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Paramagnetic ^{1}H NMR Spectrum of the Cobalt(II) Derivative of Spinach Plastocyanin

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The native type 1 copper ion of spinach plastocyanin has been substituted with Co(II). The UV/vis spectrum of this derivative is similar to those for other Co(II)-substituted cupredoxins. The paramagnetic ^{1}H NMR spectrum of Co(II) plastocyanin has been completely assigned. A number of similar studies on Co(II) cupredoxins have been published, but this is the first such analysis of a substituted plastocyanin that possesses the archetypal type 1 active site. A truly representative comparison of the available paramagnetic ^{1}H NMR data for Co(II) cupredoxins is now possible. We demonstrate in this work that there is very little difference in the metal–ligand contacts between the Co(II) derivatives of cupredoxins possessing a type 1 axial site (plastocyanin) and those having perturbed (rhombic) spectroscopic features.

Introduction

Replacement of the native mononuclear type 1 copper ion in cupredoxins with other metals has been utilized for decades to facilitate the investigation of this key family of redox metalloprotein.[1−20] In most of these approaches the introduced metal ion confers particular spectroscopic properties to the cupredoxin, which can then be used to provide information about active site structure. If the introduced metal possesses unpaired electrons, then the associated paramagnetism can be utilized to supply detailed structural information.[8,9,12,14,15,17,19,20] Herein we have utilized metal substitution to investigate the type 1 copper-containing protein plastocyanin from spinach.

The plastocyanins are cupredoxins involved in photosynthetic electron transfer between cytochrome *f* of the b_6f complex and $P700^+$ of photosystem I in plants, green algae, and cyanobacteria (blue green algae). Structures of plastocyanins from all these sources have been determined in both the cuprous and cupric forms by X-ray crystallography and also in solution by nuclear magnetic resonance (NMR) spectroscopy.[21−39] This array of structural information makes

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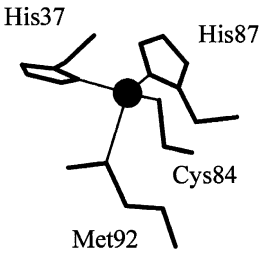

Figure 1. Active site structure of spinach Cu(II) plastocyanin (PDB accession code 1AG6[32]), drawn with MOLSCRIPT.[40] The copper ion is indicated as a black sphere.

the plastocyanins one of the best structurally characterized families of redox metalloproteins. Plastocyanin consists of a β-barrel with the copper ion buried approximately 6 Å from the protein surface in a distorted tetrahedral geometry (see Figure 1). Three ligands form strong bonds to the copper, namely the thiolate sulfur of Cys84 and the N^{δ} atoms of His37 and His87. The copper ion is slightly displaced from the plane of these three equatorial ligands toward the weakly coordinated thioether sulfur of Met92.

Paramagnetic proton NMR (^{1}H NMR) spectroscopy has recently been shown to provide detailed information about the active site structure of Cu(II) cupredoxins, including various plastocyanins.[41−49] These experiments benefit from the presence of the native metal but are hampered by the relatively slow electronic relaxation of the cupric ion which results in the isotropically shifted resonances being very broad and difficult to observe and assign. For example the resonances from the $C^{\beta}H$ protons of the Cys ligand cannot be observed directly as they are typically 10^5-10^6 Hz wide. The paramagnetic ^{1}H NMR spectra of Co(II)- and Ni(II)-substituted cupredoxins possess hyperfine-shifted resonances which are much sharper than their counterparts in the Cu(II) proteins (the $C^{\beta}H$ proton resonances are usually 2000−3000 Hz wide).[8,9,14,15,17,19,20] These sharper signals can be assigned by analyzing dipolar connectivities utilizing known structural information. Using this approach a detailed analysis of the active site structure of a number of cupredoxins has been carried out.[8,9,12,14,15,17,19,20] However, paramagnetic NMR has not been used to study a Co(II)-substituted plastocyanin which is surprising bearing in mind that the Co(II) protein from bean was probably the first metal-substituted cupredoxin to be prepared.[1] The lack of paramagnetic NMR data for a metal-substituted plastocyanin is also unexpected considering the amount of structural information available for this cupredoxin and given the accepted position of plastocyanin among the cupredoxins as possessing the "classic" type 1 copper site.[50,51] We have therefore prepared Co(II) spinach plastocyanin, assigned its paramagnetic NMR spectrum, and have made comparisons to data currently available in the literature for other Co(II)-substituted cupredoxins.

Experimental Section

Protein Isolation and Purification. The expression, isolation and purification of spinach plastocyanin was as described previously.[41] The purity of the protein was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Protein concentrations were determined from the molar absorption coefficient of 4500 M^{-1} cm^{-1} at 597 nm in the ultraviolet/visible (UV/vis) spectrum.

Preparation of Apo Plastocyanin. Apo-protein was prepared by unfolding/refolding the protein. For this procedure plastocyanin (∼1 mM) was incubated overnight under anaerobic conditions in an unfolding solution containing 6 M guanidine hydrochloride, 50 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM dithiothreitol (DTT) in 10 mM 4-(2-hydroxyethyl)piperazine-1-ethane-

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sulfonic acid (Hepes) at pH 8.0. The solution was diluted 50-fold with 10 mM Hepes pH 8.0 containing 1 mM EDTA and 2 mM DTT and incubated at room temperature for 2 h under argon to allow the protein to fold. The refolded protein was concentrated and exchanged by ultrafiltration (3 kDa MWCO membrane) into 10 mM Hepes, pH 8.0, containing 1 mM EDTA. After the removal of DTT, the protein was loaded onto a (diethylamino)ethane sepharose column equilibrated with 10 mM Hepes, pH 8.0, plus 1 mM EDTA. The protein was eluted with a 0−250 mM NaCl gradient in the same buffer, and the plastocyanin-containing fractions were combined.

