Proton–Metal Distance Determination in Cobalt(II) Stellacyanin by ^1H Nuclear Magnetic Resonance Relaxation Measurements Including Curie-Spin Effects: A Proposed Structure of the Metal-Binding Region†

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ABSTRACT: ^1H nuclear magnetic resonance (^1H NMR) experiments on Co(II)-substituted stellacyanin have been performed. Large paramagnetic hyperfine shifts are observed, the whole spectrum covering a range of 190 ppm. Experiments were mainly performed at 270 MHz from which temperature and pH* dependencies of the out-shifted resonances are reported, as well as determinations of the longitudinal (T_1) and transverse (T_2) relaxation times. These relaxation times are, among other things, dependent on the individual proton–metal distance, and the aim of this work has been to determine these distances, by use of the Solomon–Bloembergen equations modified to include the so-called "Curie spin". The application of this method to a protein has not been reported earlier. Experiments were also performed at 100, 400, and 500 MHz in order to estimate the size of the Curie spin from the field dependence of the line widths. Furthermore, determination of the values for the rotational correlation time, τ_r, and the effective magnetic moment, μ_{eff}, was necessary for the present approach. With apostellacyanin, τ_r was found to be $(6.0 \pm 0.4) \times 10^{-8}$ s. From the paramagnetic susceptibility of Co(II) stellacyanin, the value $(4.53 \pm 0.03)\beta$ was determined for μ_{eff}. The proposed assignments of several paramagnetically out-shifted resonances, the proton–metal distances obtained, and the known peptide sequence of stellacyanin have allowed us to build a three-dimensional model of the metal site and its surrounding structure consistent with all the experimental data. It is revealed that both histidine ligands bind the metal with their 3-nitrogens. Also, we find strong indications that a second sulfur atom is actually binding the metal, this being the long-sought-after fourth ligand. The model suggests that this sulfur belongs to Cys-59, which together with Cys-93 constitutes the disulfide bridge known to be present in the structure. A potential fifth ligand, an amide oxygen from Asn-47, is also found.

Stellacyanin is a member of a family of proteins which all contain a single copper ion bound in a unique site. This site is usually referred to as the type 1 or "blue" copper binding site found in an increasing number of proteins including the larger multicopper oxidases laccase, ascorbate oxidase, and ceruloplasmin. The geometry of the type 1 site is generally described as being close to a trigonal bipyramid with three ligands and the ion in a slightly distorted plane. In all known cases these three ligands have been identified as two nitrogens from histidine residues and one cysteine sulfur. The residues providing these ligands in stellacyanin are His-46, His-92, and Cys-87, respectively (Bergman et al., 1977; Engeseth et al., 1984). Charge transfer between the sulfur and the copper ion results in a large optical absorption around 600 nm, giving the type 1 proteins an intense blue color in the oxidized state. The fourth ligand, at a longer distance from the metal, is also a sulfur which in azurin and plastocyanin is contributed by a methionine. Stellacyanin, however, is known to be devoid of methionine (Bergman et al., 1977). It has been suggested that a disulfide bridge may provide the second ligating sulfur (Ferris et al., 1978), but no evidence has as yet been published. Recently, the crystal structure of the "cucumber basic blue protein" was solved (Guss et al., 1988), and from sequence homology with this protein it was put forward that the amide group of Gln-97 might replace a methionine thioether group in stellacyanin. This possibility will be discussed later. In addition, a recent X-ray crystallographic study of *Alcaligenes denitrificans* azurin presented evidence of a fifth ligand, a rather distant carbonyl oxygen (Norris et al., 1986).

This group of small proteins have peptide chain lengths ranging from approximately 80 to 150 amino acid residues, giving molecular weights from about 9000 to 23 000. Stellacyanin contains 107 amino acid residues. The molecular weight is 20 000, 40% of which is carbohydrates attached to three asparagine residues on the surface of the protein. Of the known reduction potentials in this family, the lowest is found for stellacyanin (184 mV) and the highest for rusticyanin (680 mV). Their common task is to perform electron transfer in various biochemical processes in many different organisms from the kingdoms of plants and bacteria [reviews of these proteins are given by Rydén (1984) and Adman (1984)].

Copper is a very common metal in biological systems, especially in electron-transfer proteins. The three earliest known members of the type 1 family, plastocyanin, azurin, and stellacyanin, have therefore been used extensively as models when biological electron-transfer reactions are being investigated. The aim of such research is to visualize the electron-transfer process and to correlate the spectroscopic properties of the proteins with their structure, focusing on the metal site. Many kinetic studies have therefore been performed on these three proteins (Marcus & Sutin, 1985). From X-ray crystallographic studies, the tertiary structures of plastocyanin (Guss & Freeman, 1983) and azurin from two bacterial species (Adman & Jensen, 1981; Norris et al., 1986) are known. Stellacyanin, on the other hand, has resisted laborious attempts

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of crystallization, most probably because of the high and unevenly distributed carbohydrate content.

Now, since stellacyanin has not yet been crystallized, means other than X-ray diffraction must be used to probe the structure of this protein. A possible technique is ^{1}H NMR. Experiments on native stellacyanin have been reported (Hill & Lee, 1979), but interpretation of the spectra is difficult, mainly because of the multitude of overlapping resonances normally obtained from proteins. Moreover, the situation is made even worse as the Cu(II) ion gives rise to paramagnetic broadening of resonances from protons in its vicinity (Dwek, 1973). Focusing on the metal site, one remedy for this is to exchange the native copper for another metal ion giving paramagnetic shifts instead of broadening, provided, of course, that the native structure of the site is preserved. The development of metal exchange techniques for copper proteins has been useful for this purpose (Hauenstein & McMillin, 1981). It is thus possible to "select" protons near the metal out of the large, main cluster of resonances obtained from the diamagnetic parts of the protein. A metal ion suitable for insertion is Co(II), which can bind in a trigonal-bipyramidal geometry in its high-spin state ($S = {}^3/_2$) and whose ionic radius is equal to that of Cu(II). Indeed, studies of the electronic absorption properties (McMillin et al., 1974) and the extended X-ray absorption fine structure (EXAFS) (Feiters et al., 1988) of Co(II) stellacyanin indicate very close structural similarity between this derivative and the native (oxidized) protein. In addition, large paramagnetic shifts are clearly demonstrated in a previous ^{1}H NMR study of cobalt-substituted azurin (Hill et al., 1976).

The longitudinal (T_1) and transverse (T_2) relaxation times of the resonances, as well as their positions on the shift axis, are, among other things, dependent on their individual distances to the metal ion (Solomon & Bloembergen, 1956). The combined use of the theoretical expressions for the two relaxation times, into which the effect of the so-called "Curie spin" (caused by thermal averaging in the spin levels of the paramagnetic electrons) is included, allows determination of the relevant metal–proton distances and the electronic relaxation time (see Theory). In the present work, measurements of T_1 and T_2, interpreted with the Solomon–Bloembergen equations including the Curie spin (Gueron, 1975), are presented. The proton–metal distances thus obtained and the primary structure of the protein (Bergman et al., 1977) have enabled us to build a three-dimensional model of the metal site in Co(II) stellacyanin.

