A ^{1}H NMR Study of the Paramagnetic Active Site of the Cu_A Variant of Amicyanin†

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ABSTRACT: The dinuclear paramagnetic center of the Cu_A variant of the cupredoxin amicyanin has been investigated using ^{1}H NMR. The hyperfine-shifted resonances have been assigned using a combination of 1D NOE difference and 2D WEFT-NOESY spectroscopy. The shifts experienced by the assigned resonances have been used to calculate hyperfine coupling constants for these protons from which the spin density distribution on the ligands at the Cu_A center is obtained. A comparison with published data for the paramagnetic form of wild type amicyanin highlights a number of similarities and differences between these evolutionary related sites. In both cases 50−60% of the unpaired spin density is distributed on the ligands, which in the case of the Cu_A center involves two cysteine and two histidine ligands. The two weak axial interactions at the Cu_A center carry less than 1% spin density.

The Cu_A site is found in cytochrome *c* oxidase (CCO)[1] (Dennison & Canters, 1996; Malmström & Aasa, 1993; Saraste, 1990) and nitrous oxide reductase (N_2OR) (Coyle et al., 1985; Farrer et al., 1991; Malmström & Aasa, 1993) where it is believed to function as a one electron transfer site. The structure of this unique copper center has been demonstrated in the recently published crystal structures of a bacterial CCO (Iwata et al., 1995), a mammalian CCO (Tsukihara et al., 1995; Tsukihara et al., 1996), and also in the structure of the Cu_A containing mutant of the soluble domain of subunit II of the cytochrome bo_3 quinol oxidase (CyoA) (Wilmanns et al., 1995). In all of these studies the Cu_A site is dinuclear with the two copper atoms bridged by the thiolate sulfurs of two cysteine ligands. The copper−copper distance is approximately 2.5 Å and each copper possesses a terminal histidine ligand, which coordinates via its N^δ atom. A weaker axial interaction with the thioether sulfur of a methionine is present at one copper while a backbone carbonyl oxygen of a glutamic acid is involved at the other copper atom. The structure of this metal site, as found in CyoA, is shown in Figure 1. The highly symmetric nature of the Cu_A center appears to explain why the paramagnetic form of the protein exists as a [Cu(1.5)...Cu(1.5)] mixed valence site. This latter fact led Kroneck et al. to correctly predict the dinuclear nature of Cu_A from EPR studies (Antholine et al., 1992; Coyle et al., 1985; Kroneck et al., 1988, 1990).

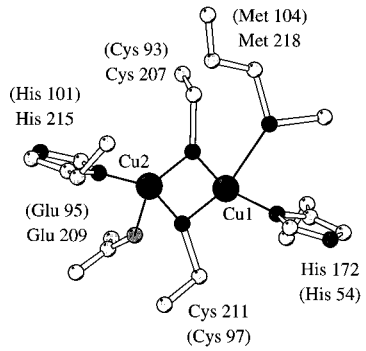

FIGURE 1: Representation of the Cu_A site as found in the purple CyoA mutant (Wilmanns et al., 1995). The residues involved in CyoA are indicated as are the corresponding amino acids for Cu_A amicyanin (in brackets, see Table 1).

Early amino acid sequence comparisons (Saraste, 1990; Steffens & Buse, 1979) and the more recent crystallographic studies have clearly demonstrated a structural relationship between the Cu_A domain of subunit II of CCO and type I blue copper proteins (cupredoxins). Cupredoxins possess an active site at which the single copper atom is strongly coordinated by a cysteine and two histidines (again the histidines coordinate via their N^δ atoms). The copper atom is usually found slightly displaced from the plane of these three equatorial ligands toward a weakly interacting methionine (Adman, 1991). Three of the amino acid residues involved at the active sites of cupredoxins, namely the cysteine, one of the histidines, and the methionine are located very close together in the primary structure of the protein and are found on one of the inter-β-strand loops (Dennison et al., 1996). The corresponding Cu_A binding loop is longer than that found in the cupredoxins and contains an extra cysteine ligand. It has previously been shown that the native loops in the cupredoxins amicyanin (Andrew et al., 1995; Dennison et al., 1995) and azurin (Andrew et al., 1995; Hay et al., 1996) can be replaced with Cu_A binding loops to produce authentic Cu_A binding proteins. The amino acid sequence of the wild type (wt) amicyanin loop and that of the Cu_A binding loop which has been introduced into

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[1] Abbreviations: WEFT, water-suppressed equilibrium Fourier transform; NMR, nuclear magnetic resonance; WT, wild type; CCO, cytochrome *c* oxidase; N_2OR, nitrous oxide reductase; 1D, one-dimensional; 2D, two-dimensional; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; EXSY, exchange spectroscopy; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; NMRD, nuclear magnetic resonance dispersion; SOD, superoxide dismutase.

