# Raman Spectroscopic Study of the Blue Copper Protein Halocyanin from *Natronobacterium pharaonis*

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ABSTRACT: Halocyanin, a blue copper protein from *Natronobacterium pharaonis* was studied by Raman spectroscopic techniques. Near-infrared Fourier transform Raman spectra, which for the first time have been employed to study copper proteins in both oxidation states, display the Raman bands of the protein and, in the oxidized state, also the preresonance-enhanced bands of the copper center. The frequency of the amide I band at 1676 cm$^{-1}$ indicates a predominant $\beta$-sheet protein structure, which is typical for small blue copper proteins. The Raman spectra provide no evidence for major redox-linked changes of the secondary structure. Resonance Raman spectra of the oxidized protein obtained upon excitation close to the 600-nm absorption band were measured in the pH range between 7.7 and 4.5. The vibrational band pattern in the Cu–cysteine stretching region is closely related to that of azurin, indicating far-reaching similarities of the coordination geometry of the copper center in both proteins. Significantly lower frequencies, however, are noted for the Cu–histidine stretches, which appear as a closely spaced doublet at ca. 260 cm$^{-1}$. Lowering the pH to 4.5 leads to an increase of this band splitting with one component shifting down to 247 cm$^{-1}$. This downshift is attributed to the rupture of a hydrogen bond between one of the histidine ligands and a nearby carboxyl group, which becomes protonated at such a low pH. On the other hand, no major changes in the Cu–cysteine stretching region are noted at pH 4.5, implying that the coordination geometry remains largely unchanged. There is no apparent correlation between the pH-dependent changes in the resonance Raman spectra and the pH dependence of the midpoint potential of halocyanin. This observation suggests that the redox potential is not exclusively controlled by the geometry of the coordination sphere of the copper center.

Small blue copper proteins adopt important roles in electron transfer reactions in plants and bacteria [for a review, see Rydén (1984) and Sykes (1991)]. The molecular masses of these proteins are in the range between 10 and 20 kDa. They fold predominantly into a $\beta$-sheet domain. The topology of the copper binding sites of these proteins are quite similar as determined by X-ray structure analysis [reviewed by Adman (1991)]. The copper ligands include generally two histidines and one cysteine in a trigonal equatorial coordination and an additional axially bound methionine.

Recently, a type I copper protein, halocyanin, has been isolated from *Natronobacterium pharaonis* and subsequently biochemically characterized (Scharf & Engelhard, 1993). It is the first example of a blue (type I) copper protein from archaebacteria. The amino acid sequence of halocyanin reveals the typical similarities to other proteins of this class with close relationships to the plastocyanin-related subgroup (Rydén, 1984; Mattar et al., 1994). Three putative copper ligands (Cys$^{124}$, His$^{127}$, Met$^{132}$) are located in the C-terminal sector, and the fourth ligand is further upstream (His$^{86}$) (Mattar et al., 1994). Further homologies are mainly found in seven regions of which $\beta$-strands are predicted. From these data and from spectroscopic analysis using Fourier transform infrared (FTIR)[1] techniques (Brischwein et al., 1993) and circular dichroism spectroscopy (Scharf & Engelhard, 1993), it was concluded that halocyanin adopts the protein fold of type I copper proteins with predominantly $\beta$-structural elements and a typical blue copper binding site.

Halocyanin can only be released from the membrane upon treatment with detergents. This is due to a lipid anchor of the protein that is similar to those found in bacterial lipoproteins. The physiological function of halocyanin remains unclear; however, the midpoint potential of 183 mV (vs SHE) at pH 7.3 (Brischwein et al., 1993) could place it in the end of the electron transfer chain with a terminal oxidase as the electron acceptor. The membrane anchor would enable halocyanin to interact optimally with partner proteins.

In the present work, we have employed Raman spectroscopic techniques to further elucidate the structure of this protein. Resonance Raman (RR) spectroscopy has widely been used to study the metal center of blue copper proteins [for a review, see Woodruff et al. (1988)]. Choosing an excitation line close to the S(Cys) → Cu$^{2+}$ charge-transfer transition at ca. 600 nm, a selective enhancement of modes involving copper-ligand vibrations is achieved. Hence, this technique holds promise to contribute to the structural analysis of the copper center in halocyanin. In particular, we were interested to investigate possible pH-dependent changes in the active site which may be associated with the pronounced pH dependence of the midpoint potential observed by Brischwein et al. (1993). In

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[1] Abbreviations: FT, Fourier transform; IR, infrared; RR, resonance Raman.

