High-Field NMR Studies of Oxidized Blue Copper Proteins: The Case of Spinach Plastocyanin

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Abstract: The 800-MHz ^{1}H NMR spectra of oxidized plastocyanin from spinach are here reported. All hyperfine-shifted signals have been assigned through saturation transfer with the reduced diamagnetic species. To detect the copper(II)-bound cysteine β-CH_2 signals, a technique has been applied which is based on irradiation of regions where such signals are expected but not detected, and the corresponding saturation transfer on the reduced species is observed. At the end, a full spectrum is reconstructed which permits, for the first time, the complete ^{1}H NMR signal assignment of an oxidized blue copper protein. These data are discussed in terms of the factors affecting the line width as related to the electronic and geometric structure of the metal center. A Karplus-type relationship is proposed between the contact shift of the Cys-84 β-CH_2 protons and the Cu−S−C−Hβ dihedral angle.

Introduction

^{1}H NMR spectroscopy of copper(II) proteins suffers due to the long electronic relaxation times of the ion.[1] The NMR lines are broad beyond detection, either because of dipolar relaxation, especially for protons that are within a sphere of 4−5-Å radius from the metal, or because of contact relaxation for protons belonging to ligand residues, or both. Tetragonal copper(II) is particularly difficult to investigate because its electronic relaxation times are 1−5 ns,[1] and the proton line width of a nucleus at, e.g., 4 Å (or at any distance if experiencing a contact hyperfine coupling of, e.g., 1 MHz) is about 10^4 Hz at a Larmor frequency of 500 MHz. The situation is somewhat better for blue copper proteins. They are characterized by the presence of a metal ion strongly bound to two histidines and one cysteine (N_2S coordination), in addition to a fourth weakly bound ligand (Met-Sδ in all cases except for stellacyanin, featuring a Gln-Oϵ bound to copper[2]) in a distorted tetrahedral geometry.[3−5] For this type of copper center the relatively small energy gap between the ground and excited states causes the electronic relaxation times to decrease about 1 order of magnitude,[1,6−8] inducing a corresponding line width narrowing of the NMR signals. Indeed, ^{1}H NMR spectra and partial signal assignments of oxidized amicyanin[9] and azurin,[10] two blue copper proteins, have been reported. However, not all the ^{1}H NMR signals from the copper(II)-bound ligands could be identified.

The present work describes a successful attempt to record the complete spectrum of a blue copper protein, which involves the exploitation of high magnetic field (800 MHz) and an audacious approach. The high field is helpful because the nuclear dipolar relaxation is much more efficient than Curie relaxation for a protein of the size of plastocyanin (ca. 10 kDa) containing a $S = {}^1/_2$ ion with relatively long electronic relaxation times; as a consequence, the NMR signal line width either decreases or remains constant with increasing field, whereas the lines appear sharper at higher field in a ppm scale.[11] The audacious approach consists of saturating a signal that is too broad to be detected, simply assuming that such signal is present in a certain region of the spectrum and by observing saturation transfer effects in the spectrum of the reduced form. In this way, the protons of the copper(II)-coordinated cysteine have been located. The investigation was performed on spinach plastocyanin, a water-soluble blue copper protein[12] involved in the photosynthetic electron-transfer chain.[13,14]

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Experimental Section

Protein Purification. Plastocyanin extraction from 1.5 kg of spinach leaves was carried out by homogenizing the leaves, previously rinsed with distilled water, in 3 L of 10 mM potassium phosphate buffer, pH 7, containing 50% acetone (v/v) using a Waring blender, and keeping the homogenate at 4 °C for 30 min. The undissolved material was removed by centrifugation (5000*g*). Protein precipitation was carried out by slowly adding cold (−20 °C) acetone (up to 75%) to the clear supernatant. The pellet obtained after centrifugation was redissolved in 20 mM potassium phosphate buffer, pH 7. The subsequent purification procedure was adapted from a previously published method,[15] modified by adding a final hydrophobic interaction chromatographic step on a Toyopearl HW-65C column. Plastocyanin was eluted using a linear gradient (60−20%) of ammonium sulfate and finally dialyzed. The purity and concentration of the protein were monitored using a CARY-3 UV/vis spectrophotometer. The absorbance ratio A_{278}/A_{597} for the purified oxidized plastocyanin was 1.15. The protein concentration was determined using a value of 4.9 mM^{-1} cm^{-1} for the extinction coefficient of oxidized plastocyanin at 597 nm.[15]

NMR Spectroscopy. NMR samples were prepared in both deuterated and nondeuterated 50 mM phosphate buffer (pH 7.5) using a Centricon-3 concentration cell. The final concentration of plastocyanin was ca. 2−4 mM. Excess of potassium ferricyanide or sodium ascorbate was added to the samples in order to achieve complete protein oxidation or reduction, respectively.

^{1}H NMR spectra were acquired using a Bruker Advance 600 and Bruker Avance 800 spectrometers operating at the Larmor frequencies of 600.14 and 800.14 MHz, respectively. All NMR measurements were performed at 298 K unless otherwise specified. The reported pH values were not corrected for the deuterium isotope effect. The spectra were calibrated at different temperatures using the residual HOD signal and the empirical relationship $\delta_{HOD} = -0.012t + 5.11$ ppm, where t is the temperature in °C.[16] Water peak suppression was achieved either by selective saturation of the water signal or by using the superWEFT pulse sequence.[17]

For oxidized plastocyanin, nonselective T_1 values were measured using the inversion−recovery method.[18] 1D saturation transfer spectra were recorded in difference mode using two reference frequencies to the immediate left and right of the irradiated signal, according to the scheme "on−off(left)−on−off(right)" as previously described.[19,20] Recycle delays (AQ + RD) and irradiation times were 30 and 30−40 ms, respectively.

1D saturation transfer spectra carried out in order to detect signals unobservable in the normal ^{1}H NMR spectrum were performed using an "on−off" scheme where "on" values were varied from 10 to 1400 ppm and "off" values were positioned symmetrically to the "on" values with respect to the frequency of either the H_2O signal or the signals to which saturation transfer is observed. This scheme was originally proposed to resolve NOEs from overlapped but observable hyperfine-shifted signals in high-spin heme proteins[21] but has never been used in an attempt to detect the presence of a signal broad beyond detection through its saturation transfer effects.

2D NOESY[22] and TOCSY[23] spectra of reduced plastocyanin were recorded at 298 and 303 K using TPPI mode,[24] $4K \times 1K$ data points, and 24 scans/experiment. The mixing time for NOESY was 100 ms, while the spin-lock time for TOCSY was 40 ms. The total recycle time (RD + AQ) was 2 s in both cases.

