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EPR characterization of ascorbyl and sulfur dioxide anion radicals trapped during the reaction of bovine Cytochrome *c* Oxidase with molecular oxygen

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ABSTRACT

The reaction intermediates of reduced bovine Cytochrome *c* Oxidase (CcO) were trapped following its reaction with oxygen at 50 μs–6 ms by innovative freeze-quenching methods and studied by EPR. When the enzyme was reduced with either ascorbate or dithionite, distinct radicals were generated; X-band (9 GHz) and D-band (130 GHz) CW-EPR measurements support the assignments of these radicals to ascorbyl and sulfur dioxide anion radical ($SO_2^{-\cdot}$), respectively. The X-band spectra show a linewidth of 12 G for the ascorbyl radical and 11 G for the $SO_2^{-\cdot}$ radical and an isotropic *g*-value of 2.005 for both species. The D-band spectra reveal clear distinctions in the *g*-tensors and powder patterns of the two species. The ascorbyl radical spectrum displays approximate axial symmetry with *g*-values of $g_x = 2.0068$, $g_y = 2.0066$, and $g_z = 2.0023$. The $SO_2^{-\cdot}$ radical has rhombic symmetry with *g*-values of $g_x = 2.0089$, $g_y = 2.0052$, and $g_z = 2.0017$. When the contributions from the ascorbyl and $SO_2^{-\cdot}$ radicals were removed, no protein-based radical on CcO could be identified in the EPR spectra.

1. Introduction

Radical-based catalysis is integral to biological processes in organisms ranging from archaebacteria [1] to mammals [2]. Protein-based radicals are essential to a variety of enzymatic systems [3], such as ribonucleotide reductases [4,5] and heme peroxidases [6–12]. Cytochrome *c* Oxidase (CcO), which catalyzes the reduction of oxygen to water, has been proposed to involve a protein-based radical [13–15] in its catalytic chemistry. Such a radical would explain how CcO sustains turnover without releasing reactive oxygen species even under conditions of limited electron availability. Despite impressive efforts and a variety of experimental approaches, the identity and role of radical species formed during the reaction of oxygen with CcO remain elusive.

Mammalian CcO is a 204 kDa membrane protein, which functions as Complex IV in the electron transport chain. Electrons derived from catabolism of fats, amino acids, and sugars enter the chain at Complex I and arrive via Cytochrome *c* (Cyt *c*) at CcO, where a bound oxygen molecule is the terminal electron acceptor. CcO catalyzes this exergonic reduction of oxygen to water, harnessing the released energy to pump four net protons across the inner mitochondrial membrane. The reaction of CcO with oxygen has been heavily studied using small molecule reductants such as ascorbate or dithionite as the external electron source for Cyt *c* and ultimately CcO. From these studies, several intermediates along the catalytic pathway have been identified. One such intermediate, termed "**P**," is believed to contain a protein-based radical, although its identity is highly debated [14,16–18].

Wilson et al. detected a radical during the reaction of oxygen with fully reduced bovine CcO; the authors concluded that it was an artifact from the reductant, although they could not rule out a protein-based radical [18]. In a radioactive iodide study, Babcock and coworkers reported a radical on the modified tyrosine, Y244, which is believed to donate a proton and an electron during catalysis [13]. In the reaction of oxygen with the fully reduced forms of two bacterial oxidases, *Paracoccus denitrificans* aa_3 and *Escherichia coli* bo_3, de Vries and coworkers suggested that as many as three radicals, ascribed to tryptophanyl and an unknown "6 ms" species, form during turnover [14,17]. However, neither the locations of the radicals nor their role in catalysis could be determined; furthermore, their formation could not be correlated with the expected steps in the catalytic cycle. In addition, L-ascorbic acid was used as the primary reductant, but its effect on the EPR spectra was not addressed.