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Table 1: Partial Amino Acid Sequence of *Thiobacillus versutus* Amicyanin Showing the C-terminal Loop Which Contains Three Active Site Ligands in wt Amicyanin and Five Ligands in the Cu_A Variant[a]

	amino acid sequence											
wt amicyanin	Cys^{93}						Thr	Pro	His^{96}	Pro	Phe	Met^{99}
Cu_A amicyanin	Cys^{93}	Ala	Glu^{95}	Ile	Cys^{97}	Gly	Pro	Gly	His^{101}	Ser	Gly	Met^{104}
CyoA	Cys^{207}	Ala	Glu^{209}	Ile	Cys^{211}	Gly	Pro	Gly	His^{215}	Ser	Gly	Met^{218}

[a] Also shown is the corresponding loop of CyoA. In all cases an additional histidine ligand is found in a different region of the primary structure of the proteins and is therefore omitted in this table. The ligands which are included are numbered.

amicyanin (along with the identical loop of CyoA) are shown in Table 1.

The study of Cu(II)-containing proteins by NMR has been limited by the long electron spin relaxation time of Cu (1−3 ns) (Bertini et al., 1989). Until recently, only in cases where this copper atom had been magnetically coupled to a faster relaxing metal atom, as in the case of the Co(II)Cu(II) superoxide dismutase (SOD) derivative, was it possible to observe the proton resonances paramagnetically shifted by Cu(II) (Banci et al., 1989; Bertini et al., 1989, 1994). Recent studies in our laboratory (Kalverda et al., 1996) have demonstrated that, by the application of tailored pulse sequences such as super-WEFT, it is possible to observe broad hyperfine-shifted signals of the paramagnetic Cu(II) forms of cupredoxins. These signals have been assigned in amicyanin by utilizing the electron self-exchange reaction and by applying 2D NMR EXSY spectroscopy to solutions containing a mixture of the reduced and oxidized protein. The observed shifts have been corrected for any pseudocontact (dipolar) contribution, which is small for Cu(II) (Kalverda et al., 1996). The resulting Fermi-contact shifts (due to the delocalization of spin density *via* covalent bonds) have been used to calculate hyperfine coupling constants for the shifted resonances, which provide estimates of the electron density on the copper ligands. This approach has proved particularly useful for quantifying the interaction between the copper and the axial methionine at the active sites of different cupredoxins.

Studies of the paramagnetic form of the Cu_A amicyanin mutant using ^1H NMR have been undertaken. This work represents the first detailed NMR investigation of a dinuclear paramagnetic metal center in a protein and the first such analysis of a mixed valence dinuclear copper center. The observed line widths of the proton resonances in the spectra of Cu_A amicyanin, compared with those of Cu(II) cupredoxins, clearly indicate that the electronic relaxation rate is considerably shorter (∼50 times, *vide infra*) for the dinuclear mixed valence site. The hyperfine-shifted resonances have been assigned and this allows an initial quantification of the spin density distribution on the ligands of the Cu_A site to be made. These results are compared with those for wt amicyanin.

MATERIALS AND METHODS

Protein. E. coli BL21 (Studier & Moffat, 1986) was transformed with a pUC18 derivative harboring the amicyanin construct which contained the Cu_A mutation under the control of the *lac* promoter (Dennison et al., 1995). The procedure used for expression and purification was a modified version of that described previously (Dennison et al., 1995). All of the precultures contained $Cu(NO_3)_2$ to a concentration of 500 μM. This resulted in a much greater yield of purple (Cu_A-containing) protein than when cultures were grown in the presence of 100 μM $Cu(NO_3)_2$. The cells were allowed to grow at 37 °C for only 3−3.5 h after induction as longer incubation times between induction and harvesting were found to result in a decrease in the amount of protein present in the cells. The cells were resuspended in sucrose buffer (20% sucrose, 30 mM Tris, and 1 mM EDTA at pH 8.0), and the periplasmic proteins were released by a single cycle of freezing and thawing. The final CM Sepharose (Pharmacia) column, at pH 4.5, used in the wt purification procedure was also used for the Cu_A amicyanin variant. This resulted in very pure protein but led to the loss of copper from the active site (approximately 20−30% of the purple Cu_A amicyanin loaded onto this column eluted as apo-protein). An alternative final purification step utilizing a Superdex S-75 (Pharmacia) gel filtration column produced slightly less pure protein (as judged by SDS−PAGE electrophoresis) but with a significant reduction in the loss of copper. There was very little observable difference between the paramagnetic ^1H NMR spectrum of the Cu_A samples purified by the two methods.

Sample Preparation. For the acquisition of NMR spectra the protein was exchanged into 25 mM phosphate buffer at pH 6.0 using ultrafiltration (Amicon, YM5 membrane). Samples were prepared in both H_2O and 99.95% ^2H_2O and typically contained between 2−5 mM protein. All of the samples used for the NMR studies had visible and EPR spectra characteristic of a Cu_A center.

NMR Spectroscopy. All ^1H NMR spectra were recorded on a Bruker DMX 600 MHz spectrometer using the super-WEFT pulse sequence (d1-180°-τ-90°-acq, where d1 is the relaxation delay and acq the acquisition time) (Inubushi & Becker, 1983). The values of τ used, which were usually equal to the total effective relaxation delay (d1 plus acq), were in the range of 10−50 ms, with the shorter times used to select for the faster relaxing resonances. 1D spectra were acquired with spectral widths up to 200 kHz and were processed using 20−100 Hz of exponential line broadening as apodization.

