Alkaline Transition of *Rhus vernicifera* Stellacyanin, an Unusual Blue Copper Protein†

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ABSTRACT: Stellacyanin from *Rhus vernificera* is a blue copper protein in which the metal is coordinated to a Cys, two His, and a Gln residue. It displays a low redox potential, a fast electron exchange rate, and a reversible alkaline transition. We have studied this transition in Cu(II)- and Co(II)-stellacyanin by means of electronic and NMR spectroscopy. The data indicate that a conformational rearrangement of the metal site occurs at high pH. A drastic alteration in the Gln coordination mode, as initially proposed, is discarded. These results show that the metal site in stellacyanin is more flexible than the sites of other blue copper proteins. The present study demonstrates that the paramagnetic shifts of the bound Cys in the Co(II) derivative are sensitive indicators of the electron delocalization and conformational changes experienced by this residue.

Blue copper proteins are ubiquitous electron transfer proteins, which are able to accommodate the metal ion in either Cu(II) or Cu(I) oxidation states (Sykes, 1991; Adman, 1991; Solomon *et al.*, 1992; Canters & Gilardi, 1993; Malmström, 1994). These proteins are characterized by an intense absorption at 600 nm, a low $A_{\|}$ copper hyperfine coupling constant, and unusually high redox potentials. The copper is bound to one cysteine and two histidine residues in a nearly trigonal-planar array, and a weakly coordinated axial methionine is the fourth axial ligand in most of these proteins (Figure 1A). Stellacyanins are an exception, since they bear a Gln as axial ligand (Figure 1B) (Vila & Fernández, 1996; Hart *et al.*, 1996). In particular, St[1] from the lacquer tree *Rhus vernicifera* has been considered an outlier among blue copper proteins owing to its low redox potential and particular spectroscopic features (Peisach *et al.*, 1967; Reinhammar, 1970). However, recent convincing evidence of a certain ubiquity of related proteins has been accumulated. The most outstanding example is cucumber St (Nersissian *et al.*, 1996). The recently reported sequences of umecyanin from horseradish roots (Van Driessche *et al.*, 1995) and mavicyanin from *Cucurbita pepo* (Schininà *et al.*, 1996) indicate that these proteins also belong to the same family. In addition, gene sequences coding for proteins displaying a high degree of similarity with *Rv* St have been characterized in a negatively light-regulated gene product from *Arabidopsis thaliana* (van Gysel *et al.*, 1993) and loblolly pine root seedlings subjected to water deficit (Chang *et al.*, 1996). It has recently been proposed that stellacyanins constitute a subclass of plant blue copper proteins (Nersissian *et al.*, 1996) characterized by being glycoproteins with particular spectroscopic features, exposed metal sites, and a Gln axial ligand. Their biological function is still unclear.

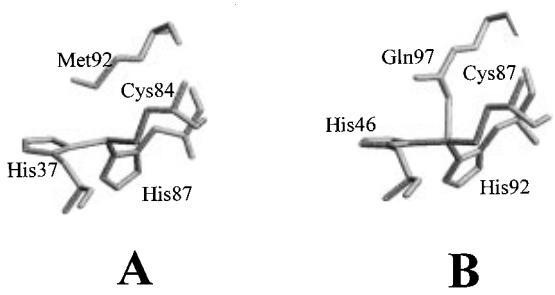

FIGURE 1: Schematic representation of the metal sites of (A) poplar plastocyanin, from Guss *et al.* (1986), and (B) cucumber stellacyanin, from Hart *et al.* (1996). The pictures were drawn with RasMol v2.6 (Sayle, 1994), Greenford, Middlesex, U.K.).

Another feature of *Rv* and cucumber Sts as well as umecyanin is that they display a reversible transition at alkaline pH characterized by a blue shift of the charge transfer bands in the visible spectrum and an alteration of the EPR spectrum (Peisach *et al.*, 1967; Paul & Stigbrand, 1970; Stigbrand & Sjoholm, 1972; Nersissian *et al.*, 1996) The structural implications of this transition have been a matter of debate (Fields et al., 1991; Strange *et al.*, 1995; Van Driessche *et al.*, 1995). A pulsed ENDOR study (Thomann *et al.*, 1991) has revealed the existence of an additional nitrogen nucleus strongly coupled to the metal center at pH 11. This fact has been interpreted as an evidence of the coordination of the Gln ligand through the nitrogen atom, in contrast to the expected oxygen-mediated coordination at neutral and acidic pH values. However, this

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[1] Abbreviations: ϵ, extinction coefficient; CBP, cucumber basic protein; EDTA, ethylenediaminetetraacetic acid; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; LMCT, ligand-to-metal charge transfer; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; *Rv*, *Rhus vernicifera*; St, stellacyanin; WEFT, water-enhanced Fourier transform.

