Electronic and Geometric Structure of the Cu_A Site Studied by ^1H NMR in a Soluble Domain of Cytochrome *c* Oxidase from *Paracoccus denitrificans*

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Abstract: An extensive assignment of the ^1H NMR spectra of a Cu_A domain from the cytochrome *c* oxidase of *Paracoccus denitrificans* has been obtained on the basis of dipolar connectivities coupled to the available structural information. The assignment has been extended with the aid of ^2H NMR spectra on a protein sample with cysteines selectively labeled at the β position. The spectra have been compared to those published earlier for a similar Cu_A fragment from the *Thermus thermophilus* oxidase and for a Cu_A construct in a blue copper protein, amicyanin. The shifts and their temperature dependence are discussed in terms of molecular orbital descriptions available for these systems. The mechanisms for the fast electron relaxation, which make the obtainment of relatively sharp NMR signals possible, are discussed. It is shown that the proton shifts of one histidine are variable from one system to the other, possibly reflecting changes in its interaction with the Cu_2S_2 diamond structure. Large electron delocalization onto the S atoms is confirmed. This is reflected in the large shifts experienced by the cysteine β-CH_2 protons, and these shifts are in turn tuned by the Cu−S−C−H torsion angles. It is suggested that the electronic structure of Cu_A is ideal for its function in electron transfer.

Introduction

The demonstration that the Cu_A center in cytochrome *c* oxidase is a dinuclear, mixed-valence copper complex constitutes one of the most intriguing recent discoveries in inorganic and bioinorganic chemistry.[1,2] The dinuclear nature of this center was first suggested by Kroneck and co-workers,[3,4] but it remained controversial,[1,2] until it was clearly established by analytical and spectroscopic work on a soluble Cu_A domain[5] and by the determination of the crystal structures of two cytochrome oxidases[6,7] as well as of the purple CyoA protein.[8] Two copper ions, formally one in the +1 and the other in the +2 oxidation states, are bridged by two cysteines. Both are coordinated by one histidine, and complete their coordination with a further weak bond each (with a methionine sulfur and a peptide carbonyl oxygen, respectively) as shown in Figure 1.

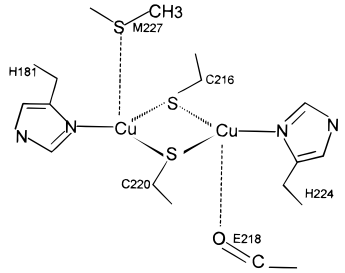

Figure 1. Schematic representation of the Cu_A domain of cytochrome *c* oxidase from *P. denitrificans*.

Several soluble proteins containing a single Cu_A center have been prepared, the first one being the CyoA protein in which a Cu_A site has been introduced in a domain of subunit II from a quinol oxidase complex.[9] Later, soluble subunit II domains with natural Cu_A sites have been prepared from three bacterial cytochrome oxidase systems (*Paracoccus denitrificans, Bacillus subtilis*, and *Thermus thermophilus*).[5,10,11] In addition, two groups have independently succeeded in engineering a Cu_A site into two blue copper proteins, amicyanin[12] and azurin.[13] The oxidase Cu_A centers are similar to one of the redox centers in

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N_2O reductase.[3] A mixed-valence dinuclear model complex has been synthesized by Houser et al.[14]

EPR[15−17] and ENDOR[18] studies have revealed essentially equivalent hyperfine coupling of the unpaired electron with the two copper ions, and these have opened a discussion on the inequivalence of the ligands. The EPR signal disappears well below room temperature,[1,19] and time-domain EPR studies have confirmed that the electronic relaxation times become fast at a relatively low temperature.[20] This induced us to perform a ^{1}H NMR investigation of the soluble Cu_A domain from *Thermus thermophilus*.[21] Indeed, relatively sharp signals are observed from the bound histidine rings, and signals were also found from the bridging cysteine ligands.[21] The spectrum recorded by us is different from a spectrum of a Cu_A domain from the *Bacillus subtilis* oxidase. The tentative assignment of the latter was based on analogy with the NMR spectrum of the Cu_A center engineered from amicyanin.[22] This assignment is discussed here in the light of the data on Cu_A domains from the *T. thermophilus*[21] and from the *P. denitrificans* oxidases presented here.

