Structure of the Metal Site in *Rhus vernicifera* Stellacyanin: A Paramagnetic NMR Study on Its Co(II) Derivative

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Abstract: The ^{1}H NMR spectrum of Co(II)-substituted stellacyanin from *Rhus vernificera* has been recorded and the hyperfine-shifted signals corresponding to metal ligands have been assigned by means of 1D and 2D NOE spectra. Two His and a Cys coordinate the metal ion, and a Gln residue is the axial metal ligand. The β-CH_2 Cys proton shifts reflect a decreased electron delocalization onto this residue when compared to azurin. Both His residues are solvent accessible. This fact could contribute to the low redox potential of this protein. This work provides the first experimental evidence of a Gln coordination to the metal in a native blue copper protein.

Introduction

Blue copper proteins (BCP's) are a paradigm in bioinorganic chemistry, since they represent one of the best examples on how the protein framework is able to tune the redox potential of a highly conserved metal site. Moreover, the copper ion is engaged in a compromise coordination geometry that is able to accommodate both oxidation states. This allows the metal site to act as an efficient redox center.[1−4]

Blue copper proteins are characterized by an intense absorption band around 600 nm, a low $A_{||}$ copper hyperfine coupling constant, and an unusually high redox potential (180−700 mV, compared to 154 mV for the aqueous Cu(II)/Cu(I) couple).[2,3] A plethora of crystallographic data on different blue copper proteins have been gathered after the report of the X-ray structure of plastocyanin in 1978.[5] These data include native,[4] mutants,[6] and metal-substituted proteins.[7−10] They reveal that the copper is bound to one cysteine and two histidines in a nearly trigonal-planar array. An axial methionine is found as a fourth axial ligand with a long Cu−S distance, which may vary between 2.6 and 3.1 Å (Figure 1A). This geometry has been proven by X-ray data for plastocyanin,[5,11] the cucumber basic protein,[12] pseudoazurin,[13,14] and amicyanin.[15] A glycine backbone carbonyl has been found to be also coordinated to the copper ion in azurin at 3.0 Å (Figure 1B).[16]

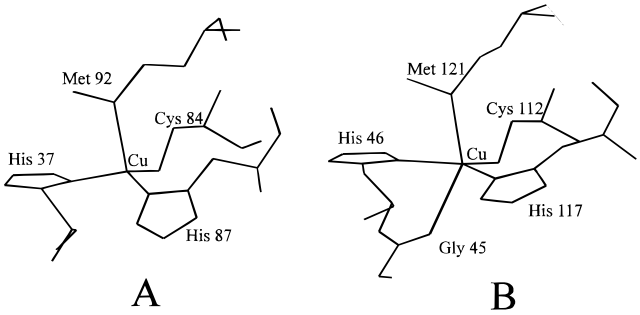

Figure 1. Schematic representations of the metal sites of (A) poplar plastocyanin and (B) *Pseudomonas aeruginosa* azurin. Data were taken from Guss et al.[11] (A) and Nar *et al*.[16] (B).

Stellacyanin (St hereafter), a blue copper protein isolated from the lacquer tree *Rhus vernicifera*,[17,18] is unusual in several respects. Stellacyanin has no methionine residues in its sequence[19] and displays the lowest redox potential in the series,[20] a fast electron exchange rate,[21−23] and particular spectroscopic features.[17,18] The structure of stellacyanin, however, is still

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unknown, since its high carbohydrate content has hitherto thwarted all crystallization attempts. The identity of the fourth ligand replacing methionine has been a matter of debate in the literature for as long as two decades, and considerable efforts have been devoted to spectroscopic and theoretical studies in this direction.

The strong blue absorption of St was taken as evidence of a Cys residue bound to the copper ion.[24,25] The presence of two His residues coordinating the metal was initially suggested by early ESEEM and ENDOR experiments,[26−28] and later confirmed by ^{1}H NMR spectroscopy.[29,30] Three structural models are available for stellacyanin. Two of them suggest that a disulfide bridge may coordinate the copper ion replacing the axial methionine. One of them is a molecular modeling analysis based on a purported homology between St and plastocyanin,[31] whereas the second one relies in a series of earlier assignments on the ^{1}H NMR spectrum of Co(II)St.[32] A more recent modeling analysis based on an earlier amino acid sequence comparison[33] with the cucumber basic protein led Freeman and co-workers to propose that Gln97 may be the elusive axial ligand.[34]

Recent studies have given experimental support to Freeman's proposal. A pulsed ENDOR study by Thomann *et al.* has revealed the existence of an amide coordinated to the metal at pH 11,[35] and new EXAFS results are consistent with the presence of a low-Z atom in the metal coordination sphere.[36] Site-directed mutagenesis studies have been revealing, since the Met121Gln mutant of azurin exhibits spectroscopic characteristics similar to those of St.[6,37,38] Even if there is a general consensus accepting that Gln97 may be the axial ligand in St, there is no direct spectroscopic evidences of it.

Metal substitution is a helpful tool for probing metal sites. In particular, when Co(II) and Ni(II) replace copper, the NMR signals of nuclei belonging to the metal ligands display considerably large shifts and line widths but can still be detected and assigned.[39,40] *Inter alia*, this strategy has proven to be fruitful for the study of wild type azurin and mutants.[38,41−45] In the case of blue copper proteins, the highly strained nature of the metal site makes it suitable to perform these substitutions without inducing substantial structural changes in the protein scaffold.[7−10]

We have recently reported the assignment of the β-CH_2 protons of Cys87 and the imidazole protons of His46 and His92 by means of steady-state NOE experiments in Co(II)St.[46] Here we report a more thorough study of these signals, the assignment of the resonances corresponding to the fourth ligand and its identification.