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transition is also displayed by CBP (also known as cucumber plantacyanin), in which a Met residue is the axial ligand (Sakurai *et al.*, 1982; Nersissian & Nalbandyan, 1988).

Paramagnetic NMR is a helpful spectroscopic tool for characterizing metal centers in proteins (Bertini *et al.*, 1993). Cu(II) is paramagnetic but displays slow electron relaxation rates, which make it not suitable for NMR spectroscopy (Bertini & Luchinat, 1996). However, ^{1}H NMR spectra can be recorded in Cu(II) blue copper proteins since the electron relaxation times are shortened owing to the existence of low-lying excited states that arise from the strained nature of the blue sites (Kalverda *et al.*, 1996). In spite of this, NMR lines are still broad. In these cases, metal substitution can be useful for probing the metal site. When Co(II) replaces Cu(II), the NMR signals of nuclei belonging to the metal ligands display narrower lines and can be better detected and assigned (Bertini *et al.*, 1993). This strategy has successfully been applied to Co(II)-substituted wild-type (Moratal Mascarell *et al.*, 1993; Salgado *et al.*, 1995) and mutant azurins (Piccioli *et al.*, 1995; Salgado *et al.*, 1996), as well as to *Rv* St (Vila, 1994; Vila & Fernández, 1996). We have studied the high-pH transition in Cu(II)- and Co(II) *Rv* St by means of electronic and paramagnetic NMR spectroscopy in an attempt to draw a structural interpretation of it.

EXPERIMENTAL PROCEDURES

Stellacyanin from *Rhus vernicifera* was obtained as previously reported (Reinhammar, 1970). The apoprotein was prepared by dialysis against thiourea, followed by dialysis against 100 mM sodium phosphate at pH 6.0 (Blaszak *et al.*, 1983). The Co(II) derivative was prepared by addition of a 4-fold excess of cobalt chloride to a buffered solution of apoprotein, followed by dialysis against EDTA to remove the excess metal ion. The metal uptake was monitored by optical spectroscopy, and the Co(II) derivative yielded an electronic spectrum similar to the one previously reported (McMillin *et al.*, 1974). The electronic spectra were recorded in Gilford Response II and LKB Ultraspec 2 spectrophotometers. The concentrated samples for NMR experiments were obtained using Centricon-10 (Amicon) concentrator units. The D_2O solutions were prepared by dissolving in deuterium oxide the lyophilized protein.

The NMR spectra were recorded on Bruker ACE 200, MSL 300, and AMX 500 spectrometers operating at proton frequencies of 200.13, 300.13, and 500.13 MHz, respectively. All chemical shifts were referenced to the chemical shift of water at the appropriate temperature, which in turn was calibrated against internal DSS. 1D experiments were performed using the superWEFT pulse sequence (180°-τ-90°) (Inubushi & Becker, 1983) or by presaturating the water resonance. Different delays (τ) were used in the superWEFT sequence to optimize the detection of the fastest relaxing signals. NOEs were performed by using a modified superWEFT sequence irradiating during the intermediate delay (Banci *et al.*, 1989).

RESULTS

Cu(II) Stellacyanin. The visible spectrum of St displays two intense thiolate-to-copper LMCT bands at 450 and 604 nm. These bands blue-shift at alkaline pH with a pK_a of 10.2 (Figure 2), as early reported (Peisach *et al.*, 1967). This transition is reversible up to pH 12. The $\epsilon_{450}/\epsilon_{600}$ ratio falls at high pH (Figure 2).

