Analysis of the Paramagnetic Copper(II) Site of Amicyanin by ^{1}H NMR Spectroscopy†

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ABSTRACT: Application of tailored pulse sequences like super-WEFT allows the direct observation of the hyperfine-shifted signals of the paramagnetic Cu(II) forms of blue copper proteins in solution. The signals can be assigned by applying 2D NMR techniques, like EXSY, to solutions containing a mixture of reduced and oxidized species. The Fermi contact shift is separated from the pseudocontact shift on the basis of the known **g**-tensor anisotropy of the Cu(II) state, allowing the determination of a number of hyperfine-splitting constants between protons on the Cu ligands and the unpaired electron. These results are used to quantify the spin density distribution over the Cu ligands. In amicyanin about 50%−60% of the unpaired electron density is found on the ligands. It appears possible to quantify the Cu−S(Met) interaction on the basis of the NMR results. Application of the technique to the wild type forms of amicyanin and azurin and to two active site mutants of amicyanin (His96Asp and a plastocyanin−amicyanin loop exchange mutant) shows that the Cu−S(Met) interaction parallels the rhombicity and axial distortion of the Cu site.

Blue copper proteins contain a redox active site that consists of a single Cu ion, immobilized within the protein framework by strong bonds with the S^{γ} of a cysteine and with the N^{δ}'s of two histidines. The copper atom resides in, or almost in, the plane of these three atoms (N_2S coordination). In most cases one of the axial positions is occupied by the S^{δ} atom of a methionine. Upon inspection of the available 3D structures of blue copper proteins (Baker, 1988; Nar et al., 1991; Durley et al., 1993; Collyer et al., 1990; Guss et al., 1988, 1992; Petratos et al., 1988; Romero et al., 1994) it appears that the copper is slightly pulled out of the N_2S plane toward this axial methionine. In a single subclass of the blue copper proteins, viz., the azurins, a second axially coordinating group is found at the opposite side of the N_2S plane. This is a backbone carbonyl group, the oxygen of which interacts with the Cu.

While the character of the three bonds that keep the Cu in the N_2S plane is fairly well understood, the nature of the interaction of the Cu with the axially coordinating groups (the methionine S^{δ} atom and, in the case of the azurins, also the carbonyl oxygen) has been a matter of strong debate in the literature, and the issue has not yet been resolved. Particular attention has been paid to the degree of covalency of these interactions (Lowery & Solomon, 1992; Solomon et al., 1992; Scott et al., 1982; Blair et al., 1982; Piccioli et al., 1995). This is understandable if one considers the special properties that have been ascribed to the axially coordinating methionine. For instance, it has been proposed that the distance from the copper to the methionine influences the spectroscopic characteristics of blue copper proteins (Han et al., 1993; Lu et al., 1993; Romero et al., 1993). Additionally the axially coordinating groups have been ascribed a decisive role in tuning the midpoint potential of these proteins (Gray & Malmström, 1983; Karlsson et al., 1989; Pascher et al., 1993; Malmström, 1994; Fields et al., 1991).

Solving the issue of the covalency of the interactions between the Cu and its axial ligands is hampered by the fact that the problem is not easily accessible spectroscopically. Techniques like EXAFS and XAS or XANES were found to have insufficient sensitivity or resolution for this purpose (Scott et al., 1982; Schmidt-Klemens et al., 1989; Shadle et al., 1993). Establishing the amount of spin density delocalized over the ligands in the oxidized Cu(II) form of the protein would provide a quantitative measure of the interaction between the Cu and its ligands, but in this case the two available magnetic resonance techniques, EPR and NMR, have met with limited success. ENDOR and ESEEM studies of blue copper proteins have focussed mainly on the study of the two ligand histidines (Roberts et al., 1984; Werst et al., 1991). The recently reported EPR and ENDOR studies of single crystals of the blue copper proteins seem to hold promise here (Coremans et al., 1994, 1995). Nevertheless, the potential to investigate the mode of binding of the axial ligands by these techniques appears limited at best.

NMR studies of the paramagnetic, oxidized [Cu(II)] form of blue copper proteins have been hampered by the long electron spin relaxation time of the Cu (1−3 ns) (Bertini et al., 1989). The considerable NMR line broadening that is induced by this slow relaxation effectively washes out the signals in the NMR spectrum from nuclei within a sphere of approximately 7 Å radius around the Cu. In the past it has been thought that this definitively precludes the study of the ligands of the Cu in the oxidized protein through direct

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[1] Abbreviations: ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; EXAFS, extended X-ray absorption fine structure; EXSY, exchange spectroscopy; Fc, Fermi contact; G, gauss; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; SCF=Xα-SW, self-consistent field Xα scattered wave; WEFT, water-suppressed equilibrium Fourier transform; XA(NE)S, X-ray absorption (near edge) spectra.

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observation by NMR (but *vide infra*). Reducing the protein to the Cu(I) state or replacing the copper by diamagnetic analogues like Zn(II) or Cd(II) abolishes the paramagnetism and restores the normal (diamagnetic) appearance of the NMR spectrum. Interpretation of the diamagnetic chemical shifts in terms of an electronic charge distribution presents a formidable theoretical challenge, however. Moreover, metal replacement will almost surely lead to small but significant changes in the coordination geometry.

