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^{1}H NMR studies of the paramagnetic Cu$_A$ center of cytochrome oxidase

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Abstract The dinuclear paramagnetic center of the soluble Cu$_A$ domain of the cytochrome *c* oxidase from *Bacillus subtilis* has been studied using ^{1}H NMR. The spectrum possesses remarkably sharp shifted resonances. Comparison with the spectrum of the Cu$_A$ amicyanin variant provides the spin density distribution in the Cu$_A$ site of cytochrome *c* oxidase. This represents the first paramagnetic NMR study of the dinuclear Cu$_A$ center from the soluble domain of subunit II of cytochrome *c* oxidase.

Key words: Copper protein; Amicyanin; Paramagnetic NMR; Cytochrome *c* oxidase

1. Introduction

The attention the Cu$_A$ center has received in recent years has been intensified by the elucidation of the structure of this unique dinuclear copper center [1–4]. The Cu$_A$ center is naturally found in subunit II of cytochrome *c* oxidase (CCO) [5] and also in the enzyme nitrous oxide reductase (N$_2$OR) [6]. It has been shown, by site directed mutagenesis, that a Cu$_A$ center can be created in the soluble domain of subunit II of the cytochrome *bo*$_3$ quinol oxidase, which naturally lacks this site [5] (the Cu$_A$ binding variant is referred to as cyoA*). The structure of the active site of cyoA* [4] is shown in Fig. 1. Authentic Cu$_A$ sites have also been introduced into the type I blue copper proteins (cupredoxins) amicyanin [7,8] and azurin [8,9]. The nature of the mutation made in the case of amicyanin is demonstrated in Table 1.

The study, by NMR, of native Cu(II) centers in proteins was thought impossible due to the relatively long relaxation time of this paramagnetic metal ion. Recently we have demonstrated that it is possible to observe broad isotropically shifted resonances of the Cu(II) forms of cupredoxins [10]. Amplifying this work it was found that the paramagnetic form of the Cu$_A$ variant of amicyanin has isotropically shifted ^{1}H NMR signals which are much sharper than the corresponding resonances in the wild-type (wt) Cu(II) protein [10]. In this letter we report how we have extended these NMR studies to the soluble Cu$_A$ domain of subunit II of the *caa*$_3$-type CCO from *B. subtilis* [11]. This represents the first documented example of an NMR study on a CCO domain. The similarity of the spectrum of the soluble Cu$_A$ domain to that of the assigned spectrum of the Cu$_A$ amicyanin variant provides a number of very interesting conclusions. These include the fact that the spin density distribution on the ligands is similar in the two Cu$_A$ centers and also that the axial Met-CuI interaction (see Fig. 1) may possess slightly more covalency in the soluble CCO domain.

2. Materials and methods

2.1. Isolation of proteins

The Cu$_A$ domain of the *caa*$_3$-type cytochrome *c* oxidase from *B. subtilis* was isolated as described previously [11]. The expression and purification of the Cu$_A$ amicyanin variant was as described elsewhere [12].

2.2. Sample preparation

NMR spectra were acquired of the soluble Cu$_A$ domain of the CCO from *B. subtilis* in 99.95% deuteriated 10 mM phosphate buffer at pH 7.3 and also in 20 mM Tris buffer at pH 8.0 (95% H$_2$O/5% ^{2}H$_2$O). The Cu$_A$ amicyanin variant was exchanged into 25 mM phosphate buffer (95% H$_2$O/5% ^{2}H$_2$O and 99.95% ^{2}H$_2$O) at pH 6.0 for the NMR experiments.

2.3. NMR spectroscopy

All ^{1}H 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) [13]. The values of τ, which were usually equal to the total effective relaxation delay (d1 plus acq), were in the range of 50 ms, with shorter times used to select for the faster relaxing resonances. 1D spectra were acquired with spectral widths ranging from 100 to 200 kHz and were processed using 20–100 Hz of exponential line broadening as apodization.

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 an interpulse delay (τ) varying between 0.1 and 70 ms. Exponential fits of the plots of peak intensity against τ yielded T$_1$ values.