Experimental Procedures

Native stellacyanin was prepared from the sap of the Japanese lacquer tree *Rhus vernicifera* by the method of Reinhammar (1970). Removal of the copper was performed as described earlier (Dahlin et al., 1984). The cobalt derivative was then produced as follows. The apoprotein, dissolved in 50 mM borate buffer, pH 9.0, was heated to 35–40 °C. One molar equivalent of Co(II), from a stock solution of 20 mM $CoCl_2$ in H_2O, was added and the mixture held at the same temperature for 15 min. After 2 h at room temperature, this process was repeated once. The protein solution was then stored at 5 °C for approximately 12 h (or overnight). Usually, addition of 2 molar equiv of the metal was sufficient to obtain a high yield, but occasionally the procedure described above was repeated for a third equivalent. After completed uptake, excess cobalt was removed by dialysis against several volumes of water, the first of which contained a 10-fold excess of ethylenediaminetetracetate (EDTA) for higher efficacy and for removal of loosely bound ions. Finally, NMR samples were transferred to pure, unbuffered ^2H_2O and concentrated to 6–7 mM. The yield was determined by the concentration ratio Co(II) stellacyanin/total protein. Both values were obtained spectrophotometrically, the largest peak in the visible region at 640 nm with a molar absorptivity of 450 M^{-1} cm^{-1} (McMillin et al., 1974) for the concentration of cobalt-containing protein and the maximum at 280 nm with a molar absorptivity of 23 200 M^{-1} cm^{-1} (Malmström et al., 1970) for the concentration of total protein being used. The yield was never lower than 80% in any preparation, typically between 90 and 95%. The protein used in the experiments presented in this paper contained 94% Co(II).

All chemicals used were of reagent grade. For use with the apoprotein and cobalt protein, all solutions and labware were made free of adventitious metals by treatment with Chelex 100 and 2 M nitric acid, respectively. Where appropriate, 1 M ^{2}HCl and 1 M NaO^{2}H were used to set the pH of the sample. (The pH is given as the meter reading uncorrected for the isotope effect and is designated pH*.)

The majority of the NMR experiments were performed at 270 MHz on a Bruker WH 270 spectrometer fitted with an Aspect 2000 computer. Experiments at 100, 400, and 500 MHz were performed on a Bruker MSL 100, Varian XL 400, and Bruker AM 500, respectively. Chemical shifts are given relative to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) with the residual H_2O resonance as internal standard at 4.65 ppm. For suppression of this peak a water elimination Fourier transform (WEFT) pulse sequence was employed. T_2 was determined from the line widths ($\Delta\nu_{1/2}$) and the relation $T_2 = 1/(\pi\Delta\nu_{1/2})$. T_1 was determined with the inversion recovery technique. Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker spectrometer equipped with an Oxford Instruments ESR-10 liquid helium cryostat.

Theory

Because of the strong distance dependence of dipole–dipole interactions, longitudinal relaxation studies of paramagnetic proteins have frequently been used in order to extract proton–metal distances. If scalar contributions (Solomon & Bloembergen, 1956) to T_1 are neglected, the dipolar part may be written as (Solomon, 1955)

$$T_1^{-1} = \Delta^2 \frac{S(S+1)}{15}\left(\frac{6\tau_1}{1+\omega_1^2\tau_1^2} + \frac{14\tau_2}{1+\omega_s^2\tau_2^2}\right) \quad (1)$$

where $\Delta = (\gamma_I\mu_0\beta\hat{g})/r^3$. Here, γ_I is the gyromagnetic ratio of the proton, μ_0 is the magnetic permeability in vacuum, β is the Bohr magneton, $\hat{g}$ is the spatially averaged electronic g values, and r is the nucleus–electron distance. The Larmor frequencies of the nucleus and the electron are represented by ω_1 and ω_s, respectively. The electronic longitudinal relaxation time (τ_1) is frequently assumed to be much larger than the transverse relaxation time (τ_2), thus leaving τ_1 and r as the only parameters to be determined.

However, there are several restrictions attached to the use of eq 1. The effects of g tensor anisotropy (Sternlicht, 1965), zero-field splitting of the electronic energy levels (Bertini et al., 1984a), and contributions to T_1 from the Curie spin (Gueron, 1975), which also may be anisotropic (Vega & Fiat, 1976), are not incorporated into the expression shown above. Furthermore, the value of τ_1 must often be assumed unless it can be independently determined from another source or calculated from a known distance.

At high magnetic fields and low molecular tumbling rates, relaxation through the thermally averaged electron spins, the Curie spin, may become important enough to affect both T_1

and T_2 in which case the Solomon equations will have to be modified (Gueron, 1975):

$$T_1^{-1} = \frac{6}{5}\Delta^2\left(S_c^2\frac{\tau_r}{1+\omega_I^2\tau_r^2} + \left[\frac{S(S+1)}{3} - S_c^2\right]\frac{\tau_1}{1+\omega_I^2\tau_1^2}\right) \quad (2)$$

and

$$T_2^{-1} = \frac{1}{5}\Delta^2 S_c^2\left(4\tau_r + \frac{3\tau_r}{1+\omega_I^2\tau_r^2}\right) + \frac{1}{5}\Delta^2\left[\frac{S(S+1)}{3} - S_c^2\right]\left(4\tau_1 + \frac{\tau_1}{1+\omega_I^2\tau_1^2}\right) \quad (3)$$

where the thermal average of the electron spins is $S_c = (B_0 S(S+1)g\beta)/3kT$ in the high-temperature limit. The magnetic field is given by B_0, k is the Boltzman constant, and T is the temperature. The equations are in their isotropic forms, and it has been assumed that $\tau_1 \gg \tau_2$. Furthermore, it should be noted that the Curie-spin effect depends on the rotational correlation time τ_r instead of τ_1. A further simplification of these equations can be made provided that $\omega_I^2\tau_r^2 \gg 1 \gg \omega_I^2\tau_1^2$ and $S_c^2 \ll S(S+1)/3$, conditions which are met in the present case. Consequently, the first term in eq 2 and 3 corresponds to relaxation by the Curie spin while the second term represents the usual mechanism.

The combined use of eq 2 and 3 obviates the need to know τ_1. Thus, by measurement of T_1 and T_2 for resonances having no scalar contributions to the relaxation rates, both r and τ_1 may be obtained. Once a value for τ_1 has been found, eq 2 may be used to calculate distances for protons exhibiting either scalar or dipolar shift since contact contributions to T_1 are negligible, which is not the case for T_2. However, τ_1 should only be regarded as an operational parameter since any anisotropy from the g values, the zero-field splitting, and the Curie spin will be contained therein. Also, T_2 will have small contributions from the diamagnetic interproton dipole–dipole interactions which cannot a priori be included in eq 3, since assignments can only be made once the correct distances are known. However, only minor errors will result from this omission.

The approach outlined above introduces two new unknown parameters, namely, τ_r and S_c. If $\tau_r > 1/\omega_I$, then τ_r may be calculated from the ratio T_1/T_2 for resonances stemming from the diamagnetic apoprotein (Dwek, 1973). The measured value of τ_r may be slightly different from the true value since cross-relaxation effects (Kalk & Berendsen, 1976) will tend to increase T_1 while T_2 is largely unaffected. The Curie spin can be obtained from magnetic susceptibility measurements since S_c also may be written $S_c = ([S(S+1)]^{1/2}\mu_{eff}B_0)/3kT$, where μ_{eff} is the effective magnetic moment in terms of Bohr magnetons. The same substitution can also be made into the $\Delta^2 S(S+1)$ term in eq 2 and 3.