Steady-state 1D NOE difference spectra were acquired using the approach of Banci et al. (Banci et al., 1989). Again, the WEFT sequence was implemented with τ values (and also total effective relaxation times) ranging between 20−50 ms. The resonance of interest was irradiated during the τ time with a selective decoupler pulse. Spectra were acquired in blocks of 8 scans alternating between on-resonance, off-resonance in an upfield direction, on-resonance, and finally off-resonance in a downfield direction (equal to the offset used in the upfield direction). The off-resonance spectra were subtracted from their on-resonance counterparts to produce the NOE difference spectrum.

2D WEFT-NOESY spectra (Chen et al., 1994) were acquired using recycle times of 20−30 ms and mixing times in the range 3−10 ms. The spectral width in both dimensions

was typically 55 kHz with 512 experiments and approximately 2048 scans per experiment. The spectra were processed into 2048 × 1024 data points with resolution enhancing windows used in both dimensions.

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 of 70 ms. The interpulse delay (τ) was varied between 0.1 and 70 ms, and an exponential fit of the plots of peak intensity against τ, for a particular proton, yielded its T_1 value.

RESULTS AND DISCUSSION

Assignment of the Paramagnetic ^{1}H NMR Spectrum of the Cu_A Amicyanin Variant. Prior to presenting the results obtained it is important to first make clear the approach which has been utilized in making the assignments of the paramagnetic ^{1}H NMR spectrum of Cu_A amicyanin. The strategy used has relied upon the observation of NOEs. Such an approach requires some knowledge of the structure of the protein being studied, especially around the paramagnetic active site. The structure of Cu_A amicyanin is not available, and the structure of CyoA (Wilmanns et al., 1995) has been used as a model (see Figure 1). Justification for this is gained from the fact that the Cu_A loop introduced into amicyanin is identical to that which was used to create the Cu_A site in the soluble domain of subunit II of the quinol oxidase (i.e., to produce CyoA) (van der Oost et al., 1992). In addition, the optical, EPR, and resonance Raman spectral features of the Cu_A amicyanin variant are identical to those of the natural Cu_A sites (from CCO and N_2OR) and the Cu_A site of CyoA (Andrew et al., 1995; Dennison et al., 1995). The investigation of the isotropically shifted resonances in Cu_A amicyanin, as has already been found to be the case for Cu(II) wt protein, is aided by the fact that the observed shifts are due mainly to Fermi-contact contributions. Therefore, the assignment of these signals is limited to protons belonging to the metal ligands. This dramatically reduces the number of possible assignments.

The 1D WEFT spectrum of Cu(II) wt amicyanin is shown in Figure 2a. From a comparison to the spectrum of Cu_A amicyanin (Figure 2b) it is apparent that there is a distinct difference in the line width of the shifted resonances. This observation is a clear indication of the shorter electronic spin relaxation time for the mixed valence dinuclear site. For wt amicyanin a value of the electron spin relaxation time, τ_s, which dominates in this case the total correlation time for the nuclear relaxation, of 1.2×10^{-9} s has been calculated from the T_1 value of the N$^{\epsilon 2}$H resonance of His54 (3.8 ms) using the Solomon−Bloembergen equation, with a Cu−H distance of 5 Å (the contact contribution to the T_1 value was found to be insignificant). This value is in good agreement with that of 8×10^{-10} s determined for the cupredoxin azurin in NMRD experiments (Kroes et al., 1996). From the T_1 value of the corresponding proton at the active site of Cu_A amicyanin (see Table 2) a τ_s value of 2.3×10^{-11} s is calculated (using distances of 4.8 and 7 Å from the two copper atoms). Therefore the rate of electronic relaxation is ~50 times faster in the dinuclear mixed valence site than in the mononuclear cupredoxin site. Fast electronic relaxation is in agreement with EPR studies where the spectrum of the Cu_A center is no longer observable at temperatures above 150 K (Malmström & Aasa, 1993) and the Cu_A signal is very difficult to saturate.

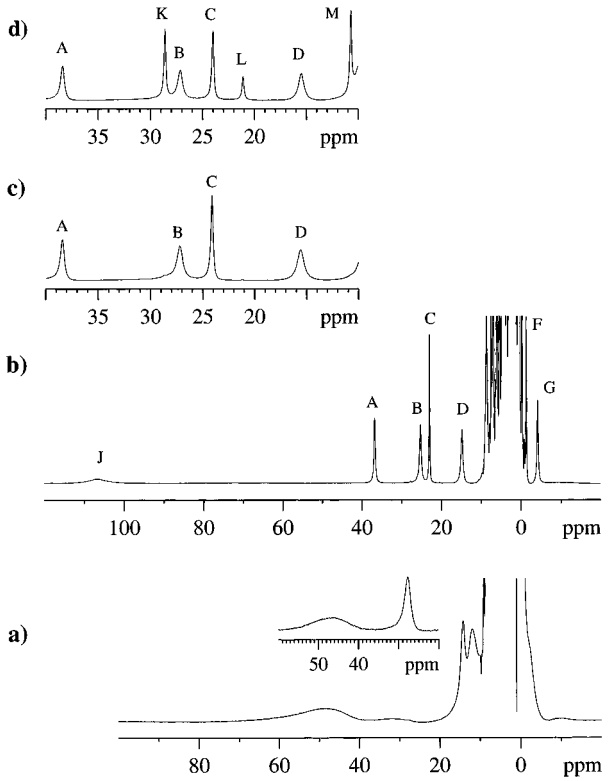

FIGURE 2: WEFT spectra of (a) wild type amicyanin in ^2H_2O at 32 °C, with the inset showing the spectrum in H_2O, (b) Cu_A amicyanin in ^2H_2O at 25 °C, (c) the region between 40 and 10 ppm in the spectrum of Cu_A amicyanin in ^2H_2O at 7 °C, and (d) Cu_A amicyanin in H_2O at 7 °C.