NMR investigations of Cu_A systems are hampered by the instability of the expressed Cu_A centers, which does not permit a thorough investigation through 2D spectroscopies. The Cu_A from *T. thermophilus* is an exception. This allowed us, through 1D and 2D experiments, to assign all cysteine β-CH_2 protons, one of the two cysteine α-CH protons, and all six ring protons of the two histidines.[21] This earlier study also permitted us to reveal that some signals have either an antiCurie or a temperature-independent behavior, attributed by us to the existence of two nearby electronic levels. We wish here to report the ^{1}H NMR spectrum of the Cu_A fragment from *Paracoccus denitrificans*. The fragment is relatively stable, though much less so than that from *T. thermophilus*, and has a 1D spectrum not directly comparable to that of the latter. In particular, fewer hyperfine-shifted signals are present, and in this respect the spectrum is reminiscent of that from the amicyanin Cu_A construct.[22] In the light of the available X-ray structure of the whole Cu_A domain from *P. denitrificans*,[6] a classification of the various types of 1D NMR spectra can be achieved. This leads to a unique structural model, whose qualitative features are here discussed.

Experimental Methods

The soluble Cu_A domain was expressed as inclusion bodies in *E. coli* BL21(DE3) by following the published procedure.[5] Purification of the protein and reconstitution with copper was also achieved following the published procedure.[5] Protein solutions (1−3 mM) for NMR experiments were prepared in 0.1 M potassium phosphate buffers within the pH range of 5.3 to 8.0. Samples in D_2O were obtained by repeated exchange with D_2O buffer with use of Centricon filters. Protein samples with cysteines specifically deuterated at the β position were prepared from the *E. coli* expression host grown in M9 media containing the deuterated cysteine and unlabeled essential amino acids.

^{1}H and ^{2}H NMR spectra were recorded on AMX 600 and Avance 800 Bruker instruments. Spectral widths of 600 and 2000 ppm were investigated for ^{1}H and ^{2}H, respectively. The ^{2}H NMR spectra were acquired for up to 600 000 scans. T_1 measurements were performed by using the inversion recovery method[23] in the nonselective version, as appropriate for paramagnetic systems.[24] Different carrier frequencies were used to cover different regions of the spectrum with the 180° pulse. 1D NOE difference spectra were obtained as previously described.[25] NOESY spectra with 5 ms mixing time were recorded in the TPPI mode.[26] A total of 1024 × 512 data points were acquired and zero filled to 2K × 1K. Cosine-squared weighting functions were used.

Results

The ^{1}H NMR spectra of Cu_A from *P. denitrificans* are shown in Figures 2A and 3, together with those of Cu_A from *T. thermophilus* (Figure 2B)[21] and from the artificial Cu_A construct in amicyanin (Figure 2C).[22] The exchangeable signals are marked with asterisks, and the NOE connectivities are shown as well. The chemical shifts, NOE connectivities, and T_1 values of the system are also reported in Table 1. The 1D NOE difference spectra have been obtained by irradiating each hyperfine-shifted signal. 2D NOESY spectra have been recorded as well. The two types of experiments provided the same information. Comparison of the *P. denitrificans* spectra, including the dipolar connectivities for each signal, with those from the *T. thermophilus* Cu_A domain shows that one histidine ring 3-signal NOE pattern (signals *f*, *i*, and *k*) is apparent (Table 1). The NH signal *i* decreases in intensity with increasing temperature (not shown) and disappears at high pH (Figure 4). This behavior is analogous to that for *T. thermophilus* for the same signal (Figure 2B) and, likewise, allows us to assign these signals to the solvent-exposed His224 (*P. denitrificans* numbering). It should be noted that signal *i* is best observed at pH 5.6, but the sample is less stable at this pH. Signals *e* and *h* (the latter exchangeable in D_2O) are also connected by NOE (Table 1). Line widths (Figure 2A) and T_1 values (Table 1) indicate their correspondence to the analogous signals in Cu_A from *T. thermophilus* (Figure 2B). In the latter case, however, the NH signal, *h*, is also dipole-coupled to *j*, which is the Hϵ1 signal. In the present system this signal is missing. On the other hand, the Hϵ1 signals are in an *ortho*-like position with respect to the coordinating nitrogens, and are therefore the broadest and have the shortest T_1 among the histidine ring protons.[24] The 1D NOEs expected on these signals by saturating the NH signals are very small, and 2D NOE experiments attempted at pH 5.3 over the limited survival time of the *P. denitrificans* Cu_A sample gave negative results. A reasonable conclusion is that the signal corresponding to signal *j* in the present system is below the diamagnetic envelope (i.e., it experiences a small hyperfine coupling) and is therefore lost.