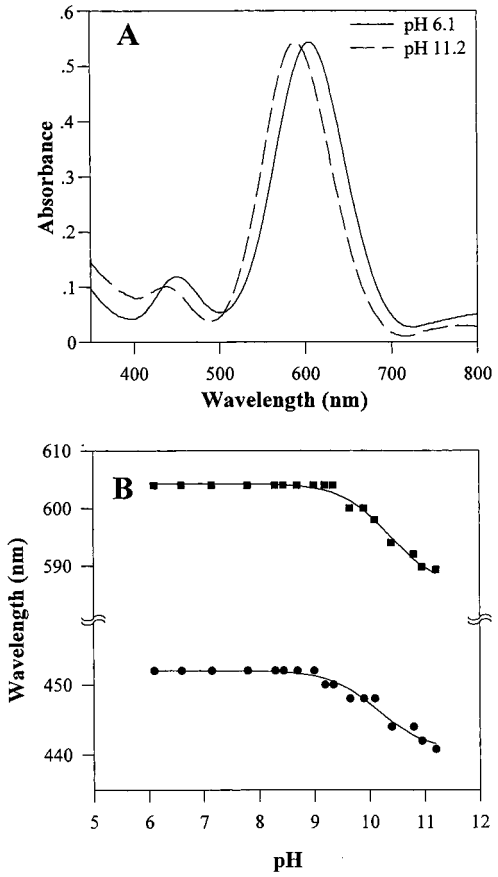

FIGURE 2: (A) Electronic spectra of *Rv* Stellacyanin at pH 6.1 and 11.2. (B) pH dependence of the charge transfer bands.

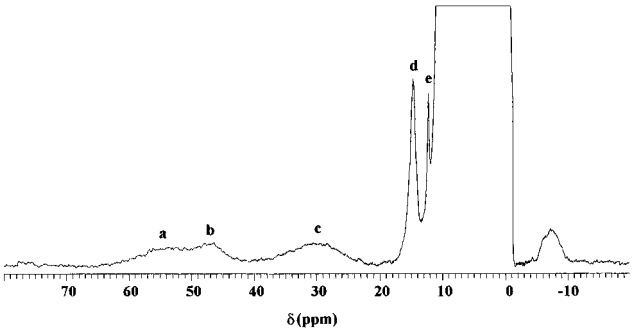

FIGURE 3: ^{1}H NMR 500 MHz spectrum (302 K) of *Rv* Cu(II) St in H_2O at pH 7.

The paramagnetic ^{1}H NMR spectrum of Cu(II)St is reported in Figure 3. The observed resonances are expected to arise from protons belonging to copper ligands located at $r_{CuH} > 5$ Å (Kalverda *et al.*, 1996). The ^{1}H NMR paramagnetic signals have unequivocally been assigned for Cu(II) amicyanin (Kalverda *et al.*, 1996). The high degree of similarity between the present spectrum and the one of amicyanin allows us to assign it on a comparative basis.

Three broad signals a−c are found at $\delta > 20$ ppm. Two of them are expected to arise from nonexchangeable His protons four bonds away from the metal ion. Since signals a and c are present in spectra recorded in the whole pH range and in D_2O solution, we assign them as the His Hδ2s. The Hϵ2 His resonances are expected to fall in the same shift range. However, we do not expect to observe these signals owing to the high degree of solvent accessibility of the metal site (Vila, 1994; Vila & Fernández, 1996). Signal b (detected only at pH < 8) may be attributed to a Hϵ2 belonging to the axial Gln ligand.

