the Ni(II) binding site is less distorted from tetrahedral geometry than that of copper(II).

Experimental:

The resonance Raman spectra were obtained using a Cary 82 spectrometer with a cooled ITT FW-130 photomultiplier and photon counting detection. Laser excitation was provided either by a Spectra-Physics 164 Krypton ion laser or 164 Argon ion laser or by a Spectra Physics 375 tunable dye laser pumped by the Ar^+ laser. Samples were contained in 1 mm id. glass capillaries. Spectra of native azurin were recorded at pH 5.5 and pH 7.5. The excitation wavelengths were near 600 nm (dye or Kr^+ laser) for native azurin in resonance with the charge transfer band at 626 nm and 457.9 nm (Ar^+ laser) for the Ni(II) derivative in resonance with its charge transfer transition at 440 nm (see Figure 1). Approximate concentrations were 6×10^{-4} M for native azurin and 2×10^{-3} M for the Ni(II) derivative. The latter was prepared by the method of Tennent and McMillin (8). Iodide was added to the Ni(II) azurin sample to reduce the luminescence from the sample. Approximate errors in peak positions are $\pm$ 2 cm^{-1}. The depolarization ratios for the low frequency band in native azurin are approximately 0.3 as expected for a symmetric vibration in low site symmetry (3). The peak intensities in the overtone region of native azurin and in the Ni(II) azurin spectrum were too low for successful measurement of their depolarization ratios.

Results and Discussion:

Figure 2 shows the resonance Raman spectra of both native azurin and its Ni(II) derivative; Table I tabulates the vibrational frequencies. Additional vibrational information in this native azurin spectrum, compared to the one previously published (3), includes the low intensity peak at 220 cm^{-1}, and the three higher frequency peaks at 748, 780 and 814 cm^{-1}. We also note that the peak at approximately 460 cm^{-1} appears weaker in our spectrum. Changing the pH of our sample (pH = 5.5 to pH = 7.5) did not affect the spectrum of native azurin. Likewise, variation of the excitation wave-

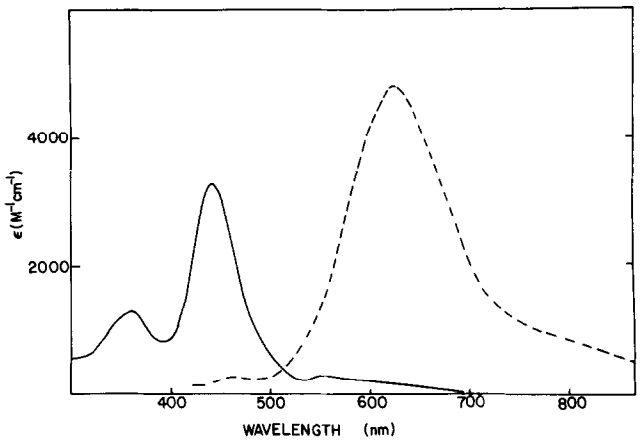

Figure 1. Absorption spectra for native azurin (------) and the Ni(II) derivative (————). Excitation wavelengths for the resonance Raman experiments were 620.1 nm for native azurin and 457.9 nm for Ni(II) azurin.

length from 568.2 nm to 647.1 nm did not change the intensities of the observed peaks relative to one another; however the absolute intensities are known to change (3) due to changes in the resonant condition with laser wavelength.

In light of the present results, x-ray crystallographic data (2) and the identification of the Cu-S (methionine) vibration in the proteins (6), we propose the following vibrational assignments in Cu(II) azurin. The 262 cm^{-1} peak is due to a Cu-S (methionine) stretch as previously assigned (6). (This assignment is also consistent with a previous resonance Raman study which concluded that the 270 cm^{-1} band in ceruloplasmin could not arise from a Cu-S (cys) vibration (9).) The three more intense peaks at at 372, 407 and 425 cm^{-1} are assigned as mixed modes composed of the remaining three Cu-L stretches (Cu-S(cys) + 2Cu-N(his)] on the basis of their strong resonance enhancement with the S(cys) → Cu(II) charge transfer transition at 626 nm. The remaining weaker bands in the 200-500 cm^{-1} frequency region of the spectrum are attributed to L-M-L deformations and/or ligand centered vibrations.