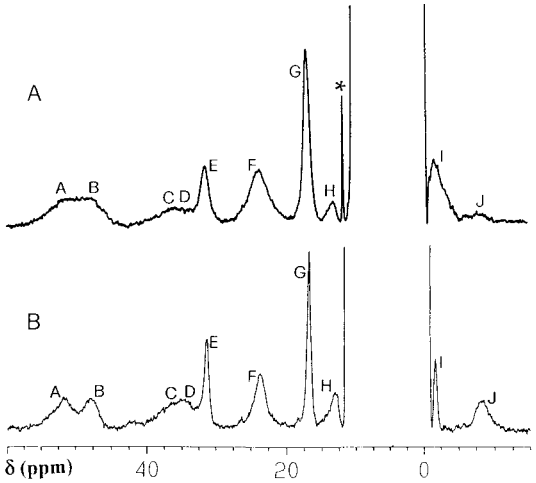

Figure 1. ^{1}H NMR spectra at 298 K of oxidized spinach plastocyanin (50 mM phosphate buffer, pH 7.5 in H_2O) at 600 (A) and 800 MHz (B). The asterisk indicates a resonance belonging to the reduced form. Signal I is barely resolved at 600 MHz. Its exceedingly large line width originates from a phasing artifact due to the proximity of intense signals from the diamagnetic envelope of the spectrum.

2D EXSY spectra were recorded on mixtures of oxidized and reduced plastocyanin in TPPI mode,[24] with $2K \times 1K$ data point matrixes in $F2$ and $F1$ dimensions, respectively, acquiring 2048 scans/experiment. The remaining water signal was eliminated by a WEFT pulse sequence (180°−τ−), which preceded the EXSY sequence.[25] The total recycle time for EXSY experiments was 40 ms, the τ delay in the WEFT sequence was 40 ms, and the mixing time was set to 4 ms.

A squared sine bell weighting function with variable phase shift was used to process all 2D spectra. The data were processed using the Bruker standard software package. Protons were added to the coordinates of oxidized spinach plastocyanin (PDB code 1AG6)[26] using the DOCK3.5 program suite.[27,28]

Results

^{1}H NMR Spectra of Oxidized Spinach Plastocyanin. To verify the small influence of the Curie contribution to the proton transverse relaxation rates, the ^{1}H NMR spectra of oxidized spinach plastocyanin were recorded at 200, 600, and 800 MHz. The spectrum acquired at 200 MHz did not reveal any resolved features outside the diamagnetic envelope. The spectrum recorded at 600 MHz is shown in Figure 1A, while the spectrum recorded at 800 MHz is shown in Figure 1B. Increase of spectral resolution upon passing from 600 to 800 MHz is evident, unambiguously indicating that the Curie contribution in plastocyanin is negligible, as expected for copper(II)-containing systems. Therefore, the analysis was performed using 800 MHz data.

The 800-MHz ^{1}H NMR spectrum clearly shows eight downfield and two upfield hyperfine-shifted signals (Table 1), which obey Curie temperature dependence (decrease in shift with temperature increasing) (Figure 2) as predicted for uncoupled systems containing a single paramagnetic metal ion. Only one signal (signal E) appears to be exchangeable, as it

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Table 1. Summary of the 800-MHz ^{1}H NMR Studies on the Hyperfine-Shifted Signals of the Oxidized Spinach Plastocyanin in H_2O[a]

signal label (OX/red)	chemical shift (ppm)	T_1 (ms)	T_2 (ms)	obsd saturation transfer (ppm)	assignment
A/a	51.6	2.0	0.14	7.01	Hδ2 His-87
B/b	47.1	2.7	0.09	7.56	Hδ2 His-37
C/c	35.6	<1	nd	7.74	Hϵ1 His-87
D/d	35.6	<1	nd	7.19	Hϵ1 His-37
E/e	31.4	nd	0.48	11.67	NHϵ2 His-37
F/f	23.5	2.3	0.23	1.70	Hγ2 Met-92
G/g	17.0	6.7	0.94	4.20	Hα Asn-38
H/h	13.0	1.3	0.29	2.25	Hγ1 Met-92
I/i	−1.5	1.1	0.71	2.80	Hβ1 His-37
J/j	−8.0	<1	0.18	5.42	Hα Cys-84
W/w	−19.0 ± 0.1	nd	7.9 ± 0.3[b]	10.20	NH Asn-38
X/x	650 ± 14	nd	519 ± 33[b]	3.26	Hβ1 Cys-84
Y/y	489 ± 5	nd	329 ± 14[b]	2.92	Hβ2 Cys-84
Z/z	19.2 ± 0.2	nd	11.8 ± 0.6[b]	6.03	Hα His-37

[a] nd = not determined. [b] In kHz.

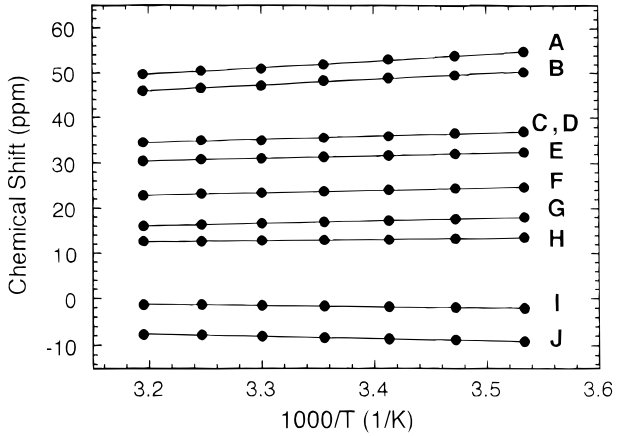

Figure 2. Experimental temperature dependence for the hyperfine-shifted signals in the ^{1}H NMR spectrum of oxidized spinach plastocyanin.

was not observed in the spectrum recorded in deuterated water. All hyperfine-shifted signals are characterized by very short longitudinal and transverse nuclear relaxation times (Table 1). Analysis of the relative signal intensities indicates that each signal accounts for one proton.

Saturation Transfer Experiments on Hyperfine Shifted Signals. The ^{1}H NMR assignment of the hyperfine-shifted signals of the oxidized spinach plastocyanin was carried out through 1D saturation transfer and 2D EXSY experiments performed on samples containing approximately equal amounts of the oxidized and reduced forms of the protein, in combination with 2D TOCSY and NOESY spectra of the reduced protein. The latter 2D spectra were recorded at two different temperatures under the same experimental conditions as the saturation transfer experiments. These spectra were thoroughly compared with the existing assignment of reduced spinach plastocyanin.[29] The NOESY and TOCSY patterns of all residues relevant to the present work could be unambiguously identified and confirmed.

1D saturation transfer and 2D EXSY experiments could be successfully performed because of the favorable electron self-exchange rate determined for spinach plastocyanin (upper limit = 1×10^7 M^{-1} s^{-1}; lower limit = 3×10^4 M^{-1} s^{-1}), in agreement with previous results.[30] 1D saturation transfer spectra obtained upon saturating signals A, B, C, D, F, H, I, and J are shown in Figure 3, while the downfield section of the 2D EXSY spectrum, showing exchange connectivities for signals E and G, is given in Figure 4.