In the 40−15-ppm region in the ^{1}H NMR spectrum of the present system, there is a signal *g* that is not connected to any other signal in this region, but is connected to signal *d* at around 50 ppm (Table 1). It is practically temperature independent, as is the homologous signal *g* that has been assigned in Cu_A from *T. thermophilus* as an α-CH of one of the two cysteines. Signal *d*, assigned as a cysteine β-CH proton in Cu_A from *T. thermophilus*, has a peculiar antiCurie temperature dependence

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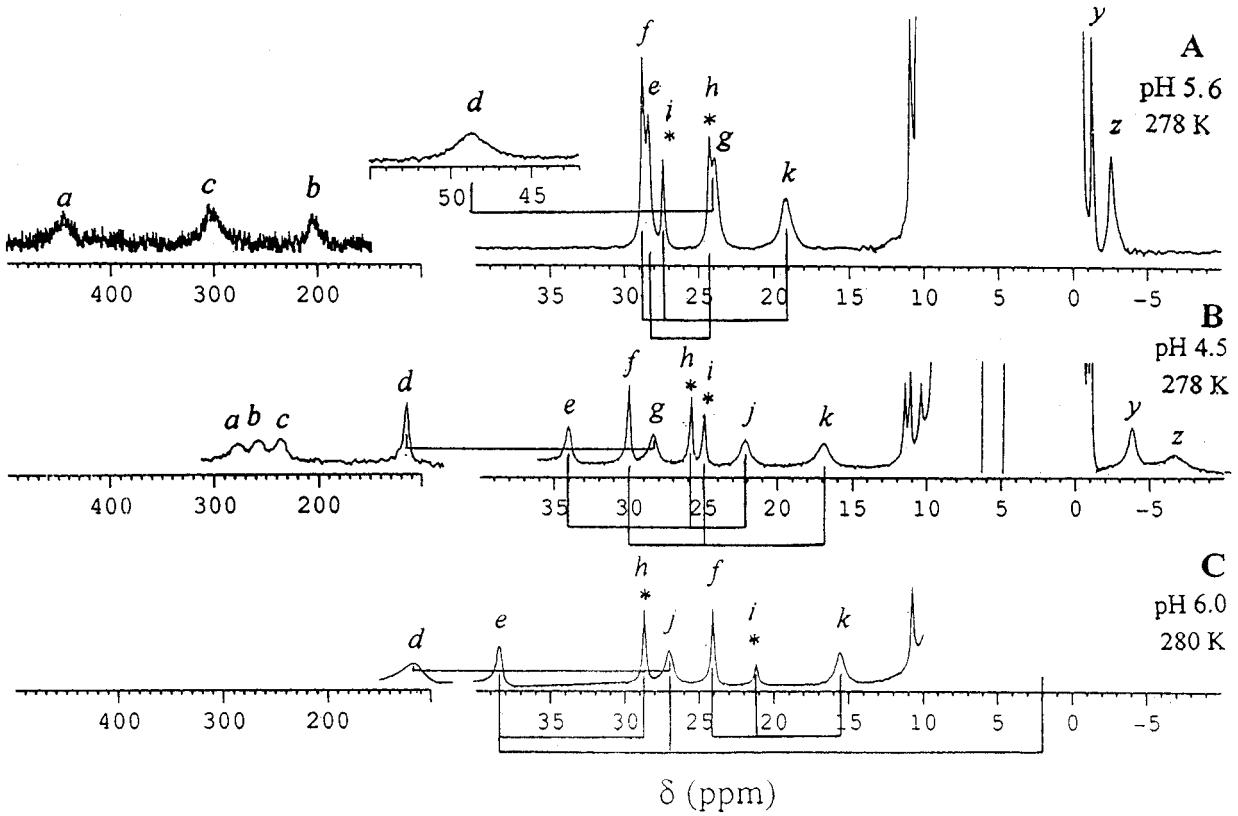