Co(II) Uptake. Co(II) uptake was performed by dialyzing 50−100 μM of apo-protein at 4 °C against 10 mM Hepes, pH 8, containing 0.2−0.4 mM $CoCl_2$ for 2 days. The Co(II) plastocyanin was filtered and exchanged into 20 mM tris(hydroxymethyl)-aminomethane (Tris), pH 8.0.

Samples for Paramagnetic ^{1}H NMR Studies. Prior to the paramagnetic NMR studies the substituted protein was purified on a Resource Q column using an AKTA prime chromatography system (Amersham Pharmacia Biotech). For this high-resolution ion-exchange chromatography step the protein was in 20 mM Tris, pH 8.0, and was eluted with an ionic strength gradient created using 20 mM Tris, pH 8.0, containing 300 mM NaCl. Paramagnetic ^{1}H NMR spectra were obtained with the protein in 10 mM phosphate in 99.9% D_2O at pH* 7.8 and in 90% H_2O/10% D_2O at pH 7.8 and 7.0. The pH values of protein solutions were measured using a narrow pH probe (Russell KCMAW11) with an Orion 420A pH meter. The pH of the samples was adjusted using 1 M NaOD or DCl in deuterated solutions and 1 M NaOH and HCl in H_2O solutions. The pH values quoted for deuterated solutions are uncorrected for the deuterium isotope effect and thus are indicated by pH*.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were acquired on a Bruker Avance 300 spectrometer. All chemical shifts are quoted in parts per million (ppm) relative to water using the relationship $\delta_{HDO} = -0.012t + 5.11$ ppm, where t is the temperature in °C.[43] One-dimensional (1D) spectra were acquired using a standard single pulse sequence employing presaturation of the H_2O or HDO resonance during the relaxation delay. The super-water-suppressed equilibrium Fourier transform (WEFT) sequence (d1−180°−τ−90°−acq, where d1 is the relaxation delay and acq the acquisition time) was used to observe fast relaxing signals. Data were acquired with spectral widths of approximately 100 kHz and were processed using 20−50 Hz of exponential line broadening as apodization. Steady-state 1D NOE difference spectra were acquired at two different temperatures (10 and 30 °C) to minimize the chance of spectral overlap. For these experiments the super-WEFT sequence was implemented with τ values and a total relaxation delay (d1 plus acq) ranging from 15 to 50 ms. The resonance of interest was irradiated during τ with a selective decoupler pulse. Spectra were acquired in blocks of eight scans alternating between on-resonance, off-resonance in an upfield direction, on-resonance, and off-resonance in a downfield direction (the offset was equal to that utilized in the upfield direction). The off-resonance spectra were subtracted from their on-resonance counterparts to produce the NOE difference spectrum.

The spin−lattice (T_1) relaxation times of the hyperfine shifted resonances were determined using an inversion−recovery experiment. For this the super-WEFT sequence was used with a total effective relaxation delay (d1 plus acq) ranging from 8 to 100 ms. The interpulse delay (τ) was varied between 0.01 ms and the total relaxation delay. An exponential fit of the plots of peak intensity against τ, for a particular proton, yielded its T_1 value. Peak widths were measured using spectra which had not undergone any baseline correction.

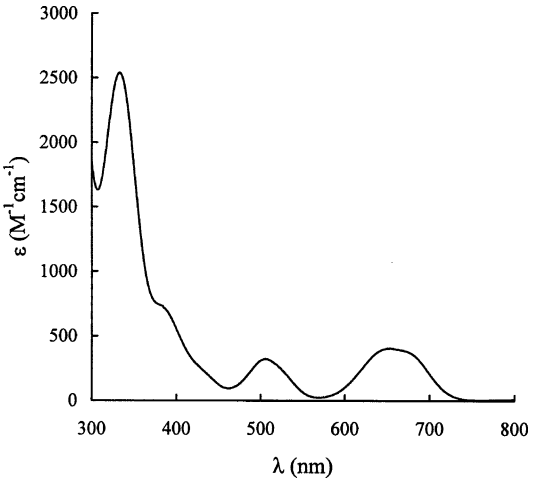

Figure 2. UV/vis spectrum of Co(II) spinach plastocyanin in 10 mM phosphate at pH 8.0 (25 °C).

Assignment Strategy. To assign the signals in the paramagnetic ^{1}H NMR spectrum of Co(II) plastocyanin we have used an approach utilizing dipolar connectivities. This method requires a structural model, and for this we have used the crystal structure of the Cu(II) protein (PDB accession code 1AG6[32]). Hydrogens were added to this structure using the program Insight II.

The NOE data were analyzed using eq 1, which is valid in the slow-motion limit:

$$\eta_{ij} = -\left(\frac{\mu_0}{4\pi}\right)^2\left(\frac{\hbar^2\gamma_H{}^4\tau_r}{10r_{ij}{}^6\rho_i}\right) \quad (1)$$

Here η_{ij} is the NOE observed for signal i upon irradiation of signal j, μ_0 is the magnetic permeability of a vacuum, $\hbar$ is Planck's constant (h) divided by 2π, γ_H is the magnetogyric ratio of the proton, τ_r is the rotational correlation time of the protein, r_{ij} is the distance between the protons i and j, and ρ_i is the longitudinal relaxation rate ($T_1{}^{-1}$) of proton i.

Results

Co(II) Substitution of Plastocyanin. The Co(II)-substituted plastocyanin has a UV/vis spectrum typical for a Co(II) cupredoxin (see Figure 2) and is sufficiently stable at pH 8.0, in the absence of excess Co(II), to allow paramagnetic NMR investigations to be undertaken. However, the sample does have a tendency to denature with time, as observed by the loss of intensity in the UV/vis spectrum and the presence of some additional peaks in the paramagnetic ^{1}H NMR spectrum (vide infra). This effect is accelerated if the sample is taken to pH 7.0 or below.