Table 2: Hyperfine-shifted Resonances of Cu_A Amicyanin at 25 °C and pH 6.0[a]

resonance	δ (ppm)	T_1 (ms)	relative intensities	assignment
J	107.3	nd[b]		Cys97 C$^{\beta}$H
A	36.9	16.3	1.0	His54 C$^{\delta 2}$H
K	27.8	15.6	1.1	His54 N$^{\epsilon 2}$H
B	25.2	3.4	0.9	His54 C$^{\epsilon 1}$H
C	23.1	15.0	1.0	His101 C$^{\delta 2}$H
L	20.5	nd		His101 N$^{\epsilon 2}$H
D	14.9	1.6	1.0	His101 C$^{\epsilon 1}$H
M	10.6	15.0	0.9	
E	−0.2	~38	~3	Ile26 C$^{\gamma}$H$_3$
F	−1.3	10.2	1.0	His54 C$^{\beta}$H
G	−4.1	3.8	1.1	His101 C$^{\beta}$H

[a] Also included are the spin−lattice (T_1) relaxation times, the relative intensities of the peaks, and the assignments that have been made. [b] Not determined.

In Figure 2c and d, the regions of the WEFT spectra of Cu_A amicyanin from 40 to 10 ppm, in ^2H_2O and H_2O respectively, are evident. Figure 3 shows the temperature dependence of the positions of all the hyperfine-shifted resonances. From this plot it is clear that all the shifted resonances exhibit Curie-type behavior. The diamagnetic chemical shifts (those at infinite temperature) obtained for resonance B, and especially for resonance J (Figure 3), are outside of the expected range. As these two protons are situated close together at the Cu_A center (*vide infra*), it is possible that a temperature-induced conformational change occurs in this specific area of the site. Experiments are currently underway to clarify this point. The assignments that have been made for Cu_A amicyanin are shown in Table

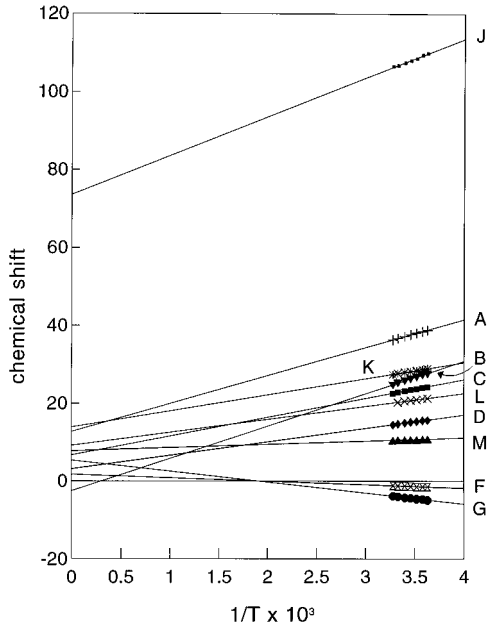

FIGURE 3: Temperature dependence of the positions of the isotropically shifted resonances of Cu_A amicyanin.

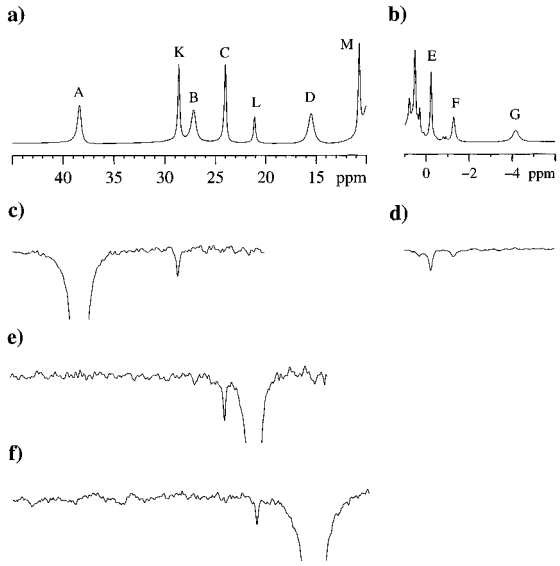

FIGURE 4: (a) The WEFT spectrum of Cu_A amicyanin in H_2O at 7 °C and (b) in ^2H_2O at 25 °C. The 1D NOE difference spectra in H_2O (7 °C) are also shown in which resonances A (4c), L (4e), and D (4f) were irradiated and also in ^2H_2O (25 °C) in which peak A was irridiated (4d).