Results

The 270-MHz ^{1}H NMR spectrum of Co(II)-substituted stellacyanin is shown in Figure 1. In addition to these peaks, the spectrum recorded at 100 MHz (not shown) revealed an extremely broad (≈15 ppm) resonance at −60 ppm. The large spread of resonances, covering almost 190 ppm, is undoubtedly due to the paramagnetic cobalt ion being in its high-spin ($S = {}^3/_2$) state. Proton groups on the metal ligands and in the vicinity of the metal center thus experience very large shifts which are of contact or pseudocontact origin, or both. The latter shift mechanism carries with it an r^{-3} distance dependence because of dipole–dipole interactions between the nuclei

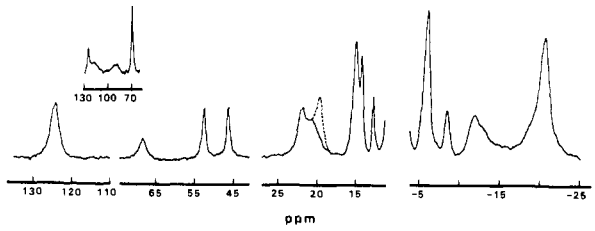

FIGURE 1: 270-MHz ^{1}H NMR spectrum of Co(II) stellacyanin, recorded at 303 K at a pH* of 7.3 in unbuffered ^2H_2O. The chemical shift is given relative to DSS. The main envelope of diamagnetically shifted resonances is omitted, as is the region between 75 and 110 ppm where the utilized quadrature detection caused an infolded image to appear. The inset shows this part of the spectrum when the center frequency is moved. The dotted peak at 19 ppm demonstrates deuterium exchange with the solvent.

under observation and the unpaired d electrons of the Co(II) ion. Difficulties in separating the two shift mechanisms, and the lack of a precise knowledge of the orientation of the electronic g tensor with respect to the molecular framework, preclude the possibility, at this stage, of using this approach to elucidate the structural surroundings of the metal. An alternative approach is to measure the longitudinal and transverse relaxation times and to extract distance information from their r^{-6} dependence. Before such measurements are discussed, however, some other properties of Co(II) stellacyanin will be touched upon.

Temperature Dependence. The temperature dependence of the resonances shown in Figure 1 (pH* 7.3) was recorded in the range 283–323 K (not shown). None of the resonances obey Curie law behavior in shift vs reciprocal temperature plots, and the intercepts on the chemical shift axis at infinite temperature deviate from the expected diamagnetic positions of the resonances. The presence of a zero-field splitting of the electronic energy levels of Co(II) stellacyanin (Solomon et al., 1976) is one likely cause for the observed behavior. However, adding a T^{-2}-dependent term to the pseudocontact shift (Bleaney, 1972) did not move the intercepts closer to the diamagnetic region. For resonances also carrying a contact shift, a temperature-dependent nuclear–electron hyperfine coupling constant (Wüthrich, 1970; Horrocks & Greenberg, 1974) may be an additional cause for the observed behavior. At this stage, however, it cannot be excluded that a slight conformational change contributes to the nonlinear Curie plots or that pH*-dependent phenomena are playing a role.

Curie-Spin Contributions. As mentioned earlier, the Curie spin may at high fields, low molecular tumbling rates, and a large S value contribute significantly to both T_1 and T_2, although the effect on the latter relaxation time usually is 2 or 3 orders of magnitude larger than that on the former. Since values of both T_1 and T_2 were used to calculate proton–metal distances, this relaxation mechanism had to be incorporated into the Solomon equations. The presence of Curie-spin contributions may be verified by varying the magnetic field, since the effect increases with the square of the field. The spectrum of Co(II) stellacyanin was therefore recorded at 100, 270, 400, and 500 MHz, and line widths for several resonances were measured as exemplified by the resonance at 68 ppm in Table I. It is seen that the increase in line width of this resonance on going from 100 to 270 MHz is approximately accounted for by the Curie spin, whereas at the higher fields the line width increases even faster. However, since the electronic relaxation times probably are frequency dependent in this range of magnetic fields (Dwek, 1973), the additional increase in line width most likely stems from this source. This also precludes the possibility of determining the electronic

Table I: Field Dependence of the Curie-Spin Contribution to the Line Width of the Hyperfine-Shifted Resonance at 68 ppm in the Spectrum of Co(II) Stellacyanin[a]

magnetic field (T)	resonance freq (MHz)	relative field	$\Delta\nu_{1/2}$ (Hz)	relative broadening	Curie-spin contribution (Hz)[b]
2.35	100	1.0	365	1.0	18
6.34	270	2.7	540	1.5	125
9.40	400	4.0	1640	4.5	281
11.74	500	5.0	3080	8.4	422

[a] Similar dependencies were found for the other resonances shown in Figure 1. [b] Calculated Curie-spin contribution to $\Delta\nu_{1/2}$ using the first term of eq 3 and the values of μ_{eff}, τ_r, and r measured at 6.34 T.

relaxation times from the field dependence of T_2 without introducing further parameters.

Determination of Rotational Correlation Time. The value of the rotational correlation time (τ_r) may often be determined by use of the Debye–Stokes relation for roughly spherical proteins. Stellacyanin, however, consists of approximately 40% carbohydrates attached to the protein at only three sites, suggesting that τ_r for this protein would deviate substantially from a value derived from the above-mentioned relation. Another approach was therefore chosen in which τ_r was calculated from the ratio T_1/T_2 for a number of resonances in the spectrum of apostellacyanin. This method is sensitive in cases where $\tau_r > \omega_I^{-1}$ since in this region $T_1 > T_2$ (Dwek, 1973). The ^{1}H NMR spectrum of apostellacyanin is not particularly well resolved (Hill & Lee, 1979), but there are some resonances for which both T_1 and T_2 may be determined accurately enough for τ_r to be calculated. In order to avoid protons with motions other than the overall tumbling of the molecule, particularly the internal rotation of methyl groups, the T_1/T_2 ratios of three resonances in the aromatic region were used in the calculation, yielding an average of $(6.0 \pm 0.4) \times 10^{-8}$ s for τ_r. Since the overall motion of stellacyanin may be anisotropic, the measured value of τ_r should be considered as the average of such anisotropic motions, if present. It may also be noted that the τ_r value for stellacyanin is about 30% higher than the value calculated from the Debye–Stokes formula for carbonic anhydrase, which has a 50% higher molecular weight. This result must be ascribed to the presence of carbohydrate chains on stellacyanin.

Paramagnetic Susceptibility of Co(II) Stellacyanin. Measurements of the room-temperature magnetic susceptibility were performed with NMR using the "internal/external reference method" as described previously (Ångström et al., 1982). Tertiary butanol (4% v/v) was used as reference substance. For a 6.23 mM protein solution with a Co(II) occupancy of 0.94, a shift difference of 56.5 Hz was found, yielding a molar magnetic susceptibility of $(1.07 \pm 0.01) \times 10^{-7}$ according to $\Delta\chi_M = 3\Delta\nu/c\nu_0$, where $\Delta\nu$ is the shift difference, c is the concentration, and ν_0 is the spectrometer frequency. This value has been corrected for the diamagnetic contribution by measuring the shift induced by apostellacyanin, which was found to be 2.1 Hz mM^{-1}. It may be noted that $\Delta\chi_M$ (SI) $= (4\pi)10^{-6}\Delta\chi_M$ (cgs). The molar magnetic susceptibility may be converted into an effective magnetic moment through the relation $\mu_{eff} = (\Delta\chi_M 3kT/N\beta^2)^{1/2}\beta$, where N is the Avogadro constant, yielding $\mu_{eff} = (4.53 \pm 0.03)\beta$ for Co(II) stellacyanin at $T = 303$ K.