3. Results and discussion

The soluble Cu$_A$ domain of the CCO from *B. subtilis* is extremely unstable. Attempts are currently under way to stabilize solutions of this protein to allow a detailed analysis of the isotropically shifted resonances to be carried out. However, the initial comparison to the assigned spectrum of the Cu$_A$ amicyanin variant (see Table 2) [12] is already quite illuminating.

In Fig. 2 the WEFT spectra (in ^{2}H$_2$O) of the Cu$_A$ amicyanin variant and of the Cu$_A$ domain from *B. subtilis* are shown. There are clearly a number of empirical similarities between these. Both spectra possess very broad resonances which are shifted beyond 50 ppm. Resonance J in the spectrum of the Cu$_A$ amicyanin variant has been assigned (see Table 1) to the C$^\beta$H$_2$ moiety of Cys97 (Cu$_A$ amicyanin numbering, see Fig. 1). Similarly, resonances i and j in the case of the Cu$_A$ domain from CCO are ascribed to C$^\beta$ protons of the cysteines.

Further similarities are apparent in the region from 40 to 10 ppm of the WEFT spectra (Fig. 2a,b). In the case of the Cu$_A$ amicyanin mutant the four resonances present in this area have been assigned to the non-exchangeable imidazole ring protons of the two histidine ligands (Table 2). The occurrence of six peaks in this region of the spectrum of the Cu$_A$ domain from *B. subtilis* is indicative of two additional shifted reso-

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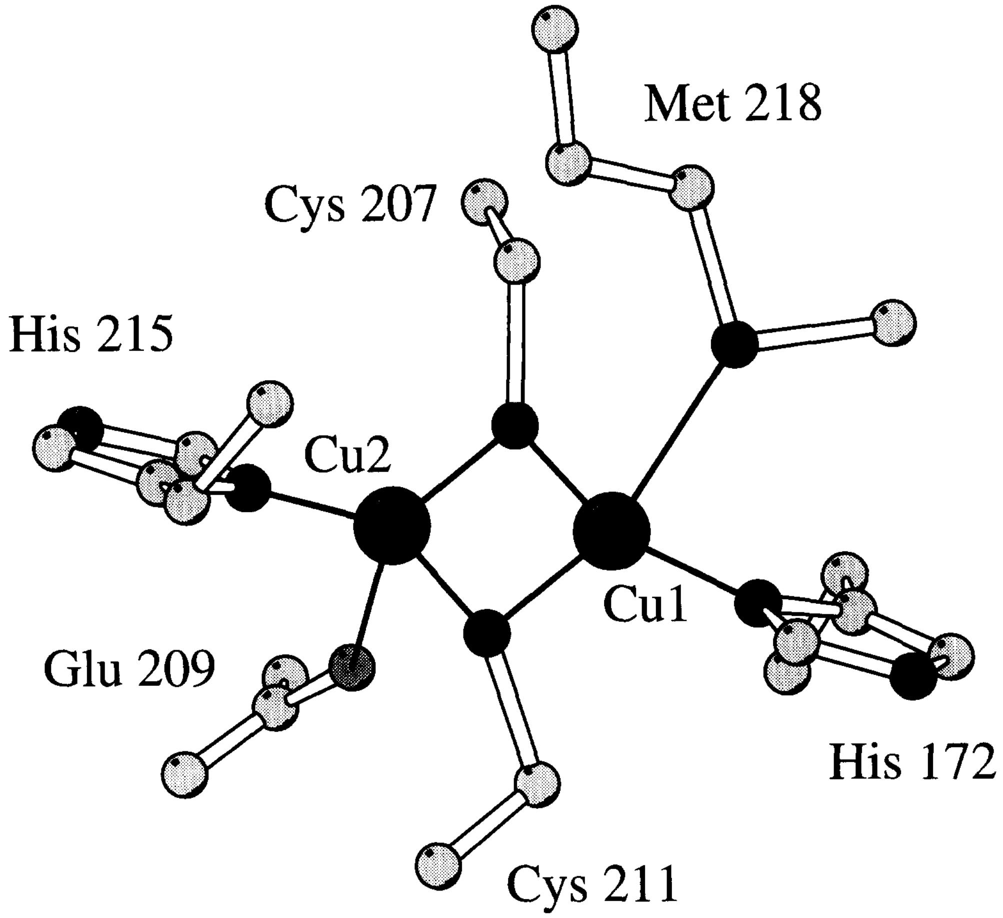

Fig. 1. Representation of the Cu_A site as found in the purple cyoA mutant [4].