Paramagnetic ^{1}H NMR Studies of Co(II) Plastocyanin. The paramagnetic ^{1}H NMR spectrum of Co(II) plastocyanin at pH 7.8 in 90% H_2O/10% D_2O is shown in Figure 3A,B. The observed resonances in the spectrum are listed in Table 1 along with their spin−lattice (T_1) relaxation times and peak widths ($\Delta\upsilon_{1/2}$). The peaks labeled a−n have properties (hyperfine shifts, line widths, and T_1 values) which identify them as arising from protons associated with the coordinating amino acid residues. The two quite sharp peaks at around

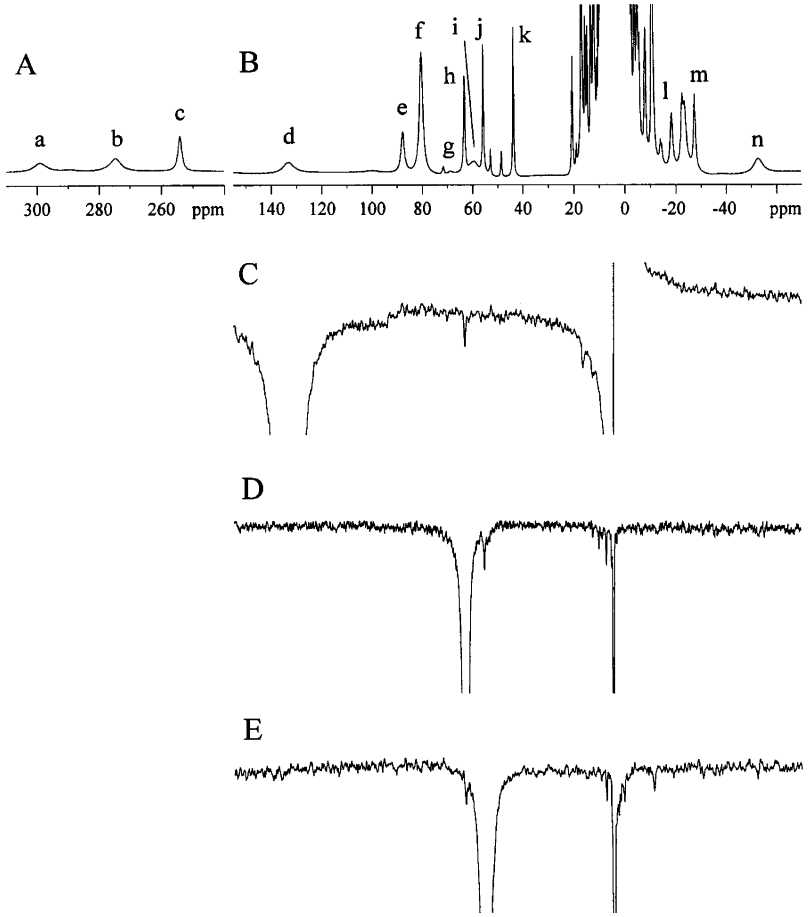

Figure 3. ^{1}H NMR spectrum (A, B) of Co(II) plastocyanin (30 °C) in 10 mM phosphate buffer in 90% H_2O/10% D_2O at pH 7.8. Also shown are 1D NOE difference experiments (C−E) in which peaks d, h, and j were irradiated.

Table 1. Hyperfine-Shifted Resonances in the ^{1}H NMR Spectrum of Co(II) Plastocyanin[a]

resonance	δ_{obs} (ppm)	T_1 (ms)	$\Delta\nu_{1/2}$ (Hz)	assignment
a	299	0.5	1400	Cys84 $C^{\beta 1}H$
b	275	0.6	1200	Cys84 $C^{\beta 2}H$
c	254	1.9	400	Met92 $C^{\gamma 1}H$
d	133	0.5	1500	His37 $C^{\epsilon 1}H$
e	87.8	1.7	420	Met92 $C^{\gamma 2}H$
f	80.4	1.9	420	Met92 $C^{\epsilon}H_3$
g[b]	75.3	5.0	230	His87 $N^{\epsilon 2}H$
h	63.2	8.3	160	His37 $N^{\epsilon 2}H$
i	60	nd[c]	∼1500	His87 $C^{\epsilon 1}H$
j	55.8	18.3	100	His37 $C^{\delta 2}H$
k	43.8	13.9	100	His87 $C^{\delta 2}H$
l	−18.4	2.7	300	Met92 $C^{\beta 1}H$
m	−27.5	4.5	220	Met92 $C^{\beta 2}H$
n	−52.5	0.5	1300	His87 $C^{\beta 2}H$

[a] Data recorded at 300 MHz and mostly at pH 7.8 and 30 °C. Included are the observed chemical shifts (δ_{obs}), the spin−lattice (T_1) relaxation times, the peak widths ($\Delta\nu_{1/2}$), and the assignments that have been made. [b] Measured at pH 7.0 and 5 °C. [c] Not determined.

50 ppm have intensities ∼10% of the other signals in the spectrum, and their relative intensities are the same when the sample is in 99.9% D_2O. The intensity of these signals increases with time (this effect is accelerated at lower pH values). We therefore assign these signals to a denatured form of the Co(II) protein.