The EPR spectrum of Co(II) stellacyanin at 11 K (Figure 2) is typical of high-spin Co(II) without any resolved hyperfine structure and with g values at 6.25, ≈2.5, and ≈1.7. The two lower values are difficult to determine accurately because of overlap between the lines. However, it is possible to reproduce these three values from the average μ_{eff} obtained from the

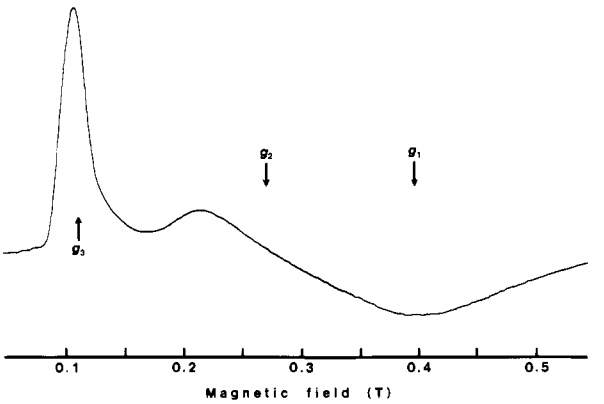

FIGURE 2: Electron paramagnetic resonance (EPR) spectrum of Co(II) stellacyanin recorded under the following conditions: microwave frequency, 9.46 GHz; microwave power, 20 dB; amplitude modulation, 2.5 mT; temperature, 11 K. The rhombic g values can be identified at ≈1.7, ≈2.5, and 6.25, indicated as g_1, g_2 and g_3, respectively.

Table II: Relative Intensities and T_1 and T_2 Values for Paramagnetically Shifted Resonances in the 270-MHz Spectrum of Co(II) Stellacyanin (See Figure 1)[a]

resonance (ppm)	relative intensity[b]	T_1 (ms)	T_2 (ms)
125	2	1.7 (0.1)	0.49 (0.05)
117[c]	1		
87[c]	1		
68	1	2.4 (0.1)	0.53 (0.03)
52	1	10.2 (0.5)	1.35 (0.24)
46	1	8.5 (0.4)	1.30 (0.20)
22[c]	1	>4.7 (0.2)	1.05 (0.21)
20[c]	1	<3.2 (0.3)	
19[c]	1		1.42 (0.18)
15	3	13.1 (0.6)	1.52 (0.13)
14	1	45.4 (2.3)	4.22 (0.76)
13	1	66.2 (6.6)	5.04 (1.46)
−6	6–7	7.3 (0.4)	1.41 (0.08)
−8	1	10.5 (0.5)	1.86 (0.24)
−11	4	2.1 (0.9)	0.84 (0.24)
−12		1.3 (0.4)	0.46 (0.09)
−20	8	4.6 (0.2)	0.79 (0.13)

[a] Absolute experimental errors are shown in parentheses. [b] Determined in relation to unambiguous one-proton resonances (46, 52, and 68 ppm, see Proposed Assignments) and rounded to the nearest integer. [c] T_1 and T_2 could not be measured accurately because of extensive overlap and deuterium exchange with the bulk solvent (22, 20, and 19 ppm, respectively; see the text) or extreme line widths (87 and 117 ppm). Because of overlap, the T_1 values for the resonances at 20 and 22 ppm should be considered as the upper and lower limits, respectively.

experimentally determined magnetic susceptibility, as given above. With these values, the low-temperature effective magnetic moment may be calculated by $\mu_{eff} = (\hat{g}[S(S+1)]^{1/2})\beta$, yielding $\mu_{eff} = 3.5$ Bohr magnetons.

Calculation of Distances. With the substitutions made in eq 2 and 3 and with the values now determined for τ_r and μ_{eff}, the only unknown parameter left besides r is τ_1. If resonances without any scalar contribution to the shift and for which both T_1 and T_2 are accurately determined are chosen, eq 2 and 3 can be solved simultaneously, yielding individual values of r and τ_1 for each resonance (see Theory). Five choices were made as follows. The intensities of the peaks at 15, −6, and −20 ppm all very strongly indicate methyl groups (see Table II). These resonances must therefore be purely pseudo contact shifted. The peaks at 13 and 14 ppm were also chosen since their line widths are much smaller than can be expected for resonances with scalar contributions. The τ_1 values for the resonances at 15, 14, 13, −6, and −20 ppm are 6.79, 5.21, 4.12, 12.8, and 11.0 × 10^{-12} s, respectively, the T_1 and T_2 values

Table III: Proton–Metal Distances Calculated with Equation 2 and Measured T_1 Values Presented in Table II[a]

resonance (ppm)	distance (Å)	assignment
125	3.24 (0.34)	His-46 2-CH, His-92 2-CH
117		Cys-87 β-CH_2
87		Cys-87 β-CH_2
68	3.43 (0.36)	His-46 4-CH
52	4.35 (0.45)	Cys-59 β-CH_2
46	4.23 (0.43)	Cys-59 β-CH_2
22	>3.83 (0.40)	His-46 1-NH
20	<3.58 (0.41)	His-92 4-CH
19		His-92 1-NH
15[b]	4.54 (0.48)	Ile-86 δ-CH_3
14[b]	5.59 (0.59)	
13[b]	5.95 (0.67)	
−6[b]	4.12 (0.43)	Val-89 γ,γ'-CH_3
−8	4.38 (0.45)	
−11	3.35 (0.58)	
−12	3.10 (0.44)	
−20[b]	3.81 (0.40)	Val-99 γ,γ'-CH_3

[a] The additional parameters used are presented in the text. The figures in parentheses show absolute errors accumulated from the estimated errors in all the experimentally determined parameters. Also given are the proposed assignments of paramagnetically shifted resonances in the 270-MHz spectrum of Co(II) stellacyanin (Figure 1); see the text for details. Numeral before an atom refers to ring position. [b] Resonances exhibiting pure pseudocontact shifts, for which both T_1 and T_2 are accurately determined. Individual values of τ_1 were calculated as detailed in the text. The average was then used for calculating the distances.

given in Table II being used. It is noticeable that the values are divided into two groups, the downfield-shifted resonances giving approximately a factor of 2 lower values than those shifted upfield. This lends support to the statement that τ_1 should be considered only as an operational parameter. The average of the above five τ_1 values is 7.98×10^{-12} s. This value was used in eq 2 in order to calculate the distances, r, for the resonances shown in Figure 1. The results are given in Table III.

A more conventional way of calculating τ_1 involves using T_1 only, for a resonance assigned to proton(s) with a known distance to the metal. After the assignments were made, we used this method to probe the field dependence of quantities used in our analysis. A value of $r = 3.1$ Å was assumed for the resonance at 125 ppm (conclusively assigned to the 2-CH protons on the ligating histidines, see below). This distance and the values of T_1 determined at 100 and 270 MHz were used with the original Solomon–Bloembergen expression (eq 1) to obtain τ_1 values at the two fields, respectively. It should be noted that the distances calculated with this method using the assumed r value were equal, within error limits, to the distances obtained in the approach with Curie spin included, as presented in Table III. Furthermore, distances in the model between the metal and atoms in its proximity (N, C, S) were in concordance with distances obtained in the EXAFS experiments mentioned earlier (Feiters et al., 1988).

pH Dependence.* Figure 3 shows the pH* dependence for some of the hyperfine-shifted resonances of Co(II) stellacyanin. Starting with the resonance at 68 ppm, assigned to a 4-CH proton stemming from His-46 or His-92 (the assignments are presented in the next section and in Table III), it is seen that it displays a typical one-proton titration curve with an estimated pK* of 7.0. This pK* is too low for a proton on a ligating histidine. The shift behavior must thus reflect a structural change due to another deprotonating group nearby.