Experimental Section

Sample Preparation. All the chemicals used were of the best available quality. Stellacyanin from *Rhus vernicifera* was obtained as previously reported[18] from the acetone powder purchased from Saito and Co. (Japan). Purity of the samples was checked by UV−vis spectroscopy[18] and PAGE. The apoprotein was prepared by dialysis against thiourea following the procedure reported by Blaszak *et al.*,[47] followed by dialysis against sodium phosphate (100 mM) at pH 6.0. All these procedures were performed at 5 °C. The Co(II) derivative was prepared by addition of a 4-fold excess of cobalt chloride to a buffered solution of apoprotein at room temperature, followed by dialysis against EDTA to remove the excess metal ion. A standard cobalt solution (Titrisol, Merck) was used for this purpose. The metal uptake was monitored by optical spectroscopy, and the Co(II) derivative yielded an electronic spectrum similar to the one previously reported.[24,48] In a standard procedure, a final yield of 90% of the Co(II) derivative was obtained after 3−4 days at room temperature.

The electronic spectra were recorded in a Gilford Response II spectrometer. The concentrated samples for NMR experiments were obtained using Centricon-10 (Amicon) concentrator units up to a final concentration of *ca.* 4 mM. The D_2O solutions were prepared by dissolving the lyophilized protein in deuterium oxide. The reported pH values measured in D_2O solutions are not corrected for the isotopic effect.

NMR Studies. The NMR spectra were recorded on Bruker ACE 200, MSL 300, and AMX 600 spectrometers operating at proton frequencies of 200, 300 and 600 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 $(180°-\tau-90°)$[49] or by presaturating the water resonance. Different delays (τ) were used in the superWEFT pulse sequence to optimize the detection of the fastest relaxing signals. The chemical shift values reported in the text were taken at 313 K, except when specified. Non-selective T_1 values were determined by means of an inversion-recovery experiment. The T_1 values were calculated from the initial slope of the semilogarithmic plots of the fractional deviation of the z-magnetization from the equilibrium *vs* the intermediate delay τ.[50,51]

The difference steady state and truncated 1D NOE spectra were recorded using the methodology reported by Banci *et al.*[52] Recycle times spanning between 82 and 16 ms were used in most of the cases, with irradiation times ranging from 0.5 to 70 ms. All the NOE and 2D experiments were performed at least at two different temperatures in order to discard accidental signal degeneracies. Interpretation of NOE data in order to retrieve structural information was performed as described in the literature under the two-spin approximation in the slow-

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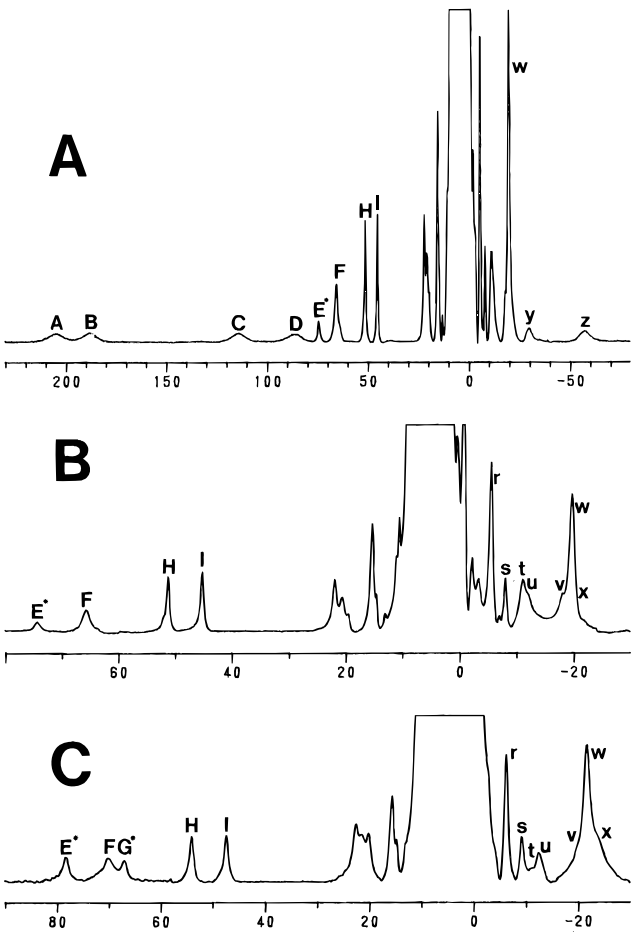

Figure 2. ^{1}H NMR 200-MHz spectra of Co(II)St: (A) spectrum recorded at pH 4.0 and 313 K under fast pulsing conditions; (B) 80 to −30 ppm portion recorded at pH 4.0 and 313 K; (C) 90 to −30 ppm portion recorded at 298 K and pH 4.0. The signals marked with an asterisk are absent when the spectra are recorded in a D_2O solution.

motion limit.[51] Distance data were retrieved from NOE experiments only in those cases where the selective relaxation rates had been determined.

2D NOESY spectra were recorded in the phase-sensitive TPPI mode.[53] In order to detect dipole−dipole connectivities corresponding to the fastest relaxing signals, recycle times of 120 ms and mixing times of 3−5 ms were used. The NMR data sets were processed on a IBM RS/600 workstation using UXNMR software (Bruker). Phase-sensitive 2D NMR data were phase-corrected and base-line leveled in both dimensions.