Figure 2. Comparison of ^{1}H NMR spectra of H_2O solutions of Cu_A domains from (A) *P. denitrificans*, 800 MHz, pH 5.6, 278 K, (B) *T. thermophilus*, 600 MHz, pH 4.5, 278 K, and (C) engineered amicyanin, 600 MHz, pH 6.0, 280 K. Asterisks denote exchangeable signals. The labeling of spectra A and C follows the assignment of spectrum B previously published by us.[21] The 100−500-ppm region of spectrum A is a ^{2}H NMR spectrum. Signals *a* and *c* are not visible in the ^{1}H spectrum. NOE connectivities are also shown.

Table 1. Chemical Shifts at 278 K, Temperature Dependence, T_1 Data, and Assignments of the Hyperfine-Shifted Signals of the *P. denitrificans* Cu_A Domain at pH 5.6

signal[a] (NOE connect.)	δ (ppm)[b]	*T* dep	T_1 (ms)[b]	assignment
a[c]	445	Curie	N.D.	Cys216 Hβ
c[c]	300	Curie	N.D.	Cys216 Hβ
b	205	T-indep.	< 1	Cys220 Hβ
d (*g*)	48.6	antiCurie	1.4	Cys220 Hβ
f (*i*)	28.6	Curie	5.3	His224 Hδ2
e (*h*)	28.3	Curie	ca. 5	His181 Hδ2
i (*f*)	27.3	Curie	> 5	His224 Hϵ2 (NH)
h (*e*)	24.2	Curie	5.0	His181 Hϵ2 (NH)
g	23.8	T-indep.	< 2	Cys220 Hα
k (*i*)	19.1	Curie	1.6	His224 Hϵ1

[a] The labeling follows the previous assignment of Cu_A from *T. thermophilus*, as shown in Figure 2. The signals in parentheses are those for which NOE connectivities have been observed. [b] Spectra recorded at 800 MHz; T_1 data obtained at 600 MHz. [c] Signals observed only in the ^{2}H spectrum.

that makes the correspondence straightforward, despite its smaller hyperfine shift in the present system.