The pK* found above is very close to the value found for one of the two free His residues, assigned to His-32 or His-100 (Hill & Lee, 1979). Inspection of the aromatic region in the spectrum of Co(II) stellacyanin at different pH* values reveals

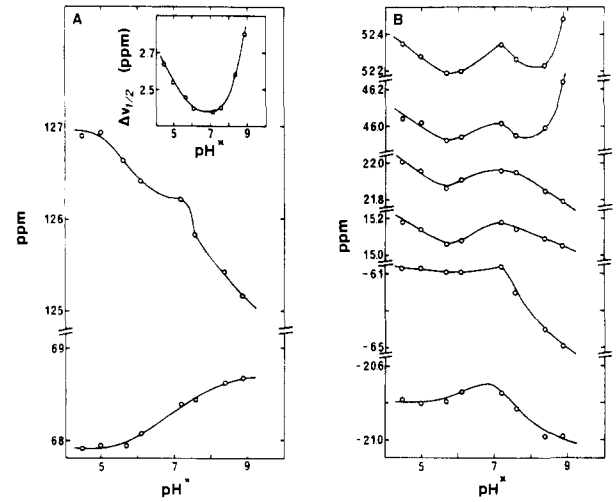

FIGURE 3: pH* dependence for some of the resonances in the ^{1}H NMR spectrum of Co(II) stellacyanin. The insert (A) shows the line width at half-height for the resonance at 125 ppm. Experimental conditions are the same as those of Figure 1.

only minor shift perturbations of the resonances belonging to these residues compared to those of the native protein. However, the observation that the 2-CH resonance, showing a pK* of 6.9, broadens significantly on going from Cu(I) to Cu(II) suggests that one of these His residues cannot be too far away from the metal center (Hill & Lee, 1979). A rough estimate from the longitudinal relaxation data for the cobalt derivative yields an approximate 2-CH proton–metal distance of 7–8 Å for the residue in question. Furthermore, from the model based on the distances extracted from the relaxation data (see Proposed Assignments), it is evident that His-100 can be built in closer to the metal than His-32, placing it in the vicinity of His-46 and Ile-86. This observation is supported by the assignment and position found for the Val-99 γ,γ'-CH_3 groups. Thus, deprotonation of His-100 with a pK* of 7.0 is most likely responsible for the pH* dependence of the resonance at 68 ppm, suggesting that this resonance should be assigned to the His-46 4-CH proton. The same arguments apply to the His NH resonance at 22 ppm, which shows a similar transition superimposed on a generally sloping pH* dependence, arguing that this resonance also should be assigned to His-46.

The resonance at 125 ppm is also affected by the deprotonation of His-100. The behavior is similar to that of the His-46 4-CH resonance up to pH* 7 whereafter a second pH*-dependent process occurs (see below). From the line width of the overlapping resonances (insert, Figure 3A) it is seen that a minimum is reached at pH* 7, suggesting that the 2-CH resonance from His-46 mainly is responsible for the shift in the lower pH* region. Other assigned resonances perturbed by the titrating His-100 residue are the Cys-59 β-CH_2 pair, Ile-86 δ-CH_3, and Val-99 γ,γ'-CH_3, all being located in the vicinity of His-100, thus lending support to these assignments (Table III, Figure 3B).

The second pH*-dependent transition, starting at pH* 7, appears not to affect His-46 since the 4-CH resonance at 68 ppm follows a simple titration curve. It must therefore be the 2-CH resonance of His-92 that is shifting in this pH* range and thus causing apparent line broadening (Figure 3A). Also, the resonances stemming from Cys-59 β-CH_2, Val-89 γ,γ'-CH_3, and Val-99 γ,γ'-CH_3 display pH* dependencies reflecting this second transition (Figure 3B). Moreover, the His-92 NH resonance at 19 ppm disappears above pH* 7, indicating that a local conformational change takes place,

involving the peptide segment 89–99 and Cys-59, caused by a titrating group with a pK^* of 9 or higher. It is also to be noted that this resonance does not show the transition with a pK^* of 7. However, the shift of the His-92 2-CH resonance is too small for this residue to be the titrating group, leaving only Lys-91 or Lys-98 as plausible candidates since there are no tyrosine residues in this region of the protein. Inspection of the model based on the relaxation data (see Proposed Assignments) shows that the amino group of Lys-91 is within hydrogen-bonding distance of the Asp-94 carboxyl group, which in turn can hydrogen bond to the His-92 NH. Thus, the deprotonation of Lys-91 offers a very logical explanation for the exchange behavior of the His-92 NH whereas no such obvious rationale can be found for Lys-98.

Proposed Assignments. The calculated distances (Table III) and the pH* dependencies discussed above were used for assigning the majority of the resonances shown in Figure 1. The relative intensities are shown in Table II. What follows is a brief motivation for each assignment, with the results summarized in Table III. (We would like to point out that preliminary results from corresponding experiments on the nickel derivative of stellacyanin, now under way in this laboratory, support the assignments given below.)

(*A*) *125 ppm.* This two-proton resonance disappears after approximately 2 months at ≈5 °C (pH* 7), indicating a slow ^2H exchange with the bulk solvent. A high degree of scalar contribution gives rise to the very large shift. The only candidates are thus the two 2-CH protons on the ligating His-46 and His-92, to which this peak can be conclusively assigned. The proton exchange phenomenon has also been observed in a resonance Raman study of Cu(II) stellacyanin (Nestor et al., 1984).

(*B*) *68 ppm.* The distance derived from the T_1 value (3.4 Å) points to either one of the His-46 or His-92 4-CH protons. From the pH* dependence it is concluded that this peak stems from the His-46 4-CH.

(*C*) *20 ppm.* The remaining 4-CH proton resonance from His-92 should most likely also be found downfield of the main resonance envelope, unless there is a very unusual orientation of this residue, resulting in a strong upfield pseudocontact shift. However, this does not appear likely as judged from similar data on Co(II)-substituted superoxide dismutase (Bertini et al., 1985). The resonance at 20 ppm is therefore the most reasonable candidate, yielding a distance (<3.6 Å) consistent with such an assignment. This proposal is further supported by our relaxation data on Ni(II) stellacyanin. Now, from the assignments of this resonance and the resonance at 68 ppm, the conclusion must be that the metal is ligated to the 3-nitrogen in both histidines since the distances to the 4-CH protons would otherwise be close to 5 Å, thus considerably longer than 3.6 and 3.4 Å as found, respectively. Both resonances have contact contributions but the large difference in shift is most likely to a great extent of pseudocontact origin.

(*D*) *52 and 46 ppm.* These two peaks show very similar pH* dependencies. From this observation and from the appearance of the resonances, as well as the appearance of the resonances at 117 and 87 ppm (see below and Figure 1), it is obvious that they originate from a CH_2 pair with scalar contributions to the shift. The derived distances (4.4 and 4.2 Å, respectively) are shorter than what is expected for histidine β-CH_2 protons, strongly suggesting β-methylene protons on a cysteine. From the EXAFS measurements mentioned above, the distance betweeen the cobalt ion and the sulfur ligand on Cys-87 is obtained. This allows estimation of the distances between the metal and the Cys-87 β-CH_2 protons which are found to be considerably shorter (3.5 Å) than the above-mentioned values, excluding this residue as a candidate. Since both the ligating histidines are binding the metal with their 3-nitrogens (see above), it is clear from structural constraints given by the peptide sequence that the only remaining cysteine with β-methylene protons within distances similar to what is found is Cys-59, half of the disulfide bridge. The sulfur–metal distance obtained from these proton–metal distances is in close agreement with the distance found from EXAFS. The scalar contribution to the shifts indicates very strongly that this residue provides the second ligating sulfur.