2. The experimental observations which led to these assignments are outlined below.

From a comparison of part of the 1D WEFT spectrum of Cu_A amicyanin in ^2H_2O (see Figure 2c) with the spectrum in water (Figure 2d) it is immediately obvious that two strongly shifted resonances (K and L) belong to exchangeable protons. The only two such protons which can exhibit sizeable Fermi-contact shifts are the $N^{\epsilon 2}H$ resonances of the two histidine ligands. Resonance L increases in intensity at lower temperatures and also at lower pH values (data not shown), which indicates that this proton is in faster exchange with the bulk solvent than resonance K. From the structure of wt amicyanin (Kalverda et al., 1994), it is known that His96 (which is located on the ligand containing loop) is more exposed than His54, as is also the case for His215 in the CyoA structure. Therefore, resonances K and L in the spectrum of the Cu_A amicyanin variant are assigned to the $N^{\epsilon 2}Hs$ of His54 and His101, respectively (for the amino acid numbering system for Cu_A amicyanin see Table 1).

In Figure 4, panels c and e, 1D NOE difference experiments are presented in which resonances A and L were selectively irradiated. It is quite clear from these spectra that resonances A and K and also resonances C and L exhibit NOEs to each other. Similar experiments in which resonances K and C were irradiated were also carried out and resulted in NOEs being observed to resonances A and L, respectively (data not shown). The NOEs between resonances A and K and resonances C and L are also observed in a 2D WEFT-NOESY spectrum (Figure 5a). These NOEs imply that resonances A and C belong to either the $C^{\delta 2}H$ or the $C^{\epsilon 1}H$ resonances of the two histidine ligands. The T_1 relaxation times of resonances A and C (see Table 2) are very similar to those of the $N^{\epsilon 2}H$ resonances of the histidine ligands. Since the $C^{\delta 2}$ and $N^{\epsilon 2}$ protons of the coordinating histidine ligands are approximately the same distance from the nearest copper atom they would be expected to have similar T_1 values. The $C^{\epsilon 1}$ protons of the histidine ligands are situated very close to the copper atoms in cases such as this when the imidazole ring coordinates via its $N^{\delta 1}$ atom and are expected to possess much shorter T_1 times. Therefore, resonances A and C are assigned as the $C^{\delta 2}H$ resonances of His54 and His101, respectively.

In Figure 4f the 1D NOE difference spectrum of Cu_A amicyanin in water is shown in which resonance D (which has a T_1 value of 1.6 ms) was selectively irradiated. The NOE between resonance D and L (the latter corresponding to the $N^{\epsilon 2}H$ of His101) is clear and leads to the conclusion that D arises from the $C^{\epsilon 1}H$ resonance of His101. The reverse NOE is not observed when resonance L is irradiated due to the short T_1 value of resonance D. In the case of the very fast relaxing peak B, the fact that it is close to the considerably sharper peaks K and C makes the 1D NOE difference experiment more demanding. The irradiation of peak B leads to considerable off-resonance effects (positive peaks in the 1D NOE difference spectrum) at resonances K and/or C both in H_2O and ^2H_2O. No NOE was observed between peak B and any other resonance which is shifted outside of the diamagnetic envelope (except to the very shifted resonance J, *vide infra*). In the case of His101 the $C^{\epsilon 2}H$ resonance has a much shorter T_1 value than both the $C^{\delta 2}$ and $N^{\epsilon 1}$ protons (Table 2). The short T_1 time of peak B coupled to its isotropic shift points to it as being the $C^{\epsilon 2}H$ resonance of His54.

In Figure 6b the 1D NOE difference spectrum obtained when the very broad peak J was selectively irradiated is shown. The strong NOE between resonances J and B is immediately apparent. The large shift experienced by resonance J coupled to its width and very small T_1 value clearly identifies it as belonging to at least one of the $C^{\beta}H$ resonances of the two bridging Cys ligands. The NOE observed between resonances J and B, the latter of which has been tentatively assigned as the $C^{\epsilon 1}H$ of His54, is consistent with the structure of CyoA (see Figure 1) since one of the $C^{\beta}Hs$ of Cys211 is only 2.2 Å from the $C^{\epsilon 1}H$ of His172. Resonance J is therefore assigned to the $C^{\beta}H_2$ moiety of the equivalent Cys97 (Cu_A amicyanin numbering).

An alternative assignment of resonance B as the $C^{\alpha}H$ resonance of the Cys ligand whose $C^{\beta}H$ moiety is peak J has also been considered. In a WEFT-NOESY spectrum of