L-Ascorbic acid (AH_2) is an endogenous water-soluble antioxidant present in up to millimolar concentrations in the human body [19]. It has pK_a values of 4.2 and 11.6 [20] and exists predominantly as the anion, AH^-, at neutral pH. Ascorbate oxidation involves the loss of a proton and an electron, which may occur via several possible mechanisms: (1) AH^- may lose an electron forming the neutral free radical, $AH^{\cdot}$, which deprotonates to form the semidehydroascorbate radical anion, $A^{-\cdot}$, (2) AH^- deprotonates to form the dianion, A^{2-}, which then releases an electron to form $A^{-\cdot}$, and (3) AH^- forms $A^{-\cdot}$ directly by a concerted proton-electron

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transfer. The first mechanism can be ruled out by its unfavorable midpoint potential (E^0 = +766 mV) [21]. The second mechanism is suggested to occur in the reduction of Cyt *c* [22]. The third mechanism has been demonstrated in cytochrome *b*561 [23]. In addition, recent energy calculations propose that a di-radical forms, although the mechanism is unclear [24,25].

Although the mechanism may vary, the end product is conventionally accepted to be $A^{-\cdot}$, which has been used as a spectroscopic indicator of oxidative stress due to its half-life of ~50 s [26,27]. $A^{-\cdot}$ has an unpaired electron in a highly delocalized π-system, conferring stability as the "terminal small-molecule antioxidant" [28]. A second one-electron oxidation produces dehydroascorbate, which is reduced and recycled in mitochondria to ascorbate [29–31]. Thus, the redox chemistry of ascorbate has immense physiological relevance as well as value as a laboratory reagent.

Sodium dithionite, which is another common reductant, has a midpoint potential of $E^0 = -420$ mV at pH 7 [32]. The dithionite ion, $S_2O_4^{2-}$ can be considered a dimer of $SO_2^{-\cdot}$ radicals, as the S–S bond is exceptionally long [33]; the reduction reactions of dithionite were suggested to occur via the $SO_2^{-\cdot}$ radical, whose EPR spectra have been studied [34,35]. $SO_2^{-\cdot}$ may arise from thermal decomposition prior to reduction or in the commercial preparation of sodium dithionite [35].

In biological systems, multiple radical species often occur in mechanisms involving radical migration between tryptophan, tyrosine, glycine, and cysteine residues as well as from a porphyrin π-cation radical in heme systems [3]. These systems give rise to complex EPR signatures. In addition, radicals originating from reductants used for reducing biological systems may further complicate EPR spectra. Thus, it is essential to identify these reductant-based radical components in order to make assignments of biologically relevant protein-based radicals.

To determine how the radicals formed from dithionite and ascorbate can affect EPR spectra obtained during the oxygen chemistry of C*c*O, we systematically investigated the reactions of these reductants with C*c*O. We found that when ascorbate/Cyt *c* or dithionite are used to reduce bovine C*c*O prior to its reaction with oxygen, ascorbyl or $SO_2^{-\cdot}$ radicals are generated and may be trapped under conditions typically used to detect reaction intermediates. We report the first multi-frequency EPR characterization of these radicals.

2. Materials and methods

C*c*O was purified from bovine heart tissue via the method described by Yoshikawa et al. [36]. Enzyme concentrations were determined by taking the optical absorption difference between fully-reduced C*c*O at 604 nm and the oxidized enzyme at 630 nm, using an extinction coefficient of 23.3 $mM^{-1}\,cm^{-1}$ [37].

Natural abundance L-ascorbic acid was obtained from Fisher Scientific. Isotopically labeled L-ascorbic acid was obtained from Omicron Biochemicals. A 1 M stock solution was prepared in degassed 0.2 M sodium phosphate buffer with the pH adjusted to 7.4 with NaOH. A final concentration of 10 mM ascorbate was added to degassed 160 μM C*c*O and incubated with 0.5 μM Cyt *c* as the electron carrier.

Sodium dithionite was obtained from Sigma. A 1 M stock solution was prepared in degassed 0.2 M sodium phosphate buffer at pH 7.4. A final concentration of 6 mM dithionite was added to degassed 600 μM C*c*O without Cyt *c*.

