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MULTI-FREQUENCY EPR EVIDENCE FOR A BINUCLEAR Cu_A CENTER IN CYTOCHROME *c* OXIDASE: STUDIES WITH A ^{63}Cu- AND ^{65}Cu-ENRICHED, SOLUBLE DOMAIN OF THE CYTOCHROME ba_3 SUBUNIT II FROM *Thermus thermophilus*

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We have recorded multi-frequency EPR spectra of ^{63}Cu- and ^{65}Cu-labeled, water-soluble Cu_A-protein from the cytochrome ba_3 of *T. thermophilus*. The spectrum taken at the highest frequency (34.03 GHz) shows no hyperfine structure and is nominally axial with apparent g_z ~ 2.18 and g_{xy} ~2.00 The spectrum taken at the lowest frequency (3.93 GHz) shows a rich hyperfine structure. Analyses of the spectra show that the observed splitting arises from an interaction of the unpaired electron with two Cu nuclei and support the notion that Cu_A is a mixed-valent [Cu(II)/Cu(I)] complex in which the unpaired electronic spin is distributed evenly over the two Cu ions.

The nature of the Cu_A center of cytochrome *c* oxidase has been a mystery since its discovery by Beinert, Griffiths, Wharton and Sands in 1962 [1]. Its defining features, as observed in the purified enzyme, are an unusual $S = 1/2$ EPR spectrum and an intense absorption in the near infrared. The Cu_A center is associated with subunit II, a polypeptide of ~150 - 250 residues depending on species. Near the N-terminus, there are normally two closely spaced hydrophobic stretches of sequence with the remainder forming a water soluble domain. It is

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widely believed that the hydrophobic regions anchor the protein to the membrane with the N- and C-termini protruding into the intermembrane (or periplasmic) space. Near the C-terminus there exists a conserved sequence, called the 'CuA motif', containing 1 methionine, 2 histidine and 2 cysteine residues which appear to serve as ligands to the Cu_A center.

Recently, recombinant genetic techniques have been used to express in *Escherichia coli* the water soluble portion of subunit II from various heme-copper oxidases. This has been done for the subunits II of *Paracoccus denitrificans* [2], *Bacillus subtilis* [3], and *Thermus thermophilus* [4]. In addition, the quinol oxidase subunit II of *E. coli* was converted by mutagenesis to restore the Cu_A ligands, and this also forms a Cu_A center (purple CyoA) [5, 6]. These proteins all show similar spectral features: a characteristic Cu_A EPR spectrum with g-values near 2.18 and 2.00; an optical absorption spectrum consisting of a near infrared band at ~800 nm, two stronger bands near 530 nm and 480 nm and a much weaker band near 360 nm; and a unique magnetic circular dichroism (MCD) band centered at ~500 nm [7]. The site can accept a single electron thereby becoming colorless and diamagnetic.

There has been considerable discussion during the past decade about how many Cu ions are present in the Cu_A site. It has been known for some time that the Cu_B center is mononuclear [8], and it was observed as early as 1976 [9] that the total copper content was closer to 3 Cu per enzyme molecule containing one Cu_A site and one Cu_B site. Nevertheless, the traditional view has been that Cu_A is also mononuclear. The first suggestion that Cu_A might contain 2 Cu came from the efforts of Kroneck and Antholine and their co-workers who noted the great similarity of the Cu_A EPR signal to that of N_2O reductase [10]. These workers recorded EPR spectra of N_2O reductase and Cu_A in bovine cytochrome *c* oxidase at several microwave frequencies. The N_2O spectra clearly showed the presence of a 7-line hyperfine pattern that most likely arises from a mixed-valent [Cu(I)/Cu(II)] metal cluster, and this conclusion was confirmed by spectral simulations. The results with cytochrome *c* oxidase were not so clear because the broad $g_y = 2.21$ signal from cytochrome *a* overlaps with the g_z region of Cu_A. Interference from this signal prevented a detailed analysis of this region of the spectrum and detracted from the author's conclusion that the Cu_A EPR signal also arises from a binuclear Cu center.