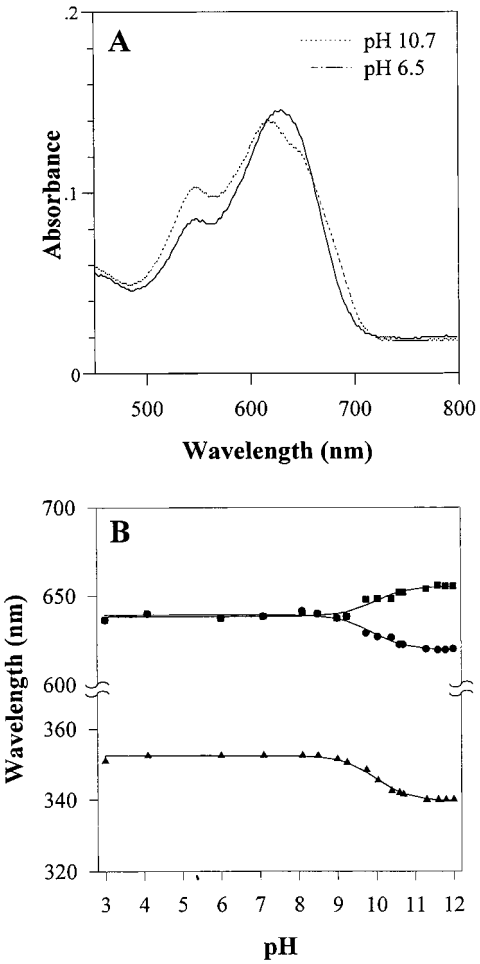

FIGURE 4: (A) Electronic spectra of *Rv* Co(II)-stellacyanin at pH 6.5 and 10.7. (B) pH dependence of the charge transfer and ligand field bands.

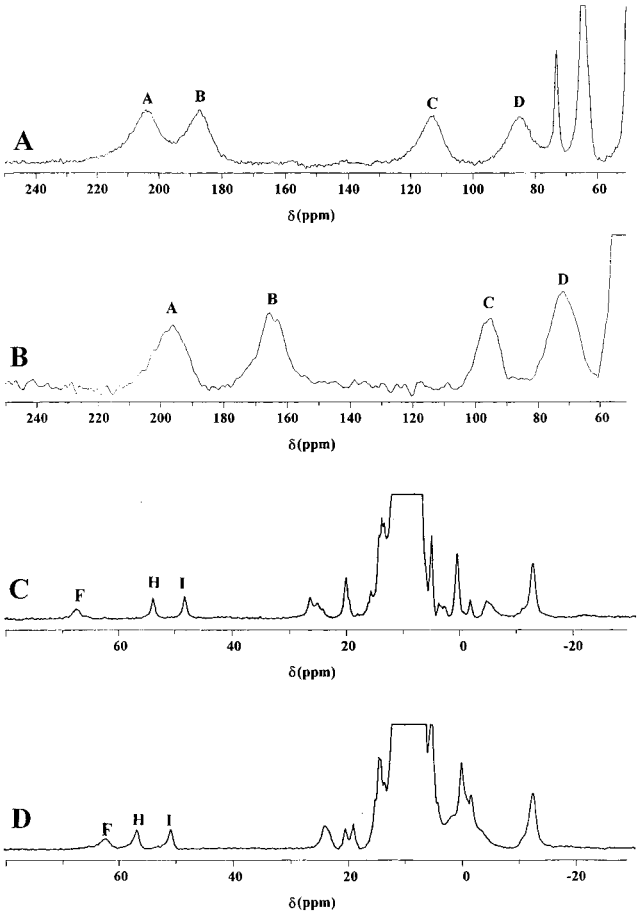

FIGURE 5: ^{1}H NMR spectra (313 K, D_2O) of Co(II) St: At 200 MHz, enlarged views of the most downfield-shifted signals recorded at (A) pH 4.0 and (B) pH 11.3 are shown. At 300 MHz, the 80/−30 ppm region recorded at (C) pH 4.0 and (D) pH 11.0 is shown. The signal lettering is taken from Vila and Fernández (1996).

We assign resonance e to the Hα of Cys 87 and the composite signal d to the two Hγs of Gln 97, after having compared the present spectrum to the spectra of other blue copper proteins (Kalverda *et al.*, 1996). The spectrum is insensitive to pH changes in the pH 5−8 range. Both signals d and e experience upfield shifts at high pH (e moves from 12.1 to 11.9 at pH 11, whereas signal d shifts from 14.6 to 13.2 ppm).

Co(II) Stellacyanin. The electronic spectrum of Co(II)-substituted St displays an intense thiolate-to-cobalt(II) LMCT band at 350 nm and Laporté-forbidden ligand field transitions in the visible range (McMillin *et al.*, 1974). The charge transfer band is blue-shifted at high pH. The intensity of the ligand field transitions in the visible spectrum does not change at alkaline pH, and a splitting of the 640 nm band is observed, as shown in Figure 4A. The pK_a of this transition is 10.1 (Figure 4B).