Rapid freeze-quench (RFQ) is a novel technology used to trap transient radicals formed at room temperature for spectroscopic characterization at low temperature. RFQ samples were prepared using a custom-built device described by Lin et al. [38] and Egawa et al. [39]. Resting C*c*O, reduced with either ascorbate or dithionite in a gas-tight syringe, was mixed with oxygenated buffer in the RFQ device at room temperature. Samples were freeze-quenched on a timescale of 50 μs–6 ms. The frozen powder was packed into 4-mm OD (X-band) and 0.55 mm OD (D-band) precision-bore quartz EPR tubes immersed in liquid nitrogen. Hand-quenched samples were prepared in similar EPR tubes and frozen in liquid nitrogen on the timescale of minutes.

X-band (9 GHz) measurements were made on a Varian E-line spectrometer. A finger Dewar was filled with liquid nitrogen and inserted into the EPR cavity preserved the sample at 77 K. Experimental conditions were: modulation amplitude, 3.2 G; microwave power, 1 mW; receiver gain, 2.5×10^4; microwave frequency, 9.107 GHz.

D-band (130 GHz) two pulse echo-detected spectra were obtained on a spectrometer described elsewhere [40,41] using the following parameters: temperature, 7 K; repetition rate, 30 Hz; 30 averages per point; 90° pulse, 50 ns; time τ between pulses, 130 ns. For both X- and D-band spectra, the field was calibrated using Mn^{2+} doped in MgO [42].

Low temperature optical absorption was measured at 77 K on a modified Linkam THMS600 microscope stage system with a halogen light source.

3. Results and discussion

3.1. Single-turnover reaction of O_2 with ascorbate-reduced C*c*O

Fig. 1A shows optical absorption measurements of the reaction intermediates freeze-quenched at 50–6000 μs. The visible band exhibits a red-shift from 603 nm (50 μs) to 604 nm (150 μs), where it remains for ~1000 μs until shifting to 602 nm and finally to 599 nm (6000 μs). These shifts suggest progression from the fully reduced form, **R**, to the pulsed oxidized form of the enzyme, $\mathbf{O_H}$. We find that the visible α-band of the pulsed oxidized form at 599 nm is red-shifted relative to the resting oxidized form at 597 nm, consistent with that reported in the literature [43–45]. The Soret band is saturated at the high enzyme concentration needed for EPR measurements and hence, is not shown.

In the corresponding X-band CW-EPR spectra for the RFQ samples, a narrow radical with a linewidth of 12 G overlies features of cupric Cu_A in all samples containing oxygen-containing intermediates (Fig. 1B). The isolated Cu_A^{2+} signal is seen clearly in the RFQ resting C*c*O sample and can be simulated with the parameters, $g_x = 1.99$, $g_y = 2.02$, and $g_z = 2.18$, consistent with reported values [46–48]. To demonstrate that the narrow radical was not formed due to passing a non-specific protein solution through the RFQ device, resting C*c*O and BSA were mixed with buffer and freeze-quenched in a similar fashion. As shown in Fig. 1B, the narrow radical was not generated in these controls.

High-frequency EPR was used to determine the *g*-tensor of the radical. The radical has a powder pattern consistent with near-axial symmetry and can be simulated with $g_x = 2.0068$, $g_y = 2.0066$, and $g_z = 2.0023$ (Fig. 2a). The isotropic *g*-value calculated from the trace of the *g*-tensor matches values reported for the ascorbyl radical at room temperature, $g = 2.0052$ [49].

Annealing experiments were performed, in which the 150 μs sample was warmed to either 180 K or 298 K then cooled back to 7 K for EPR measurements. The paramagnetic species diminishes in the D-band spectra after annealing (Fig. 2b and c). The stability of this radical species is consistent with its assignment as the ascorbyl radical. The same radical species could be observed by hand-quenching the C*c*O reaction during multiple-turnover conditions as shown in Fig. 3.