Here we describe the EPR properties of a Cu_A fragment obtained from the cytochrome ba_3 of *T. thermophilus* and prepared with the stable isotopes ^{63}Cu and ^{65}Cu, which have different magnetic moments. The EPR spectra recorded at 34.03, 9.45 and 3.93 GHz are unequivocally interpreted as arising from a mixed-valent, binuclear Cu center in this protein.

Materials and Methods

Isotopically enriched Cu (as CuO) was obtained from Oak Ridge National Laboratory: 99.70 % ^{65}Cu plus 0.3 % ^{63}Cu; and 99.89 % ^{63}Cu plus 0.11 % ^{65}Cu. The CuO was dissolved into a small amount of concentrated HCl to yield a green solution. This was diluted with a small amount of water to form a blue solution. It was then converted to the bis-histidine complex at pH 7 by addition of concentrated L-histidine followed by pH adjustment with concentrated NaOH. A portion of this solution was added to *E. coli* lysate containing apo-Cu_A protein, and the holoprotein was purified as will be described elsewhere [4].

EPR samples enriched with ^{63}Cu or ^{65}Cu were prepared by freeze-drying and redissolving the protein into water or 40 % ethyleneglycol to give a final buffer concentration of ~100 mM ammonium succinate at pH 4.6. Protein concentrations were 1 - 2 mM in Cu_A as determined by double integration of the EPR spectra. In all cases, the samples were characterized by Cu and protein analyses, SDS-PAGE, and EPR and optical spectra. We focused on the EPR spectra of the samples containing ethylene glycol because they showed narrower lines. The samples were prepared in San Diego and shipped on dry ice to Göteborg for EPR measurements.

EPR spectra at 3.93 and 9.45 GHz were recorded with a Bruker ER 200D-SRC spectrometer equipped with an Oxford Instruments ESR-9 helium flow cryostat. 3.93 GHz spectra employed an ER 061 SR microwave bridge, an ER 6102 SR reentrant cavity and a homemade quartz insert. Spectra at 34 GHz were recorded with a Bruker ESP 380 spectrometer using an ER 050 QG bridge and an ER 5103 QT cavity. Low temperature was obtained with a Bruker Flexline ER 4118 CF helium cryostat. Quantitations of EPR spectra were performed under nonsaturating conditions as described earlier [11], with Cu(II) in 2 M $NaClO_4$ (pH 2) as reference.

Results and Discussion

We will describe elsewhere [4] the properties of the water soluble Cu_A protein obtained by cloning a portion of the subunit II gene of cytochrome ba_3 from *T. thermophilus* (*cf.* [12]). Briefly, we have demonstrated that the protein contains ~2 Cu ions per molecule and has spectral properties very similar to those of native cytochrome ba_3, those of soluble subunits II from *P. denitrificans* [2] and *B. subtilis* [3] and to the engineered 'purple CyoA' fragment from *E. coli* [6]. Further, the ^1H and ^{14}N ENDOR spectra of the water soluble domain were the same as those previously reported for native cytochrome ba_3 [13] (P. Doan *et al.* unpublished observations).

EPR spectra were recorded at microwave frequencies of 34.03, 9.45 and 3.93 GHz. The spectra are dominated by the signal from Cu_A, but quantitation showed that the ^{63}Cu-enriched sample contained 15 % of a Type 2 Cu(II) signal while the ^{65}Cu sample contained very little extraneous signal. The experimental spectra are shown in Fig. 1 where they are displayed on a common g-value scale. At 34 GHz there is no evident hyperfine structure and is used primarily to obtain the g_z-value, which occurs at 2.187 and is marked with a vertical dashed line. At 9.45 GHz, the spectrum shows considerable hyperfine structure on the low-field side of g_z. As seen earlier by Fronsicz *et al.* [14] and by Antholine, Kroneck and co-workers [10], the hyperfine structure is resolved better at lower microwave frequencies. Thus, one can count 5 lines on the low-field side and 4 lines on the high-field side of the 3.93 GHz spectrum. The hyperfine structure is more pronounced in a second-derivative presentation. The data of Fig. 2 were obtained by numerical differentiation of first-derivative spectra using EXCEL. Fig. 2A and B show the low- and high-field portions, respectively, of the 3.93 GHz spectra of ^{63}Cu- and ^{65}Cu-enriched Cu_A proteins. Because the experimental, first-derivative spectra show peaks in these regions, the second-derivative spectra have a first-derivative shape.