Peaks g and h are resonances from exchangeable protons and therefore must belong to the $N^{\epsilon 2}H$ protons of the two His ligands. Peak g is in fast exchange with the bulk solvent at pH 7.8 and 30 °C and thus is hardly observed. Upon lowering of the pH to 7.0 and decreasing of the temperature to 5 °C, the intensity of peak g increases considerably (see Figure 4A). These observations are consistent with peaks g and h arising from the $N^{\epsilon 2}H$ protons of His87 and His37, respectively (the His87 residue is much more solvent exposed than His37). Irradiation of the broad signal d gives rise to a nuclear Overhauser effect (NOE) to peak h, while NOEs are observed between peaks h and j (see Figure 3C−E). This pattern of NOEs, along with the observed line widths and T_1 values of these signals, is consistent with peak d being the $C^{\epsilon 1}H$ resonance of His37 (this proton is only 3.1 Å from the metal and therefore is broad and has a short T_1) and peak j arising from the $C^{\delta 2}H$ proton of His37 (this proton is 5.1 Å from the metal and thus is sharper and has a longer T_1). Irradiation of peak j also gives rise to an NOE to a signal at −11.2 ppm (see Figure 3E) which is assigned to the $C^{\beta 2}H$ proton of His37. [The His37 $C^{\delta 2}H$ and $C^{\beta 2}H$ protons are 2.7 Å apart in the structure of Cu(II) plastocyanin, and the $C^{\beta 2}H$ proton is situated 4.5 Å from the metal which is consistent with the relaxation properties of the resonance at −11.2 ppm.]

At pH 7.0 and 5 °C (in 90% H_2O/10% D_2O) NOEs are observed between signals g and k (see Figure 4B,C), and thus peak k can be assigned to the His87 $C^{\delta 2}H$ proton resonance. The broad signal i (see Figure 3B) must belong

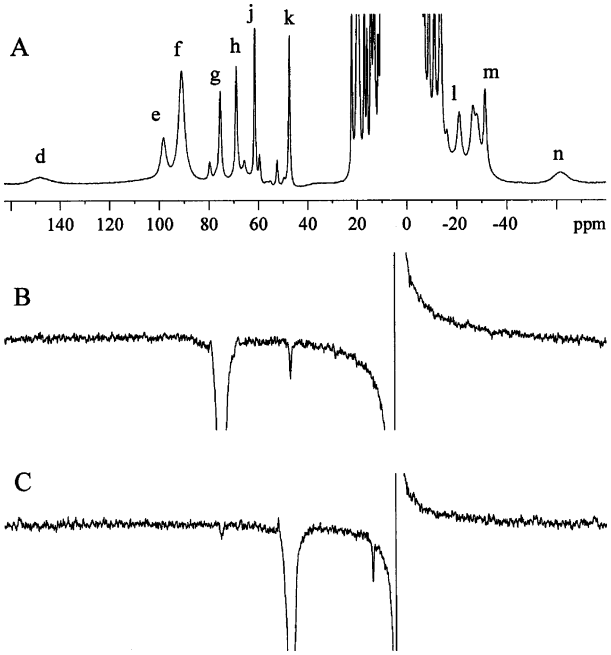

Figure 4. Reference (A) and 1D NOE difference spectra (B, C) of Co(II) plastocyanin (5 °C) in 10 mM phosphate in 90% H_2O/10% D_2O at pH 7.0. In the 1D NOE difference experiments peaks g (B) and k (C) were irradiated.

to the $C^{\epsilon 1}H$ proton of His87, but because it overlaps with a number of signals, and is difficult to observe at 5 °C, it was not possible to irradiate this peak and observe the NOE to signal g (His87 $N^{\epsilon 2}H$). Irradiation of the His87 $C^{\delta 2}H$ resonance (peak k) also gives rise to an NOE to a relatively sharp peak at 14.2 ppm (see Figure 4C) which possibly arises from the $C^{\alpha}H$ proton of His87 [these two protons are 2.5 Å apart in the structure of Cu(II) plastocyanin]. The irradiation of peak n did not give rise to NOEs to any other isotropically shifted resonance in the spectrum of Co(II) plastocyanin. Given the relaxation properties of signal n (see Table 1), we have tentatively assigned it to the His87 $C^{\beta 2}H$ proton which is situated 2.8 Å from the metal ion in the crystal structure of oxidized spinach plastocyanin.

The very large isotropic shifts and line widths and the short T_1 values of peaks a and b identify them as arising from the $C^{\beta}H$ protons of the Cys84 ligand. Attempts to observe the NOE between these two signals were unsuccessful due to their very short T_1 values, their broad nature, and their similar chemical shifts which lead to considerable off-resonance effects in the NOE difference experiments (this is exacerbated by the high power levels required for the irradiation of these peaks). Irradiation of peak a gives rise to a weak NOE to signal e and a slightly stronger NOE to signal l (see Figure 5B), while the irradiation of peak b results in a weak NOE to peak e (data not shown). These NOEs will be discussed further below.

The assignment of resonances belonging to the axial Met92 ligand begins at peak f which has an intensity equivalent to three protons. Such a signal can only arise from the $C^{\epsilon}H_3$ group of the Met92 ligand. Irradiation of peak f does not give rise to NOEs to any other shifted signals in the spectrum of Co(II) plastocyanin. Likewise, irradiation of all of the other shifted signals does not result in any NOEs being observed