The previous resonance Raman studies of some of the blue copper proteins noted vibrations in the 750 cm^{-1} spectral region as well as in the 1240 and 1650 cm^{-1} regions (3,4) although none of these vibrations were

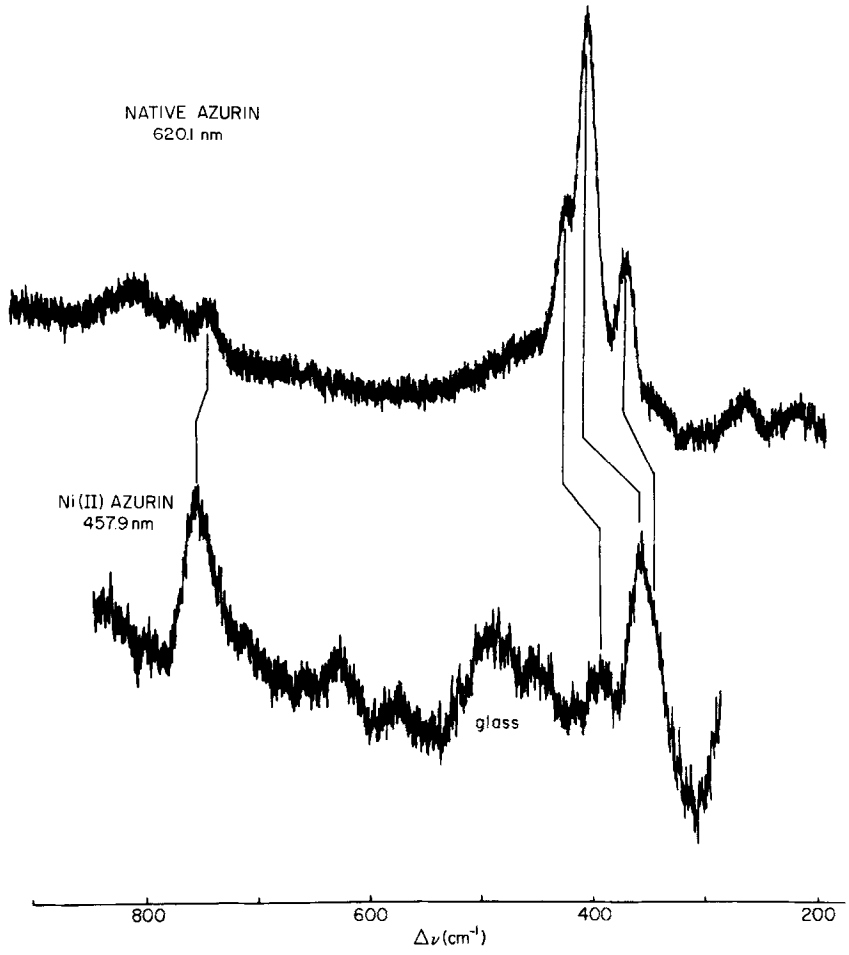

Figure 2. Resonance Raman spectra of native azurin and its Ni(II) derivative. Spectral conditions were as noted in the experimental section.

reported for azurin. The vibration at 750 cm^{-1} has been variously assigned as an amide group vibration (4) or a C-S stretching mode from a coordinated cysteine residue (3). The higher frequency modes were also assigned to amide group vibrations (4) and, based on this, the coordination of a peptide group to copper was proposed. The crystal structures have since shown that deprotonated peptide nitrogen is not bound to copper in either plastocyanin or azurin and, therefore, resonance enhancement of amide group vibrations is not expected.

In the 750-800 cm^{-1} region of the native azurin spectrum, we observe three vibrations at 748, 780 and 814 cm^{-1}. The band at 780 cm^{-1} is assigned as

TABLE I

Vibrational Frequencies for Native Azurin

From P. Aeruginosa and its Ni(II) Derivative

Native Azurin (cm^{-1})	Ni(II)Azurin, (cm^{-1})	Assignments
~220		Deformation
262		Cu - S(met) stretch
~340(sh)		Deformation
372	~345(sh)	M - S⁻(cys) stretch
	356	plus
407	395	M - N(his) stretch
425		Primarily Cu - N(his) stretch
	453	Ligand deformation mode
~460(sh)		" " "
	575	" " "
	625	" " "
748	758	Overtone (2 x 372 cm^{-1}) + C - S stretch or Im[a] ring mode in native Azurin; C - S stretch or Im ring mode in Ni(II) Azurin
780		Combination (372 cm^{-1} + 407 cm^{-1})
814		Overtone (2 x 407 cm^{-1})
	1230	Im. ring mode

[a]Im = Imidazole

a combination band involving M-L modes (372 + 407 cm^{-1}) and the band at 813 cm^{-1} (approximately 2 x 407 cm^{-1}) is assigned as an overtone of the 407 cm^{-1} fundamental. The overtone of the 372 cm^{-1} fundamental is expected at approximately 744 cm^{-1}. However, this region of the spectrum also contains a resonance enhanced ligand mode. The ligand mode and the overtone together result in a broad peak at ∿748 cm^{-1}. A vibration assigned as C-S stretch has been observed at ∿750 cm^{-1} in the spectra of a number of blue copper proteins (4) and in the spectrum of Ni(II) azurin, vide infra. We favor the C-S stretch assignment (3), but note that an imidazole ring mode of a

coordinated histidine cannot be excluded (11). We do not observe vibrations at 1240 or 1650 cm^{-1} in our spectra of native azurin, in agreement with previous work (3).