The nuclear magnetic moments of ^{63}Cu and ^{65}Cu are 2.226 and 2.385 nuclear magnetons respectively, giving a '65/63' ratio equal to 1.071. Thus, one expects the Cu hyperfine structure, centered around a common g-value, to have A-values with the same ratio. Fig. 2 also shows spectra, derived from the ^{63}Cu spectra, but "stretched" about the g-position on the field axis to match the ^{65}Cu spectra as closely as possible, thereby producing a "simulated" ^{65}Cu spectrum. The

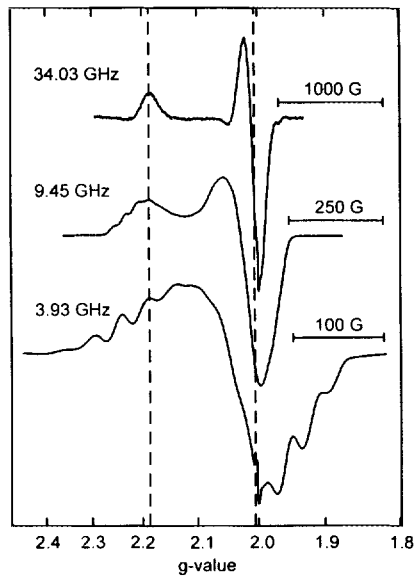


Fig. 1. Q-band (34.03 GHz), X-band (9.45 GHz) and S-band (3.93 GHz) EPR spectra of the ^{65}Cu enriched, soluble Cu_A-protein from the cytochrome ba_3 *of T. thermophilus*. The spectra are shown on a common *g*-value scale. The left dashed line indicates the position of g_z (=2.187) and the right dashed line is placed on the fourth extremum in the perpendicular region. The narrow signal at g = 2.00 is due to a free-radical impurity. Experimental conditions for Q-, X- and S-band spectra, respectively: microwave power (mW), 0.66, 0.2, 2; modulation amplitude (mT), 1.8, 1.0, 0.63; time constant (ms), 82, 200, 100; sweep time (s), 41, 200, 100 and number of scans, 10, 1, 32. Protein concentration, 1.3 mM. All spectra were recorded at 20 K. The same tube was used in X- and S-band.

center of the stretching is used as a variable in this simulation, thus giving an independent measure of the corresponding *g*-value. In both low- and high-field regions, the best "fit" is obtained with a stretching factor of 1.067, very close to the magnetic moment ratio for the two isotopes. This analysis shows that the observed splitting is derived primarily from the magnetic hyperfine interaction with copper nuclei, and rules out major contributions from copper quadrupole effects and other nuclei, such as ^{14}N or ^{1}H. The field axes of the experimental and simulated ^{65}Cu spectra coincide at the dashed lines in Fig. 2, thus showing the centers of the stretching. On both sides of the spectrum these occur in the middle of a 7-line multiplet. At the low-field side the center coincides with the g_z-value derived from the 34 GHz spectrum. At the high-field side the center should be at one or both of the 'perpendicular' *g*-values. The same positions are marked with vertical dashed lines in Fig. 1.

It is possible that the apparent hyperfine structure in the spectra could arise from either forbidden transitions and/or a rare combination of *A*- and *g*-tensor orientations. To test these possibilities, we attempted to simulate a 7-line multiplet in the *z*-direction of the 'powder' spectrum using a single Cu nucleus but with different axes of the *g*- and *A*-tensors but were unsuccessful. Further, microwave saturation studies (data not shown) indicated that forbidden transitions do not contribute to the number of lines. However, as predicted from the work of Antholine *et al.* [10],