Another way to circumvent the problem posed by the intrinsically slow electron spin relaxation of Cu(II) has been either to magnetically couple the Cu to a second metal with very fast electronic spin relaxation or to replace the metal altogether by a fast-relaxing paramagnetic ion. The latter approach has led to success in cases where Cu(II) was replaced by Co(II) or Ni(II), for instance (Moratal et al., 1993a,b; Villa, 1994). The former approach has been successfully applied to the study of Cu−Zn superoxide dismutase where the Zn ion in the binuclear Cu−Zn site was replaced by Co(II) (Bertini et al., 1989, 1992, 1994). The drawback of these approaches is that, apart from possible distortions of the site caused by the metal replacement, Co(II) and Ni(II) may exhibit a sizable **g**-tensor anisotropy which may lead to considerable pseudocontact shifts. Interpretation of the shifts observed in the NMR spectra may therefore be difficult, and this will eventually hamper the determination of the electron spin density on the ligands.

In recent years, thanks to the development of new multidimensional NMR techniques and their adaptation to the study of paramagnetic proteins, the latter are slowly beginning to yield their structural and mechanistic secrets. Yet, Cu(II) proteins up till now were considered difficult, if not impossible, to tackle by means of NMR spectroscopy. Here we show that NMR spectra of Cu(II) proteins, when recorded under special conditions, do display clearly visible contact-shifted signals. Furthermore, it appears possible by means of 2D EXSY NMR spectroscopy on partly oxidized solutions to correlate the signals of the paramagnetic form of the protein with those of the diamagnetic species. Since the NMR spectra of the latter have been assigned for a number of blue copper proteins, the assignment of the contact-shifted signals in the NMR spectra of the Cu(II) forms of these proteins is a relatively easy task.

A further advantage compared with the metal replacement techniques mentioned above is that the **g**-tensor anisotropy in the Cu(II) state is small and can be easily established from EPR spectroscopy, and since a fairly good idea exists about the location of the **g**-tensor axes with respect to the molecular frame-work, in a number of blue copper proteins, it is possible to correct the observed shifts for their pseudocontact contribution. In this way, the magnitude of the Fermi contact interaction and an estimate of the spin density on the ligands can be obtained. Thus, although the spectra of the paramagnetic Cu(II) species lack structure as compared to those of the Co(II) or Ni(II) analogues, for instance, they provide in a fairly direct and simple way information about the electronic structure of the Cu site.

The present report focuses on the study of wild type (wt) and mutant amicyanins and of wt azurin. It will be shown how the strength of the Cu−S(Met) interaction at least qualitatively correlates with previously established measures of the rhombicity and axial distortion of the Cu site.

MATERIALS AND METHODS

Proteins. Four proteins were used in this study. Wild type amicyanin was expressed and purified as described previously (Kalverda et al., 1994). Wild type azurin was obtained as described previously (van de Kamp et al., 1990a) and was a kind gift of Ms. Gertie van Pouderoyen. Furthermore, two mutants of amicyanin were used. The first was the H96D mutant in which one of the histidine ligands has been replaced with an aspartate. In the second mutant the loop between His96 and Met99 was exchanged for the corresponding loop from plastocyanin, $H^{96}PFM \rightarrow H^{96}QGAGM$. The construction of these two mutants will be described elsewhere. Purification was performed in a way similar to the wt amicyanin purification.

Sample Preparation. NMR samples in 99.95% ^2H_2O were prepared by using ultrafiltration equipment and contained 6 mM (CuII) amicyanin and 50 mM potassium phosphate (pH 7.0). Samples of the mutant amicyanins were 1 mM in protein. The azurin sample contained 2 mM protein.

NMR Spectroscopy. The NMR methodology developed to study paramagnetic systems, principally iron proteins (heme or Fe−S proteins) and cobalt- and nickel-substituted proteins (mainly metalloderivatives of zinc enzymes), is used here to study paramagnetic Cu(II) proteins. 1D ^1H NMR spectra are recorded by means of the super-WEFT sequence, which combines very rapid pulsing conditions with an inversion recovery sequence ($180°-\tau-90°$) (Inubushi & Becker, 1983). An appropriate choice of τ and the recycle time results in efficient quenching of the intensity of slowly relaxing (diamagnetic) signals, while paramagnetically affected signals are emphasized. Using this approach has the advantage of dramatically shortening the recycle time and allowing the collection of many more scans per unit of time, thus improving the signal-to-noise ratio for paramagnetic resonances. Additionally it allows the detection of fast-relaxing signals within the diamagnetic envelope and the reduction of the water line in H_2O solvent samples.

All ^1H NMR spectra were collected on a Bruker DMX 600 MHz spectrometer. 1D spectra were recorded with the super-WEFT sequence (Inubushi & Becker, 1983) to suppress the diamagnetic resonances including H_2O. An interpulse delay of 40 ms was used with an acquisition time of 50 ms to give a repetition rate of 10 s^{-1}. The spectra were recorded with a spectral width of 120 kHz. They were processed by using 20 Hz of exponential line broadening as apodization. The WEFT sequence can be combined with 2D EXSY or NOESY to give WEFT−EXSY and WEFT−NOESY spectra (Chen et al., 1994). The initial 90° pulse is then replaced by $[180°-\tau_{rd}-90°]$. With τ_{rd} of the order of 24 ms and an acquisition time of 25 ms, a repetition rate of 17 s^{-1} is reached. This fast repetition makes it possible to acquire the EXSY spectrum with 256 t_1 values and 4096 scans per t_1 point in about 17 h. The mixing time for the EXSY spectrum was 3 ms. A total of 2000 points were collected over a bandwidth of 28 kHz in t_2. The spectral width in t_1 was set to 60 kHz in order to include all the shifted resonances. 2D spectra were processed with cosine apodization windows in both directions.