Upon saturating signals A, B, and the two overlapped signals C, D, saturation transfer with signals a−d at 7.01, 7.56, 7.74 and 7.19 ppm, respectively, was observed. The chemical shift of signals a−d in the spectrum of the reduced diamagnetic form immediately indicates that signals A−D belong to the nonexchangeable aromatic protons Hδ2 and Hϵ1 of His-37 and His-87 in the paramagnetic oxidized protein. In the TOCSY spectrum, scalar connectivities between signals pairs (a,c) and (b,d) were observed, allowing their pairwise assignment to the two different histidines. The longitudinal relaxation times of signals A−D can be used to perform the assignment of signals A−D to Hδ2 and Hϵ1 His protons using the structural data available on plastocyanin from spinach[26] and other sources.[31−39] In all blue copper proteins, the two histidines are bound to the metal through the Nδ1 nitrogen, so that both Hϵ1 protons lie significantly closer to the copper(II) ion than Hδ2 protons (ca. 3 vs ca. 5 Å); therefore, their T_1 values should be much shorter than those corresponding to the Hδ2 protons. The longitudinal relaxation times for signals A and B were determined with reasonable accuracy (Table 1), while those of signals C and D are so short that they are difficult to measure. Therefore, pairs A,C and B,D correspond to pairs of Hδ2,Hϵ1 protons of the two copper(II)-bound histidines. This assignment is unequivocally confirmed by the observation of strong NOESY cross-peaks between signals c and d, which, according to the available structural data, should be observed between His-87 Hϵ1 and His-37 Hϵ1.

The only exchangeable hyperfine-shifted signal E gives an EXSY cross-peak with signal e at 11.67 ppm (Figure 4). This

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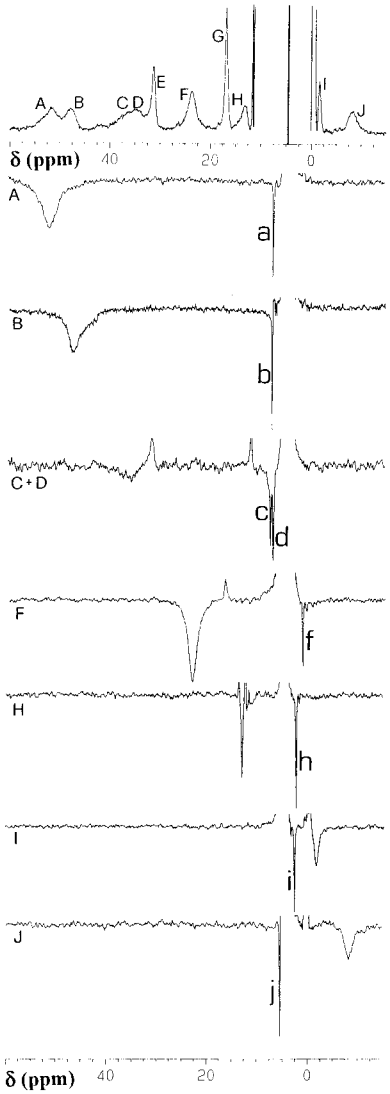

Figure 3. 800-MHz ^{1}H NMR 1D saturation transfer difference spectra at 298 K of spinach plastocyanin (50 mM phosphate buffer, pH 7.5 in H_2O). The sample contained approximately equal amounts of oxidized and reduced forms of the protein. The upper trace represents the reference spectrum. The other traces are 1D saturation transfer spectra, labeled according to the saturated signals. These difference spectra were obtained using the "on−off(left)−on−off(right)" scheme already reported for 1D NOE experiments.[19,20] The "off(left)" and "off(right)" irradiation positions were 51.6 ± 6.2, 47.1 ± 5.6, 35.6 ± 1.9, 23.5 ± 3.8, 13.0 ± 0.6, −1.5 ± 0.4, and −8.0 ± 1.2 ppm for traces A, B, C + D, F, H, I, and J, respectively. Positive signals are artifacts due to off-resonance effects.

signal is assigned to the exchangeable NHϵ2 proton of one of the two copper-bound histidines. As evidenced from the structures of plastocyanins, His-87 NHϵ2 lies on the protein surface and is probably in fast exchange with bulk water. This fact accounts for the observation of only one histidine NHϵ2 signal in the NMR spectra of all reduced plastocyanins investigated so far at neutral pH and room temperature (Table 2). Attempts to lower this proton exchange rate for spinach plastocyanin by decreasing the pH of the solution resulted in protein precipitation. Following the above considerations, signal E is assigned to His-37 NHϵ2 proton, in agreement also with the observed chemical shift of signal e in other plastocyanins (Table 2).

Signals F and H show saturation transfer with signals f (1.70 ppm) and h (2.25 ppm), which, in turn, are correlated by TOCSY

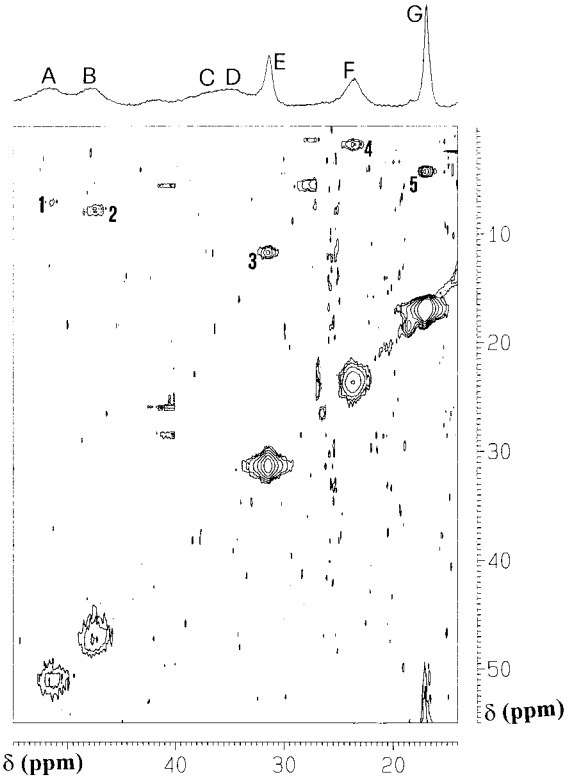

Figure 4. 800-MHz ^{1}H NMR EXSY spectrum at 298 K of spinach plastocyanin (50 mM phosphate buffer, pH 7.5 in H_2O) obtained using a mixing time of 4 ms. Other conditions are described in the Experimental Section. The sample contained approximately equal amounts of oxidized and reduced forms of the protein. The upper trace represents the reference spectrum. The exchange cross-peaks are due to interactions between the following signals (uppercase letter stands for the oxidized form, whereas the lowercase for the reduced form): (1) A−a, (2) B−b, (3) E−e, (4) F−f, (5) G−g. For the assignment of these signals, see Table 1.

cross-peaks. Therefore, signals F and H belong to the same spin pattern.