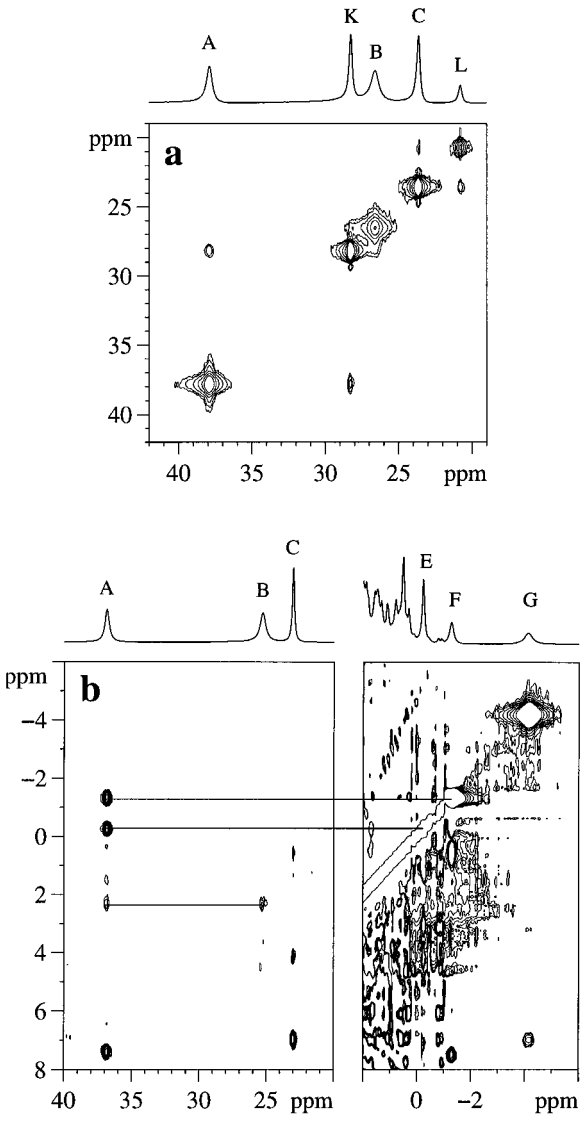

FIGURE 5: (a) Part of the WEFT-NOESY spectrum of Cu_A amicyanin in H_2O at 11 °C with a mixing time of 5 ms and (b) sections of the WEFT-NOESY spectrum of Cu_A amicyanin in ^2H_2O at 25 °C with a mixing time of 7 ms.

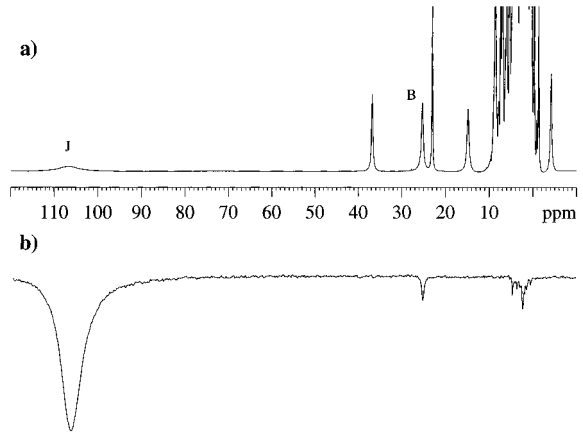

FIGURE 6: (a) The WEFT spectrum of Cu_A amicyanin in ^2H_2O at 25 °C and (b) the 1D NOE difference experiment is shown in which resonance J was irradiated.

Cu_A amicyanin (see Figure 5b) an NOE is seen between a peak at 2.1 ppm and resonances A and B. The observation of an NOE between a Cys $C^{\alpha}H$ and an aliphatic resonance which gives an NOE to the $C^{\delta 2}H$ resonance of His54 (His172 in CyoA) is inconsistent with the structure of the Cu_A center. On the other hand, the assignment of B to the $C^{\epsilon 1}H$ of His54 is consistent with the structure of amicyanin, in which the $C^{\delta 2}H$ (peak A) and $C^{\epsilon 1}H$ (peak B) of His54 are both reasonably close to C^{β} and C^{γ} protons of Met52, either of which could give rise to the resonance at 2.1 ppm (Lommen et al., 1991). We therefore favor the latter assignment. However, as the choice between the two assignments is based in part on structural information of a related domain (CyoA) rather than the amicyanin Cu_A itself, the assignment of B to the Cys $C^{\alpha}H$ cannot be ruled out at this stage. A third possible assignment of resonance B as one of the $C^{\gamma}H$ resonances of Met104 has also been considered. An NOE between the Cys $C^{\beta}H$ and at least one of the side chain protons of the weakly interacting axial ligand (which is usually a Met but in some cases is a Gln) is always observed in NMR studies on Co(II)-substituted cupredoxins (Piccioli et al., 1995; Salgado et al., 1995, 1996). Resonance B is a single proton (see Table 2), which makes this option less likely, since if one of the $C^{\gamma}H$ of Met104 experiences such a large Fermi-contact shift then it would be expected that the second $C^{\gamma}H$ should also appear outside of the diamagnetic envelope.

From a comparison to the WEFT spectrum of Cu(II) wt amicyanin, resonances F and G in the spectrum of Cu_A amicyanin are thought to originate from the C^{β} protons of histidine ligands, since the sign of the shifts is in agreement with the presence of negative spin density on the adjacent carbon atom (Kalverda et al., 1996). In a WEFT-NOESY experiment (see Figure 5b), and also a 1D NOE difference experiment (see Figure 4, panels b and d), the observed NOE between peaks F and A is consistent with resonance F belonging to one of the C^{β} protons of His54. Resonances F and G do not show an NOE to each other and hence the latter is assigned to one of the C^{β}Hs of His101. In the WEFT-NOESY spectrum (Figure 5b) it is interesting to note that peak E also gives an NOE to peak A. This NOE is also observed in the 1D NOE difference experiment when resonance A is irradiated (Figure 4, panels b and d). The exchangeable resonance K also gives an NOE to peak E (data not shown). The intensity of peak E is consistent with it belonging to a methyl group, and from the structure of wt amicyanin; a potential candidate is the $C^{\gamma}H_3$ group of Ile26. Resonance E shows very little shift with changing temperature (data not shown) consistent with a chemical shift of 0.77 ppm for the $C^{\gamma}H_3$ group of Ile26 in the diamagnetic Cu(I) wt protein (Lommen et al., 1991).