In line with the observations given, the assignment of *P. denitrificans* Cu_A in the 40 to 15 ppm spectral region matches that of *T. thermophilus* Cu_A, except for the lack of signal *j*. The overall signal spreading is somewhat smaller than that in *T. thermophilus* Cu_A (compare parts A and B of Figure 2). The Cu_A amicyanin construct contains the same number of signals as the present system (compare parts A and C of Figure 2) while, in terms of signal spreading, it is more similar to the Cu_A from *T. thermophilus* (compare parts B and C of Figure 2). The difference between the construct and the two natural proteins, besides the absence of the signals at ≥200 ppm, is intriguing. Below 100 ppm there are seven signals in both natural systems, but the NOE patterns, line widths, and T_1 values lead to two different assignments: three signals for each histidine ring plus one α-CH of a cysteine in the *T. thermophilus* protein,[21] and three signals from a histidine ring, two from the other, and one α-CH and one β-CH of a cysteine in the *P. denitrificans* protein. In the artificial construct, six signals are observed in the same 0−100-ppm region, which the authors assign as signals of the two histidines.[22] If this is the case, NOE between signals *j* and *h* should have been observed. Their assignment is based on the observation of a NOE between signals *j* and *d* and on the assumption that a β-CH_2 proton and an Hϵ1 of a histidine are close to each other, as observed in the structure of CyoA.[8] In the case of *T. thermophilus*, no NOE is observed between the two protons mentioned, probably as a result of short T_1s and a not-so-close distance. An alternative assignment would be that of assigning *j* as *g*, i.e., to a cysteine α-CH which gives rise to a dipolar connectivity with its own β-CH. In this case *j* would be, as in the case of *P. denitrificans*, below the diamagnetic envelope. With the present experimental data, we favor the assignment of the 27-ppm signal, in amycyanin Cu_A, as an α-CH signal of a cysteine if no dipolar coupling is present between this signal and signal *h*.

Very far downfield in the ^{1}H NMR spectrum of the present system, only signal *b* is observed at 205 ppm, whose shift is temperature independent (not shown). This signal can be assigned to another cysteine β-CH proton. In *T. thermophilus*, there are three signals above 200 ppm assigned as cysteine β-CH protons. Two of these signals may be either too broad or too far shifted (or both) to be observed in the ^{1}H spectrum of the present system. To resolve this ambiguity, we have recorded ^{2}H NMR spectra of a sample with cysteines selectively deuterated at the β position. ^{2}H NMR spectra are expected to show smaller paramagnetic broadening of the lines due to the smaller gyromagnetic ratio of the deuteron with respect to the proton.[24,27] The resulting ^{2}H NMR spectra show all four cysteine β-CH_2 signals *a*−*d*, those missing in the ^{1}H NMR