The paramagnetic ^{1}H NMR spectrum of Co(II) St shows 20 signals shifted from their diamagnetic positions spanning from 210 to −60 ppm. The resonances corresponding to the metal ligands have been recently assigned (Vila, 1994; Vila & Fernández, 1996). The NMR spectrum is not substantially altered in the pH 4−9 range, but major changes do occur at higher pH values (Figure 5). The broad resonances A and B corresponding to the β-CH_2 protons of Cys 87 shift upfield at high pH. It is interesting to note that signal B experiences a larger shift than signal A does. This results in a larger frequency separation between signals A and B at alkaline pH. The His resonances experience shifts in different directions: the Hδ2 resonances H and I are downfield-shifted at alkaline pH, whereas the broad His signals C and D (Hε1s) shift upfield (*cf.* Figure 5). Resonance F, which stands for a proton belonging to the axial Gln ligand, is also affected. Signal F displays two transitions with different pK_as: the first one characterized by a small downfield shift (pK_a ∼7) and a larger upfield one occurring at alkaline pH. Since the signal corresponding to the geminal proton of F (signal q) is buried within other signals in the upfield region of the spectrum (Vila & Fernández, 1996), we were able to monitor this resonance by irradiating F at different pH values (Figure 6). Fitting the pH-induced shift changes of these resonances (A−D, F, H, I, and q) to a common sigmoideal equation yields a pK_a = 10.2 for this transition.

DISCUSSION

Analysis of Spectroscopic Data. The cysteine-copper interaction in St gives rise to two LMCT bands at 450 and 604 nm, which are altered at high pH. The intensity ratio of the LMCT bands in blue copper proteins has been correlated with the strength of the axial ligand (Lu *et al.*, 1993). A Gln ligand is able to displace the metal out of the plane defined by the three equatorial ligands (Cys_2His), giving rise to a perturbed type 1 site with respect to azurin and a larger $\epsilon_{450}/\epsilon_{600}$ ratio (Romero *et al.*, 1993; Salgado *et al.*, 1996; Vila & Fernández, 1996; Hart *et al.*, 1996). The purported binding of a deprotonated amide group at high

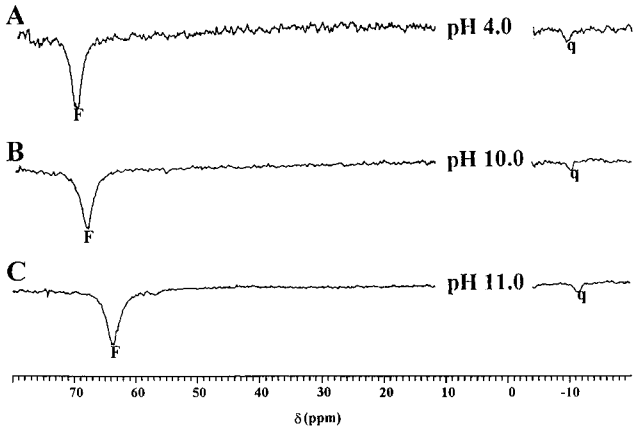

FIGURE 6: NOE experiments on Co(II) St recorded at 313 K and 300 MHz in D_2O solution by saturation of signal F at (A) pH 4.0, (B) pH 10.0, and (C) pH 11.0. An irradiation time of 30 ms was used in all cases.

pH (Thomann *et al.*, 1991) should provide an even stronger axial ligand field and give rise to a larger $\epsilon_{450}/\epsilon_{600}$ ratio, but this does not seem to be the case in St.

The LMCT band at 350 nm in Co(II) St shifts with a similar pK_a value, indicating that both Cu(II) and Co(II) St experience the same transition. The splitting observed in the visible region for Co(II) St suggests that a conformational change is occurring in the active site. However, the unaltered spectral intensity indicates that the flattened tetrahedral geometry is maintained (Bertini & Luchinat, 1985).