Results

The ^{1}H NMR Spectrum of Cobalt(II) Stellacyanin. The spectrum of Co(II)St shows at least 20 signals shifted from their diamagnetic positions (Figure 2A,B), spanning from 210 to −60 ppm. Their corresponding shifts, line widths, and T_1 values are reported in Table 1. These spectral features are typical of paramagnetic Co(II) metalloproteins.[39,40] In particular, five of these signals (A−D, z) show extremely large line widths and short T_1 values. All the hyperfine shifted signals show an inverse linear temperature dependence, some of them exhibiting intercepts at $T^{-1} = 0$ outside the diamagnetic region.[54]

Signals E and G, which are absent when the spectra are recorded in D_2O solution, have been previously assigned as the Hϵ2 protons of the metal-bound histidines (Table 1).[46] These

Table 1. ^{1}H NMR Isotropic Shifted Signals at 313 K and pH 4.0

			$\Delta\delta$ (Hz)		
signal	δ (ppm)	T_1 (ms)[a]	200 MHz	600 MHz	assignment
A	206	<0.1	1850		Hβ1 Cys87
B	180	<0.1	1750		Hβ2 Cys87
C	115	<0.1	1400		Hϵ1 His46
D	86	<0.1	1800		Hϵ1 His92
E	74.4	4.3	400	1040	Hϵ2 His46
F	66.2	2.7	380	1450	Hγ1 Gln97
G	63.4[d]	2.5	450		Hϵ2 His92
H	51.4	11.5	220	650	Hδ2 His92
I	45.3	9.6	200	670	Hδ2 His46
p	−1.1	66.0[c]	80	200	Hα Gln97
q	−5.8	7.2[c]	180	800	Hγ2 Gln97
r	−5.9	*b*	200	550	
s	−8.3	*b*	200	400	
t	−11.5	*b*	350	1700	
u	−12.3	*b*	*b*	*b*	
v	−18.6	*b*	400	*b*	Hβ1 Gln97
w	−20.2	4.4	300	1100	
x	−21.8	*b*	*b*	*b*	
y	−29.7	1.2	800		
z	−58	<0.1	1600		

[a] Nonselective relaxation times at 200 MHz, except when specified. [b] Overlap prevented measuring this value. [c] Selective relaxation time, as determined from the NOE buildup experiment. [d] Measured at 303 K.

signals are present in the spectra of H_2O solutions only at low pH values (*cf.* Figures 2B,C). In particular, signal G (Hϵ2 His92) broadens beyond detection above 308 K. This behavior is indicative of a fast exchange of this proton with the bulk solvent even at pH 4. On the other hand, the intensity of signal E (Hϵ2 His46) falls at higher pH values, whereas its line width is reduced when the temperature is raised at acidic pH. This situation has been met also for the Hϵ2 proton of His117 in Co(II) azurin.[43,83] We interpret this as an increase of the exchange rate of this proton with increasing pH due to a general base catalysis mechanism, being in a fast-exchange regime at high pH values.

Except for the His NH signals, the spectrum of Co(II)St is almost pH independent in the 4−9 pH range.[46] This observation seems to be in conflict with data reported by Dahlin *et al.*[32] However, this purported pH dependence of the chemical shifts is significant (*i.e.*, larger than the signal line widths) only for resonance F, which is shifted 0.8 ppm in the 4−9 pH range.[32]

Spectra at different fields were recorded. All the hyperfine shifted resonances broadened with increasing magnetic fields. However, only the broadest resonances (A−D, z) could not be detected at 600 MHz. These line width differences, reported in Table 1, are attributable to the Curie relaxation mechanism.[55,56] In order to circumvent the limitations imposed by the large line widths, we decided to perform most of the experiments at 313 K, taking advantage of the thermal stability of St.

1D and 2D NOE Experiments. As previously reported,[46] signals (E;I) and (G;H) are pairwise dipole−dipole connected. This fact has allowed us to assign signals H and I as the Hδ2 of the coordinated histidines. These correlations were checked and confirmed by saturating the four signals one by one (data not shown). We also performed a series of truncated NOE experiments by irradiating signals E and G. By assuming an interproton distance of 2.4 Å for vicinal protons in an imidazole ring, we were able to calculate $\tau_r = 2 \pm 0.4 \times 10^{-8}$ s at 301 K and $\tau_r = 9.8 \pm 1.1 \times 10^{-9}$ s at 313 K. Similar values (within the experimental error) were obtained for the (E;I) and (G;H)

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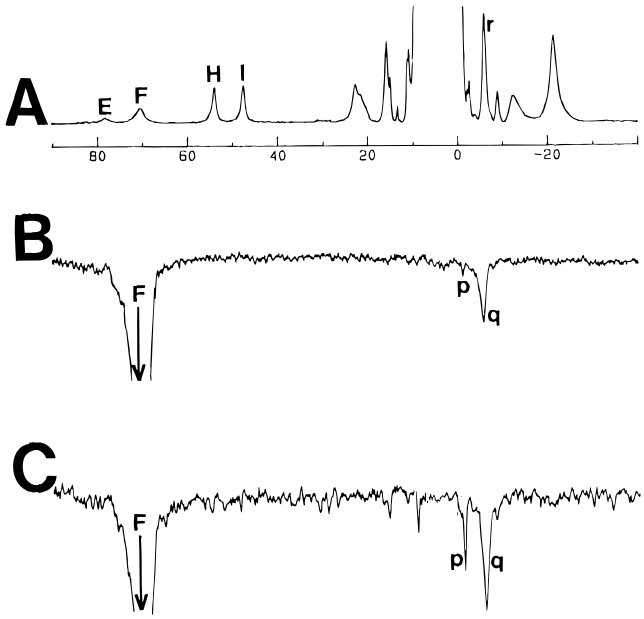

Figure 3. ^{1}H NMR 300-MHz spectrum (A) of Co(II)St and NOE difference spectra in the same experimental conditions obtained by saturation of signal F at (B) 0.5 ms and (C) 40 ms. The spectra were recorded at 313 K and pH 4.0. Arrows indicate the irradiated signals.

proton couples, indicating a negligible (if any) anisotropy in the τ_r values in the metal coordination environment. Hence, these values may be used with confidence to interpret NOE data from other metal ligands.