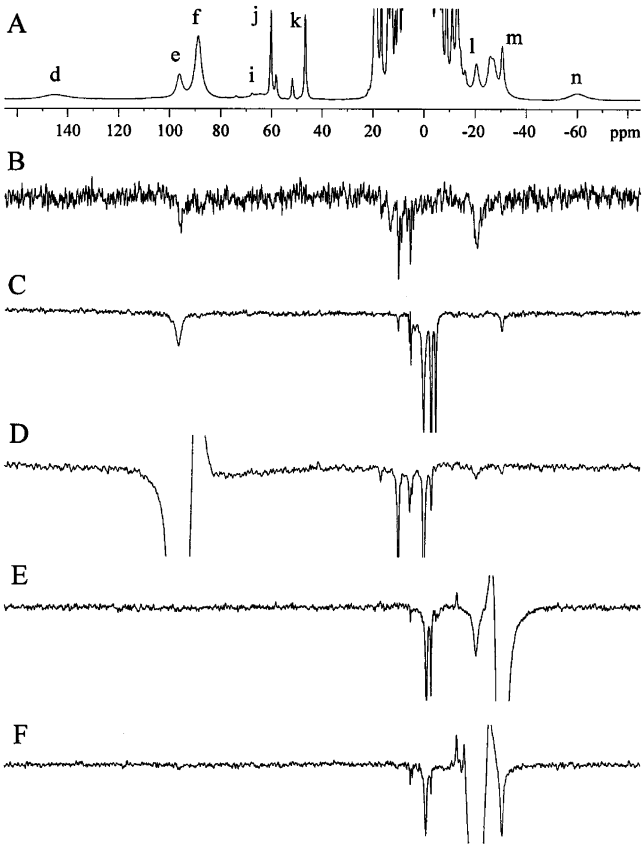

Figure 5. Reference (A) and 1D NOE difference spectra (B−F) of Co(II) plastocyanin (10 °C) in 10 mM phosphate in 99.9% D_2O at pH* 7.8. In the 1D NOE difference experiments peaks a (B), c (C), e (D), m (E), and l (F) were irradiated.

to peak f. Given the distance between the Met92 $C^{\epsilon}H_3$ protons and the other protons of the Met92 side chain and the T_1 values for these resonances, it is not surprising that such NOEs are difficult to observe (see Table 2; vide infra). Irradiation of peak c gives rise to a strong NOE to peak e (see Figure 5C) indicating that these signals arise from a geminal pair of protons (the reverse NOE is observed when peak e is irradiated, data not shown). A weaker NOE is observed to peak m upon irradiation of peak c, while weak NOEs are observed to signals l and m upon irradiation of peak e (see Figure 5D). Signals l and m exhibit strong NOEs to each other (see Figure 5E,F) indicative of them belonging to geminal protons. This pattern of NOEs, along with the dipolar connectivity observed between peak a (a Cys84 $C^{\beta}H$ proton resonance) and signals e and l (Figure 5B), is consistent with resonances c and e being from the Met92 $C^{\gamma}H$ protons and peaks l and m arising from the $C^{\beta}H$ protons of this ligand. The NOE observed between peaks a and l (vide supra) identifies the former as the Cys84 $C^{\beta 1}H$ resonance and peak l as the Met92 $C^{\beta 1}H$ signal [these protons are only 2.2 Å apart in the structure of the Cu(II) plastocyanin while the other Met92 $C^{\beta}H$ to Cys84 $C^{\beta}H$ distances range from 3.7 to 4.9 Å]. Consequently, peak e must arise from the Met92 $C^{\gamma 2}H$ proton which is situated 2.8 Å from the Cys84 $C^{\beta 1}H$, and peak c must be the Met92 $C^{\gamma 1}H$ resonance and peak m the Met92 $C^{\beta 2}H$ signal. Finally, peak b must be the Cys84 $C^{\beta 2}H$ which is consistent with the observation of

Table 2. Calculated[a] (Calcd) and Observed (Obsd) NOE[b] Intensities for the Hyperfine-Shifted Resonances of Co(II) Plastocyanin

	peak to which NOE is obsd																					
resonance	a		b		c		d		e		g		h		j		k		l		m	
irradiated	calcd	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd	obsd
a			0.6	no[c]					0.1	0.2									0.8	0.5		
b	0.5	no[c]							0.1	0.2												
c									2.3	1.8											0.5	0.5
d													0.9	0.8								
e					2.6	3.6													0.4	0.5	0.2	0.2
f					0.05	no[c]			0.03	no[c]												
g																	1.5	1.3				
h															2.0	2.1						
j													0.9	0.8								
k											0.5	0.5										
l																					4.4	5.2
m																			2.6	3.4		

[a] Using eq 1, a τ_r value of 5.8 ns,[39] the T_1 values and assignments listed in Table 1 and interproton distances derived from the structure of Cu(II) plastocyanin (PDB accession code 1AG6 with protons added in Insight II). [b] The intensities of the NOEs are reported as percentages with the minus sign omitted. [c] Not observed due to an insufficient signal-to-noise ratio.

an NOE to the Met92 $C^{\gamma 2}H$ (peak e; videsupra) as these protons are 2.9 Å apart in the structure of Cu(II) plastocyanin. NOEs are observed to a relatively sharp signal at −3.1 ppm upon irradiation of peaks c, e, l, and m (see Figure 5C−F), which thus can be assigned to the $C^{\alpha}H$ proton of Met92.

The results of all of the 1D NOE difference experiments that have been carried out on Co(II) plastocyanin are listed in Table 2. Also included in this table are the calculated NOE intensities determined using eq 1, a τ_r value of 5.8 ns [as found for the Cu(II) plastocyanin from *Synechocystis*[39]], the T_1 values and assignments listed in Table 1, and interproton distances derived from the crystal structure of oxidized spinach plastocyanin.[32] From the values listed in Table 2 it is clear that the agreement between calculated and observed NOE intensities is very good, thus confirming all of the assignments that we have made. It should be noted that the observation of the very weak NOEs (0.2%) required very large numbers of accumulations (typically 1 000 000) on concentrated samples.

Discussion

Type 1 copper sites have unique spectroscopic properties due to the coordination environment of the cupric ion.[50,51] This includes an intense S(Cys) → Cu(II) LMCT band at ∼600 nm in their UV/vis spectra, with a second LMCT band at ∼450 nm. The relative intensities of these two LMCT bands is found to vary in cupredoxins. The electron paramagnetic resonance (EPR) spectra of cupredoxins differ in the separation between the g_x and g_y values. Cupredoxins having EPR spectra with a small difference between the g_x and g_y values usually have little absorption at ∼450 nm in their UV/vis spectra and are said to possess type 1 axial copper sites.[52] Plastocyanin, which is referred to as possessing the classic cupredoxin active center,[50,51] has an axial type 1 copper site as do azurin and amicyanin. Pseudoazurin and rusticyanin possess type 1 rhombic (sometimes also referred to as perturbed) sites having a larger separation between g_x and g_y in their EPR spectra and increased absorption at ∼450 nm in their UV/vis spectra.