Signal G, characterized by the longest T_1 and T_2 among all hyperfine-shifted signals (Table 1), yields saturation transfer with signal g at 4.20 ppm, whereas signals I and J show saturation transfer with signals i and j at 2.80 and 5.42 ppm, respectively.

Detection of Signals Broadened "Beyond Detection". A downfield contact shift of a few hundred ppm is expected[11] for the β-CH_2 protons of the copper(II)-bound cysteine because of the large direct electron spin delocalization observed in plastocyanins.[40−42] According to this estimate, none of the observed hyperfine-shifted signals correspond to the Cys-84 β-CH_2 protons. Furthermore, none of the signals of reduced plastocyanin, detected by saturation transfer experiments, display chemical shifts expected for these protons (Table 2). Increasing the spectral window of the ^{1}H NMR spectra of oxidized plastocyanin to a very large frequency range (down to 1200 ppm downfield) did not result in the direct detection of the Cys-84 β-CH_2 proton signals. However, "blind" high-power irradiation in this spectral region was expected to result in the observation of saturation transfer to the Cys-84 β-CH_2 protons in the reduced form, because of the favorable electron self-

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Table 2. ^{1}H NMR Assignments for Cu-Bound Residues in Reduced Plastocyanins[a]

reduced plastocyanin	french bean, 303 K, pH 7 (ref 43)	parsley, 308 K, pH 7.3 (ref 37)	*Anabaena variabilis*, 298 K, pH 7.0 (ref 38)	pea, 303 K, pH 6.0 (ref 44)	spinach, 308 K, pH 7.3 (ref 29)	spinach, 298 K, pH 7.5 (this work)
			His 37			
Hα	5.93	5.70	5.96	5.95	5.98	6.03
Hβ1	2.77	3.53	(2.58)	(2.77)	(2.70)	2.80
Hβ2	3.51	2.61	(3.54)	(3.48)	(3.48)	3.53
Hδ2	7.57	7.70	7.50	7.53	7.59	7.56
Hε1	7.16	7.06	7.05	7.15	7.13	7.19
NHε2	11.50	11.50	11.52	11.62	11.58	11.67
			Cys 84			
Hα	5.33	5.21	5.28	5.35	5.36	5.42
Hβ1	3.22	2.93	(3.33)	(3.18)	(3.21)	3.26
Hβ2	2.92	3.24	(2.88)	(2.87)	(2.88)	2.92
			His 87			
Hα	5.17	5.18	5.14	5.20	5.16	5.21
Hβ1	3.89	3.79	(3.80)	(3.88)	(3.86)	3.93
Hβ2	3.43	3.40	(3.38)	(3.45)	(3.44)	3.48
Hδ2	7.00	7.00	7.18	7.08	7.00	7.01
Hε1	7.67	7.59	7.65	7.72	7.66	7.74
			Met 92			
Hα	4.78	4.65	4.50	4.67	4.59	4.65
Hβ1	(2.31)	(1.81)	(1.88)	(2.31)		1.40
Hβ2	(2.31)	(2.28)	(2.18)	(2.31)		2.36
Hγ1	(1.98)	(1.65)	(1.44)	(2.20)		2.25
Hγ2	(2.15)	(2.07)	(1.44)	(2.20)		1.70
ε−CH_3	0.54	0.51		0.57	0.57	0.55

[a] The chemical shifts of protons for which stereospecific assignment was not performed are reported in parentheses.

exchange rate found in this system. Indeed, a sample irradiation at 500 ppm downfield with 65 W for 30 ms resulted in a difference 1D spectrum showing two saturation transfer signals x (3.26 ppm) and y (2.92 ppm). The shift of these two signals is in very good agreement with the reported shifts of Cys-84 β-CH_2 protons in reduced plastocyanins (Table 2). The precise location of the corresponding resonances in the oxidized form was then achieved by designing a "saturation transfer profile" experiment: a classical NMR NOE difference spectroscopy scheme was used,[21] where "on" and "off" pulses were centered at regular frequency intervals and positioned symmetrically with respect to the average shift of the saturation transfer signals x and y (3.09 ppm). A very large spectral window (from +1400 to −1400 ppm) was sampled in steps of 50−150 ppm. Observation of the build-up and decay of the intensity of signals x and y as a function of the sampled frequency provided the location of extremely broad signals. The traces of the 1D saturation transfer experiments as a function of decoupler position are shown in Figure 5. The corresponding intensities of signals x and y are reported in Figure 6A as a function of the irradiation frequency. In this way, the positions of signals X (at 650 ppm) and Y (at 489 ppm) for Cys-84 β-CH_2 in the oxidized protein could be obtained (Figure 6 and Table 1). The very large line width of signals X (519 kHz) and Y (329 kHz), determined using a Lorentzian fit to the curves shown in Figure 6A, prevents their direct observation in the 1D ^{1}H NMR spectrum.

The assignment of signals X and Y as Cys-84 β-CH_2 protons is further supported by the presence of a TOCSY pattern comprising signals x, y, and j. The presence of signal j in this pattern indicates unambiguously that signals j and J correspond to the Cys-84 Hα proton in the reduced and oxidized species, respectively. To our knowledge, this is the first time ever that NMR signals of nuclei belonging to copper(II)-bound cysteine have been located in the ^{1}H NMR spectrum of blue copper proteins.

The presence of additional broad signals in the near downfield

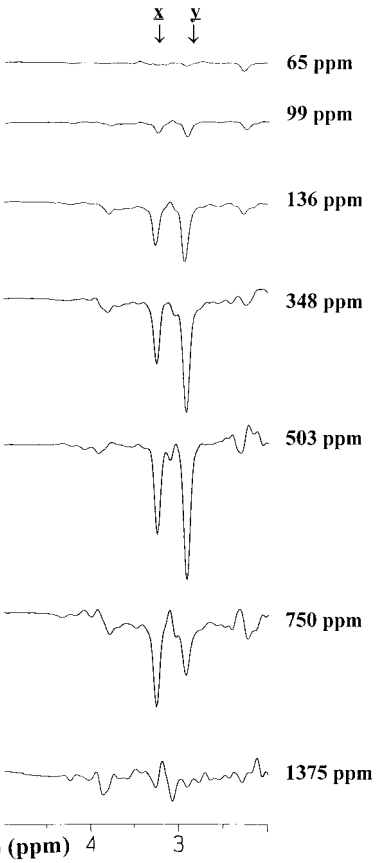

Figure 5. Portions of the 800-MHz ^{1}H NMR saturation transfer spectra at 298 K of spinach plastocyanin (50 mM phosphate buffer, pH 7.5 in D_2O), revealing positions of signals x and y. The sample contained approximately equal amounts of oxidized and reduced forms of the protein. The symmetric "on−off" scheme used to acquire these spectra is described in detail in both the Experimental Section and the Results and Discussion. Each trace arises from setting the decoupler irradiation at a different downfield position, as indicated.