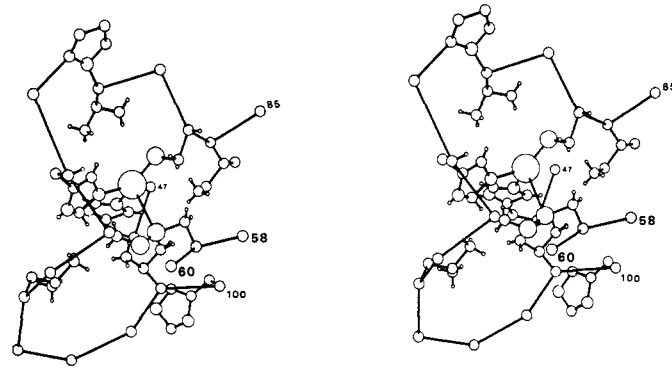

FIGURE 4: Stereo drawing of the model of the metal site in Co(II) stellacyanin. Only α-carbons are included, except for residues of interest for the results presented in this paper. The ends of the included peptide chains are given their sequence number, except the α-carbon of His-46 which is hidden behind the sulfur of Cys-93. His-92 is the ligand closest to the protein surface.

(*E*) *117 and 87 ppm.* The extreme line widths (approximately 10 ppm) and the very large shifts are, again, caused by large scalar contributions. These resonances are therefore assigned to the β-CH_2 protons on the ligating Cys-87. Extremely broad resonances from β-methylene protons on ligating cysteines are also found in a ^1H NMR study of Co(II)-substituted liver alcohol dehydrogenase (Bertini et al., 1984b).

(*F*) *19 ppm.* This resonance is exchangeable and can therefore unambiguously be assigned to a histidine NH proton. The magnitude of the shift strongly indicates one of the ligating histidines. From the pH* dependence it is evident that the correct assignment is to His-92 1-NH.

(*G*) *22 ppm.* From the identical shift of the two His-92 and His-46 2-CH protons (125 ppm), it is expected from symmetry reasons that the respective 1-NH protons will exhibit very similar shifts. This observation, together with the calculated distance (>3.8 Å), suggests that the His-46 1-NH proton gives rise to the resonance at 22 ppm. This tentative assignment is further supported by the pH* dependence. The nonexchangeability of this proton may be explained by hydrogen bonding, since it is known from the structure of poplar plastocyanin that the corresponding NH proton participates in a hydrogen bond with a carbonyl oxygen from the polypeptide backbone (Guss & Freeman, 1983).

With the above, all resonances shown in Figure 1 expected to exhibit contact shift contributions, i.e., protons situated on ligating residues, are assigned, albeit some with a lesser degree of certainty. The proton–metal distances obtained for these resonances (Table III), together with the primary structure of stellacyanin (Bergman et al., 1977), allowed us to build a three-dimensional model of the metal site and its surroundings (Figure 4). When this model was being built, the short Co–S distance only was taken from EXAFS data of Co(II) stellacyanin (Feiters et al., 1988). To corroborate the folding of the peptide backbone around the metal, qualitative assessments of the pseudocontact shift contributions to the resonances were

made. In order to do this, it was assumed that the *x,y* plane of the Co(II) *g* tensor in stellacyanin has the same orientation relative to the trigonal ligand plane as found for the Cu(II) *g* tensor in plastocyanin (Penfield et al., 1985). The positive shift sphere is then coincident with the *x,y* plane while the negative spheres are placed in the axial direction (Bleaney, 1972). In no case were the observed pseudocontact shifts, for resonances lacking scalar contributions, in conflict with those deduced from the model based on distance and sequence information.

The remaining resonances are thus purely pseudocontact shifted.

(*H*) *15 ppm.* This is a three-proton resonance and must thus originate from a methyl group. The model shows that the only methyl group that can be positioned within the positive pseudocontact shift sphere at the required distance (4.5 Å) is Ile-86 δ-CH_3.

(*I*) *−6 ppm.* The intensity of this peak (see Table II) corresponds to two methyl groups and, possibly, one additional proton. Again, the model reveals that the only possible residues providing these methyl groups are Val-89 and Val-99. Given the structural constraints of the peptide loop between Cys-87 and His-92 (see Figure 4), it is evident that the methyls of Val-89 cannot be positioned closer than 4 Å to the metal without introducing strain in the structure. The calculated distance (4.1 Å) is thus in very high agreement with this observation.

(*J*) *−20 ppm.* Upon integration, this resonance was found to contain eight protons (see Table II). Furthermore, it was observed that, in spectra recorded after the sample was heated to about 308 K, a one-proton resonance had emerged slightly on each side of the main peak centered around −20 ppm. It is thus very reasonable to assume that the remaining six protons stem from two methyl groups. The only logical assignment is therefore to Val-99, whose larger shift is consistent with a shorter distance to the metal compared with Val-89.

The structure of the metal site in Co(II) stellacyanin, on the basis of the assignments of the contact shifted resonances, represents a solution consistent with the experimental data given in the foregoing sections. Several other configurations, e.g., ligation of one or both histidines with their 1-N atoms and/or the Cys-93 sulfur instead of Cys-59, could not be reconciled with the distance information derived from the relaxation data, chemical shifts, and pH* dependencies. Since the assignments for some of the paramagnetically shifted resonances are not entirely conclusive, additional future evidence might conceivably alter some aspects of the structural model as presented in Figure 4.

Discussion

Methodology for Distance Determinations. The idea of using relaxation measurements to investigate structural features in molecules with paramagnetic centers is certainly not new. However, the approaches generally adopted in earlier work utilize the original Solomon–Bloembergen expressions in which the Curie spin is not accounted for. Since the Curie-spin contribution to T_2 usually is several orders of magnitude larger than that to T_1, such analyses are confined to using the latter relaxation time only. But even if the Curie-spin contribution to the longitudinal relaxation time is considered small enough to be disregarded, it is however still there and will cause larger errors in the interpretations, particularly in highly anisotropic systems, compared to an approach where the Curie spin is included. Another obstacle toward a successful analysis using this approach is the determination of the electronic relaxation time, τ_1. Generally, this is accomplished in a semiempirical manner by the use of the T_1 value for a proton whose distance to the metal is already known. If such a distance can be obtained by some other means and the proton can be assigned to a shifted resonance in the NMR spectrum, this "classical method" is perfectly feasible and may be a powerful tool in structure analysis. Excellent examples of this are the works performed by Bertini et al. (1983) and Khalifah et al. (1984) on cobalt-substituted carbonic anhydrase.