The NMR data in the Cu(II) and Co(II) proteins are more revealing. We were able to detect the Cys ^{1}H NMR resonances in both Cu(II) and Co(II) *Rv* St. Upfield shifts were observed at high pH for the β-CH_2 in the Co(II) derivative and for the α-CH in the Cu(II) protein. The paramagnetic shifts are the result of several contributions:

$$\delta_{obs} = \delta_{dia} + \delta_{con} + \delta_{pc} \quad (1)$$

where δ_{dia} is the shift that would be observed in an equivalent diamagnetic molecule, δ_{con} is the Fermi contact shift owing to the unpaired spin density on the nucleus of interest, and δ_{pc} represents the pseudocontact contribution originated in the nucleus−electron dipolar interaction (Bertini & Luchinat, 1996). The latter term has been shown to be small for protons at $r_{CuH} > 5$ Å in blue copper proteins (Kalverda *et al.*, 1996), and its magnitude may be estimated under the point-dipole approximation by means of

$$\delta_{pc} = \left(\frac{\mu_0}{4\pi}\right)\frac{{\mu_B}^2 S(S+1)}{9kT}[{g_{||}}^2 - {g_{\perp}}^2]\left(\frac{3\cos^2\theta - 1}{r^3}\right) \quad (2)$$

where μ_0 is the magnetic permeability in vacuum, μ_B is the electron Bohr magneton, S is the electron spin, k is the Boltzmann constant, $g_{||}$ and $g_\perp$ are the parallel and perpendicular components of the **g** tensor, and θ is the angle between the magnetic z axis and the metal−proton vector **r**. We have calculated approximate δ_{pc} and δ_{con} values for the Cys and Gln proton resonances from eqs 1 and 2 for *Rv* St (Table 1). Since the available ligand field calculations (Gewirth *et al.*, 1987) only predict the orientation of the magnetic z axis, we have assumed an axially symmetric **g** tensor. In the case of St, this approximation introduces an error < 5% in the calculated δ_{pc} values.

In the case of wt azurin the proton Met resonances are not shifted outside the diamagnetic envelope, whereas in amicyanin (with a shorter Cu−S_δ Met bond) there is some spin density delocalized onto this residue (Kalverda *et al.*, 1996). The estimated δ_{con} for St indicates a more covalent Cu−Gln bond in St compared to the Cu−Met bond in amicyanin (*cf.* Table 1). Instead, a decreasing trend is observed for the Hα Cys contact shifts, thus indicating a concomitant reduction of the Cu(II)−Cys covalence. This is a direct proof of the interplay between the axial ligand and the Cys residue in tuning the electronic structure of blue sites (Guckert *et al.*, 1995). The present data also indicate that δ_{con} is reduced in *ca.* 3% in St at high pH. A weakening of the Cu(II)−Cys bond has also been observed in resonance Raman spectra of the high-pH form of cucumber St (Nersissian *et al.*, 1996).

Table 1: Experimental Shifts and Calculated Contact Shifts for Selected Protons in Amicyanin and Stellacyanin Recorded at 305 K

proton	protein	δ_{obs}	δ_{pc}	δ_{con}
Hα Cys	azurin	18.8[a]	*b*	*b*
Hα Cys	amicyanin	14.1[a]	−1.1[a]	10.7[a]
Hα Cys	stellacyanin (pH 7)	12.1[c]	−0.8[d]	8.2[d]
Hγ Met	azurin	<8.0[a]	*b*	*b*
Hγ Met	amicyanin	12.0/11.1[a]	2.0/1.9[a]	7.3/6.7[a]
Hγ Gln[e]	stellacyanin (pH 7)	14.6[c]	2.2[d]	10.0[d]

[a] From Kalverda *et al.* (1996). [b] Not calculated. [c] The present work. [d] Calculated by using the coordinates from the structure of cucumber St (Hart *et al.*, 1996) and the orientation of the magnetic z axis predicted by ligand field calculations (Gewirth *et al.*, 1987). The diamagnetic shifts were taken from random coil values. [e] The mean value for the two Gln γ protons is reported.