The appearance of an overtone progression in the native azurin spectrum aids in specifying more precisely the distribution of the three stretching vibrations (Cu-S(cys) + 2 Cu-N(his)] among modes observed at 372, 407 and 425 cm^{-1}. The vibronic theory of the resonance Raman effect (12,13) concludes that the intensity of overtone progressions, as well as fundamental vibrations, is related to the effectiveness of the vibrational mode in enhancing Franck-Condon overlap for the resonant electronic transition. Since the resonant electronic transition is S(cys) → Cu(II) charge transfer, the equilibrium Cu-S(cysteine) bond length of the excited state should differ appreciably from that of the ground state, so that the Cu-S stretching vibration should be quite strongly resonance enhanced. On the other hand, the other three M-L vibrations, all of which involve neutral ligands which are not major contributors to the resonant charge transfer transition, are expected to be correspondingly less resonance enhanced. Although the three strongest observed normal modes in the native azurin spectrum probably each contain a mixture of Cu-S (cys) + 2 Cu-N(his), we suggest that the 372 cm^{-1} and the 407 cm^{-1} modes which exhibit higher order effects (i.e., overtones and the combination band)contain the major fraction of the Cu-S(cys) stretching vibration. The remaining intense mode at 425 cm^{-1} is then assigned as a predominantly Cu-N stretching mode.

Previous work on the nickel(II) analogue of azurin has indicated that the 440 nm electronic transition in the modified protein originates from a S(cys) → Ni(II) charge transfer transition with S(met)→Ni(II) character (7,8) suggesting that these two ligands are the same for both native azurin and the Ni(II) derivative. Figure 2 shows the resonance Raman spectrum of Ni(II) azurin excited at 457.9 nm near the 440 nm charge transfer transition No vibration below 345 cm^{-1} was observed indicating that if Ni(II) is bound

to methionine sulfur the Ni-S(met) stretch is too weak to be seen on the sharply rising background near the laser line. The major vibrations which are observed include a group of low frequency peaks (345, 356 and 395 cm^{-1}) which correspond in approximate relative intensities to the most intense low frequency modes in native azurin. In addition, relatively strong modes higher in frequency than the Ni(II)-L bands are observed, which are assignable to ligand centered vibrations. A broad peak due to the glass capillary material is also observed at approximately 475 cm^{-1}. The 758 cm^{-1} band can be assigned to the C-S stretch as discussed above. By analogy to the 1240 cm^{-1} vibration observed in some of the blue copper proteins, we assign the 1230 cm^{-1} band observed in Ni(II) azurin as an imidazole ring mode. The fact that these two resonance enhanced ligand modes are observed in the Ni(II) azurin spectrum at approximately the same frequencies as those observed in some blue copper proteins (4) is strong evidence that Ni(II) is bound to at least two of the same ligands as Cu(II) in native azurin (i.e., S(cys) and at least one N(his).). Taking the present Raman study with the electronic spectral study (8), at least three and possibly all four of the ligands in the native azurin are implicated in Ni(II) azurin. The greater relative intensity of the ligand versus M-L modes in Ni(II) azurin compared to the native blue copper proteins may be due to a resonant condition (or preresonant condition) with a transition involving more histidine character.

Vibrational data on isostructural complexes of Ni(II) and Cu(II) reveal that Ni(II)-L stretching frequencies are generally as high as, or higher than, Cu(II)-L stretching frequencies (11,14,15,16). In contrast, the protein Ni(II)-L frequencies are substantially lower than the corresponding Cu(II)-L frequencies. However, Ni(II)-L stretching frequencies have been shown to be very sensitive to coordination geometry, tending to decrease as the geometry changes from planar to tetrahedral (17). Assuming the same ligands are involved (10), the M-L frequency shift between native and Ni(II) azurin therefore suggests that the coordination geometry about Ni(II) in the

protein is more nearly tetrahedral than in native azurin. The crystal structure of native plastocyanin indicates the L-Cu-L angles may be distorted up to 50° from the tetrahedral angle (2), and a similar distortion is predicted on the basis of low energy d-d transitions in the other blue copper proteins (19). We infer that the metal-free protein structure favors a more nearly tetrahedral geometry about the metal, and that a significant fraction of the distortion toward square planar coordination in native azurin is due to the unique stereochemical preferences of the Cu(II) ion (18) which are absent in the Ni(II) protein.

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