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addition to the conventional RR spectroscopic approach, we have also employed the near-infrared Fourier transform (FT) Raman technique (Schrader et al., 1990). This method, which for the first time has been applied to blue copper proteins, provides complementary information about the protein structure by probing the vibrational modes of both the copper center and the apoprotein.

## MATERIALS AND METHODS

*Materials.* Halocyanin was isolated and purified as described previously (Scharf & Engelhard, 1993). The protein (82 mg/mL) was solubilized in 1% *n*-octyl glucopyranoside (pH > 6) or 0.1% dodecyl maltoside (pH < 6) using 20 mM phosphate buffer. Hydrogen/deuterium exchange was achieved by repeatedly lyophilizing and resolubilizing the sample with the appropriate $D_2O$ buffer. Reduction of halocyanin was achieved by the addition of appropriate amounts of dithionite and was monitored by the disappearance of the absorption band at 600 nm.

*Methods.* RR spectra were obtained with 568-nm excitation (Krypton ion laser) using a scanning double monochromator. Details of the equipment are described elsewhere (Heibel et al., 1993). The spectra were measured at ambient temperature with 2-cm$^{-1}$ spectral resolution and an increment step width of 0.2 cm$^{-1}$. The sample solution containing about 0.5 mM halocyanin was deposited in a spinning cell, and the power of the laser beam was 200 mW focused onto the sample by a 10-cm lens. No time-dependent changes of the RR spectra were observed during the measurements except for a significant decrease of the background scattering. The integrity of the protein was checked by comparing the individual scans and the absorption spectra before and after the RR experiments. Up to 20 scans were combined to yield the spectra which are displayed in Figures 1–3. Thus, the total accumulation time was up to 20 s/increment.

Near-infrared FT Raman spectra were obtained with 1064-nm excitation using a Bruker IFS 66 spectrometer equipped with the Raman module FRA-106. A four-term Blackman–Harris apodization and a zero-filling factor of 2 were used. The spectral resolution was 4 cm$^{-1}$. The experimental setup is described in detail by Schrader et al. (1990). For these experiments, the protein was lyophilized either in its oxidized or its reduced state and inserted into an open semi-spherical metal cup. The laser power focused onto the sample was about 500 mW. No time-dependent changes in the FT Raman spectra were observed. The total accumulation time was about 4 h for each experiment.

*Spectral Analysis.* From both the RR and the FT Raman spectra, the structureless background was removed by polynomial subtraction. The RR spectra were further analyzed by using a band-fitting program as described by Heibel et al. (1993). The fitting procedure took advantage of the fact that the number of the band components and their approximate positions could be obtained by a careful inspection of the raw spectra. The standard errors of the individual spectral parameters obtained by the band fitting strongly depended on the relative intensities of the bands. For the strong bands (nos. 8, 10, and 13—see Table 1), the errors were ±0.2 cm$^{-1}$ for the frequencies, ±0.6 cm$^{-1}$ for the half-widths, and ±4.5% for the relative intensities. For the medium bands (nos. 7 and 14) and the remaining weak bands, the errors increased by a factor of 2 and 7.5, respectively. In order to reliably determine the spectral changes induced upon H/D exchange or by alteration of the pH (p*D*), the same frequencies and half-widths were used as initial parameters for the band fitting of all the measured spectra and kept constant during the first iteration cycles, allowing only the relative intensities to vary. In subsequent iteration cycles, the half-widths and frequencies were progressively released to optimize the fits (Hildebrandt et al., 1993).