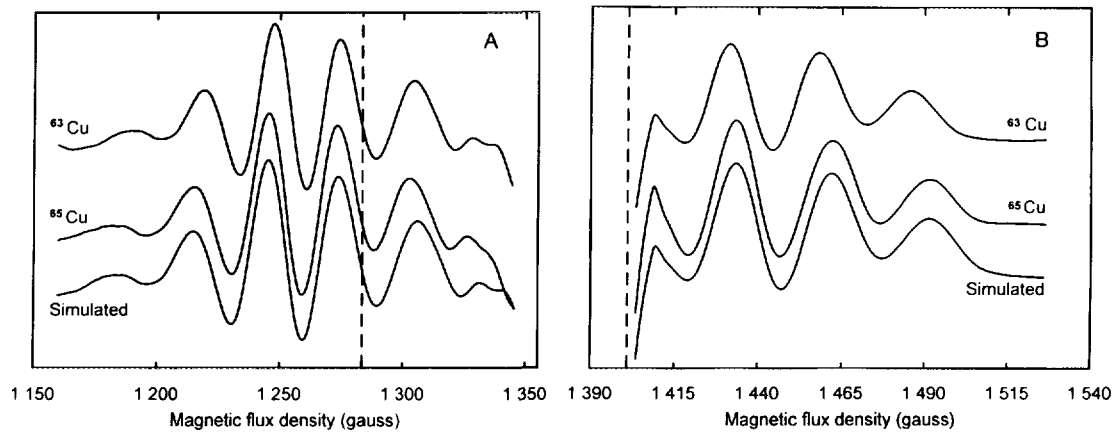
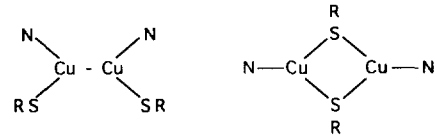


Fig. 2. Experimental and "simulated" S-band (3.93 GHz) EPR spectra of ^{63}Cu and ^{65}Cu EPR spectra of the soluble Cu_A-protein from the cytochrome *ba*$_3$ *of T. thermophilus*, presented as second derivatives. (A) The low field portion of ^{63}Cu, ^{65}Cu and "simulated" ^{65}Cu spectra (see text). The deviation between the experimental and "simulated" ^{65}Cu spectrum, seen in the parallel region at around 1300 Gauss, is caused by contributions from the outermost hyperfine lines around the other *g*-values. (B) The high field region of the spectra shown in (A). The very left of the spectra in (B) is dominated by the free radical also seen in Fig. 1. The dashed lines show where the field axes of the experimental and "simulated" ^{65}Cu spectra coincide (see text). Experimental conditions are the same as in Fig. 1.

the spectra can be fitted rather closely using two identical *A*-tensors (Karpefors *et al.*, unpublished observations). In addition, X-band and Q-band ENDOR spectra show that the coupling constants could only differ by ~10 MHz or less (~3 Gauss) (P. Doan *et al.*, unpublished observations). Taken together, the present data therefore proves beyond any doubt that the multiplet must originate in the coupling to two closely equivalent Cu nuclei (resulting in a 1:2:3:4:3:2:1 septet).

The near identity of the two Cu *A*-values suggests that the unpaired electron is equally distributed over both Cu ions. This signifies that the complex is a Class III, mixed-valent center as discussed by Robin and Day [15] (*cf.* Westmoreland *et al.* [16]), and any proposed structure must satisfy this condition. Mononuclear models are clearly no longer relevant (*cf.* [17]), and the possibility that the site could be binuclear has already led to several suggestions for its structure [6, 18, 19]. Two recently suggested models are shown in Scheme I.

Blackburn, Woodruff and co-workers [20] have interpreted EXAFS spectra of the *B. subtilis* Cu_A fragment as indicating the presence of a 2.5 Å metal-metal bond between the two Cu ions with one Cu ion being coordinated to 1 thiolate, 1 histidine and 1 methionine while the other Cu ion is coordinated to 1 thiolate, 1 histidine and an unidentified ligand, X; both Cu ions are thought to be tetrahedrally coordinated. Larsson *et al.* [21] extended this idea (Scheme I *left*), suggesting a planar Cu-Cu bonding model in which both the N and S ligands are in *cis* positions and proposed a molecular orbital scheme to account for the observed optical absorption properties. Thomson and co-workers [22, 23, personal communication] have proposed that the Cu ions are bridged by the 2 thiolate sulfur atoms while the 2 histidines serve as terminal ligands; no direct role for the conserved methionine was proposed (Scheme I *right*). Both structures include the presence



Scheme I

of two histidine residues, as previously determined by ^{14}N and ^{15}N ENDOR [13] and would satisfy the condition set by the present EPR, namely that the electron should be nearly equally distributed over both Cu ions.

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