The final peak shifted outside of the diamagnetic region, which has not been discussed so far is resonance M. This peak is only present in the spectrum when the sample is in water and therefore belongs to an exchangeable proton. The small shift of this resonance and the fact that no NOEs were observed from it to other shifted resonances make a definite assignment difficult. One intriguing possibility is that this peak belongs to the amide proton of Ile96 which is adjacent to Glu95 whose carbonyl oxygen acts as a weak axial ligand to Cu2. Alternatively, it could belong to the amide proton of one of the Cys ligands.

Hyperfine Coupling Constants Determined from the Isotropic Shifts. As has already been stated, the observed isotropic shifts in the ^1H NMR spectrum of the Cu_A amicyanin mutant are thought to be mainly due to Fermi-contact shifts. This conclusion is made from a comparison

Table 3: Estimated Fermi-Contact Shifts and Hyperfine Coupling Constants for the Isotropically Shifted Resonances of Cu_A Amicyanin at 25 °C and pH 6.0[a]

resonance	assignment	δ_{Fc} (ppm)	a_H (gauss)	a_H (gauss) (wt amicyanin)[b]
J	Cys97 $C^{\beta}H$	104	1.40	
A	His54 $C^{\delta 2}H$	30	0.40	0.50
K	His54 $N^{\epsilon 2}H$	15	0.20	0.21
B	His54 $C^{\epsilon 1}H$	18	0.24	
C	His101 $C^{\delta 2}H$	16	0.22	0.60 (His96)
L	His101 $N^{\epsilon 2}H$	8	0.11	
D	His101 $C^{\epsilon 1}H$	8	0.11	

[a] The values are compared with those for Cu(II) wt amicyanin (Kalverda et al., 1996). [b] 32 °C, pH 7.0.

with Cu(II) wt amicyanin, where the pseudocontact contributions to the shifts have been found to be small and on the basis of the small anisotropy of the *g*-tensor for the Cu_A center ($g_{||} - g_{\perp} = 0.180$), which is even less than the value of 0.193 found for wt amicyanin. Moreover, most of the assigned protons are not within 4 Å of either of the copper atoms (as judged from the CyoA structure) which will reduce their pseudocontact shifts even further. Consequently, the observed shifts are used directly as Fermi-contact shifts, with diamagnetic positions estimated from what is usually observed for the particular type of proton in NMR studies on wt Cu(I) amicyanin (Lommen et al., 1991). From eq 1 these Fermi-contact shifts can be used to calculate hyperfine coupling constants.

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

In eq 1 ΔH_{Fc} is the Fermi-contact shift in magnetic field units, H is the externally applied magnetic field, a is the isotropic Fermi-contact coupling constant in magnetic field units (Gauss), and the other symbols have their usual meanings. The Fermi-contact shifts determined for the assigned protons around the active site of Cu_A amicyanin are shown in Table 3 along with the calculated hyperfine coupling constants. The values previously determined for corresponding protons at the active site of Cu(II) wt amicyanin are also listed.

From Table 2 it is apparent that no protons associated with the two weak axially interacting amino acids at the Cu_A center are shifted outside of the diamagnetic envelope. In the case of wt Cu(II) amicyanin the $C^{\gamma}H$ resonances of Met99 are considerably shifted whereas, in the case of azurin, which from crystallographic studies is known to have a longer Cu−S(Met) distance (Nar et al., 1991), the corresponding signals are found inside the diamagnetic envelope. Therefore, it would appear that the Cu−S(Met) interaction in Cu_A amicyanin has very little or no covalent character. This conclusion thus poses the question as to the role of the weak axial ligands at the Cu_A center. It has been proposed that, as in the cupredoxins (Malmström, 1994; Pascher et al., 1993), these axial ligands function in tuning the reduction potential of the site (Zickermann et al., 1995).

The Fermi-contact shifts, and subsequently the hyperfine coupling constants, experienced by protons on the two histidine ligands of the Cu_A site are a very intriguing result of this study. The ratio between the coupling constants for the $C^{\delta 2}H$, $N^{\epsilon 2}H$, and $C^{\epsilon 1}H$ resonances is the same for both His54 and His101. This observation further strengthens the assignment of resonance B as belonging to the $C^{\epsilon 1}H$ resonance of His101 as the observed Fermi-contact shift is in line with that observed for the corresponding imidazole ring proton of His54. The coupling constants calculated clearly indicate that there is approximately twice as much electron spin density on His54 as compared to His101. These observations agree with previously reported ENDOR studies on the Cu_A sites in three different CCOs (Gubriel et al., 1993) which identified two strongly coupled signals with hyperfine coupling constants ranging from 15 to 17 MHz for one nitrogen and from 6 to 10 MHz for the other nitrogen.[2] It was assumed that these two signals arose from the two coordinating nitrogen atoms. The corresponding signals are also observed in ENDOR spectra of cupredoxins (Werst et al., 1991), and values of 22 MHz have been reported for the N^{δ} atoms of the two histidine ligands in plastocyanin. The NMR data for the Cu_A site in amicyanin and for wt amicyanin (see Table 3) clearly agree with the ENDOR studies in that His54 possesses approximately the same amount of spin density in both sites and that His101 has approximately half that amount.