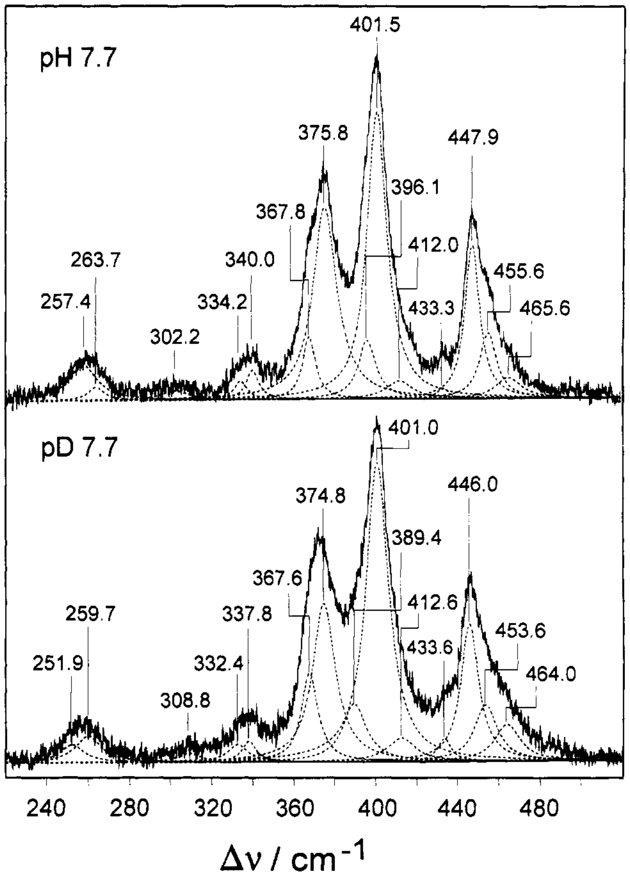

FIGURE 1: RR spectra of the oxidized halocyanin dissolved in $H_2O$ and $D_2O$ at pH (p*D*) 7.7. The excitation line was 568 nm. The dashed lines represent the fitted Lorentzian line shapes.

## RESULTS

*Resonance Raman Spectra.* Halocyanin is a blue copper protein, exhibiting a characteristic absorption band at 600 nm, due to a S(Cys) → $Cu^{2+}$ charge-transfer transition (Sykes, 1991; Solomon et al., 1992). Therefore, excitation of the RR spectrum close to this transition should lead to a preferential enhancement of those RR bands which originate from modes including large contributions from the Cu–S(Cys) stretching vibration (Blair et al., 1985; Han et al., 1991; Dave et al., 1993). As demonstrated in previous studies, the Cu–S stretching vibration is strongly coupled to deformation vibrations of the cysteine side chain, yielding a large number of modes with various contributions from the Cu–S stretch coordinate (Han et al., 1991, 1993; den Blaauwen et al., 1993). The 568-nm excited RR spectra of the oxidized halocyanin at pH 7.7 is displayed in Figure 1. The spectrum is dominated by three strong bands at 375, 401, and 447 cm$^{-1}$, which consequently are assigned to modes exibiting major Cu–S stretching character. These three bands are accompanied by seven somewhat weaker bands in the range between 330 and 470 cm$^{-1}$. Most of them may be due to deformation modes of the Cys side chain with minor admixtures of the Cu–S stretch coordinate. Such an interpretation is based on previous studies by Blair et al. (1985), who suggested a proportionality