RESULTS

Amicyanin is a type I blue copper protein found in the methylotroph *Thiobacillus versutus*. In the cupric state the

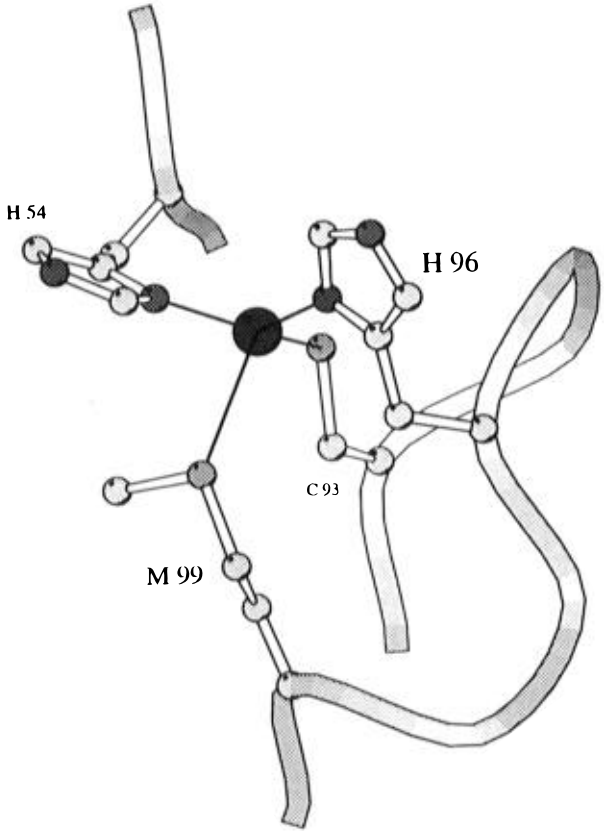

FIGURE 1: Representation of the Cu site in amicyanin. The heavy atoms of the ligand side chains are shown starting with the C^{α} atoms. The copper atom is indicated by the dark sphere in the center of the figure.

protein has an axial ($g_{\|} = 2.239$, $g_{\perp} = 2.046$) X-band EPR spectrum with a small hyperfine coupling constant ($A_{\|} = 5.6 \times 10^{-4}$ cm^{-1}) and an intense absorption at 596 nm ($\epsilon = 3900$ M^{-1} cm^{-1}) (van Houwelingen et al., 1985). The structure of the protein has been determined by NMR and X-ray crystallography for the Cu(I) and Cu(II) forms, respectively (Kalverda et al., 1994; Romero et al., 1994). The copper is strongly coordinated by a sulfur from Cys93 and two nitrogens from His54 and His96. A fourth, weaker interaction with the sulfur of Met99 is also present. The copper has moved 0.4 Å out of the plane of the three strong ligands toward the axial methionine. The structure of the active site of amicyanin is shown in Figure 1.

Assignment of Hyperfine-Shifted Resonances of Wild Type Amicyanin and the H96D Mutant. The 1D WEFT spectrum of amicyanin is reproduced in Figure 2A. It shows a number of very broad peaks that are shifted outside the diamagnetic envelope. Their widths and the magnitude of their shifts characterize them as contact-shifted signals. Since the *g*-anisotropy of Cu(II) in amicyanin is small (0.193), the pseudocontact interaction is also small, and the experimentally observed shifts are dominated by the Fermi contact interaction. This means that the shifted resonances originate from protons of one or more of the copper ligands, viz., His54, Cys93, His96, and Met99. The line widths of these peaks vary from about 600 Hz for the signals at 12 ppm to over 4000 Hz for the peak at 47 ppm.

The assignment of these peaks is achieved by correlating them to their diamagnetic counterparts, which have been assigned previously. Amicyanin has a high electron self-exchange rate constant of about 5×10^4 M^{-1} s^{-1} at pH 7.0