Irradiation of resonance F yielded a strong NOE with a signal at −5.8 ppm and smaller NOEs with several other signals (*cf.* Figure 3C). By using a shorter irradiation time (0.5 ms), only the NOEs with the signals at −5.8 and −1.1 ppm (p) could be detected (Figure 3B). The intense NOE observed between signal F and the −5.8-ppm resonance suggests that they may correspond to geminal protons. However, an inspection of the 1D spectrum (Figure 3A) indicates that the upfield signal r may represent at least two protons due to its intensity. Spectra and NOE experiments performed at different temperatures in the 298−313 K temperature range did not allow us to clarify whether two signals were overlapping or not at this frequency.

Two additional experiments were enlightening in this respect. First, irradiation of the intense resonance w (at −20.2 ppm) yielded an NOE at −5.9 ppm with signal r which is shifted with respect to the NOE observed by irradiating signal F (Figure 4). In addition, in spite of the unfavorable large line width of signal F at 600 MHz, we performed a truncated NOE experiment at this field with a short irradiation time (2 ms). The observed strong NOE corresponds to a signal with a line width of 800 Hz (not shown) whereas the −5.9-ppm resonance observed in the reference spectrum (r) is 550 Hz broad (Table 1). This allows us to conclude that the resonance dipole−dipole connected to signal F is a one-proton resonance lying below resonance r, and we will refer to it as signal q hereafter. The line width difference between signals q and r becomes more evident at 600 MHz, and it is therefore attributed to the Curie relaxation mechanism.[55,56]

We decided to characterize more in detail the (F;q) dipolar connectivity by performing NOE buildup experiments on signal F at different temperatures. (Figure 5). From the NOE buildup curve, we calculated the following values at 313 K: $\sigma_{q_F} = -16.4 \pm 1.3\ s^{-1}$; $\rho_q = 131 \pm 13\ s^{-1}$; $\sigma_{p_F} = -1.2 \pm 0.1\ s^{-1}$; $\rho_p = 15.4 \pm 4\ s^{-1}$, where σ_{ij} is the cross relaxation rate between protons i and j, and ρ_i is the selective relaxation rate of proton i. From these values, an internuclear distance of 1.8 ± 0.2 Å is calculated

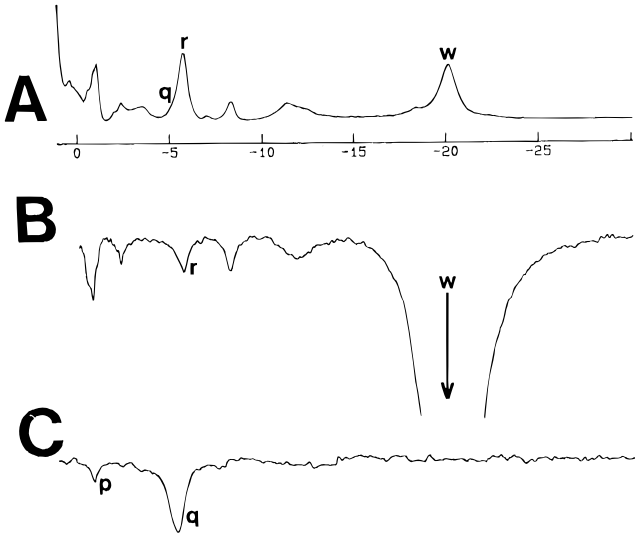

Figure 4. Upfield hyperfine shifted portions of ^{1}H NMR spectra of Co(II)St at 313 K in D_2O. (A) 1D reference spectra at 300 MHz. Traces B and C show the NOE difference spectra upon steady-state irradiation of signal w (B) and signal F (C) at 300 MHz. Arrows indicate the irradiated signals.

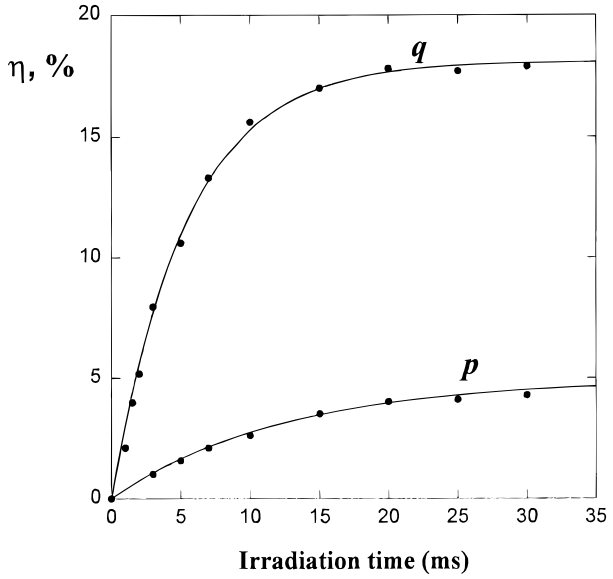

Figure 5. Time-dependent buildup of the NOEs observed after saturation of resonance F at 313 K and 300 MHz. The fittings and experimental values correspond to NOEs on signals q and p.

for the proton couple (F;q), whereas the distance between protons giving rise to signals F and p is of 2.5 ± 0.2 Å.