UV/Vis Spectrum of Co(II) Plastocyanin. The UV/vis spectrum of Co(II) spinach plastocyanin shown in Figure 2 is almost identical with those published previously for the Co(II) proteins from bean[1] and cucumber[4] and also to those of other Co(II) cupredoxins.[4−6,15] The main S(Cys) → Co(II) LMCT bands are at 334 and 385 nm (these transitions are at 333 and 385 nm in bean plastocyanin[1] and at 336 and 380 nm in the cucumber protein[4]) with LF transitions at 501, 641, and 675 nm. The intensity of the LF transitions in Co(II) spinach plastocyanin and the other Co(II)-substituted plastocyanins[1,4] are in the range expected for Co(II) in a distorted tetrahedral environment ($\epsilon > 250\ M^{-1}\ cm^{-1}$).

^{1}H NMR Spectrum of Co(II) Plastocyanin. The assignments that we have made for the isotropically shifted resonances in the ^{1}H NMR spectrum of Co(II) plastocyanin are consistent with the structural model that we have used [Cu(II) spinach plastocyanin[32]] (see Table 2). The assigned spectrum provides detailed structural information about the active site of plastocyanin. Furthermore, a comprehensive comparison to published data for other Co(II)-substituted cupredoxins which have been investigated by paramagnetic ^{1}H NMR (see Table 3) can be undertaken. The observed shifts (δ_{obs}) for the signals in the NMR spectrum of a paramagnetic molecule arise from three contributing factors as shown in the eq 2:

$$\delta_{obs} = \delta_{dia} + \delta_{pc} + \delta_c \qquad (2)$$

Here δ_{dia} is the shift in an analogous diamagnetic system and δ_{pc} and δ_c are the pseudocontact (through space) and Fermi contact (through bond) contributions, respectively, to the δ_{obs}. The δ_{dia} values are usually in the range of 0−10 ppm in diamagnetic cupredoxins and are not considered here (the isotropic shift, δ_{iso}, is $\delta_{pc} + \delta_c$). The δ_{pc} contribution to δ_{iso} arises from the magnetic anisotropy and can be quite large for Co(II) systems.[14,17] To determine the contribution of δ_{pc} to the isotropic shifts requires the orientation of the magnetic susceptibility (χ) tensor and the axial ($\Delta\chi_{ax}$) and rhombic ($\Delta\chi_{rh}$) magnetic susceptibility anisotropy compo-

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Table 3. Observed Hyperfine Shifts (δ_{obs}) in the ^{1}H NMR Spectrum of Co(II) Plastocyanin Compared with the δ_{obs} Values for the Corresponding Resonances in the Spectrum of Other Co(II)-Substituted Cupredoxins

		Co(II) plastocyanin[b]	Co(II) azurin[c]					Co(II) rusticyanin[f]		
ligand[a]	proton	δ_{obs} (ppm)	δ_{obs} (ppm)	δ_{pc} (ppm)	δ_c (ppm)	Co(II) Ami[d] δ_{obs} (ppm)	Co(II) PAz[e] δ_{obs} (ppm)	δ_{obs} (ppm)	δ_{pc} (ppm)	δ_c (ppm)
His	$H^{\delta 2}$	55.8	50.6	7.8	37.3	52.6	53.1	59.7	−1.9	53.6
	$H^{\epsilon 1}$	133	97	85.7	4.4	118/38	57/146			
	$H^{\epsilon 2}$	63.2	74.9	16.6	46.9	62.3	61.9	69.2	10.3	44.2
Cys	$H^{\beta 1}$	299	232	5.4	223.2	285	315/267	287/260	69.9	205
	$H^{\beta 2}$	275	285	−5.3	287.4	285	315/267	287/260	59.7	184
His	$H^{\delta 2}$	43.8	56.4	7.2	42.3	51.0	43.6	48.7	3.4	38.5
	$H^{\epsilon 1}$	60	75	23.3	45	118/38	57/146			
	$H^{\epsilon 2}$	75.3	65.8	7.5	46.8	74	71.4	80.3	19.9	48.9
Met	$H^{\beta 1}$	−18.4	−18.9	−25.9	5.3	−18.6	−31.2	−31.0	−23.2	−9.8
	$H^{\beta 2}$	−27.5	−18.5	−26.9	6.1	−16.1		−24.4	−13.5	−17.4
	$H^{\gamma 1}$	254	45.3	−19.5	64.4	132.5	105.8	122.9	−11.5	132.5
	$H^{\gamma 2}$	87.8	−19.1	−20.2	−0.3	10.0	271.3	285.2	−19.2	301.6
	$C^{\epsilon}H_3$	80.4	−7.3	−31.2	24	74.5	90.2	103.3	−16.9	130.3
Gly	$H^{\alpha 1}$		47.8	−10.5	54.2					
	$H^{\alpha 2}$		−29.4	−26.7	−5.9					