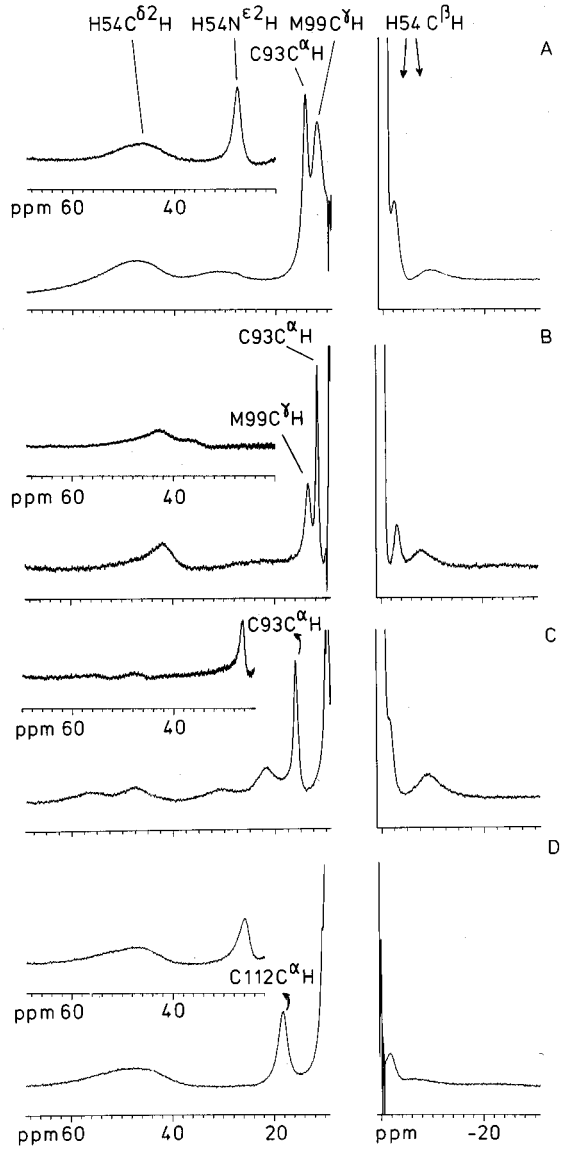

FIGURE 2: 1D WEFT spectra of (A) wild type amicyanin; (B) the amicyanin mutant H96D; (C) the amicyanin mutant H^{96}PFM → H^{96}QGAGM; (D) wild type *Pseudomonas aeruginosa* azurin. The diamagnetic region from 9 to 1 ppm has been deleted from the center of the figures. The insets show the spectra in H_2O, where an additional peak from an exchangeable proton appears. Peak assignments for wild type amicyanin are given at the top of panel A. Spectra were collected at 32 °C on samples containing 50 mM phosphate, pH 7.0, and 6 mM (wt amicyanin), 2 mM (azurin) or 1 mM (ami mutants) protein. Between 8000 and 20 000 transients were collected for each spectrum.

and 32 °C (Lommen & Canters, 1990). The resonances of the Cu(I) and Cu(II) form are, however, in slow exchange on the chemical shift time scale at the concentrations used for the experiments. In principle it should be possible, therefore, to infer the assignments from exchange cross-peaks in the EXSY spectrum of a partially reduced solution of the protein, provided the pulse sequence is adapted to the paramagnetic nature of the Cu(II) form of the amicyanin. A combination of the EXSY and WEFT sequences appeared effective for this purpose as shown in Figure 3. Exchange cross-peaks are found for a large number of resonances both within and outside the diamagnetic envelope. In this report we concentrate on the latter signals. A more complete analysis will be reported elsewhere.

Table 1: Hyperfine-Shifted Resonances of Cu(II) Amicyanin

assignment	δ_{obs} (ppm)	δ_d[a] (ppm)	R (Å)	θ (deg)	δ_{pc} (ppm)	δ_{Fc} (ppm)	a_H (G)
H54 $C^{\delta 2}H$	43	7.41	5.23	86.5	−0.97	36.6	1.0
H54 $N^{\epsilon 2}H$	27.5	13.4	5.00	71.2	−0.77	14.9	0.41
H54 $C^{\beta 2}H$	−9.5	3.17	3.36	94.4	−3.65	−9.0	−0.25
H54 $C^{\beta 3}H$	−2.5	2.44	4.61	101.0	−1.28	−3.7	−0.10
C93 $C^{\alpha}H$	14.1	4.53	4.87	99.4	−1.13	10.7	0.30
M99 $C^{\gamma}H$	12	2.72	4.64	25.5	2.03	7.3	0.20
M99 $C^{\gamma}H$	11.1	2.46	5.22	7.5	1.93	6.7	0.19
H96 $C^{\delta 2}H$	50[b]	7.38	5.34	95.6	−0.90	43.5	1.2

[a] Chemical shift in the reduced protein; values taken from Lommen et al. (1991) and Kalverda et al. (1994). [b] Tentatively assigned; see text.

When the assignment of the NMR spectrum of the diamagnetic amicyanin is used as a starting point, it turns out that the peak at 14.1 ppm corresponds to the $C^{\alpha}H$ of Cys 93, the two overlapping peaks at 12 and 11.1 ppm to the two $C^{\gamma}H$ protons of Met 99, and the peak at 43 ppm to the $C^{\delta 2}H$ of His54. The peak at −2.5 ppm originates from one of the $C^{\beta}H$ protons of His 54, the sign of the shift being in accordance with the (expected) presence of negative spin density on the adjacent carbon atom. Similarly the peak at −9.5 ppm must originate from the $C^{\beta}H$ proton of a ligand histidine. Since no cross-peak could be observed for this peak, the signal may originate from either His54 or His96. An additional peak appears at 27.5 ppm when the 1D WEFT spectrum is recorded in H_2O (Figure 2A). This resonance has not yet disappeared in a sample that is freshly dissolved in D_2O, and thus the exchange correlation between the Cu(I) and Cu(II) forms is picked up in the EXSY spectrum (Figure 3). This peak is assigned to the $N^{\epsilon 2}H$ of His54. The $N^{\epsilon 2}H$ of His96 is located on the surface and is in fast exchange with H_2O. It is therefore not observable at pH 7.0. The positions of the hyperfine-shifted resonances are summarized in Table 1.