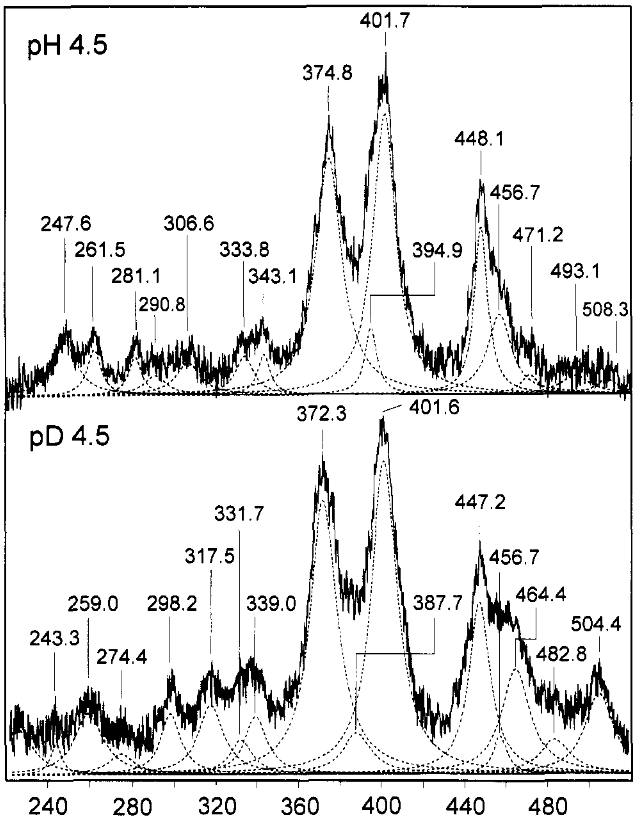

FIGURE 2: RR spectra of the oxidized halocyanin dissolved in $H_2O$ and $D_2O$ at pH (p*D*) 4.5. The excitation line was 568 nm. The dashed lines represent the fitted Lorentzian line shapes.

between the RR intensity and the degree of Cu–S stretch character of a mode. Support for this idea comes from a recent RR study of azurin mutants with isotopically labeled sulfur (Dave et al., 1993), although excited-state displacements of the coupled internal coordinates of the Cys may also contribute to the RR intensity (Han et al., 1991).

The RR bands of halocyanin in the region between 330 and 470 $cm^{-1}$ exhibit only small, if any, frequency shifts ($\leq 2$ $cm^{-1}$) in $D_2O$.[2] These shifts can readily be attributed to effects of H/D exchange on the strength of the hydrogen-bonding network and may reflect a structural alteration in the Cu environment (Han et al., 1991; Hildebrandt et al., 1993).

The band doublet at ca. 260 $cm^{-1}$ (Figure 1) is attributed to modes predominantly including the Cu–N(His) stretching (Nestor et al., 1984, 1986; Woodruff et al., 1984). The relatively large downshift upon H/D exchange should mainly be due to the isotopic mass effect although contribution from modified hydrogen-bonding interactions cannot be ruled out.

The RR spectra of halocyanin vary with the pH. Between 7.7 and 6.2, these variations are relatively small (Table 1). Above 300 $cm^{-1}$, frequencies and the H/D-dependent shifts are constant within 1 $cm^{-1}$. The larger deviations for the bands at 340, 412, and 465 $cm^{-1}$ may reflect the lower accuracy of the frequency determination due to the weak intensity of these components. Additionally, the half-widths and the relative intensities do not differ significantly. Below 300 $cm^{-1}$, we note a further splitting of the bands at ca. 250 $cm^{-1}$, leading to a new but relatively weak component at 246.6 $cm^{-1}$ at pH 6.2.

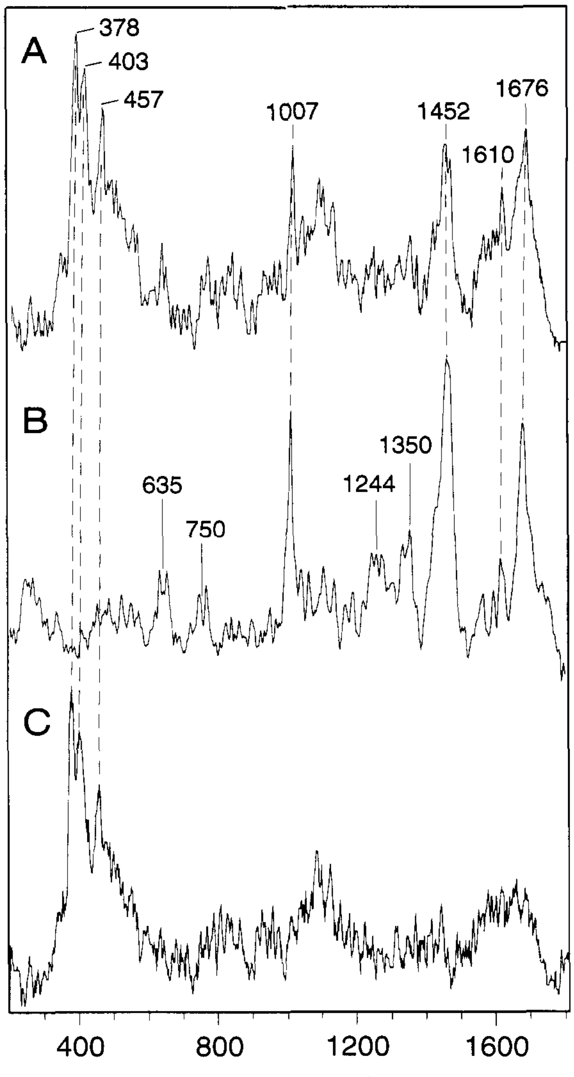

FIGURE 3: Fourier transform Raman spectra of the oxidized (A) and the reduced halocyanin (B). C represents the difference spectrum (oxidized minus reduced). The excitation line was 1064 nm.