Table 1
Partial C-terminal amino acid sequence of *Thiobacillus versutus* wild-type (wt) amicyanin

	Amino acid sequence											
wt amicyanin	Cys^{93}	–	–	–	–	–	Thr	Pro	His^{96}	Pro	Phe	Met^{99}
Cu_A amicyanin	Cys^{93}	Ala	Glu	Ile	Cys^{97}	Gly	Pro	Gly	His^{101}	Ser	Gly	Met^{104}
cyoA*	Cys^{207}	Ala	Glu	Ile	Cys^{211}	Gly	Pro	Gly	His^{215}	Ser	Gly	Met^{218}
COII	Cys^{195}	Ala	Glu	Leu	Cys^{199}	Gly	Pro	Ser	His^{203}	Ala	Leu	Met^{206}

Also shown is the sequence introduced into the soluble domain of subunit II of the cytochrome bo_3 quinol oxidase to produce the Cu_A binding variant (cyoA*, see Fig. 1) and the corresponding sequence as found in subunit II of the CCO from *B. subtilis* (COII). The metal ligands are provided with their sequence numbers.

Table 2
Hyperfine-shifted resonances, and their spin-lattice relaxation times (T_1) of Cu_A amicyanin (25°C) at pH 6.0 and also of the Cu_A domain of *B. subtilis* at pH 7.3 (10°C)

Cu_A amicyanin				Cu_A domain		
Resonance	δ (ppm)	T_1 (ms)	Assignments	Resonance	δ (ppm)	T_1 (ms)
J	107.3	n.d.	Cys97 $C^{\beta}H$	i	90.1	n.d.
A	36.9	9.1	His54 $C^{\delta2}H$	j	78.6	n.d.
K	27.8	11.5	His54 $N^{\epsilon2}H$	a	34.5	4.3
B	25.2	2.5	His54 $C^{\epsilon1}H$	b	33.9	4.3
C	23.1	12.0	His101 $C^{\delta2}H$	c	28.9	3.0
L	20.5	n.d.	His101 $N^{\epsilon2}H$	d	26.9	4.8
D	14.9	1.5	His101 $C^{\epsilon1}H$	k	25.5	n.d.
M	10.6	14.0		e	23.9	1.3
F	−1.3	7.0	His54 $C^{\beta}H$	f	15.0	0.7
G	−4.1	3.0	His101 $C^{\beta}H$	m	11.1	n.d.
				g	−0.7	n.d.
				h	−2.4	1.3

Also included are the known assignments for the Cu_A amicyanin variant [12].