Conclusions concerning the Cys ligands from the NMR studies on Cu_A amicyanin are less straightforward. Only one Cys $C^{\beta}H_2$ moiety is observed in the NMR spectrum. Attempts to observe additional $C^{\beta}H$ resonances were carried out on a 300 MHz spectrometer using a sample containing 5 mM protein using a spectral width of 600 kHz and utilizing an interpulse delay in the WEFT sequence of 10 ms. After 1 000 000 scans there was still no evidence of additional resonances. Peak J is assigned to the $C^{\beta}H_2$ group of Cys97. The estimation of the hyperfine coupling of this proton (1.40 G) is in good agreement with the range 5−19 MHz quoted for $C^{\beta}Hs$ at the Cu_A site in the ENDOR studies on the complete CCOs (Gubriel et al., 1993). The fact that the hyperfine coupling value of the only observed $C^{\beta}H$ resonance of the Cu_A site is at the low end of the range of coupling values seen for these protons in the ENDOR studies agrees with the proposal that the other $C^{\beta}H$ resonances are shifted further downfield and are too broad to be observed. This fact has recently been confirmed by ^1H NMR studies, in our laboratory, on the native Cu_A domain of the aa_3-type CCO from *T. versutus*, where additional $C^{\beta}H$ resonances are observed in the 200−300 ppm region (Salgado & Canters).

In the ^1H NMR spectrum of Cu_A amicyanin the $C^{\alpha}H$ resonances of the Cys ligands are probably not shifted outside of the diamagnetic envelope contrary to what has previously been observed for Cu(II) wt amicyanin. The hyperfine coupling constant for the $C^{\alpha}H$ resonance of Cys93 in wt amicyanin is consistent with the presence of appreciable electron density on the $C^{\beta}Hs$. ENDOR studies on plastocyanin (Werst et al., 1991) have identified hyperfine couplings for the two $C^{\beta}H$ resonances of the Cys ligand of 16 and 27 MHz. The hyperfine coupling constant for the $C^{\beta}H$ of Cys97 in Cu_A amicyanin (1.40 G) and the fact that the $C^{\alpha}H$ resonances are within the diamagnetic envelope indicates that each Cys ligand possesses less electron density than the single Cys present at the mononuclear cupredoxin site.

[2] To convert hyperfine coupling constants in MHz into the unit of gauss, the following relationship should be used:

$$A(\text{MHz}) = 2.80247(g/g_e)a(\text{gauss}).$$

CONCLUSIONS

The observation of relatively sharp hyperfine-shifted resonances in the ^{1}H NMR spectrum of Cu_A amicyanin clearly demonstrates that the mixed valence dinuclear nature of this active site results in faster relaxation of the unpaired electron as compared to the case for the mononuclear cupredoxins. A similar enhancement of electronic relaxation has also been observed in moderately coupled dinuclear Cu(II) complexes (Brink et al., 1996; Satcher & Balch, 1995). The assignment of the 1D WEFT spectrum of Cu_A amicyanin has provided detailed information about the spin density distribution over the ligands at this unique, and up until now, little studied copper center. The main conclusions from these results are the following:

(1) In wt Cu(II) amicyanin approximately 40% spin density is delocalized on the sulfur of the cysteine ligand (Cys93) (Solomon & Lowery, 1993) and NMR studies have demonstrated that the $C^{\alpha}H$ resonance of Cys93 is shifted outside of the diamagnetic envelope (Kalverda et al., 1996). In this study we find that the $C^{\alpha}H$ resonances, of the two bridging cysteine ligands at the Cu_A center, are probably not so shifted. This indicates that the cysteine ligands possess less spin density than their counterpart at the wt active site. The shift experienced by the $C^{\beta}H_2$ moiety of Cys97 indicates that there is approximately 20% spin density on this ligand.

(2) The two histidine ligands possess different amounts of spin density. One of the His ligands in Cu_A amicyanin (His54) carries a similar amount of spin density as compared to the two His ligands in the Cu(II) wt amicyanin (5%), while the second His (His101) possesses half this amount.

(3) The two weak axial interactions present at the Cu_A center possess little or no covalency (<1% spin density). This is particularly apparent from the above studies for Met104.

(4) The hyperfine coupling constants determined for protons at the active site of Cu_A amicyanin have a number of similarities to what has been previously observed for the mononuclear cupredoxins. It appears, in both cases, that the ligands possess approximately 50−60% of the unpaired spin density, but in the case of the Cu_A center, this is distributed over two His and two Cys ligands. This results in less spin density per ligand than in their cupredoxin counterparts.

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