To check the assignments a 1D WEFT spectrum was recorded of the site-directed mutant H96D, in which one of the ligands, His96, is replaced by an aspartate. The spectrum of this mutant, shown in Figure 2B, is quite similar to that of the wild type protein. A comparison of the two D_2O spectra shows that the very broad resonances at 50 and 31 ppm have disappeared in the spectrum of the mutant. These resonances are therefore tentatively assigned to His96. According to this interpretation the large, very broad peak stretching from 38 to 57 ppm in the wt amicyanin spectrum is composed of at least two overlapping peaks deriving from His54 and His96. The signal at −9.5 ppm was assigned (see above) to a $C^{\beta}H$ of either His54 or His96. Since it appears to be only marginally affected by the mutation we can now assign it to a $C^{\beta}H$ proton of His54. Thus, the two peaks observed at −9.5 and −2.5 ppm are ascribed to the two $C^{\beta}H$ protons of His54. Their stereospecific assignment will be discussed in the next section. The exchangeable $N^{\epsilon 2}H$ proton of His 54 at 27.5 ppm in the wild type protein is not seen in the mutant. This may be due to a faster exchange with water.

Methionine Resonances. To check the applicability of the technique described above, especially in relation to the study of the strength of the Cu−S(Met) interaction, spectra were recorded of a loop exchange mutant of amicyanin, $H^{96}PFM \rightarrow H^{96}QGAGM$, in which the loop from His96 to Met99 of amicyanin was exchanged for the corresponding loop of plastocyanin. This mutant was considered appropriate since a shorter Cu−S(Met) bond was indicated by (a) the increased absorption in the visible spectrum at 460 nm compared to wild type amicyanin and by (b) the rhombic character of the EPR spectrum of the mutant (*vide infra*). Spectra were also recorded of the oxidized form of wild type azurin. The spectra are reproduced in Figure 2C,D, respectively. No EXSY spectra were recorded of the amicyanin loop mutant or the H96D mutant, since the spectra of the Cu(I) forms of these proteins have not been assigned. The NMR spectrum of reduced wt azurin has been assigned (van de Kamp et al., 1992), but in this case the EXSY spectrum appeared very difficult to observe. Possibly the high electron self exchange (ESE) rate of azu [an order of magnitude higher than for amicyanin (Groeneveld & Canters, 1988)] pushes the interconversion between reduced and oxidized azurin into the "intermediate exchange" regime, leading to excessive broadening and weak signal intensity. This is the subject of further research.

A full assignment of the paramagnetically shifted peaks in Figure 2B−D has not been attempted, but the strong similarity of the spectra to the spectrum of the oxidized wt amicyanin (Figure 2A) may be used for a first analysis. In doing so we shall restrict our attention to the signals of the methionine and cysteine ligands in each case. The relatively sharp peak at 14.1 ppm in the spectrum of oxidized wt amicyanin (Figure 2A) was found to correspond (*vide supra*) to the $C^{\alpha}H$ of the ligand cysteine. The position of this peak is dominated by the Fermi contact (Fc) interaction (see next section), the magnitude of which depends on the configuration of the cysteine side chain, i.e., on the dihedral angles along the chain Cu-S^{γ}-C^{β}-C^{α}-H^{α}. One of the features of the type 1 Cu centers in blue copper proteins is that this configuration is remarkably constant (Han et al., 1991). We therefore expect the cysteine $C^{\alpha}H$ signal of oxidized blue copper proteins to occur at approximately the same position in the spectrum. Thus, the fairly sharp peaks at 12.5, 17.3, and 18.8 ppm in the spectra of Figure 2B−D, respectively, are ascribed to the $C^{\alpha}H$ of the ligand cysteine. The somewhat broader peak at 11.6 ppm in Figure 2A was found to consist of the overlapping signals at 11.1 and 12.0 ppm of the C^{γ} protons of the ligand methionine. In the spectrum of H96D amicyanin (Figure 2B) this peak occurs at 13.8 ppm, and in the spectrum of the ami loop mutant (Figure 2C) the peak at 21.5 ppm is assigned to the $C^{\gamma}H_2$ pair of the ligand methionine. It is conceivable that the peak at 28.5 ppm in Figure 2C is also derived from this pair and that the peaks are split further apart than in the case of the wt amicyanin. Finally, in the wt azurin spectrum (Figure 2D) no contact-shifted signal of the Met121 protons is observed. The results for the assignment of the ligand methionine $C^{\gamma}H_2$ signals have been collected in Table 2.

DISCUSSION

Contact Shifts. The contact shifts observed in the spectrum of Cu(II) amicyanin (Figure 2A, Table 1) are the sum of a Fermi contact (δ_{Fc}) and a pseudocontact (δ_{pc}) contribution. The Fermi contact shift is due to delocalization of the spin density of the unpaired electron through the network of covalent bonds. The pseudocontact shift is caused by the through space (dipolar) interaction of the nucleus with the magnetic moment of the unpaired electron. The charge difference between the Cu center in reduced and oxidized amicyanin provides for an additional, electrostatic contribution to the shift, but its magnitude is small compared to the