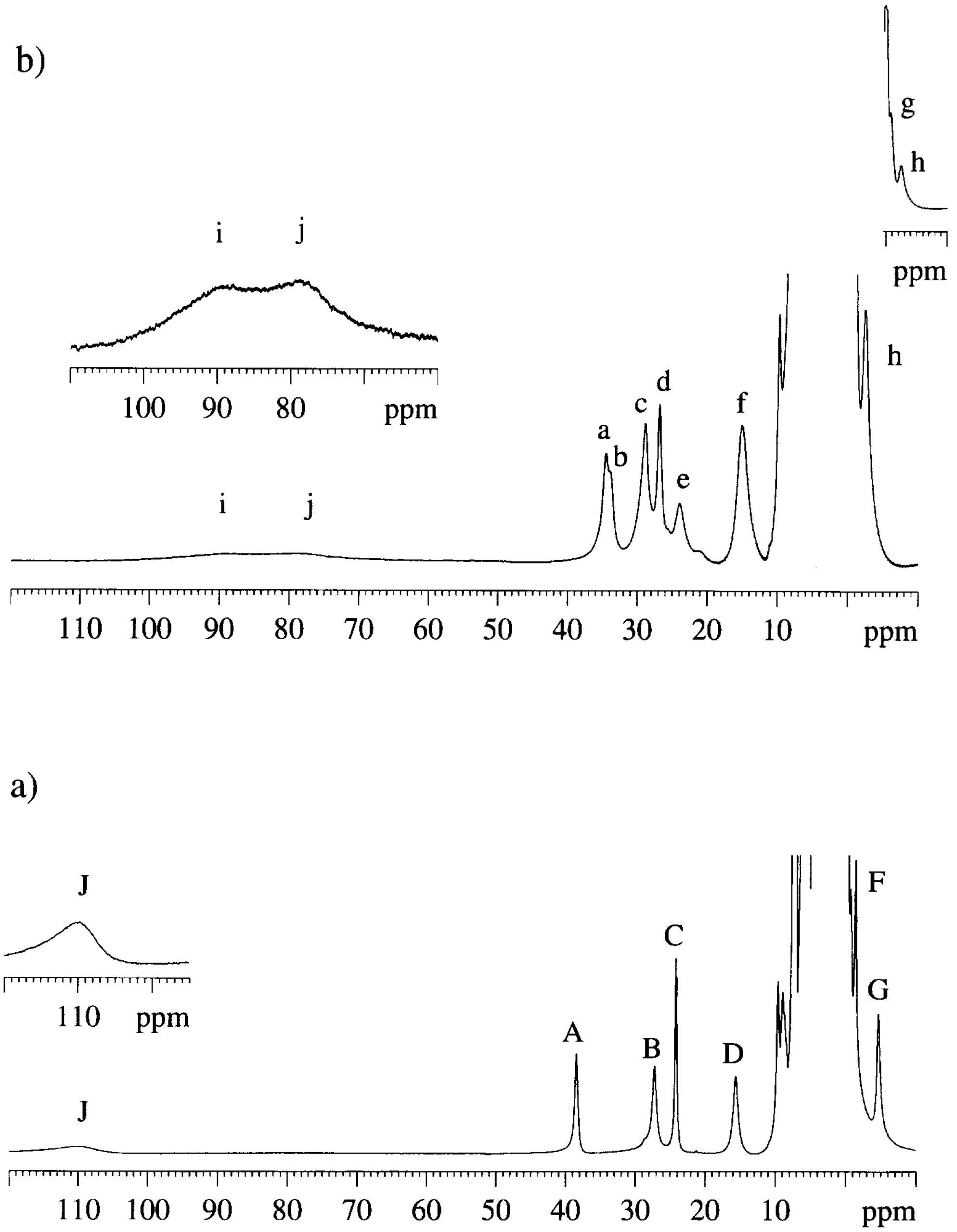

Fig. 2. WEFT spectra of (a) Cu_A amicyanin (7°C) at pH 6.0 in 25 mM phosphate buffer (^2H_2O) and (b) the Cu_A domain of the CCO from *B. subtilis* (10°C) at pH 7.3 in 10 mM phosphate buffer (^2H_2O) using an interpulse delay (τ) in the WEFT sequence of 10 ms.

nances. Potential protons which could give rise to such resonances include the $C^{\gamma}H$ resonances of the axial Met ligand involved in coordinating Cu1 (see Fig. 1). This would indicate subtle differences in the two Cu_A sites with respect to the strength of this interaction. Similar differences have been well documented for cupredoxins [14–16] and it has been shown that ^1H NMR of the Cu(II) forms of these proteins is a very sensitive indicator of the strength of this interaction [10,16]. Another possibility is that the extra resonances present in this region of the spectrum of the Cu_A domain are due to the C^{α} protons of the two bridging Cys ligands. Further experiments are in progress to clarify this issue.