Table 1: RR Band Frequencies (Given in $cm^{-1}$) of Oxidized Halocyanin at Different pH (p*D*)

| band no. | pH 7.7 (p*D* 7.7) | pH 6.2 (p*D* 6.2) | pH 4.5 (p*D* 4.5) |
|---|---|---|---|
| 1 | | 246.6 (249.7) | 247.6 (243.3) |
| | 257.4 (251.9) | 257.3 | |
| 2 | 263.7 (259.7) | 262.9 (259.0) | 261.5 (259.0) |
| | | | 281.1 (274.4) |
| 3 | | (296.5) | 290.8 (298.2) |
| 4 | 302.2 (308.8) | 300.0 (310.6) | 306.6 (317.5) |
| 5 | 334.2 (332.4) | 333.9 (332.3) | 333.8 (331.7) |
| 6 | 340.0 (337.8) | 341.2 (339.4) | 343.1 (339.0) |
| 7 | 367.8 (367.6) | 367.7 (368.1) | |
| 8 | 375.8 (374.8) | 375.4 (374.6) | 374.8 (372.3) |
| 9 | 396.1 (389.4) | 395.4 (389.2) | 394.9 (387.7) |
| 10 | 401.5 (401.0) | 401.4 (400.9) | 401.7 (401.6) |
| 11 | 412.0 (412.6) | 409.9 (409.1) | |
| 12 | 433.3 (433.6) | 433.5 (433.7) | |
| 13 | 447.9 (446.0) | 447.6 (446.4) | 448.1 (447.2) |
| 14 | 455.6 (453.6) | 455.4 (454.3) | 456.7 (456.7) |
| 15 | 465.6 (464.0) | 466.9 (464.6) | 471.2 (464.4) |

At pH 4.5, the spectral changes are clearly more pronounced. Figure 2 shows the RR spectra of halocyanin at pH 4.5 (p*D* 4.5). The Cu–N(His) stretching region now displays two clearly separated bands at 261.5 and 247.6 $cm^{-1}$. While the former is at a position very similar to the high-frequency

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[2] The band fitting reveals a larger isotopic shift for the 396-$cm^{-1}$ band (Table 1). However, the accuracy of the frequency determination for this band is too low to allow for a detailed discussion of this effect.

component of the doublet at neutral pH, the latter is significantly downshifted with respect to the low-frequency component (257.4 cm$^{-1}$ at pH 7.7). In addition, there are two further peaks at 281.1 and 290.8 cm$^{-1}$ which fall within the range of Cu–N(His) stretching vibrations and which have no counterparts in the RR spectrum of halocyanin at neutral pH. The spectral region above 300 cm$^{-1}$ reveals a different behavior. We note frequency shifts only for the bands at 340, 396, and 465 cm$^{-1}$, whereby the latter shift is less certain due to the low intensity at pH 4.5. The frequencies of the strongest bands remain largely unchanged.

In the entire frequency range, there are distinct intensity changes at pH 4.5. Taking the 401-cm$^{-1}$ band as a reference, the intensities of most of the bands increase upon lowering the pH from 7.7 to 4.5. The only exceptions are the bands at 367, 412, and 433 cm$^{-1}$ which are already weak at neutral pH and cannot be detected anymore at low pH.