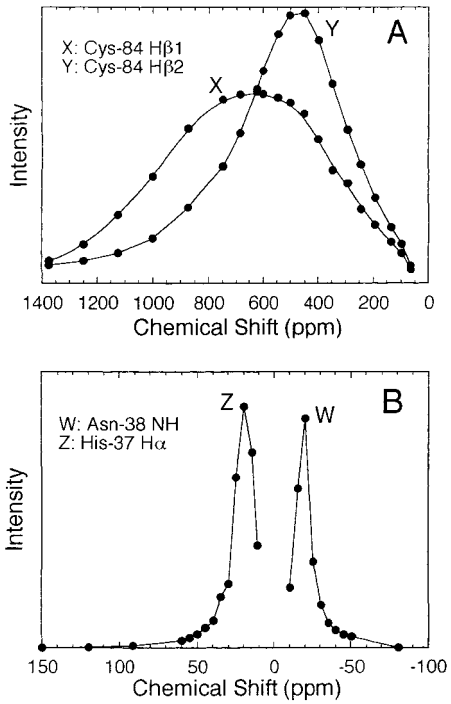

Figure 6. ^{1}H NMR spectrum of oxidized spinach plastocyanin containing signals not observable in the normal spectrum. The positions and line widths of signals X, Y (panel A) and W, Z (panel B) were obtained using saturation transfer experiments (see Experimental Section and Results and Discussion) by plotting the intensity of the respective exchange connectivities with the reduced species as a function of the decoupler irradiation frequency.

and upfield regions of the spectrum, possible obscured by signals A−H and I, J, was checked by performing a similar saturation transfer profile using lower irradiation power (28 W) and finer sampling (5 ppm, with "off" irradiation placed symmetrically with respect to the residual water signal). All saturation transfer signals from resonances A−J that were observed using the conventional 1D NOE irradiation scheme (Figure 3) were also observed in this saturation transfer profile experiment (those from upfield signals appeared as positive in the difference spectra). In addition, two new saturation transfer signals were observed at 10.20 (signal w) and 6.03 ppm (signal z) whose intensity profiles pointed to undetected signals W and Z, centered at −19.0 and 19.2 ppm, with line widths of 7.9 and 11.8 kHz, respectively (see Figure 6B and Table 1).

Full Sequence-Specific Assignment of Copper Ligand Protons. Chart 1 shows the environment of the copper ion in spinach plastocyanin (PDB code 1AG6). In reduced plastocyanin, a TOCSY cross-peak pattern which includes signals z (6.03 ppm), i (2.80 ppm), and k (3.53 ppm) is observed. NOESY connectivities are also observed among these signals and signals b, d (previously assigned to Hδ2 and Hϵ1 of either His-37 or His-87). Therefore, signals z (Hα), and i, k (β-CH_2), can be assigned as the AMX pattern of a coordinated histidine. Dipolar connectivities are observed between signals z and x, and between signals k and j. Signals x and j have been previously assigned to Cys-84 Hβ and Hα protons, respectively. From the available structural data, it clearly appears that the observation of the dipolar connectivities with Cys-84 is expected for His-37 but not for His-87. Therefore, it is possible to assign signals b, d, i, k, and z to His-37. By looking at the dipolar connectivities, the stereospecific assignment of signals x and y as Cys-84 Hβ1 and Hβ2, respectively, as well as signals i and k to His-37 Hβ1 and Hβ2, respectively, is also obtained. The much larger line

Chart 1

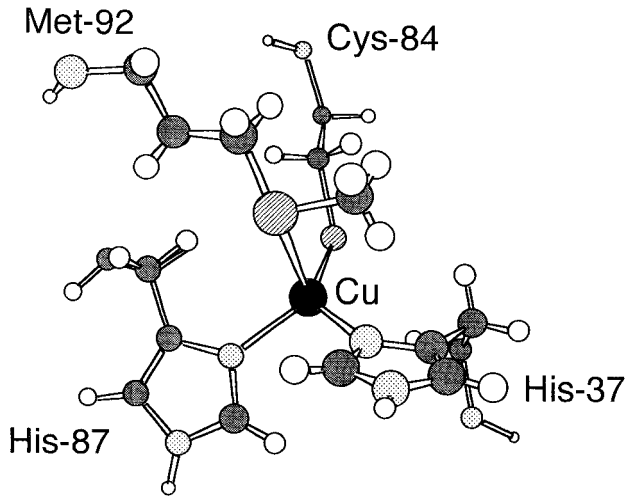

Table 3. Factorization of the Observed Chemical Shifts for Oxidized Spinach Plastocyanin

	angle θ (deg)[a]	Cu−H distance (Å)[a]	δ_{obs} (ppm)	δ_{dia} (ppm)	δ_{dip} (ppm)	δ_{con} (ppm)	A_c (MHz)
			His 37				
Hα	142.3	2.8	19.2	6.03	5.2	8.0	0.30
Hβ1	107.7	4.4	−1.5	2.80	−1.1	−3.2	−0.11
Hβ2	98.0	3.3		3.53	−3.4		
Hδ2	94.3	5.0	47.1	7.56	−1.0	40.5	1.45
Hϵ1	72.8	3.0	35.6	7.19	−3.5	31.9	1.14
NHϵ2	80.4	4.9	31.4	11.67	−1.0	20.7	0.74
			Asn 38				
NH	117.2	3.9	−19.0	10.2	−0.8	−28.4	−1.02
Hα	112.9	6.5	17.0	4.2	−0.3	13.1	0.47
			Cys 84				
Hα	86.0	4.9	−8.0	5.42	−1.1	−12.3	−0.44
Hβ1	62.2	3.4	650	3.26	−1.1	648	23.2
Hβ2	58.6	3.2	489	2.92	−0.7	487	17.4
			His 87				
Hα	87.6	5.2		5.21	−0.9		
Hβ1	66.0	2.8		3.93	−3.0		
Hβ2	62.6	4.0		3.48	−0.7		
Hδ2	96.9	5.1	51.6	7.01	−0.9	45.5	1.63
Hϵ1	123.9	3.1	35.6	7.74	−0.3	28.2	1.01
NHϵ2	115.5	5.0			−0.5		
			Met 92				
Hα	20.7	6.6		4.65	0.7		
Hβ1	30.7	4.7		1.40	1.5		
Hβ2	38.7	4.4		2.36	1.3		
Hγ1	22.5	4.5	13.0	2.25	2.2	8.6	0.31
Hγ2	4.9	5.1	23.5	1.70	1.9	19.9	0.71
ϵ−CH_3	32.0	3.9		0.55	2.5		

[a] These parameters were obtained using the X-ray structure of oxidized spinach plastocyanin (PDB code 1AG6).[26]

width of signal Z in the oxidized form with respect to signal I is fully consistent with the distances from the metal estimated from the available structural data (Hα 2.8 Å, Hβ1 4.4 Å, Hβ2 3.3 Å, see also Table 3) and confirms the stereospecific assignment of signal I as Hβ1. Signal K, corresponding to Hβ2, is expected to be almost as broad as signal Z and is likely to be hidden in the diamagnetic region of the spectrum. Only for reduced plastocyanins from French bean[43] and parsley[37] is the stereospecific assignment of the methylene protons of His-37 and Cys-84 available, but the two assignments are in disagreement with one another. The stereospecific assignment presented

(43) Chazin, W. J.; Wright, P. E. *J. Mol. Biol.* **1988**, *202*, 623−636.

in this work for His-37 and Cys-84 methylene protons is in agreement with the former and in disagreement with the latter.