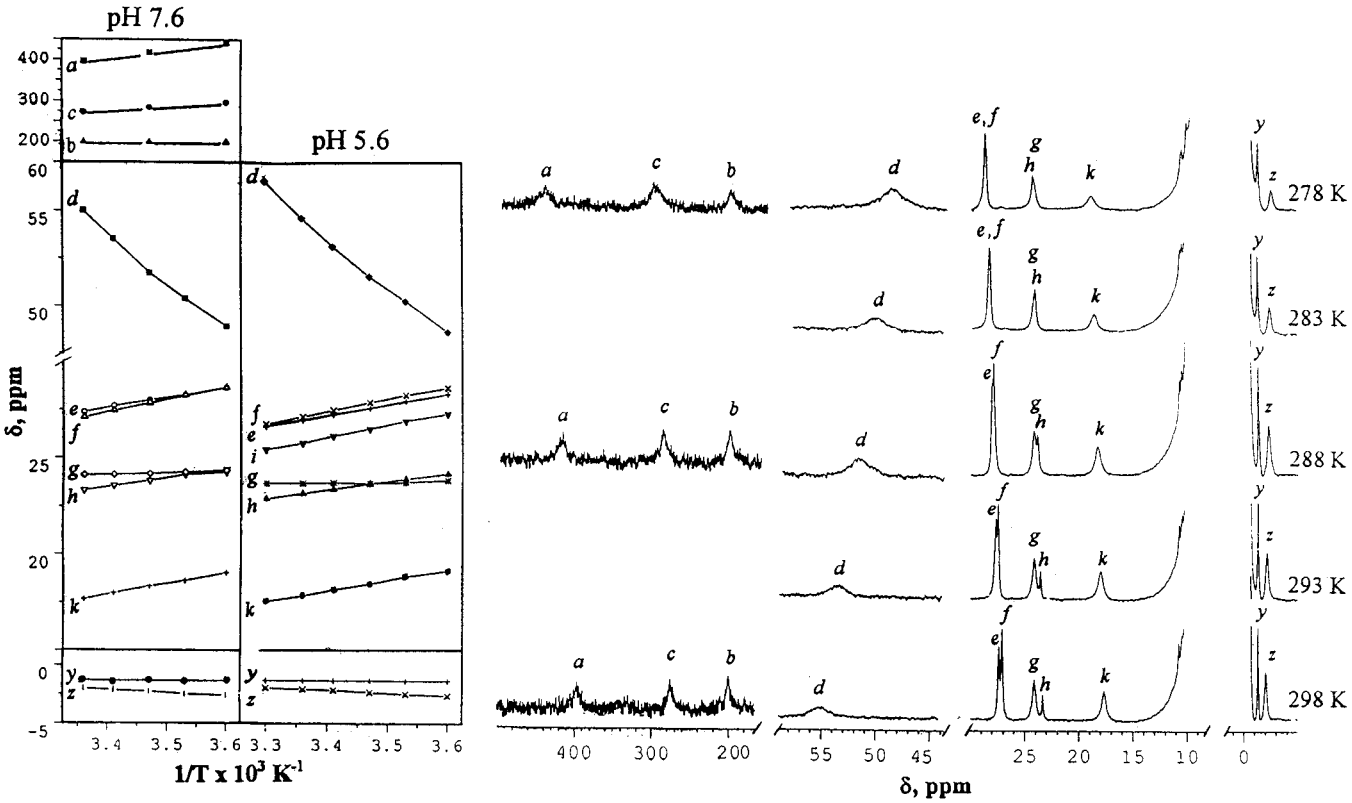

Figure 3. 800-MHz ^{1}H NMR spectra of H_2O solutions of the Cu_A domain from *P. denitrificans* as a function of temperature at pH 7.6. The 160−500-ppm regions are ^{2}H NMR spectra. Note the antiCurie behavior of signal *d* and the temperature independence of signals *b* and *g*. The same behavior is observed at pH 5.6 (inset) where the temperature dependence of signal *i* is also observable.

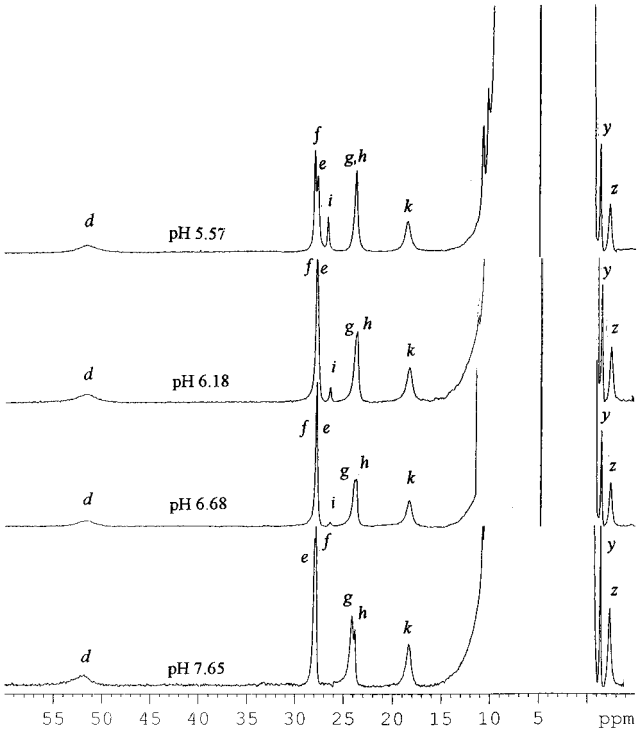

Figure 4. 800-MHz ^{1}H NMR spectra of H_2O solutions of the Cu_A domain from *P. denitrificans* as a function of pH at 288 K. Note the decrease in intensity of signal *i* with increasing pH and the substantial pH independence of the spectra.

spectrum being at 300 and 445 ppm (Figure 2A). The latter two signals have a Curie-type temperature dependence (Figure 3). The temperature dependence of signals *a*−*d* suggests an assignment analogous to that of *T. thermophilus*, i.e., signals *a* and *c* belong to one cysteine and signals *b* and *d* belong to the other cysteine. The proposed assignment is summarized in Table 1. The inequivalence between signals *a* and *c* on the one hand and signal *b* on the other is apparently stronger in terms of ^{1}H relaxation than it is in terms of hyperfine shifts.