*FT Raman Spectra.* The FT Raman spectra of the oxidized and the reduced halocyanin are displayed in Figure 3. In the oxidized state, the FT Raman spectrum displays three prominent bands at 378, 403, and 457 cm$^{-1}$ in the low-frequency region which are readily attributed to the Cu–S stretch containing modes. This implies that the energy of the 1064-nm excitation line is still sufficient to provide an appreciable resonance enhancement of the metal–ligand modes. Although a detailed spectral analysis is not possible due to the poor S/N ratio, it is evident that the relative intensities of these three bands are quite different from those under rigorous resonance enhancement. The intensity ratio of the 403- and 378-cm$^{-1}$ bands (band nos. 8 and 10, see Table 1) is reversed, and the 457-cm$^{-1}$ band, which exhibits only a weak intensity at 568-nm excitation, has gained considerable intensity upon near-infrared excitation. It may be that in the latter case low-energy electronic transitions contribute to the resonance enhancement and, thus, affect the relative band intensities of these modes. In addition to these low-frequency bands, there are a number of prominent bands in the region between 600 and 1800 cm$^{-1}$ that can readily be identified as (nonresonance) Raman bands of the apoprotein.

In the reduced state, where due to the lack of a S → Cu charge-transfer transition no resonance enhancement of the Cu-ligand vibrations can occur, only the protein bands are observed in the FT Raman spectrum. According to Mattar et al. (1994), halocyanin contains eight phenylalanine, three tyrosine, and two tryptophan residues. These aromatic amino acid residues are known to exhibit the largest Raman cross sections (Lord & Yu, 1970). In fact, the strong band at 1007 cm$^{-1}$ is assigned to the Phe mode F1, which is known to be the most intense Raman mode of Phe under nonresonance conditions (Lord & Yu, 1970; Harada & Takeuchi, 1986). Also other bands in the spectrum such as those at 635, 750, 1350, and 1610 cm$^{-1}$ can be assigned to normal modes of the aromatic amino acids (Harada & Takeuchi, 1986). In addition, the strong band at ca. 1452 cm$^{-1}$ is assigned to C–H deformation modes, originating from all amino acid residues (Lord & Yu, 1970).

Raman-active modes of the peptide backbone are expected in the region between 1240 and 1300 cm$^{-1}$ (amide III) and between 1640 and 1700 cm$^{-1}$ (amide I, Figure 4) (Tu, 1986). While the former region is only poorly structured in both the oxidized and reduced halocyanin so that the identification of individual bands is difficult, the amide I region displays a sharp peak with a maximum at 1676 cm$^{-1}$ in both oxidation states.

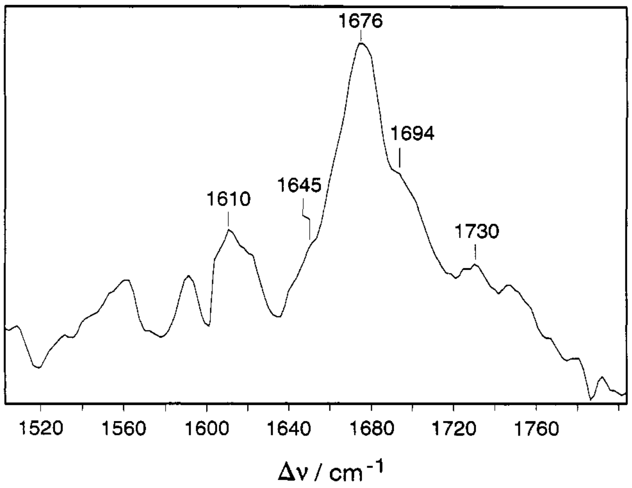

FIGURE 4: Expanded view of the Fourier transform Raman spectrum of the reduced halocyanin (see Figure 3B) in the region of the amide I bands.

## DISCUSSION

*Secondary Structure.* The amide modes of proteins appear at characteristic frequencies depending on the secondary structure of the polypeptide backbone (Tu, 1986). The amide I mode is at ca. 1645 cm$^{-1}$ in $\alpha$-helices, at ca. 1655 cm$^{-1}$ in random coils, and above 1670 cm$^{-1}$ in $\beta$-sheet structures. The FT Raman spectra of both the oxidized and the reduced halocyanin reveal an intense peak at ca. 1676 cm$^{-1}$, indicating a predominantly $\beta$-sheet structure of the protein. On the low-frequency side of this peak, there may be a weak shoulder at 1645 cm$^{-1}$ that would correspond to the frequency characteristic for an $\alpha$-helix structure (Figure 4). However, the intensity of this feature is within the noise level so that only an upper limit of about 5% for a possible $\alpha$-helix content can be given. These results agree very well with those obtained by the analysis of the UV CD spectra (Scharf & Engelhard, 1993). A high $\beta$-sheet content is typical for blue copper proteins (Sykes, 1991). The FT Raman spectra reveal no change of the secondary structure upon changing the oxidation state. The difference spectrum "oxidized minus reduced" as displayed in Figure 3C does not exhibit any distinct positive or negative peaks above the noise level except for the positive peaks of the Cu center in the low-frequency region. These observations are in line with those obtained by the significantly more sensitive IR difference technique, which revealed only minor changes in the amide I region (Brischwein et al., 1993).