These values suggest that resonances F, q, and p belong to the same residue. In particular, we are able to state that signals F and q correspond to geminal protons of the fourth ligand bound to the metal ion. This fact was further confirmed by performing a NOESY experiment with a short mixing time (5 ms). This spectrum clearly shows a cross peak between signals F and q (Figure 6) which is not detectable at longer mixing times (due to the fast relaxation rates of these protons) but may be observed by using shorter values. We could not detect further cross peaks of resonance q with any other signals in these experiments. Some connectivities are seen when recording spectra at longer mixing times, but they correspond to the sharper signal r. On the other hand, the large number of broad resonances overlapping in this region of the spectrum rendered futile any attempt of extracting significant information by irradiating at the frequency of signal q.

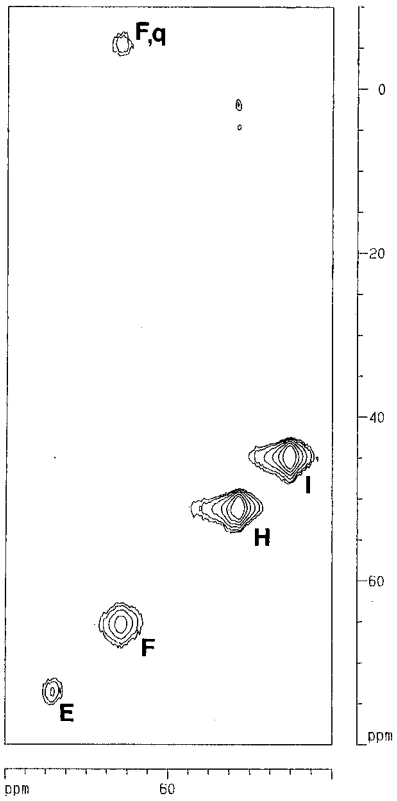

Figure 6. Portion of a NOESY spectrum of Co(II)St recorded at 600 MHz and 313 K with a mixing time of 5 ms and a recycle time of 150 ms.

The broad signals A and B, already assigned to the β-CH_2 couple of the coordinated Cys87,[46] were also irradiated. No NOEs between signal A and any other resonance could be found. However, signal B revealed a dipole−dipole connection with the upfield signal v (Figure 7A), a one-proton resonance partially overlapping with the intense signal w. Candidates for this signal are protons in the vicinity of the Cys ligand or the Hα of Cys87 itself. However, since the Cys Hα has not been located in Co(II) azurin,[44,45] we discard this possibility due to the larger line widths observed in Co(II)St. An inspection of the crystallographic structures of several blue copper proteins reveals that the Cys β-CH_2 protons are close to the axial ligand side chain (*cf.* Figure 1).[4] Indeed, NMR studies on wild type and Met121Gln Co(II) azurin have revealed NOEs between the Hβ2 Cys resonance and proton(s) belonging to the axial ligand.[38,44,45] Hence, we tentatively assign signal v to a proton belonging to the axial ligand. A dipolar connection to either signal F or q would be required to verify this hypothesis.

When irradiating signal v, we faced the problem of avoiding off-resonance spillage to signal w. We therefore carry on a series of irradiation experiments by stepping the decoupler frequency at constant power in the region around resonance v, as described by Lecomte *et al.*[57] The measured NOEs were plotted as a function of the decoupler offset with respect to signal v (Figure 7B). The intensities of the overlapping signals q and r in each experiment were calculated through computer simulation of the experimental spectra using Lorentzian line shapes from the known line widths and chemical shifts of these resonances. Figure 7B shows that maxima in the NOEs on signals q and p are observed when the decoupler is positioned at the frequency corresponding to signal v, whereas the other NOEs exhibit maxima when the irradiation frequency is that of

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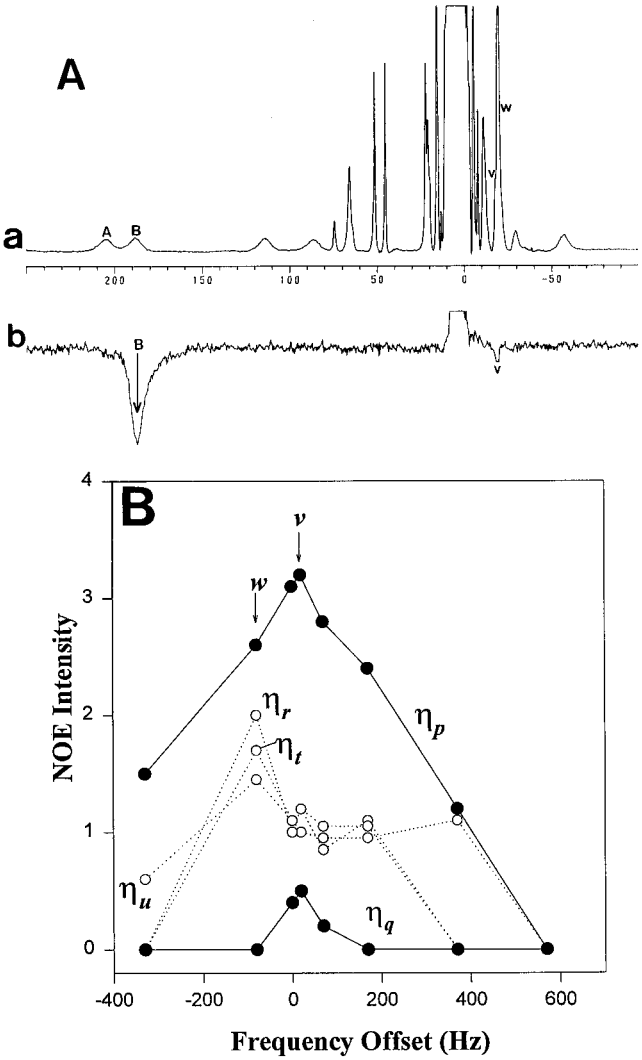