By including the Curie spin in the interpretation of relaxation data, it is possible to make use of the extended expressions for both T_1 and T_2 (Gueron, 1975). The problem of determining τ_1 can thus be circumvented, for instance as presented in this paper, and distances may be obtained from structures of which very little is known. Thus, the usefulness and generality of the method is increased since no proton–metal distance is needed beforehand. A good demonstration of the Curie-spin approach is a recent publication on the structure of the dysprosium–glycocholate complex, where also chemical exchange with the environment was included in the interpretations (Mukidjam et al., 1987). This system is however considerably different from ours, both in size and in behavior. The results presented by these authors included, among other things, a three-dimensional model of the complex. In the present work, we have used cobalt-substituted stellacyanin as a further example of an interpretation with the Curie spin included. It is interesting to note that the operational value found for τ_1 (8×10^{-12} s) is of the same order of magnitude as the value determined by Bertini et al. (1983) (2.5×10^{-12} s) for cobalt-substituted human carbonic anhydrase. Very little structural evidence have been gathered on any stellacyanin derivative, but we are able to present a model of the metal-binding site totally compatible with the relaxation data and the assignments presented above. We do acknowledge, however, that further experiments with some complementary techniques are desirable to verify this structure.

Geometry of Metal Site. From sequence homology studies (Rydén, 1984; Adman, 1984) and the finding that Cys-59 and Cys-93 form the disulfide bridge in stellacyanin (Engeseth et al., 1984a), it has been clear that three of the metal ligands are given by His-46, Cys-87, and His-92. Since stellacyanin lacks methionine (Bergman et al., 1977), the question of a fourth ligand has been a matter of debate, and the participation of sulfur(s) from a disulfide bridge in the ligand arrangement of metalloproteins has hitherto not been found, although it has been suggested (Ferris et al., 1978). The present results strongly indicate that the sulfur of Cys-59 makes up the fourth ligand at a distance (3.2 Å) comparable to those found by X-ray crystallography for the S(Met)–Cu bond in azurin (3.13 Å) from *A. denitrificans* (Norris et al., 1986) and plastocyanin from *Populus nigra* (Guss & Freeman, 1983). It proved impossible to fit the β-CH_2 pair of Cys-93 to the required distances (Table III), and furthermore, a very unusual metal–sulfur bond angle vis-á-vis the trigonal ligand plane would then have to be introduced. From the large hyperfine shifts of the β-CH_2 protons of Cys-59, it is also quite clear that the metal–sulfur interaction represents a true bond, as has been argued for the methionine ligand in plastocyanin (Guss & Freeman, 1983; Live et al., 1985).

As an alternative to coordination by a sulfur, it was recently suggested that the amide group of Gln-97 might be the fourth ligand in stellacyanin (Guss et al., 1988). The possibility of a glutamine amide in this position cannot be discarded from the NMR data alone. The EXAFS data on Cu(I) and Cu(II) stellacyanin (Feiters et al., 1988), on the other hand, show

conclusively that a second sulfur is present at a distance of about 3 Å, and no indications could be found for any additional nitrogen or oxygen atoms close to the metal (contrary to the cobalt and nickel derivatives, see below). These results seem to contradict the proposal of an amide ligand in the fourth position.

Another feature that makes stellacyanin stand out is that both of the histidine ligands bind the metal with their 3-N atoms instead of the 1-N atoms as in plastocyanin (Guss & Freeman, 1983) and azurin (Norris et al., 1986). This fact is also borne out by the ^{1}H NMR spectrum of Co(II) azurin from *Pseudomonas aeruginosa* (Hill et al., 1976), which is quite different from that presented in Figure 1. Although no assignments of the hyperfine-shifted resonances were done in this case, the His β-CH_2 resonances are now expected in the low-field part of the spectrum, since these groups will be directed toward the metal at a much shorter distance. The appearance of the Co(II) azurin spectrum seems to support this line of reasoning. However, the change in the nitrogen binding to the metal appears not to have had any profound influence on the metal–ligand bond angles as compared to those found for azurin (Norris et al., 1986) and plastocyanin (Guss & Freeman, 1983). Severe restrictions are imposed by the presence of Pro-90 and the requirement of the Val-89 γ,γ'-CH_3 groups being at a distance of 4.1 Å from the metal on the S(87)–Co–N(92) angle (≈130°), while the N(46)–Co–S(87) and N(46)–Co–N(92) angles cannot be ascertained with the same degree of precision. However, the possibility of interactions between Asn-47 and Cys-87 (see below) suggests that the latter two angles should be approximately the same as in plastocyanin and azurin at ≈130° and ≈95°, respectively (Guss & Freeman, 1983; Norris et al., 1986).

A computer study on stellacyanin has recently been presented where energy minimization and graphics modeling were used to predict the folding of the peptide chain (Wherland et al., 1988). The sequence homology with plastocyanin was used as input, and it was assumed that both ligating histidines are binding the metal with their 1-nitrogen and that Cys-93 provides the second ligating sulfur. The authors did also perform calculations, however without extensive refinement, based on our proposal that both histidines ligate with the 3-nitrogens. They found the energy of this structure to be only 100 kcal/mol higher than that for the plastocyanin analogy, thus insignificantly different regarding conformational strain. Hence, this technique cannot reliably distinguish between these two alternatives. In addition, the computer calculations gave indications of a carbonyl oxygen within binding distance (3 Å) to the metal.

Comparison with EXAFS Data. Parallel to the study presented here, a comparative EXAFS study on native stellacyanin and the Co(II)- and Ni(II)-substituted proteins (Feiters et al., 1988) has been completed. The coordination spheres of the Cu(II) and Co(II) proteins are found to be almost identical, a fact which strongly supports the model derived from the NMR data as being valid also for the native protein. Apart from the short ligand–metal bond distances in the cobalt derivative (2-N at 1.94 Å and 1-S at 2.21 Å), evidence was also found for a second sulfur at 3.12 Å, which must undoubtedly stem from the Cys-59–Cys-93 disulfide bridge. The distance is, within experimental error, identical with that found by NMR. Previous EXAFS studies of stellacyanin, however, did not indicate any sulfur atoms at longer distances (Tullius et al., 1978). It may be noted that in Ni(II) stellacyanin this sulfur moves in toward the metal by ≈0.4 Å compared to Co(II) substituted protein. Also, in the NMR spectrum of the nickel protein (Dahlin and Ångström, unpublished results), an increased shift is observed for the resonances assigned to the β-CH_2 proton pair of Cys-59. This significant difference between the two derivatives observed with both techniques is most likely originating from the same source. One notable dissimilarity between the native protein and the metal-substituted derivatives is that, in the recent EXAFS analysis of the latter, an additional oxygen or nitrogen, approximately 2 Å from the metal, had to be included. A possible reason for this observation is discussed below.

Magnetic Susceptibility of Co(II) Stellacyanin. The effective magnetic moment (μ_{eff}) obtained by the NMR method at 303 K is 4.53 Bohr magnetons. This value is intermediate in the range of values expected for four- and five-coordinated Co(II) complexes (Figgis & Lewis, 1964). A previous study (Solomon et al., 1976), made in the low-temperature range (2–50 K), indicated a very low zero-field splitting from which it was concluded that the metal site would have a distorted tetrahedral geometry without any orbital contributions to the susceptibility. Our results, however, suggest a much higher zero-field splitting since both the room-temperature susceptibility and the relaxation data are compatible with five ligands. A comparison with low-temperature susceptibility measurements of the azetazolamide complex of Co(II) carbonic anhydrase, where the zero-field splitting was found to be 33 cm^{-1} (Aasa et al., 1976) and in which the metal is believed to be five-coordinated, indicates that Co(II) stellacyanin has a zero-field splitting of the same magnitude. This shows that the earlier experiments on stellacyanin mentioned above are probably misinterpreted due to a paramagnetic impurity in the sample.