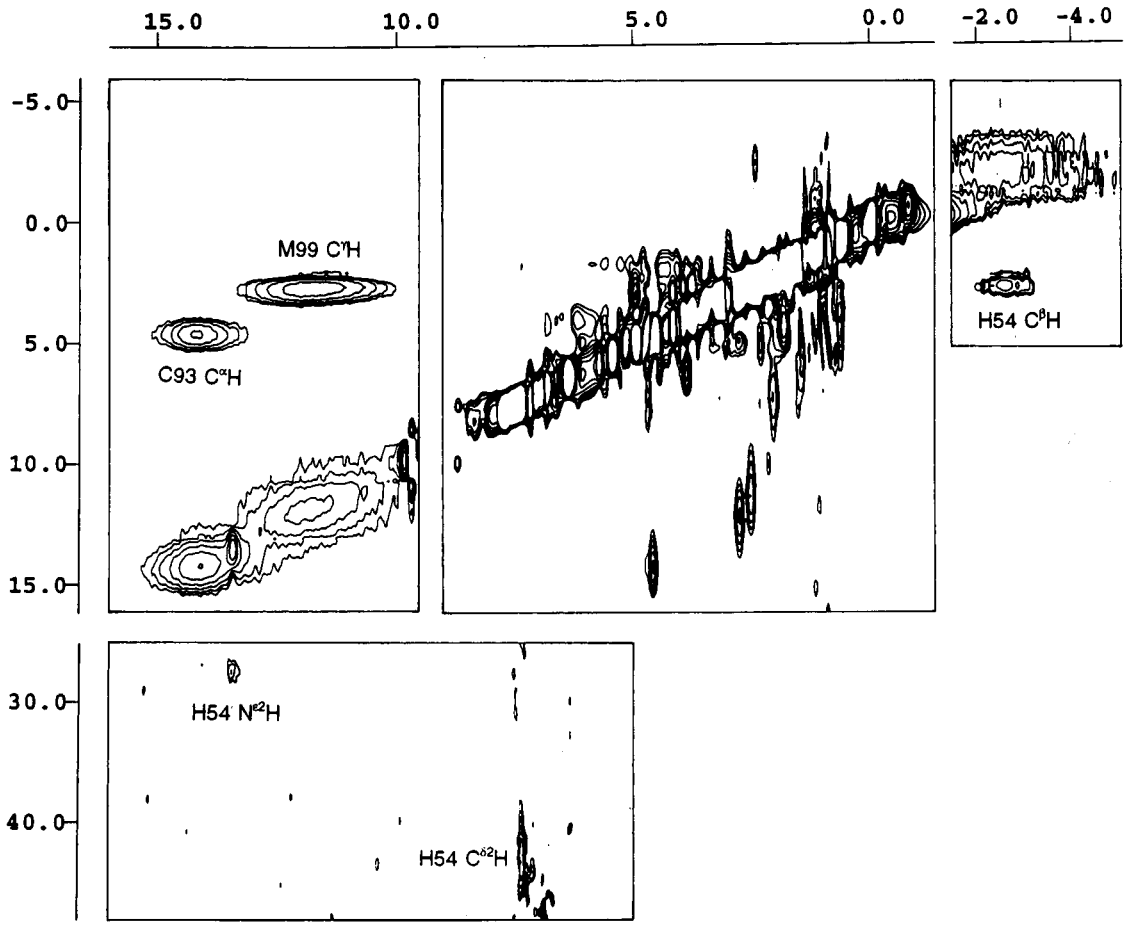

FIGURE 3: Regions of the 2D WEFT−EXSY spectrum of a 50/50 mixture of Cu(I) and Cu(II) amicyanin. The exchange peaks between the Cu(I) and Cu(II) form are labeled with their assignments. Spectra were recorded at 32 °C with a mixing time of 3 ms on a 50/50 mixture of Cu(I) and Cu(II) amicyanin in D_2O/50 mM phosphate, pH 7.0. Both forms were present at 3 mM concentration.

Table 2: Methionine $C^{\gamma}H$ Signals of wt and Mutant Cu(II) Amicyanin and Azurin

	paramagnetic shift[a] (ppm)	$\Delta g_{\perp}$[b]	spectral ratio[c]
wt azurin	<8	0.0175[d]	0.035[e]
wt amicyanin	8.6/9.3	nd[f]	0.10[g]
H96D amicyanin	11	nd	0.14[h]
loop mutant amicyanin	19	>0.050	0.26[i]

[a] $\delta_{obs} - \delta_d$. [b] $|g_{xx} - g_{yy}|$. [c] Ratio of intensities of optical absorption bands at around 400−450 nm and around 600 nm. Intensities are taken at wavelengths of maximum absorption. [d] Taken from van de Kamp et al. (1990b). [e] I_{481}/I_{628}. [f] nd, not determined. [g] I_{460}/I_{596}. [h] I_{405}/I_{607}. [i] I_{460}/I_{593}.

contact shifts and it is ignored here. The magnitudes of the first two contributions are given (in ppm) by

$$\delta_{Fc} = 10^6\,(\Delta H_{Fc}/H) = 10^6[(g\beta)^2/4kT]\{a/\gamma_N\hbar\} \quad (1)$$

$$\delta_{pc} = 10^6\,(\Delta H_{pc}/H) = 10^6\,[(g_{||}{}^2 - g_{\perp}{}^2)\beta^2/4kT]\{(3\cos^2\theta - 1)/3R^3\} \quad (2)$$