The WEFT spectra of the two Cu_A centers contain exchangeable resonances in the region from 40 to 10 ppm. In the case of Cu_A amicyanin (see Fig. 3a,b) it is known that resonances K and L belong to the $N^{\epsilon2}$ protons of His54 and His101, respectively [12]. At higher temperatures, and more alkaline pH values, resonance L decreases in intensity (data not shown) consistent with this resonance belonging to the more exposed histidine (His215 is more exposed in the cyoA* structure, see Fig. 1). The spectrum of the Cu_A domain in water (see Fig. 2d) was acquired with the sample at pH 8.0 (the protein becomes much more unstable at lower pH values). Under these experimental conditions the $N^{\epsilon2}H$ of the exposed histidine will be exchanging too rapidly to be observed. Resonance k is therefore assigned as the $N^{\epsilon2}H$ of the more buried histidine in the soluble Cu_A domain of *B. subtilis* (His156). A comparison of the isotropic shift of reso-

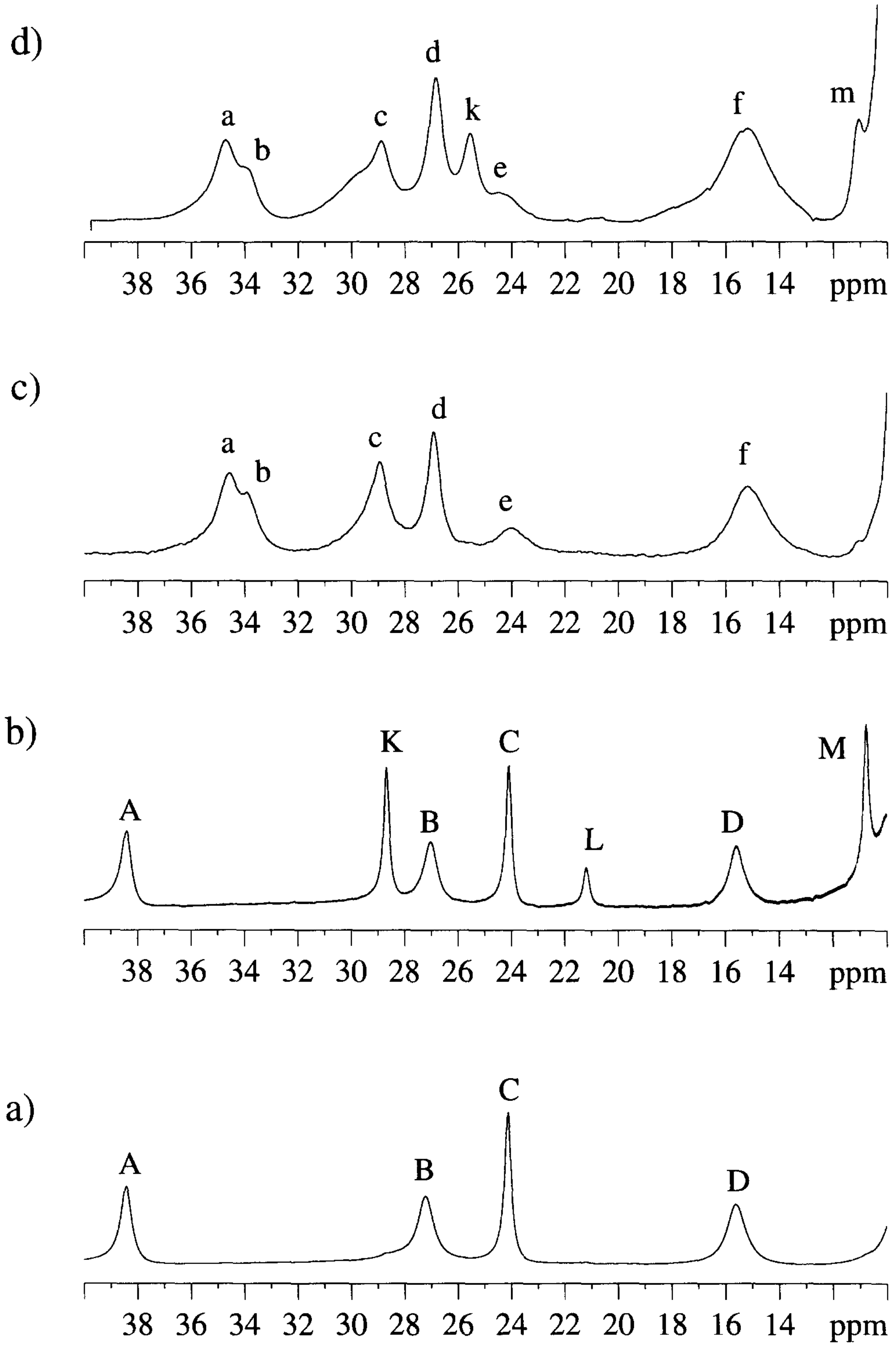

Fig. 3. WEFT spectra of (a) Cu_A amicyanin (7°C) at pH 6.0 in 25 mM phosphate buffer (^2H_2O), (b) Cu_A amicyanin (7°C) at pH 6.0 in 25 mM phosphate buffer (H_2O), (c) the Cu_A domain of the CCO from *B. subtilis* (10°C) at pH 7.3 in 10 mM phosphate buffer (^2H_2O) using an interpulse delay (τ) in the WEFT sequence of 25 ms and (d) the Cu_A domain of the CCO from *B. subtilis* (10°C) in 20 mM Tris buffer at pH 8.0 (H_2O) using an interpulse delay (τ) in the WEFT sequence of 45 ms.

nance k with that of its counterpart in the spectrum of Cu_A amicyanin (peak K) clearly indicates similarities between the two sites, with respect to the histidine ligands. From the analogy to studies on wt Cu(II) cupredoxins [10] it is known that the pseudo-contact (dipolar) contribution to the observed shifts is small. The isotropic shifts are therefore primarily due to Fermi contact contributions (the delocalization of spin density via covalent bonds) and hence detailed information regarding the spin density distribution on the ligands is directly obtained. The amount of spin density on the less exposed histidine ligand (His54 in Cu_A amicyanin and His156 in the *B. subtilis* domain) in these two centers is therefore almost the same. This argument can be further extended to include both of the histidine ligands because of the similarities between the spectra of the two Cu_A centers in the region from 40 to 10 ppm (see above).