Role of Asn-47. This residue is conserved in the small blue copper proteins sequenced to date. In the crystal structure of plastocyanin, the backbone amide group of this residue (Asn-38) forms a hydrogen bond with the thiolate sulfur of Cys-84, while the side chain forms further hydrogen bonds with the peptide amide and side chain of Ser-85 (Guss & Freeman, 1983). Corresponding interactions have been found in the azurin structure (Adman & Jensen, 1981). In stellacyanin, however, the fact that His-46 binds to the metal with the 3-N atom precludes any interaction between the backbone amide of Asn-47 and the sulfur of Cys-87. Furthermore, Ser-85 in plastocyanin is in stellacyanin replaced by Gly-88, which is adjacent to the bulky side chain of Val-89, disallowing any interactions with the peptide loop.

Due to these structural differences, it is now possible for the side chain of Asn-47 to form a hydrogen bond with the sulfur of Cys-87 instead. This will in turn bring the amide oxygen within binding distance to the metal. There are good reasons to believe that this is the case: first, EXAFS measurements of Co(II) stellacyanin indicate the presence of an extra oxygen or nitrogen at a distance of 2 Å from the metal; second, the susceptibility is consistent with a five-coordinated complex; third, in the 100-MHz NMR spectrum a new, very broad (≈15 ppm), resonance at −60 ppm was detected which most likely originates from an additional ligand. Furthermore, the fastest relaxing resonances in the 270-MHz NMR spectrum at −11 and −12 ppm (Figure 1) yield distances close to 3 Å from the metal (Table III), consistent with the description of the Asn-47 side-chain orientation given above. Moreover, according to EXAFS this ligand seems to have a different position in the native protein, probably at a longer distance to the metal. It is now easy to rationalize this difference as being due to a reorientation of the Asn-47 side chain while still retaining the hydrogen bond to Cys-87. It should also be

noted that in a resonance Raman study of Cu(II) stellacyanin a fifth ligand was suggested, a side-chain amide group of Asn or Gln (Siiman et al., 1974). Furthermore, in azurin from *A. denitrificans* a carbonyl oxygen from the backbone forms a potential fifth ligand to copper at a distance of 3.2 Å (Norris et al., 1986), and in a comparative ^{113}Cd NMR study of Cd(II)-substituted blue copper proteins (Engeseth et al., 1984b), the spectra of stellacyanin and azurin were found to be similar, while the spectrum of the plastocyanin derivative showed deviations from the other two. These findings are also in line with our reasoning since the crystal structure of native plastocyanin did not reveal a fifth ligand (Guss & Freeman, 1983).

Reduction Potential of Stellacyanin. The reduction potential of native stellacyanin, 184 mV (Reinhammar, 1972), is the lowest found so far within the blue copper protein family. This value can be compared with, e.g., those of plastocyanin from different sources at roughly 350–400 mV (Ryden, 1984; Adman, 1984), which are intermediate in the range of values found to date. The metal site geometry of poplar plastocyanin is not altered much by insertion of the copper ion into the apoprotein (Garrett et al., 1984). The protein thus forces the metal into a strained configuration which has a profound influence on redox and spectral properties. In this context it has been proposed (Gray & Malström, 1983) that the axial ligand(s) play(s) a crucial role in tuning the reduction potential. Our finding of a slightly longer axial metal–sulfur bond than in plastocyanin and the likely presence of a fifth ligand in Co(II) stellacyanin, as recently suggested for the native protein (Ainscough et al., 1987), supports this view and may explain the low value found for stellacyanin. However, the recent suggestion of a glutamine in the fourth ligand position (Guss et al., 1988) is also compatible with the unusually low potential.

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Registry No. L-His, 71-00-1; L-Cys, 52-90-4; L-Asn, 70-47-3; Co, 7440-48-4.

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X-ray Absorption Spectroscopy of the [2Fe-2S] Rieske Cluster in *Pseudomonas cepacia* Phthalate Dioxygenase. Determination of Core Dimensions and Iron Ligation†

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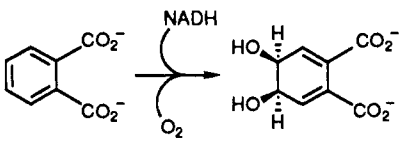
ABSTRACT: We have employed X-ray absorption spectroscopy to obtain structural information about the Rieske Fe/S center in the phthalate dioxygenase (PDO) from *Pseudomonas cepacia*. Native PDO contains a dinuclear Rieske Fe/S center and an additional mononuclear Fe site. In order to study selectively the Fe/S cluster, we measured data for samples in which the mononuclear site was either depleted of metal or reconstituted with Co or Zn. Our results demonstrate that the iron environment in the Rieske cluster is structurally indistinguishable from that found in other Fe/S clusters, thus strongly supporting the suggestion that the unusually high reduction potentials for Rieske clusters are due to electrostatic rather than structural effects. The average Fe–Fe distance is 2.68 (3) Å for both oxidized and reduced Rieske clusters. The average Fe–S distance is 2.24 (2) Å in the oxidized cluster and 2.28 (2) Å in the reduced cluster. Careful analysis of the EXAFS Debye–Waller factors suggests that the bridging and terminal Fe–S distances for the oxidized cluster are 2.20 and 2.31 Å, respectively. Taken together with recent ENDOR results, these studies provide a detailed structural model for the Rieske [2Fe-2S] centers.

Phthalate dioxygenase (PDO) is a stable protein that can be isolated in sufficient quantities for detailed structural characterization (Batie et al., 1987). In analogy to other such oxygenases, it catalyzes the first step in the metabolism of an unactivated aromatic compound, in this case phthalate, yielding the 4,5-*cis*-dihydrodiol of phthalate.



(1)

There are four identical subunits in PDO, each of which contains an Fe/S cluster that is spectroscopically indistinguishable from the "Rieske" Fe/S cluster (see below). In addition to the Fe/S cluster, ferrous ion is required for full activity (Batie et al., 1987). If the enzyme is dialyzed against EDTA to completely remove the ferrous ion, 1 equiv of Fe^{2+} must be added to reactivate the enzyme. Although other divalent metal ions will bind in place of Fe^{2+}, none will restore activity. The ferrous (mononuclear) site, which is >10 Å from the Rieske center (C. J. Batie, W. R. Dunham, and D. P. Ballou, unpublished work), has been proposed as the site of oxygen activation (Batie et al., 1987). We have employed EXAFS spectroscopy to obtain structural information about the Rieske center and to characterize the structural differences between Rieske clusters and the plant-type ferredoxins.

The Rieske-type cluster was first discovered (Rieske et al., 1964) in the mitochondrial ubiquinol–cytochrome *c* reductase system and was recognized by its unusual EPR spectral properties. Rieske clusters have since been found in photosynthetic electron-transfer proteins (Nelson & Newmann, 1972; Hurt & Hauska, 1981), in bacterial respiratory chains (Bowyer & Crofts, 1981; Gabellini et al., 1982), and in several bacterial oxygenases (Geary et al., 1984; Axcell & Geary, 1972; Gibson et al., 1970; Sauber et al., 1977; Batie & Ballou, 1987). The structure of the Rieske-type [2Fe-2S] center is of substantial interest because proteins containing this center have properties that are distinctively different from those of the ferredoxins. These properties include anomalous EPR, visible, and CD spectra and unusually high reduction potentials (−150 to 150 mV) [Kuila & Fee (1986) and references cited therein].

Characterization of the structure of the Rieske-type cluster is one important step toward understanding its properties. The Rieske cluster is believed to contain an Fe_2S_2 core similar to that found in the plant-type ferredoxins; however, in contrast to the ferredoxins, which have four symmetrically placed cysteine sulfurs surrounding the [2Fe-2S] core of the center,

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