The 1676-cm$^{-1}$ band of the reduced halocyanin exhibits a shoulder on the high-frequency side at 1694 cm$^{-1}$. We have no unambiguous assignment for this band. It is at the same position as an IR signal observed in the oxidized minus reduced difference spectrum of halocyanin by Brischwein et al. (1993). These authors tentatively assigned this difference band, which is also found in other blue copper proteins, to the C═O stretching of a conserved Asn (Gln) residue which in azurin is located at 5.7 Å away from the Cu. However, if the same assignment were valid for the 1694-cm$^{-1}$ shoulder in the FT Raman spectrum (Figure 4), it would imply an unusual enhancement of this band by a factor of about 20 relative to the other amide I modes.

The above results have shown that FT Raman spectroscopy is appropriate to probe the Raman bands of the apoprotein, in particular, those of the aromatic amino acids and the amide I, which in the case of an improved S/N ratio would provide further structural data about blue copper proteins, complementary to those obtained by RR and IR spectroscopy.

*Structure of Copper Center.* An unique feature of type I blue copper proteins is the trigonal (equatorial) coordination environment of the Cu ion involving two histidines and one cysteine, which is essentially the same for all members of this protein family (Sykes, 1991). Within the resolution of the available X-ray structures, the Cu–Cys and Cu–His bond lengths and bond angles are identical (Han et al., 1991, and references cited therein). Contrary to these data, the RR spectra of the various Cu proteins differ significantly both with respect to band frequencies and intensities, implying that the RR spectra constitute a characteristic fingerprint for conformational differences of the copper centers beyond the resolution of the X-ray structures.

Comparing the RR spectra of various Cu proteins (Miskowski et al., 1975; Siiman et al., 1976; Woodruff et al., 1984; Nestor et al., 1984, 1986; Blair et al., 1985; Han et al., 1991, 1993; McManus et al., 1992; den Blaauwen et al., 1993; Dave et al., 1993), the closest resemblance in the region between 300 and 450 cm$^{-1}$ is noted between halocyanin and azurin. While the intensity pattern is quite similar for both species, the frequencies differ by up to 10 cm$^{-1}$. Such an agreement is not found for halocyanin and other Cu proteins. The only obvious structural pecularity in the Cu center of azurin compared to other Cu proteins is the axial coordination (Sykes, 1991). The methionine ligand is at a considerably longer distance from the Cu ion, presumably due to the additional weak coordination by a carbonyl oxygen at the second axial site. In fact, it was shown that the distortion of the axial ligation pattern by the replacement of the Met ligand by glutamine, which binds considerably stronger to the Cu ion, has a pronounced effect on the RR band intensities and, to a minor extent, on the frequencies (den Blaauwen et al., 1993). These changes in the RR spectrum could convincingly be ascribed to an alteration of the coupling between the Cu–S(Cys) and Cys deformation modes and at a slight increase of the Cu–S bond length, which is far beyond the resolution of the X-ray structure. Thus, we conclude that the good agreement between the RR spectra of halocyanin and azurin indicate far-reaching similarities of the coordination geometry of the Cu centers, including a comparably long Cu–Met distance and an additional Cu–carbonyl interaction. It should be noted that the IR oxidized minus reduced difference spectra exhibit positive and negative peaks between 1630 and 1650 cm$^{-1}$ that have been attributed to a peptide carbonyl ligated to the copper ion at the (second) axial coordination site similar to azurin (Brischwein et al., 1993).