Figure 7. Steady-state NOE experiment of Co(II)St performed at 200 MHz: (A) 1D reference spectrum (trace a) and NOE difference spectrum recorded at a 30 ms irradiation time of resonance B (trace b). The arrow indicates the irradiated signal. (B) Intensity plot of the observed NOEs upon irradiation in the upfield region around resonances v and w by stepping the frequency offset at constant power (see text). The vertical scale is in arbitrary intensity units. The arrows indicate the frequency corresponding to signals v and w. The continuous lines show the NOE profile for resonances presenting maximum NOE upon irradiation on signal v. The dotted lines show the NOE profile for resonances presenting maximum NOE upon irradiation on signal w.

resonance w. By these means, we conclude that signal v is dipole−dipole connected to signals p and q. We recall at this point that resonance p corresponds to a proton located 2.5 Å from the nucleus giving rise to signal F. We therefore feel confident to propose that signals v and p both stand for protons belonging to the axial metal ligand. Even though we cannot measure the absolute NOEs, the $\eta_{p\{v\}}$ and $\eta_{q\{v\}}$ values hold a 1:13 ratio. The selective relaxation rates for signals p and q have been determined from the buildup experiment. Therefore, from the relation $\eta_{p\{v\}}/\eta_{q\{v\}} = (\sigma_{pv}/\sigma_{qv})\cdot(\rho_q/\rho_p)$, a σ_{pv}/σ_{qv} ratio of 1.5:1 is determined. Due to the r^{-6} dependence of the cross relaxation term, we may conclude that $r_{pv} \approx r_{qv}$.

Discussion

The Fourth Ligand. Since the signals corresponding to the bound Cys and His residues are assigned, signal F corresponds by exclusion to a proton belonging to the fourth ligand. The NOE and NOESY data clearly indicate that signals F and q

Table 2. Isotropic Shifts for Axial Ligands in Co(II)-Substituted Blue Copper Proteins

	Co(II)St[a]			Co(II)Met121Gln azurin[b]			Co(II) wt azurin[b]		
proton	δ (ppm)	T_1 (ms)	$\Delta\nu$ (Hz)	δ (ppm)	T_1 (ms)	$\Delta\nu$ (Hz)	δ (ppm)	T_1 (ms)	$\Delta\nu$ (Hz)
Hγ1	66.2	2.7	380	46.4	4.2	320	39.2	4.8	245
Hγ2	−5.8	7.2	200	−12.8	6.8	140	−14.7	5.3	
Hβ1	−18.3	*c*	400	−15.0	3.2	280	−15.0	6.4	
Hβ2				−22.7	4.0	250	−15.0	5.0	
Hα	−1.1	66.0	80	−3.2					

[a] Measured at 313 K and 200 MHz, this work. [b] Measured at 310 K and 400 MHz in *A. denitrificans* azurin, from ref 38. [c] Overlap prevented measuring this value.

stand for a geminal couple of protons belonging to this residue. We have shown that signals p and v may well correspond to protons from the same ligand. Moreover, resonance v represents a proton equidistant to those giving rise to signals p and q. Signals F and q may be safely attributed to a $-CH_2$ moiety of this residue close to the metal. We therefore propose the $-CH(p)-CH(v)-CH_2(F,q)-X$ assignment pattern for the axial ligand, with X being the donor group coordinated to the metal ion. This moiety is found in Gln, Glu, or Met residues. According to the reported sequence data, there are no Met nor Glu residues in St.[19] In this way, we are able to affirm that a Gln residue is the fourth metal ligand in St. Resonances F and q are the γ-CH_2 protons, signal v is one of the β-CH_2 protons, and p stands for the α-CH proton.

The relaxivity data (Table 2) are in agreement with this proposal. The assignment of resonance p as the α-CH proton is plausible since this proton is the one located farthermost from the metal ion, showing therefore a reduced line width, a longer relaxation time, and a smaller Curie relaxivity at higher fields (*cf.* Table 1). Table 2 displays the corresponding assignments for the axial ligands in Co(II)St, wild type Co(II) azurin, and Met121Gln Co(II) azurin.[38,45]

The γ-CH_2 Gln protons (F,q) display quite distinct shifts, with $\Delta\delta_{gem} = 72.0$ ppm. Similar $\Delta\delta$ values have been found for the corresponding γ-CH_2 protons in Met121Gln Co(II) azurin[38] and even for the methionine γ-CH_2 resonances in wild type Co(II) azurins (Table 2).[43−45] The γ-CH_2 signals in Co(II)St are shifted downfield with respect to the other two cases, suggesting a larger electron delocalization onto the Gln residue in St. The matching shift and relaxation patterns summarized indicate that a similar conformation of the axial ligand occurs in the three proteins. However, the coordinating glutamine residue in St is found in a subtly different orientation with respect to Met121Gln azurin. In the latter protein, the γ protons of Gln121 are closer to the β-CH_2 of Cys112,[6,38] whereas in the native protein the Cys protons are found facing the β-CH_2 Met couple.[16,44,45] The Cys-Gln dipole-dipole connectivity found in St is the same as in wild type azurin. The different conformation of the glutamine ligand in the azurin mutant may be ascribed to a rearrangement in order to accommodate the carbonyl moiety at a reasonable bond distance from the metal.[6]

In the case of Met121Gln azurin, an upfield signal corresponding to an exchangeable proton has been assigned to the amide Hϵ21 of the Gln ligand.[38] We have not observed an analogous resonance in Co(II)St. We attribute this fact to the higher degree of exposure of the metal site in St (see below), which may enhance the exchange rate of the Gln amide protons.