The β-CH_2 Cys signals in Co(II)-substituted blue copper proteins are another probe of the delocalized spin density onto the S_γ−Cys atom (Vila & Fernández, 1996). This is dictated by the following equation (Bertini & Luchinat, 1996):

$$\delta_i = [A\cos^2\theta_i + B]\rho_S = [\cos^2\theta_i + B/A][A\rho_S] \quad (3)$$

where A and B are constants, θ_i is the M−S−C_β−$H_{\beta i}$ dihedral angle (i = 1, 2), and ρ_s is the spin density localized in the coordinating sulfur atom. The different Cys shifts may be attributed to changes in these dihedral angles or to distinct electron spin densities on the sulfur atom. For a given β-CH_2 couple we define, from eq 3

$$\delta_{1/2} = \frac{\delta_1 + \delta_2}{2} = \frac{A\rho_S}{2}[\cos^2\theta_1 + \cos^2\theta_2 + 2B/A] \quad (4)$$

$$\Delta\delta = \delta_1 - \delta_2 = A\rho_S[\cos^2\theta_1 - \cos^2\theta_2] \quad (5)$$

In blue copper proteins, the ($\cos^2\theta_1 + \cos^2\theta_2$) term ranges between 0.5 and 0.63, whereas ($\cos^2\theta_1 - \cos^2\theta_2$) oscillates between 0 and 0.5. The average shift $\delta_{1/2}$ is therefore less sensitive to conformational changes and may then be useful for reflecting the electron delocalization on the Cys ligand. On the other hand, the shift difference ($\Delta\delta$) should be more sensitive to changes in the Cys dihedral angles, even if ρ_S is a weighting factor. In St, $\delta_{1/2}$ shifts from 197 ppm (at pH 4) to 191 ppm (at pH 11.3), whereas $\Delta\delta$ increases from 18 to 30 ppm in the same pH range. Both parameters follow a sigmoidal dependence with pH (not shown), thus indicating that these trends are meaningful. Since the major changes are observed in $\Delta\delta$, we conclude that the Cys conformation is being altered at alkaline pH in St. The NMR data on the Cu(II) protein (*vide supra*) indicate that a minor

change in ρ_S occurs at high pH, thus confirming that the alteration of the Cys dihedral angles is the main factor in the $\Delta\delta$ change at high pH.

The Gln and His ligands experience a conformational change at high pH, as monitored through NMR spectroscopy of Cu(II) and Co(II)St. Recent electron spin echo envelope modulation (ESEEM) studies on cucumber St have suggested a reorientation of the His imidazole rings at high pH (Nersissian *et al.*, 1996). All the herein reported data clearly indicate that the Cu(II) and Co(II) coordination environments in *Rv* St are altered by this transition and that all the four ligands are experiencing a conformational rearrangement.

Comparison with Other Blue Copper Proteins. This transition is absent in Met121Gln azurin (Romero *et al.*, 1993; Salgado *et al.*, 1996). However, it has been found in CBP, in which a Met coordinates the metal ion (Sakurai *et al.*, 1982; Nersissian *et al.*, 1988). These facts, together with the herein discussed data, allow us to discard the possibility that the deprotonated side-chain nitrogen of Gln 97 binds the metal at high pH.

Several authors have suggested that this transition may be triggered by the deprotonation of a Lys adjacent to the Gln ligand (Romero *et al.*, 1993; Van Driessche *et al.*, 1995). Since this Lys residue is also present in Met121Gln azurin, some other reasons may be responsible for this change. A closer look at the crystal structures of blue copper proteins reveals that cucumber St (Hart *et al*., 1996) and CBP (Guss *et al*., 1996) exhibit a common folding topology, which deviates from the typical β-barrel motif. Both proteins possess a higher portion of α-helices than any other blue copper protein. A CD study on CBP (Nersissian *et al.*, 1988) early predicted this observation, and it also revealed that raising the pH results in an increase of the α-helix content. This indicates an overall flexibility of the protein structure, not only restricted to the metal site. We therefore attribute the herein studied alkaline transition to the deprotonation of an exposed residue, which triggers a change in the protein secondary structure. The protein framework should be flexible enough to allow the metal site distortion to occur, without altering its "blue" features. This phenomenon is a common feature of these proteins, generically termed as "phytocyanins" (Rydén & Hunt, 1993). The flexibility of the metal site in native blue copper proteins has been studied in plastocyanin, amicyanin, and pseudoazurin, in which a histidine residue is detached from the metal in the acidic form of the Cu(I) proteins (Guss *et al*., 1986; Lommen & Canters, 1990; Dennison *et al.*, 1994). Stellacyanins and plantacyanins seem to be the only native blue copper proteins that display a conformational equilibrium in the Cu(II) form. It still remains to be established whether or not this flexibility is related to the hitherto unknown biological role of these proteins.

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