In contrast to the modes involving the Cu–S stretching, the Cu–N(His) modes of halocyanin differ from those of azurin. The RR spectrum of halocyanin reveals a closely spaced doublet at 260 cm$^{-1}$ that is at a similar position as the (only partially resolved) peaks in the RR spectra of other blue copper proteins such as plastocyanin or pseudoazurin (Han et al., 1991). The doublet structure of this peak in halocyanin and the comparable H/D isotopic shifts for each component point to two Cu–N stretching modes. In azurins from various species, two well-separated bands are observed in this region (Blair et al., 1985). For azurin from *Pseudomonas aeruginosa*, the higher frequency component has unambiguously been assigned to an almost pure Cu–N stretching mode of His-117 based on the results from azurin mutants with substitution of the histidine ligands (den Blaauwen et al., 1993). Presumably, the lower frequency band at 266 cm$^{-1}$, which is close to the doublet observed in halocyanin, originates from the Cu–N stretch of the second histidine ligand (His-46). This relatively large splitting points to different Cu–N bond strengths for both ligands which may be due to stronger hydrogen-bonding interactions of His-117 with adjacent amino acids. In halocyanin, however, both histidine ligands are structurally similar at neutral pH as judged from the closely spaced doublet.

*pH-Dependent Conformational Changes.* The most pronounced spectral changes at low pH are observed in the spectral region below 330 cm$^{-1}$. This clearly rules out a tetragonal distortion of the coordination geometry due to the dissociation of one histidine ligand as it is observed, for example, in plastocyanin (Sykes, 1991). Such a distortion is expected to affect the Cu–Cys stretching region significantly, which is not observed for halocyanin. On the other hand, the significant downshift of the lower frequency component of the Cu–N stretching doublet in halocyanin from 257.4 to 247.6 cm$^{-1}$ indicates that one of the histidine ligands is involved in the acid-induced conformational changes at pH 4.5 while the other histidine is essentially unaffected as judged from the much smaller frequency shift of the high-frequency component. It is well known that the frequency of a metal–histidine stretching strongly depends on the electron density distribution within the imidazole ring, which in turn is controlled by hydrogen-bonding interactions with the protein environment (Smulevich et al., 1988). Strong hydrogen bonding of the N(His)–H group with negatively charged amino acid side chains would increase the electron density in the imidazole ring and, hence, strengthen the Cu–N(His) bond. On the other hand, the rupture of such hydrogen-bonding interactions would cause a weakening of the metal–ligand bond, which is reflected by a downshift of the stretching frequency. Thus, the significant frequency lowering of the 257-cm$^{-1}$ band at pH 4.5 is interpreted in terms of the protonation of a nearby amino acid residue (Asp, Glu) which, in the deprotonated state, acts as a potent hydrogen bond acceptor for the N–H group of one of the histidine ligands. In fact, IR difference spectra have revealed a carboxyl group which, at low pH, is protonated in the oxidized but deprotonated in the reduced state (Brischwein et al., 1993). Based on this redox-linked (de)protonation process, it was suggested that this amino acid side chain may be located in close vicinity to the copper center. Thus, this residue is a likely candidate for the hydrogen bond acceptor interacting with one of the histidine ligands.

However, one should keep in mind that acidification of the solution down to pH 4.5 may also lead to a loosening of the protein structure, causing a conformational heterogeneity. There are several RR bands between 330 and 270 cm$^{-1}$ and between 480 and 520 cm$^{-1}$ in the spectra at pH 4.5 and, even more pronounced, at p*D* 4.5 which have no counterparts in the spectra of the neutral form.

The redox potential of halocyanin varies considerably with the pH, ranging from +0.333 V (vs SHE) at pH 4 to +0.119 V at pH 10 (Brischwein et al., 1993). The decrease of the potential is nearly as steep between pH 4.5 and 6.2 as between pH 6.2 and 7.7. Thus, the pH dependence of the redox potential between pH 6.2 and 7.7 is not correlated with the pH-dependent structural changes which are visible in the RR spectra. This implies that the redox potential is not exclusively governed by the geometry of the coordination sphere of the copper ion. Moreover, the present results suggest that other parameters such as the hydrophobicity of the solvent accessibility of the active site may also sensitively control the redox potential [cf. Kassner (1972)].

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