The Histidine Signals. The most relevant finding regarding the His ligands concerns the exchange rates of the Hϵ2 protons. In the case of Co(II) azurin, the signal corresponding to the Hϵ2 proton of the exposed histidine (His117) has been shown to exchange rapidly at pH values higher than 6.5,[43] whereas the Hϵ2 signal for His46 is in slow exchange. A slow exchange regime for both Hϵ2 His protons has been observed in Cu(I) azurin.[58] In Co(II)St, both NH His signals are in a fast exchange regime at pH 7.0. This fact indicates that both His residues are solvent accessible. A similar situation is met in the case of the cucumber basic protein, where the two metal-bound His have been found to be solvent exposed by means of X-ray crystallography.[12]

Signal G (Hϵ2 His92) is in fast exchange even at pH 4.0. We calculated a lower limit of 1.5×10^5 s^{-1} for the proton exchange rate at 308 K, *vs* a value of 5.3×10^4 s^{-1} for the Hϵ2 of His117 in Co(II) azurin.[83] It has been suggested that the solvent-exposed His117 in azurin may play a key role in electron transfer.[59] Hence, the exposed site in St may explain the fast electron-transfer kinetics observed with inorganic redox partners, as well as the possibility of performing redox potential measurements in St without mediators.[22,23]

The shift pattern observed for the six histidine signals is strikingly similar to that of Co(II) azurin.[43,45] This similarity could be even fortuitous, since the contact and pseudocontact contributions to the observed shifts may be altered in such a way that they are giving rise to opposite shifts and canceling mutually. However, it is relevant to note that in the case of azurin, replacement of Met121 residue by a Gln does not alter the His shift pattern, as it is observed also in this case.[38] A different situation is met in the Co(II) Cys112Asp mutant protein.[44] The His shift pattern seems therefore to be more sensitive to structural changes in the equatorial metal ligands rather than to variations in the axial coordinating groups.

The Cysteine Signals. Even though the histidine ligands are conserved among all the hitherto known blue copper proteins, the copper−cysteinate bond is the most outstanding feature of the type 1 Cu site.[1,2,60−62] The strong Cys(S) → Cu(II) charge transfer bands dominate the visible spectrum of these proteins[2] and replacement of this residue by an aspartic acid has yielded a non-blue copper protein.[63]

Signals A and B are assigned as the β-CH_2 protons of Cys87.[46] Their extreme downfield shifts, large line widths and short T_1 values are characteristic of cysteine residues bound to Co(II) ions.[44,64,65] These shifts are mainly due to a contact contribution,[40,54] so that it may be inferred that the electron delocalization onto the cysteine moiety is large. This is

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Table 3. Correlation between Spectroscopic and Structural Parameters for the Cys Ligands

	Stellacyanin	Met121Gln azurin	wt azurin
δ β-CH_2 shifts in Co(II) proteins	207; 183[a]	224; 183[b]	262; 222[c]
$[A\rho_s]$		175.2	484.8
[B/A]		0.90	0.25
M−S−C_β−$H_{\beta i}$ dihedral angle		67.9; −52.2[d]	62.8; −57.3[e]
$\epsilon_{450}/\epsilon_{600}$, Cu(II) proteins[j]	0.27[f]	0.20[d]	0.11[f]
$g_{\|\|}$, $A_{\|\|} \times 10^4$ cm^{-1} (EPR)	2.287; 37[g]	2.287; 35[d]	2.258; 62[f]
ν(Cu−S), cm^{-1}	386[h]	412[i]	414[i]

[a] Measured at 310 K and 200 MHz, this work. [b] Measured at 310 K and 400 MHz, from ref 38. [c] Measured at 310 K and 400 MHz, from ref 38. [d] From ref 6. [e] From ref 16b. [f] From ref 62. [g] From ref 20. [h] From ref 70. [i] From ref 61. [j] Ratio of absorbances for the two ligand-to-metal charge transfer bands in the Cu(II) proteins, at ca. 450 and 600 nm, defined according to ref 62.

consistent with results coming from ^{1}H ENDOR studies of the Cu(II) proteins,[35,66] and with the existence of a high degree of covalence of the metal−cysteine bond.[2]

The β-CH_2 proton shifts in wild type Co(II) azurin are found considerably more downfield than in the spectra of the Met121Gln mutant and St. These differences have been generically accounted for by a reduced metal−cysteine interaction in the latter proteins.[38,46] These shifts are expected to follow a general equation of this type:[54,67,68,69]

$$\delta_i = [A\cos^2\theta_i + B]\rho_s = [\cos^2\theta_i + \mathrm{B/A}]\cdot[A\rho_s] \quad (1)$$