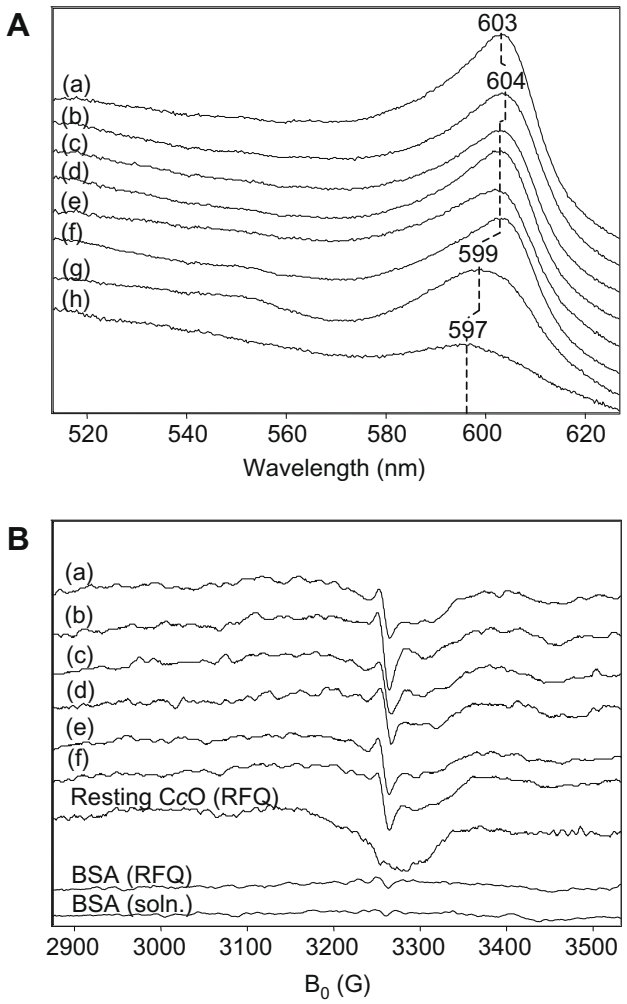

Fig. 1. Low-temperature optical absorption and X-band CW-EPR measurements of bovine C*c*O. (A) C*c*O (80 μM) completely reduced by 10 mM ascorbate and catalytic amounts of Cyt *c* under Ar then mixed with O_2-saturated buffer and rapid freeze-quenched at time points (a) 50 μs, (b) 150 μs, (c) 300 μs, (d) 400 μs, (e) 540 μs, (f) 1 ms, (g) 6 ms. (h) is a sample of the resting enzyme. Optical absorption measurements were made at 83 K. Solutions were prepared in 200 mM NaPi buffer, pH 7.4, containing 0.2% w/v *n*-decyl-β-D-maltoside (DM). (B) X-band EPR under the same conditions with quench times of (a) 50 μs, (b) 150 μs, (c) 300 μs, (d) 540 μs, (e) 1 ms, (f) 6 ms. Directly below (f) is a sample made by running resting enzyme through the RFQ device. The bottom two spectra are control samples of BSA run through the RFQ device (upper) and simply frozen in solution (lower). The conditions of EPR spectroscopy were: microwave power, 1 mW; microwave frequency, 9.1 GHz; modulation amplitude, 3.2 G; temperature, 77 K.