After performing the assignment of the complete His-37 spin pattern, signals A/a and C/c can be immediately assigned to His-87 Hδ2 and Hϵ1 protons, respectively. The combined analysis of TOCSY and NOESY spectra resulted in the assignment of the complete pattern of His-87 in reduced plastocyanin: β-CH_2 protons at 3.48 (signal l) and 3.93 (signal m) ppm and Hα at 5.21 ppm (signal n). The observed dipolar connectivities which involve His-87 β-CH_2 protons resulted also in stereospecific assignment of signals m and l to Hβ1 and Hβ2 protons of His-87. Such stereospecific assignment is in agreement with that derived for French bean and parsley plastocyanins (Table 2). None of the aliphatic His-87 protons are detected outside the diamagnetic region in the spectrum of oxidized spinach plastocyanin.

Once the sequence-specific assignment of His-37, Cys-84, and His-87 was achieved, the careful analysis of dipolar connectivities expected between the protons belonging to these residues and those belonging to Met-92, as well as NOESY and TOCSY connectivities among the Met-92 proton signals, permitted the identification of the complete spin pattern and stereospecific assignment of signals belonging to Met-92 in reduced plastocyanin: Hα at 4.65 ppm, Hβ1 at 1.40 ppm, Hβ2 at 2.36 ppm, Hγ1 (signal h) at 2.25 ppm, Hγ2 (signal f) at 1.70 ppm, and ϵ-CH_3 at 0.55 ppm. The assignment for Hα and ϵ-CH_3 is consistent with that previously reported for reduced spinach plastocyanin (Table 2), whereas signal assignment for Hβ and Hγ protons is novel. In conclusion, signals H and F in the ^{1}H NMR spectrum of the oxidized protein belong to Met-92 Hγ1 and Hγ2 protons.

In summary, all ^{1}H NMR hyperfine-shifted signals belonging to copper(II)-bound amino acid residues in oxidized spinach plastocyanin have been assigned by observation of exchange, dipolar, and scalar connectivities. The reported assignment is in good agreement with the proton relaxation rates and is consistent with the available ^{1}H NMR assignments of the reduced plastocyanins from spinach[29] and other sources[37,38,43,44] (Table 2).

The least paramagnetic signal G should belong to an amino acid residue not directly coordinated to copper(II), because it does not show any saturation transfer with signals assigned to copper(I)-bound residues, for which the assignment has been fully performed. However, the pseudocontact contribution that can be calculated for any of the protons in the neighborhood of the copper(II) ion, based on the known structures of plastocyanins, cannot justify the observed chemical shift for signal G. Therefore, signal G is shifted not only by a pseudocontact contribution but also by some contact interaction with the copper(II) ion. A scalar connectivity between signals g and w (Table 1) is detected in the TOCSY spectra. These two signals belong to a four-signal TOCSY pattern identified as arising from Asn-38.[29,37,38,43,44] Signal w corresponds to the backbone NH proton and signal g to the Hα proton. The X-ray structure of spinach plastocyanin[26] indicates the presence of a strong hydrogen bond between Asn-38 NH and the copper(II)-bound Cys-84 Sγ (the N−S distance is 3.4 Å). This observation may explain the sizable contact shifts experienced by the Asn-38 NH and Hα protons.

Comparison with Literature Data on Other Blue Proteins. By comparing the ^{1}H NMR 800-MHz spectrum of oxidized spinach plastocyanin to the 600-MHz spectra of amicyanin, azurin and their mutants reported recently,[9,10] some similar features can be observed, consistent with the similar coordination environment in all these proteins. The spectrum of oxidized amicyanin[9] shows two signals at 43 and 27.5 ppm assigned to Hδ2 and NHϵ2 protons of His-37 (spinach consensus sequence), a signal at 50 ppm assigned to His 87 Hδ2, and two signals at 12 and 11.1 ppm assigned as geminal γ-CH_2 Met-92 protons, in agreement with the present assignment. Two upfield signals at −2.5 and −9.5 ppm were assigned as Hβ2 and Hβ1 protons of His-37, respectively. If the similarity in shifts between these proteins holds, then the most upfield signal should be assigned as the Hα proton of Cys-84, while the stereospecific assignment of the signal at −2.5 ppm should be reversed. Finally, a signal at 14.1 ppm with line width similar to that of the signal G observed for spinach plastocyanin is assigned as the Hα proton of Cys-84. This is inconsistent with the present assignment, as signal G belongs to Asn-38 Hα, and the Hα proton of Cys-84 is here unambiguously attributed to signal J. In the case of azurin, the assignment of signals corresponding to the spinach plastocyanin signals G and J was analogous to that of amicyanin[10] and, therefore, equally inconsistent with the present data.

Discussion

Separation of Contact and Pseudocontact Contributions to the Hyperfine Shifts. The isotropic chemical shift of the hyperfine shifted signals is composed of three contributions: (i) the Fermi contact shift, due to delocalization of unpaired spin density on the nuclei of the coordinating residues, (ii) the pseudocontact shift (or rotational average of the dipolar shift) due to the anisotropy of the magnetic moment of the unpaired electron on the metal ion interacting with nuclear magnetic moments through space, and (iii) the diamagnetic shift, i.e., the shift of the nuclei in the diamagnetic system:

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

Often, the lack of knowledge of either pseudocontact or diamagnetic contributions prevents determination of the contact contribution, which directly reflects the distribution of unpaired spin density over the nuclei of the coordinating residues, providing information on the electronic structure of the metal site. In our case, the diamagnetic contribution is directly available from the spectrum of the reduced form. The pseudocontact contribution can be calculated using the structure of the protein [26] and the magnetic susceptibility tensor. The latter is not available, but in copper(II) systems its parameters are approximately proportional to the square of the **g**-tensor parameters.[11] The **g**-tensor for spinach plastocyanin was determined and shown to have axial symmetry.[45] Assuming that, in solution, this tensor has the same direction, the pseudocontact contribution to the chemical shift can be determined using the following equation, valid for axial systems.[46,47]

$$\delta_{pc}(\text{ppm}) = \frac{\mu_0}{4\pi}\frac{{\mu_B}^2 S(S+1)}{9kTr^3}(3\cos^2\theta - 1)({g_{||}}^2 - {g_{\perp}}^2) \times 10^6 \quad (2)$$

where μ_0 is the magnetic permeability of vacuum, μ_B is the electron Bohr magneton, k is Boltzmann's constant, T is the

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absolute temperature, r is the distance between the proton and the copper ion, and θ is the angle between the metal−proton vector and the $g_{\|}$ axis.