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 Cys shift variations may then originate from changes in this dihedral angle or to distinct electron spin densities on the sulfur atom. The $[A\rho_s]$ and $[B/A]$ parameters were calculated for wild type *A. denitrificans* azurin and the Met121Gln mutant by considering the dihedral angles as derived from the X-ray data (Table 3)[6,16] The $[A\rho_s]$ value is largely reduced in the mutant protein, suggesting a reduced electron spin density in the Cys sulfur atoms. Since the dihedral angles in wild type and Met121Gln azurin differ by ±5°,[6] we attribute the shift differences mainly to a distinct electron delocalization. Unfortunately, the dihedral angles are not known for St. However, one should take into account that the Cu−S−C_β−C_α angles in BCP's do not change substantially, ranging from 168.0° in poplar plastocyanin to 178.7° in *Alcaligenes faecalis* pseudoazurin.[4] Resonance Raman studies have shown that a similar Cu−S−C_β−C_α angle should be expected as well in St.[60,70,71] We therefore interpret the smaller shifts values observed in Co(II)St as arising from a reduced electron delocalization onto the bound Cys residue.

This weakened metal−Cys interaction may be caused by a strong coordination to the axial Gln ligand. Following this reasoning, the presence of a strong axial ligand would displace the Co(II) ion somewhat from the plane defined by the equatorial ligand triad reducing the Co(II)−S orbital overlap. This is consistent with the larger downfield shift observed for the Hγ1 Gln97 resonances. Moreover, in the X-ray structure of the Met121Gln mutant the Cu(II) ion is found 0.26 Å out of the plane defined by the three equatorial ligands. We propose that the hyperfine shifted resonances of the β-CH_2 protons of the bound Cys (and the metal−Cys interaction) are highly sensitive to the nature of the axial ligand. This is in agreement with recent conclusions of a series of elegant resonance Raman studies on native and mutant copper proteins.[60−62]

The Paramagnetic Shifts. The relative contact and pseudocontact contributions to the shifts in Co(II)−BCP's have been a matter of debate.[43−45] The observed pseudocontact shifts in Co(II)St are significant (as in Co(II) azurin[45]), since several signals corresponding to non-coordinating residues fall in the 20 to −20 ppm range (Figure 2). It is well known that the magnetic anisotropy giving rise to pseudocontact shifts is larger in trigonal planar than in tetrahedral Co(II) complexes.[39,40,54] This indicates that in Co(II)St the strong equatorial ligand trial constrains the metal ion to adopt a nearly planar trigonal geometry. A tetrahedral geometry would be irreconcilable with the existence of a type 1 copper center.[62] Even if these data do not allow us to estimate the contact and pseudocontact contribution to the measured shifts in Co(II)St, we conclude that the rigidity of the metal site gives rise to similar geometries and therefore to pseudocontact shifts of the same order in Co(II)-substituted BCP's. This means that the shift differences may be mainly attributable to the contact term. Our attempts to rationalize the Gln and Cys sihfts in the previous sections seem to confirm this statement, as well as the constancy of the His shift pattern among the different Co(II)−BCP's. The case of Cys112Asp Co(II)azurin, in which the type 1 center is disrupted, is the only exception.[44]

The Low Redox Potential of Stellacyanin. It has been claimed that the axial coordination of an amide oxygen group in St instead of a thioether sulfur may be the main cause of the low redox potential of this metal center by stabilizing the Cu(II) oxidation state.[34,72] It is intriguing, however, that the Met-to-Gln replacement in azurin has provided a good spectroscopic and structural model for St, but has not been sufficient to induce such a low redox potential in the copper center.[6] Moreover, site-directed mutagenesis studies on the other copper ligands and even of nonbound residues in azurin have rendered azurin mutants with redox potentials even lower than that of the Met121Gln mutant.[73] (For a detailed discussion on this point, see refs. 1b and 73.)

On the other hand, recent studies seem to indicate that the case of St is far from being an exception in the field of blue copper proteins: the recently sequenced cucumber peelings cupredoxin (CPC)[74] and umecyanin from horseradish roots,[75] which share some spectroscopic features with St, do have a Gln residue in their sequences which line up with Gln97 in St. Notwithstanding, the redox potential of umecyanin is 283 mV,[76] a value that is closer to that of Met121Gln azurin.

We have shown that both coordinating His are solvent exposed in St. Solvent accessibility has been cited in several opportunities as a contributing factor to the redox potential of

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metalloproteins.[77,78] In particular, in the case of BCP's, a study in a series of Met121 mutants of azurin gives strong support to this hypothesis: the presence of water molecules in the vicinity of the copper site lowers the redox potential, whereas hydrophobic residues favor the Cu(I) state, thus raising the potential.[73] In the case of rusticyanin, which displays the largest redox potential observed in BCP's, the metal site seems to be shielded from the solvent environment.[79−82] These facts situate rusticyanin in the antipodes of St, suggesting a correlation between solvent accessibility to the metal center and redox potential in the BCP series.

Concluding Remarks

The present work demonstrates that this strategy is useful for probing the metal site structure in copper proteins whose structures are not known, and that subtle changes in the structure of blue copper proteins can be monitored by changes of the shifts induced by the Co(II) ion. This work constitutes the first direct evidence of the coordination of a glutamine residue to the metal in St, which is highly consistent with all the data coming from other spectroscopies, and confirms the predictions of Freeman's model for St. Additional paramagnetic NMR studies on site-directed mutants of azurin would be of considerable interest in order to better explore the sensitivity of the isotropic shifts (in particular, those of the Cys residue) upon structural changes in the metal coordination environment.

Further studies aimed to the establishment of structure of the noncoordinating environment of the metal ion are underway. These may be of help in expanding the knowledge of the 3-D picture of the protein around the metal ion.

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