Discussion

From the present investigation it would appear that the structure of Cu_A may be variable, at least at the ^{1}H NMR resolution level. The ^{1}H NMR spectrum of the Cu_A from *T. thermophilus*, previously reported,[21] and the present investigation suggest that the two histidines may be more inequivalent in the present Cu_A than they are in the Cu_A from *T. thermophilus*. It is possible that the differences are not so large, but a slightly smaller hyperfine coupling for the Hδ2 and Hϵ2 of His181 and a significantly smaller shift for Hϵ1 may be indicative of weaker ligation in the present system. This statement cannot be made quantitative, because the hyperfine coupling of histidine ring protons depends on several contributions which may even cancel one another: direct σ delocalization, π delocalization, and $\sigma-\pi$ spin-polarization. The three contributions are sensitive to π and σ coordination bonds. Comparison of the two systems indicates that the involvement of the sulfurs seems similarly asymmetric in the two systems. Perhaps one of the β-CH protons, corresponding to signal *d*, is geometrically slightly different in the two compounds. It is confirmed that delocalization onto sulfur is very high. Therefore, despite the difference in the NMR spectra, the overall picture seems to be very similar. This may also extend to Cu_A sites in amicyanin and *B. subtilis* Cu_A fragments.[22]

The similarity of the various electronic structures is confirmed by the fact that, with the exception of signals *a* and *c* that are too broad to be observed in the ^{1}H spectrum of the present system, the line widths and T_1 values are similar, and therefore the electronic relaxation times must also be similar in the various

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systems. The electronic relaxation times, in turn, depend on the separation between two or more doublets. Tetragonal copper(II) has electronic relaxation times in the range of 10^{-9}–10^{-8} s.[28] It can be estimated that in the systems discussed here, including the amicyanin construct, the electronic relaxation times are about 10^{-11} s. This estimate derives from use of the Solomon equation, appropriately scaled by a factor of 1/2 to account for the dimeric structure,[28,29] with the experimental T_1 values and metal−proton distances from the X-ray structure.[6] It is most probable that a number of excited doublet levels, relatively close in energy to the ground state,[30,31] makes the Raman type of electron relaxation[32] more efficient than in tetragonal copper complexes, so that two orders of magnitude of electronic relaxation rates are gained. There is an alternative electron relaxation mechanism (Orbach relaxation[28,33]), which would be efficient when the first excited doublet is within the thermal bath at room temperature. This mechanism would be appealing, because it could explain the temperature independence of signal *b* and the antiCurie behavior of signal *d*, assigned as a pair of β-CH_2 protons of one cysteine. However, the requirement of an energy level within the thermal bath is perhaps too stringent, if we consider that several systems, even with different electronic structures,[31] have similarly short electronic relaxation times.