in which ΔH_{Fc} and ΔH_{pc} are the Fermi contact and pseudocontact shifts in magnetic field units, respectively, H is the externally applied magnetic field, g is the electronic g-factor, β is the Bohr magnetron, k is the Boltzmann constant, T the absolute temperature, a is the isotropic Fermi contact coupling constant in magnetic field units (Gauss), γ_N is the gyromagnetic ratio of the proton, $\hbar$ is Planck's constant divided by 2π, $g_{||}$ and $g_{\perp}$ are the parallel and perpendicular components of the **g**-tensor, θ is the angle between the z-axis of the **g**-tensor and the vector connecting the Cu with the proton, and R is the distance between the proton and the Cu. Equation 2 is based on the point dipole approximation and disregards the delocalization of the unpaired electronic spin over the ligands. To get an idea of the orders of magnitude involved, with $\Delta g = 0.193$ (wt amicyanin), δ_{pc} may vary between −5.3 and 10.6 ppm for $R = 3$ Å and between −1.1 and 2.2 ppm for $R = 5$ Å. Thus, for most cases ($R \geq 5$ Å) the pseudocontact contribution to the shift will be small compared to the Fc contribution. For detailed calculations values of R were obtained from the 3D structure of amicyanin (Romero et al., 1994). To obtain θ the orientation of the z-axis of the **g**-tensor with respect to the molecular framework must be known. In azurin this axis makes an angle of 15° with the Cu−S(Met) bond vector (Coremans et al., 1994), and in plastocyanin it amounts to 4° (Penfield et al., 1981). For the present case the angle is not known, and it is set to 0°. This introduces an error in the calculation of θ, but since amicyanin is more similar to plastocyanin than to azurin the error is thought to be small. The values of R and θ are quoted in Table 1. Values of δ_{pc} were subsequently estimated with the help of eq 2; they are also presented in Table 1. By correcting the measured shift, δ_{obs}, for δ_{pc}, δ_{Fc} was obtained, and from this the values of a were calculated with the help of eq 1. They are presented in Table 1.

In principle the hyperfine splitting constants derived from the spectra of wild type amicyanin can be analyzed in terms of the spin density distribution of the unpaired electron, since

a is proportional to the probability of finding the electron at the nucleus either by direct delocalization or by correlation effects. The analysis starts with His54. First, the stereospecific assignment of the $C^{\beta}H$ protons for this residue is derived from an evaluation of their hyperfine splitting constants. The assignment proposed in Table 1 leads to hyperfine splitting constants of −0.25 and −0.10 G. With the reverse assignment the values of δ_{pc} would have to be reversed too, leading to hyperfine splitting constants of −0.32 and −0.04 G. The hyperfine splitting constants of the $C^{\beta}H$ protons of a histidine are proportional to $\cos^2 \phi$, in which ϕ is the dihedral angle defined by the normal to the histidine ring and the $C^{\gamma}-C^{\beta}-H^{\beta}$ bonds (Heller & McConnel, 1960; Derbyshire, 1962). According to the crystal structure these angles are 29 and 89° for the $C^{\beta 2}H$ and $C^{\beta 3}H$ protons, respectively. These values are compatible with the assignment as proposed in the table but not with the reversed assignment.

The *a* values for the $C^{\delta 2}H$ and $N^{\epsilon 2}H$ protons of His54 reported in Table 1 amount to one-tenth of the hyperfine splitting constants reported in the literature for the imidazole radical (Quoc-hai Ngo et al., 1974; Wu & Kuntz, 1989; Box et al., 1967). This shows that about 10% spin density is delocalized into the π-system of the imidazole ring. The sign and magnitude of the *a* values of the $C^{\beta}H_2$ protons are also in accordance with this conclusion.

The contact shifts observed for the $C^{\gamma}H$ protons of Met99 (see Table 1) reflect a small but definite amount of spin density present on the methionine sulfur. This demonstrates that the orbital in which the unpaired electron is found does extend to the Met ligand. Taking into account that the hyperfine splitting constants of protons on aliphatic groups adjacent to a thioether radical may vary between 10 and 20 G (Muster & Woolford, 1976; Kou & Box, 1976; Wells & Budzinski, 1973), the spin density on the methionine sulfur can be roughly estimated as being of the order of 0.5%−2%.

As for the third ligand, Cys93, the spin density on the C^{α} of Cys93 must originate from spin density which is transmitted to it from the S^{γ} via the C^{β} atom. Propagation of spin density along an alkyl chain through spin polarization leads to attenuation factors of 13−40 per carbon−carbon bond (de Boer & MacLean, 1965; Fessenden & Schuler, 1963; Takeda & Williams, 1969). The contact shift of Cys93 $C^{\alpha}H$ must therefore reflect the presence of considerable spin density on the cysteine sulfur. This is in accordance with results obtained about the electronic structure of the blue copper site in plastocyanin (Werst et al., 1991) and SCF-Xα-SW calculations (Penfield et al., 1985; Shadle et al., 1993). The covalency of the Cu−Cys interaction could in principle be estimated from the contact shift of the $C^{\beta}H$ protons, but these are too broad to be observed in Cu(II) amicyanin. Selective deuteration of the β-positions of the cysteine should allow the observation of the hyperfine shift for these protons, as has recently been demonstrated in a study of rubredoxin (Xia et al., 1995).

Finally, for His96 no cross-peaks are observed in the EXSY spectra that might allow the assignments of its hyperfine shifted resonances. This is probably related to the pH-dependent conformational equilibrium in which His96 is involved in the reduced protein ($pK_a = 6.8$) and which broadens the signals from His96 in this form of the protein (Lommen & Canters, 1990). More information on the nature of the Cu site could be obtained by extending the present study of hyperfine-shifted resonances to include other nuclei such as ^{15}N, ^{13}C, and ^{2}H (Banci et al., 1995; Xia et al., 1995).