[a] From top to bottom: His37, Cys84, His87, and Met92 for plastocyanin; His46, Cys112, His117, Met121, and Gly45 for azurin; His54, Cys93, His96, and Met99 for Ami; His40, Cys78, His81, and Met86 for PAz; His85, Cys138, His143, and Met148 for rusticyanin. [b] This study at 30 °C and pH 7.8 (5 °C and pH 7.0 in the case of the His87 $N^{\epsilon 2}H$ resonance). [c] Azurin from *Pseudomonas aeruginosa* at pH 4.5 and 37 °C.[8,14] [d] Amicyanin from *Paracoccus versutus* at 40 °C and pH 8.0 (the His96 $N^{\epsilon 2}H$ resonance was observed at 22 °C and pH 5.0).[15] [e] Pseudoazurin from *Achromobacter cycloclastes* at 40 °C and pH 8.0.[20] [f] Rusticyanin from *Thiobacillus ferrooxidans* at pH 6.0 and 20 °C.[17]

nents to be calculated. These features have been determined for Co(II) azurin[14] and Co(II) rusticyanin[17] and are similar in both of these derivatives. The orientation of the χ tensor is not known for Co(II) plastocyanin although a similar orientation of the *z* component, relative to the axial Cu−S(Met) bond, has been determined for both Cu(II) plastocyanin[53] and Cu(II) azurin[54] [this is also very similar to what is found for Co(II) azurin[14] and Co(II) rusticyanin[17]]. The orientation of the *z* component of the χ tensor in Co(II) azurin and rusticyanin results in negative δ_{pc} contributions for protons oriented toward the axial positions, and in Co(II) rusticyanin these are less negative.[17] The *xy* plane of the χ tensor is almost in the plane defined by the N_2S equatorial ligands in both Co(II) azurin[14] and Co(II) rusticyanin[17] but exhibits a different orientation with respect to the Co(II)−S(Cys) bond in the two proteins. This altered orientation, along with the different $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ values, results in different δ_{pc} contributions for the $C^{\beta}H$ protons of the Cys ligand in the two proteins (see Table 3). The active site structure of plastocyanin is more similar to that of rusticyanin, rather than azurin (distorted tetrahedral rather than trigonal bipyramidal), so we assume that the orientation of the χ tensor and the $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ values for Co(II) plastocyanin will be comparable to those for Co(II) rusticyanin [a similar supposition has recently be made in the case of Co(II) pseudoazurin[20]]. We therefore believe that the δ_{pc} values listed for Co(II) rusticyanin in Table 3 are similar to those for Co(II) plastocyanin. The dissection of the δ_{pc} values from δ_{iso} provides the δ_c contribution which is a direct measure of the spin density present at a particular proton. Since we do not know the exact δ_{pc} values for Co(II) plastocyanin, we will compare the substituted proteins listed in Table 3 on the basis of δ_{obs} values, keeping in mind the key points which have been discussed in the present paragraph.

The δ_{obs} values listed in Table 3 for the two His ligands are very similar in all of the Co(II) cupredoxins. The only exceptions to this are the His $C^{\epsilon 1}H$ protons which are situated close to the metal and thus experience large δ_{pc} values [see data for Co(II) azurin in Table 3]. The pattern of δ_{obs} values for the His $C^{\epsilon 1}H$ protons are similar in Co(II) plastocyanin, Co(II) pseudoazurin,[20] and also Co(II) amicyanin,[15] which indicates similarities in the orientation of the *xy* component of the χ tensor in the N_2S plane in these three proteins. Unfortunately the His $C^{\epsilon 1}H$ protons are not observed in Co(II) rusticyanin.[17]

The $C^{\beta}H$ protons of the Cys ligands all exhibit very large δ_{obs} values in the Co(II)-substituted cupredoxins (see Table 3) which are similar. The δ_c values (and therefore δ_{obs}) for the Cys $C^{\beta}H$ protons are influenced by the Co(II)−S^{γ}−C^{β}−H^{β} dihedral angles, whereas the average shift ($\delta_{\beta,av}$) is not prone to such angular dependencies.[55] The $\delta_{\beta,av}$ values are 277 ppm for Co(II) plastocyanin (at 40 °C), 260 ppm for Co(II) rusticyanin (at 40 °C[20]), 259 ppm for Co(II) azurin,[14] 285 ppm for Co(II) amicyanin,[15] and 291 ppm for Co(II) pseudoazurin.[20] In Co(II) azurin the δ_{pc} values for these two protons are small,[14] but in Co(II) rusticyanin[17] and probably also all of the other proteins listed in Table 3, the δ_{pc} contribution to δ_{obs} is much larger (this results in average δ_c values of 255.3 and 194.5 ppm in Co(II) azurin[14] and rusticyanin,[17] respectively). Thus, it is likely that the spin density on the Cys $C^{\beta}H$ protons is greater in Co(II) azurin than in all of the other Co(II)-substituted cupredoxins. Furthermore, the spin densities on this ligand in Co(II) plastocyanin, Co(II) rusticyanin, Co(II) amicyanin, and Co(II) pseudoazurin are very similar and follow the pattern Co(II)

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pseudoazurin > Co(II) amicyanin > Co(II) plastocyanin > Co(II) rusticyanin.