By now, several investigations are available,[31,34] which describe the MO schemes of Cu_A under different symmetries. A common qualitative finding is that indeed the first excited state is somewhat closer than it is in blue copper proteins.[35] An MO which is essentially antibonding in character and made up by contributions from $d_{x^2-y^2}$, d_{xy}, p_x, and p_y of the four centers (as long as the $Cu_2S_2N_2$ moiety is planar) bears the unpaired electron.[16,17,23,33,34] This conclusion is based on symmetry considerations and should be true at any level of computational sophistication. The large shifts of the β-CH_2 cysteine protons are consistent with a large fraction of unpaired spin density on the S−Cu units. On the assumption that the spreading of the shifts within a β-CH_2 pair depends on the inequivalence of the Cu−S−C−H dihedral angles, and that this spreading is maximal for the present *P. denitrificans* Cu_A, we may exploit the available structural information to assign tentatively the two strongly inequivalent β-CH_2 protons (signals *b* and *d*) as belonging to Cys 220, and the more symmetric β-CH_2 pair (signals *a* and *c*) as belonging to Cys 216. Apparently, the σ-bonding p orbitals of the histidine nitrogens are less involved in the MO bearing the unpaired electron.[16,31] Nevertheless, the different shifts and shift patterns of the protons of His181 may indicate inequivalence in the imidazole−metal binding in the various systems. The diamond structure is possibly stabilized by π bonds involving the d_{xz} and d_{yz} copper orbitals and the p_z sulfur orbitals. Furthermore, one copper ion is also loosely bound to a methionine, which may be capable of affecting the energy of the antibonding MO. However, the fact that we do not observe any hyperfine shifted CH_3 signal suggests a very modest involvement of methionine in the Cu_A electronic structure.

The temperature dependence of the shifts of the cysteine proton signals does not follow the simple Curie law (Figure 3). In the *T. thermophilus* and *P. denitrificans* Cu_A, one cysteine β-CH proton shows antiCurie behavior. This was earlier attributed to the different populations of two levels close in energy.[21] An alternative explanation would be that temperature-dependent conformational changes induce this kind of shift. In the amicyanin Cu_A construct, there are different temperature dependences of the shifts but not just a simple T^{-1} dependence for some signals.[22] This observation would support the conformational change hypothesis. MO calculations show that the individual contributions of the three sulfur p orbitals involved in the orbital containing the unpaired electron change drastically with small geometric changes.[31,34] Therefore, minor variations in the Cu_2S_2 moiety with temperature may strongly affect the hyperfine coupling of the β-CH_2 (and even of the α-CH) of cysteines, as such shifts depend on the overlap of the 1s orbital of hydrogen with sulfur p orbitals. However, the shifts of the other β-CH_2 and of the α-CH are temperature independent, which makes the conformational change hypothesis less attractive.

In conclusion, we have shown that ^{1}H NMR is a very powerful tool for the investigation of the electronic and geometric structures of Cu_A centers. In particular, the large hyperfine shifts of cysteine β-CH_2 protons indicate large electron delocalization on sulfur and involvement of sulfur orbitals in a diamond structure, as found from X-ray[6−8] and spectroscopic measurements.[15−17] The highly delocalized electronic structure of the Cu_A site, coupled to the reduced reorganization energy due to its dinuclear nature,[36] may favor the function of this center as a mediator of electron transfer from cytochrome *c* to the proton-pumping machinery of cytochrome *c* oxidase. The hyperfine shifts of cysteine β-CH_2 protons depend critically on the Cu−S−C−H torsion angle. Apparently, the detectability itself of these signals also depends on this angle, to the point that in the Cu_A of *P. denitrificans*, the two β-CH_2 of Cys 216 can only be observed on the deuterated analogue, whereas all four β-CH_2 signals can be observed in the ^{1}H NMR spectrum of *T. thermophilus* Cu_A protein.[21] The unpaired electron is largely delocalized on the two copper as well as on the two sulfur atoms, but the delocalization on the latter two atoms seems not to be equal. This may arise from the geometric difference in the C_α−C_β−S−Cu torsion angles for the two ligands resulting in the different involvement of the p orbitals of these two atoms in the frontier MO of the system.[21] Despite the large electronic delocalization on the Cu_2S_2 moiety, the two histidines experience hyperfine coupling of more or less normal magnitude, and may also reveal inequivalency possibly due to the nonplanarity of one of them with respect to the Cu_2S_2 moiety. Finally, the sharp NMR line widths of the histidine proton signals reflect fast electron relaxation at room temperature, in turn arising from the proximity of doublet levels, but are not sensitive to slight inequivalencies in histidine binding which are, on the other hand, reflected in the shift patterns.

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