Methionine Signals. Azurin possesses a Cu−S(Met) distance of 3.12 Å (Nar et al., 1991). [Crystallographic distances are quoted, here, to two decimal places, following common practice, although it should be realized that the resolution of X-ray diffraction data of blue copper proteins often does not warrant a better precision than 0.1 Å for the Cu−ligand distances. This point is highlighted in a recent 1.33 Å resolution structure of poplar plastocyanin, in which the Cu−S(Met) bond is 2.82 Å compared with 2.90 Å in the 1.6 Å resolution structure (Guss et al., 1992).] It has been suggested from studies on Co(II)-substituted azurin that the Cu−S(Met) interaction lacks covalency (Piccioli et al., 1995). In amicyanin and plastocyanin the Cu−S(Met) distance is shorter [2.84 and 2.82 Å, respectively (Romero et al., 1994; Guss et al., 1992)]. These two proteins, along with azurin, have axial EPR spectra and a weak optical absorption at around 460 nm. Cucumber basic protein has a significantly shorter Cu−S(Met) distance of 2.63 Å (Fields et al., 1991) and has a rhombic EPR spectrum and a relatively intense visible absorption band at 460 nm (Guss et al., 1988). Thus, a tendency has been noted that an increased axial interaction (shorter Cu−ligand bond) is paralleled by an increase in the rhombicity in the EPR spectrum (Solomon et al., 1992; Penfield et al., 1981). Concomitantly the intensity of the optical transition around 400−480 nm in the spectrum of the oxidized protein increases. Recent resonance Raman studies confirm these conclusions (Han et al., 1993; Andrew et al., 1994) as do spectroscopic studies on Cu site mutants of blue copper proteins (Karlsson et al., 1991; Romero et al., 1993; Chang et al., 1991). The present study demonstrates how the distribution of the unpaired electron over the copper ligands in the Cu(II) state of the protein can be established by NMR spectroscopy, and the question of the strength of the Cu−S(Met) interaction can now be addressed directly.

Remarkable differences between the spectra in Figure 2 are found in the positions of the Met $C^{\gamma}H$ resonances. As noted above, the shifts of these peaks reflect the strength of the interaction between the copper and the axial ligand. In wild type azurin the $C^{\gamma}H$ resonances appear not to be shifted outside the diamagnetic envelope (Figure 2D). The Cu−S(Met) bond must therefore have very little bonding character. In wild type amicyanin the Met99 $C^{\gamma}H$ resonances are found at 11.1 and 12 ppm (Figure 2A). The Fermi contact shifts amount to 6.6 and 7.3 ppm (see Table 1), reflecting a spin density of approximately 1% on the methionine sulfur. In the two amicyanin mutants (H96D, Figure 2B, and the loop mutant, Figure 2C) it is seen that the $C^{\gamma}H$'s have a larger shift [when referenced to the same diamagnetic position (2.5 ppm) as in wt amicyanin], viz., 11 ppm for H96D and 19 ppm for H^{96}PFM → H^{96}QGAGM. (As noted before, in the latter case it is possible that the two $C^{\gamma}H$ resonances have separated and that the new resonance at 28.5 ppm derives from the second C^{γ} proton.) Thus, along the series azurin, wt ami, H96D ami, and H^{96}PFM → H^{96}QGAGM ami, there is an increase in the strength of the Cu−S(Met) axial interaction. This confirms a trend that is also apparent from the available optical and EPR data on these proteins (see Table 2).

CONCLUSION

In this study hyperfine-shifted resonances have been observed for the first time in the NMR spectrum of a type I copper protein in its paramagnetic Cu(II) form. Studies of hyperfine-shifted resonances have previously always relied on nickel or cobalt substitution for the copper (Moratal et al., 1993a−c; Villa, 1994; Piccioli et al., 1995; Dahlin et al., 1989). Studying the protein in its native, copper-bound form has the advantage that conclusions drawn about the coordination of the metal site are not affected by possible structural complications brought about by metal substitution. Moreover, due to the small *g*-anisotropy of type I copper sites, the anisotropic dipolar interaction in most cases contributes less than 2 ppm (pseudocontact shift) to the hyperfine shift. It is therefore relatively easy to interpret the observed hyperfine shifts in terms of approximate Fermi contact shifts.

Owing to the fast electron self-exchange reaction of amicyanin it has proved possible to assign a number of hyperfine-shifted signals from the WEFT−EXSY spectra of a solution containing equal amounts of Cu(I) and Cu(II) amicyanin. Starting with these spectra, assignments could be made for most of the observed hyperfine-shifted signals.

The most important conditions for detecting hyperfine shifted signals in the NMR spectra of oxidized type I copper proteins are that they must have a hyperfine splitting constant that is less than 1.5 G and that they should be at a distance of >4 Å from the copper, otherwise the signals become too broad to be observable. These conditions will be met by a substantial number of signals in most type I copper proteins. The study of these hyperfine-shifted signals may provide valuable information about the coordination geometry of the metal sites and the amount of covalency of the metal−ligand bonds in different type I copper proteins and site-directed mutants. In this study, for example, it could be shown that the H^{96}PFM → H^{96}QGAGM loop mutant of amicyanin has a considerably stronger interaction of the Cu with the axial methionine than the wild type protein.

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