The observed shifts of the axial Met ligand protons in the Co(II) proteins provides an insight into the strength of the axial interaction in the various substituted cupredoxins. The δ_c values for the Met $C^{\gamma}H$ protons are influenced by the Co(II)$-S^{\delta}-C^{\gamma}-H^{\gamma}$ dihedral angles, whereas the average shift of these protons ($\delta_{\gamma,av}$) will not be so affected. The $\delta_{\gamma,av}$ values are 13.1, 71.3, 163.2, 188.6, and 191.5 ppm for the Co(II) derivatives of azurin,[14] amicyanin,[15] plastocyanin (at 40 °C), pseudoazurin,[20] and rusticyanin (at 40 °C[20]), respectively. It should be remembered that the azurin value includes a considerable negative δ_{pc} contribution and the average δ_c value for these protons is 32.1 ppm.[14] In rusticyanin[17] and probably all of the other cupredoxins listed in Table 3, the δ_{pc} contribution for these protons is less significant. The small $\delta_{\gamma,av}$ value for Co(II) azurin can be attributed to the very long Co(II)−S(Met) bond length identified in the structure of this substituted cupredoxin (3.49 Å).[13] The bond length to the other axial ligand (backbone carbonyl oxygen of Gly45) is only 2.15 Å in Co(II) azurin[13] [as compared to 2.97 Å in the Cu(II) protein[56]], and thus, the introduced metal has a preference for the harder oxygen ligand. This results in the large δ_c value for one of the $C^{\alpha}H$ protons of Gly45 (see Table 3). For this reason azurin is excluded from the current comparison. The altered preference for the harder axial ligand in the Co(II) derivative as observed in azurin will not occur in the other cupredoxins as the corresponding backbone carbonyl oxygen is much further away from the active site and will not interact with the metal. Of the remaining $\delta_{\gamma,av}$ values the outlier is that for Co(II) amicyanin (71.3 ppm), which is consistent with the fact that the conformation of the Met side chain is grossly different in amicyanin compared to plastocyanin, pseudoazurin, and rusticyanin.[57] This argument is supported by the δ_{obs} values for the Met $C^{\epsilon}H_3$ signals (whose δ_c contributions will not exhibit an angular dependence) in the Co(II) cupredoxins which, excluding azurin, are all alike. The conclusion therefore is that the Co(II)−S(Met) interactions are comparable in plastocyanin, pseudoazurin, and rusticyanin (and probably also amicyanin). The $\delta_{\gamma,av}$ value for Co(II) plastocyanin is a little smaller than those of Co(II) rusticyanin and Co(II) pseudoazurin (by ∼30 ppm). This indicates a slightly decreased Co(II)−S(Met) interaction in the axial type 1 site (plastocyanin) as compared to those cupredoxins having distorted centers. However, this is not consistent with the observed Cu(II)−S(Met) bond lengths which are 2.88, 2.71, and 2.88 Å in plastocyanin,[32] pseudoazurin,[58] and rusticyanin,[59] respectively.

Comparison to the Cu(II) Cupredoxins. The conclusions about the interaction between the metal and the His ligands from paramagnetic NMR studies on the Cu(II) proteins are very similar to those discussed above from investigations of the Co(II) derivatives.[39,41−49] Thus, the M(II)−N(His) interactions appear to be very similar in all cupredoxins. The shifts observed for the Cys $C^{\beta}H$ protons are significantly smaller in the Co(II) cupredoxins than in their Cu(II) counterparts.[43,45,49] For example, these protons in spinach Cu(II) plastocyanin are found at 650 and 490 ppm[43] compared to 299 and 275 ppm in the Co(II) protein. This is indicative of the Cys $C^{\beta}H$ protons possessing much smaller δ_c values even though the Co(II) centers have three unpaired electrons compared to one in the Cu(II) forms. Thus the Co(II)−S(Cys) bond is being much less covalent than the Cu(II)−S(Cys) bond.[16] The range of shifts observed for the Cys $C^{\beta}H$ protons in the cupredoxins listed in Table 3 is considerably smaller than for the Cu(II) proteins.[43,45,49] The average shifts of the Cys $C^{\beta}H$ protons is 825 ppm in Cu(II) azurin,[45] 570 ppm in Cu(II) plastocyanin,[43] 450 ppm in Cu(II) pseudoazurin, and 270 ppm in Cu(II) rusticyanin,[49] and these differences have been attributed to variations in the Cu−S(Cys) distances and the angle between the $CuN_{His}N_{His}$ plane and the Cu−S(Cys) vector.[49] The decreased spin density on the Cys ligand in the Co(II) proteins results in the $\delta_{\beta,av}$ values being a less sensitive measure of differences in M(II)−S(Cys) interactions in the substituted cupredoxins. Furthermore, it would appear that the variations apparent in the Cu(II) proteins are not present in the Co(II) cupredoxins.

In certain Cu(II) cupredoxins (azurin and rusticyanin) isotropically shifted resonances from the axial Met are not observed shifted outside of the diamagnetic envelope.[45,49] In the case of spinach Cu(II) plastocyanin the $C^{\gamma}H$ protons of the Met ligand are observed at 23.5 and 13.0 ppm,[43] whereas in Cu(II) amicyanin they are found at 12.0 and 11.1 ppm.[42] In Cu(II) pseudoazurin a signal observed at 12.2 ppm has been assigned as either a Met $C^{\gamma}H$ proton[48] or as the $C^{\epsilon}H_3$ resonance of the axial ligand.[49] In the Co(II) cupredoxins shifted resonances can be observed for most of the axial Met protons, and the δ_{pc} contributions to the δ_{obs} values of these resonances are quite small. Thus, detailed information can be gleaned about the axial M(II)−S(Met) interaction from the Co(II) proteins. The drawback of the studies on the Co(II) derivatives is that the metal−ligand bond lengths may be altered in the substituted proteins. Thus, the observations of similar $\delta_{\gamma,av}$ values for Co(II) plastocyanin, Co(II) pseudoazurin, and Co(II) rusticyanin may reflect different coordination geometry preferences in the substituted proteins which are not present in the Cu(II) forms.

Various proposals have been made concerning the structural features which result in type 1 copper centers being axial (classic) or rhombic (perturbed).[50−52,60] In all of these the strength of the Cu−S(Met) interaction is increased in rhombic sites resulting in a lengthening of the Cu−S(Cys) bond. From the studies that we have carried out it is apparent that the active site structures of Co(II) cupredoxins which

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possess either axial or rhombic type 1 sites [when in their native Cu(II) forms] are very similar.

Conclusions

Co(II)-substituted spinach plastocyanin has a UV/vis spectrum like those of other Co(II) proteins from this class. The paramagnetic ^{1}H NMR spectrum of Co(II) plastocyanin is remarkably similar to those of the cupredoxins pseudoazurin and rusticyanin. Plastocyanin possesses a type 1 axial copper center whereas the other two cupredoxins have distorted (rhombic) type 1 sites. The results of this study therefore suggest that the active site variations which result in these differences in the Cu(II) proteins are either not present in the Co(II) derivatives or do not significantly alter their paramagnetic NMR spectra.

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