As expected, the calculated pseudocontact contribution to the observed chemical shifts is rather small (Table 3). Its magnitude varies from +5.2 ppm for Hα of His-37 to −3.5 ppm for His-37 Hϵ1. The contact contribution, δ_{con}, to the observed chemical shifts for the assigned proton signals in oxidized spinach plastocyanin can then be obtained from eq 1, and the values are reported in Table 3. It appears that the contact shift is by far the major contribution to the overall chemical shift. The value of the hyperfine coupling constant, A/h, can be obtained from the following equation:[46,47]

$$\frac{A}{h} = \frac{1}{2\pi}\frac{\delta_{con}3\gamma_N kT}{g_{av}\mu_B S(S+1)} \quad (3)$$

where γ_N is the nuclear magnetogyric ratio, g_{av} is the average g value, and the other symbols have been already defined. The hyperfine coupling constants, calculated from the contact contribution to the hyperfine shifts, are reported in Table 3.

Electronic and Steric Considerations. The hyperfine constants observed for the copper(II)-bound cysteine are much larger than in the case of His and Met residues, indicating that a much larger fraction of unpaired spin density is delocalized over Cys-84. This result is in agreement with theoretical studies on plastocyanin which indicate the presence of a strong π interaction between the singly occupied copper $d_{x^2-y^2}$ orbital and cysteine S p orbital, together with a weaker and equivalent σ overlap between the same metal orbital and the two imidazole rings of histidines, and a similarly weak interaction with the methionine ligand.[48]

The values of the hyperfine coupling constants displayed by the imidazole ring protons Hϵ1 and Hδ2 are similar for both His-37 and His-87, indicating similar spin delocalization on these residues. These values agree well with ^{14}N ENDOR data on poplar plastocyanin, interpreted as indicating a spin delocalization of ca. 5% on each His residue.[40]

The very large values of the hyperfine coupling constants for Cys-84 β-CH_2 protons obtained using NMR (23.2 and 17.4 MHz for Hβ1 and Hβ2, respectively) agree well with those obtained by ^{1}H ENDOR on poplar plastocyanin (16 and 27 MHz),[40,49] except for the stereospecific assignment. The present assignment is based on NOEs from the two methylene protons to residues located at opposite sides of the β-CH_2 pair, and therefore it is unambiguous. The structures of spinach and poplar plastocyanins indicate that the two Cu−S−C−Hβ dihedral angles are similar in magnitude and opposite in sign (+70°/+66° for Hβ1 and −50°/−50° for Hβ2 in spinach/poplar plastocyanins), consistent with the observation of similar hyperfine shifts for cysteine Hβ1 and Hβ2. If this structural feature is conserved in solution for spinach plastocyanin, and given the electronic structure of this site, then the angular dependence of the shift is of the type

$$\frac{A}{h} = (\sin^2\theta + C/B)(B\rho_s) \quad (4)$$

where ρ_s is the total spin density on sulfur, and B and C are parameters related to π and σ spin distribution mechanisms, respectively. A similar angular dependence for the hyperfine coupling constant of cysteine methylene protons has been also reported for Fe−S proteins.[50] Using the structural data for spinach plastocyanin and the hyperfine coupling constants determined here by NMR, a parametrization for eq 4 gives $B\rho_s = 19.6$ MHz and $C/B = 0.30$. Equation 4 accounts also for the ^{1}H ENDOR data on poplar plastocyanin, which were interpreted using a $\cos^2\theta$ angular dependence,[40] thereby explaining the reversed stereospecific assignment.

Sulfur K-edge XAS studies[41] indicate that, in plastocyanin, the copper site has ca. 38% S−Cys character in the highest energy half-occupied molecular orbital. Cu L-edge absorption XAS studies[42] indicate that this blue copper site is very covalent with only ca. 40% Cu $d_{x^2-y^2}$ character in the half-occupied molecular orbital. These experimental data[51] are in excellent agreement with the results of EPR and SCF-Xα-SW calculations.[45,48] An electron spin density of 1.6% and 1.2% can be estimated to be localized on the 1s orbital of Cys-84 Hβ1 and Hβ2, respectively, using the values of the hyperfine coupling constants of the β-CH_2 protons determined by NMR and that of the free hydrogen atom (1420 MHz[52]). These high values are qualitatively in agreement with all the above results but do not allow a quantitative estimate of the electronic delocalization on the copper(II)-bound cysteine because spin polarization effects could also be operative. It is noticeable that the hyperfine constant of the Cys-84 Hα proton is much smaller that that observed for the β-CH_2 protons, and negative in sign. This can be largely explained using eq 4 applied to the Cα atom, which leads to a small spin density delocalization; the negative sign can be interpreted as revealing the presence of spin polarization.

Some electron density is also present on Asn-38, possibly due to the H bond between the peptide NH group of this residue and the copper(II)-bound cysteine sulfur (see hyperfine coupling constants in Table 3). The sizable value of the hyperfine coupling constant of the NH proton may be accounted for by the high spin density on the cysteine sulfur. The reversal in sign of the hyperfine coupling constant upon passing from the NH to the Hα proton of Asn-38 is consistent with the presence of a spin-polarization mechanism.

It is interesting to compare the hyperfine shifts of plastocyanin with those observed in Cu_A systems (purple copper site), the only other oxidized copper center, related to that of plastocyanin, for which ^{1}H NMR studies are available.[53−57] The structure of the Cu_A center shows the presence a $Cu_2(SCys)_2$ cluster in which each copper ion is further coordinated to a histidine and weakly interacting with a fourth ligand (a methionine sulfur or a peptide carbonyl oxygen)[58−62] In the oxidized state, a single electron is

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fully delocalized over the mixed-valence Cu^{2+}/Cu^{+} chromophore.[53,63] Considering that most of the unpaired electron in oxidized plastocyanin is delocalized between one copper and one sulfur atom, whereas the unpaired electron in Cu_A is shared among two copper and two sulfur atoms, the observation that the shifts of cysteine protons in Cu_A[53,55,57] are about half of those in spinach plastocyanin indicates that the covalency of the Cu−SCys bond is essentially the same in the two systems and the smaller shift is due to the halved unpaired electron density on the sulfur atoms. The same argument holds for the shifts of the histidine ligands, which are approximately half in Cu_A with respect to those in plastocyanin. These observations are consistent with ENDOR data.[64] The peculiarity of the Cu−SCys bond is further evidenced by comparing the NMR properties of copper(II) plastocyanin with those of metal-substituted blue copper proteins. The Cys β-CH_2 hyperfine shifts of cobalt(II)- and nickel(II)-substituted blue copper proteins[10,65−70] are much smaller than those observed here for copper(II) plastocyanin, despite the larger number of unpaired electrons on the metal center, directly indicating that metal substitution modifies the covalency of the M−SCys bond and, as a consequence, the degree of spin delocalization.