The presence of an exchangeable resonance at around 11 ppm in the WEFT spectra of the two Cu_A centers is a further intriguing aspect of this work. In Cu_A amicyanin resonance M has been assigned to either the amide proton of Ile96 (this is involved in a peptide bond with Glu95 whose carbonyl oxygen is the axial ligand to Cu2, see Fig. 1) or the amide proton of one of the two cysteine ligands [12]. The presence of the exchangeable resonance m in the spectrum of the soluble Cu_A domain, in a similar position, clearly points to this as a conserved feature of the Cu_A coordination environment in the two centers.

A final likeness between the spectra shown in Fig. 2 is found in the upfield shifted region. Resonances F and G in the spectrum of Cu_A amicyanin have been assigned as C^β protons of the histidine ligands (see Table 2). The presence of matching resonances in the spectrum of the Cu_A domain from CCO (peaks g and h) further indicates the comparable nature of the paramagnetic centers in these two proteins.

In conclusion, it appears that the WEFT spectra of the Cu_A site in the amicyanin variant and in the soluble domain of the CCO from *B. subtilis* are very similar. The resonances in the soluble Cu_A domain have shorter T_1 values than their counterparts in Cu_A amicyanin (see Table 2). The Cu_A domain from the CCO is larger (M_r 17000) than the Cu_A amicyanin variant (M_r 12000). However, the overall correlation time of these Cu_A-containing systems is governed by τ_s (the rate of electronic relaxation) and the difference in molecular weight will have a minimal effect on the relaxation times. Therefore, the longer T_1 values for the Cu_A amicyanin variant are indicative of slightly faster electronic relaxation in this site.

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