Considerations on Nuclear Relaxation and Signal Observability in Copper Proteins. Signal detection in copper(II) proteins, i.e., signal line widths, depends on both dipolar and contact contributions to transverse relaxation through the relationship $\Delta\nu = 1/\pi T_2$). In turn, dipolar relaxation depends on the reciprocal of the sixth power of the metal−nucleus distance, while contact relaxation depends on the square of the hyperfine coupling constant. Both depend on the electronic relaxation time of the metal, which, in the case of copper(II) plastocyanin, is the dominant correlation time. The relevant equations, due to Solomon and Bloembergen,[71,72] are

$$T_2^{-1}(\mathrm{dip}) = \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left(4\tau_{s_1} + \frac{3\tau_{s_1}}{1+\omega_I^2\tau_{s_1}^2} + \frac{13\tau_{s_2}}{1+\omega_S^2\tau_{s_2}^2}\right) \quad (5)$$

$$T_2^{-1}(\mathrm{con}) = \frac{1}{3}\left(\frac{A}{\hbar}\right)^2 S(S+1)\left(\tau_{s_1} + \frac{\tau_{s_2}}{1+\omega_S^2\tau_{s_2}^2}\right) \quad (6)$$

where τ_{s_1} and τ_{s_2} are the longitudinal and transverse electronic relaxation times, respectively, and r is the metal−nucleus distance. Another source of dipolar relaxation is Curie spin relaxation.[73,74] However, Curie spin relaxation is important only when electronic relaxation is at least 2−3 orders of magnitude faster than the rotational correlation time of the molecule, which is not the case here. Ligand centered terms may be important[75] but are difficult to assess: they are somehow related to the presence of unpaired spin density delocalization, and therefore their effect can hardly be separated from contact relaxation. Nuclear longitudinal relaxation in copper(II)-containing systems, at variance with transverse relaxation, is affected only by dipolar relaxation:[1]

$$T_1^{-1}(\mathrm{dip}) = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left(\frac{3\tau_{s_1}}{1+\omega_I^2\tau_{s_1}^2} + \frac{7\tau_{s_2}}{1+\omega_S^2\tau_{s_2}^2}\right) \quad (7)$$

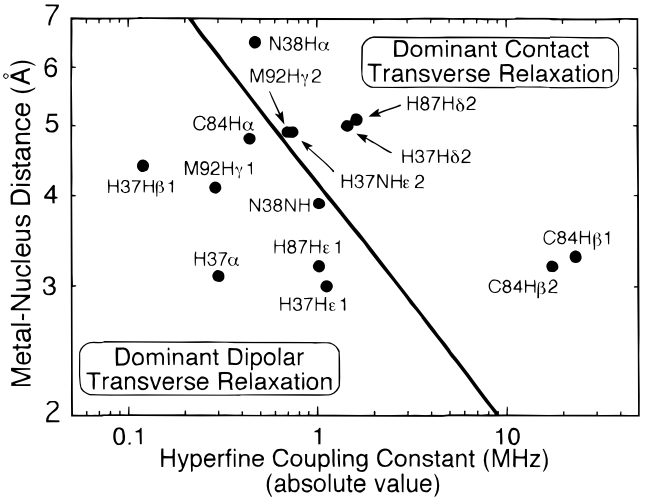

Figure 7. Metal−proton distances vs hyperfine coupling constants for ligand protons in plastocyanin. Protons falling to the left of the line experience dominant dipolar relaxation, while protons to the right experience dominant contact relaxation.

Therefore, a simultaneous analysis of both nuclear relaxation rates may provide a reasonably complete picture. The electronic relaxation time of copper (assuming $\tau_{s_1} = \tau_{s_2}$), estimated using Table 3, ranges between 0.2 and 0.8 ns, in reasonable agreement with the values previously proposed for other blue copper proteins.[6−8] It is remarkable that, among the 14 protons for which the assignment was performed, seven are relaxed mainly by dipolar contributions and the other seven by contact contributions (Figure 7). For protons with relatively large hyperfine couplings, contact relaxation is dominant. The most striking example is provided by the β-CH_2 protons of the copper(II)-coordinated cysteine, whose large line widths (300−600 kHz) are totally determined by the contact interaction, with a dipolar contribution accounting only for 5−6 kHz.

In conclusion, NMR signals from residues noncoordinated to copper(II) centers, i.e., experiencing only dipolar broadening, should not be too difficult to observe: the dipolar line width of a proton 6 Å from the metal is predicted to be only about 100 Hz and, therefore, already amenable to the classical multidimensional experiments used for solution structure determination.

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The situation will be even more favorable for heteronuclei, whose dipolar relaxation is reduced with respect to that of protons by factors of 16 for ^{13}C, 42 for ^{2}H, and 97 for ^{15}N, because of their smaller magnetogyric ratios.

Concluding Remarks

The accomplishments of the present work are summarized below:

(1) The 800-MHz ^{1}H NMR spectra of oxidized spinach plastocyanin were recorded, and relatively sharp hyperfine shifted signals were observed. The complete assignment of these signals was performed using 1D and 2D NMR experiments tailored for detecting fast relaxing signals.

(2) The β-CH_2 proton signals for the coordinated cysteine residue were detected for the first time in a copper(II) protein by devising a "blind" saturation transfer experiment that exploited the favorable electron exchange properties between oxidized and reduced proteins.

(3) The contact hyperfine coupling constants for protons belonging to the copper(II)-bound protein residues were calculated from the contact shifts, after correcting for the small pseudocontact contributions. The dependence of the contact shift on the Cu−S−C−Hβ dihedral angle is of the $\sin^2\theta$ type. Insights into the electronic structure of the system have been gained that complement data obtained from ENDOR experiments. A relatively large share of unpaired spin density is delocalized on the sulfur of the copper(II)-bound cysteine. The analogies found with the Cu_A center indicate a similar nature of the Cu−S bond in these two systems.

(4) Nuclear relaxation properties reveal a large contribution of contact interactions in Cys β-CH_2 protons and His Hδ2 protons, whereas dipolar coupling is more important for His Hϵ1 as well as for the remaining aliphatic protons.

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