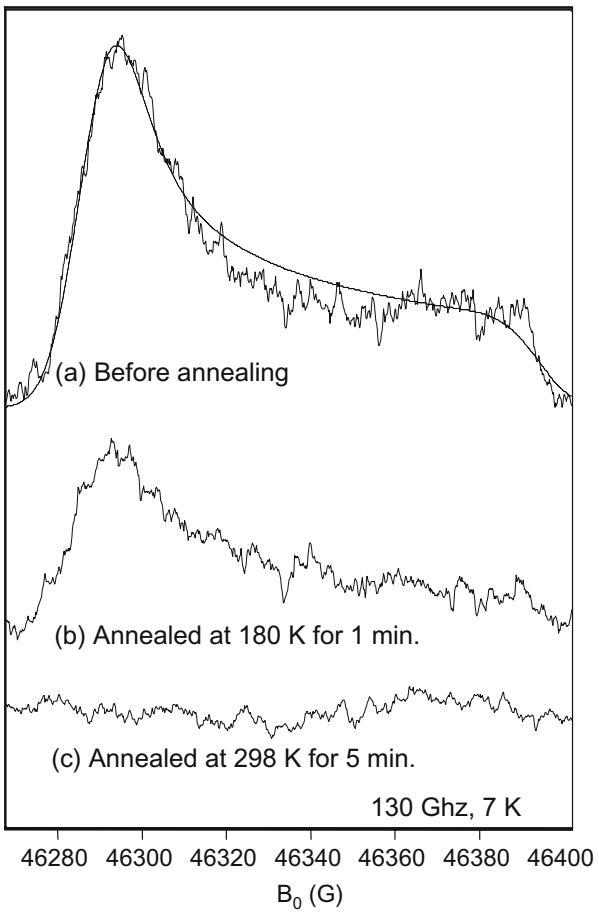

Fig. 2. D-band EPR spectra of RFQ C*c*O prepared with ascorbate. (a) D-band EPR measurements of 80 μM C*c*O, completely reduced by 10 mM ascorbate and catalytic amounts of Cyt *c* under Ar, then mixed with O_2-saturated buffer and rapid freeze-quenched at 150 μs. A simulation with $g_x = 2.0068$, $g_y = 2.0066$, and $g_z = 2.0023$ is the dotted line. Sample from (a) annealed at the conditions: (b) 180 K for 1 min and (c) 298 K for 5 min. The conditions for the D-band Hahn echo-detected spectra were: microwave frequency, 129.998 GHz; repetition rate, 50 Hz; averages per point, 100; 90° pulse, 50 ns; time between pulses, 130 ns; temperature, 7 K.

3.2. Multiple-turnover reaction of O_2 with ascorbate-reduced C*c*O

The stability of the radical observed in the single-turnover reaction suggested that large amounts of radical could be trapped in the multiple-turnover reaction. In particular, an increase in radical would be seen if its production depended on the C*c*O reaction—either in the reduction of C*c*O to produce **R** or in the oxygen chemistry to produce **P**, **F**, or $\mathbf{O_H}$. To test this, we prepared hand-quenched samples in which the ascorbate-reduced C*c*O was mixed with O_2-saturated buffer and allowed to turn over for several minutes. An accumulation of the relatively stable radical species was detected by X-band CW-EPR. Fig. 3 shows the amount of unpaired spin in the multiple-turnover compared to the single-turnover samples. The signal-to-noise is higher in the multiple-turnover samples, which are frozen liquid, compared to the RFQ samples, which are frozen powders. The origin of the feature at ~3360 G in the RFQ sample spectrum is unknown at present but it does not interfere with our analysis of the signal at $g \sim 2.005$.

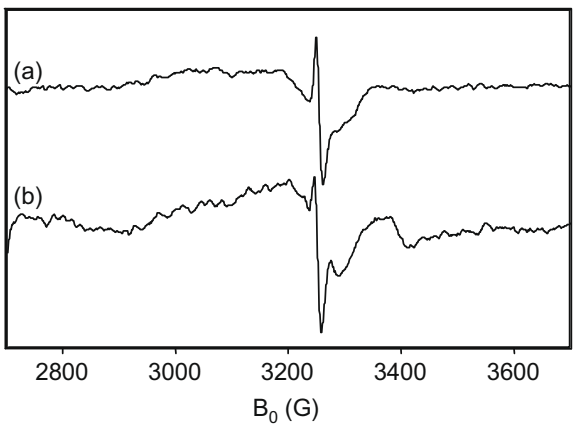

Fig. 3. X-band EPR spectra of C*c*O under multiple-turnover and single-turnover. (a) C*c*O (150 μM) completely reduced by 10 mM ascorbate and catalytic amounts of Cyt *c* under Ar then mixed with O_2-saturated buffer and hand-quenched at 5 min. (b) C*c*O (150 μM) completely reduced by 10 mM ascorbate and catalytic amounts of Cyt *c* under Ar then mixed with O_2-saturated buffer and rapid freeze-quenched at 150 μs. EPR conditions as in Fig. 1.

The principal g-values and stability of the radical species at room temperature were consistent with the ascorbyl radical rather than a protein radical arising from C*c*O. To confirm the assignment, experiments were carried out with isotopically-labeled ascorbate. L-[1-^{13}C]-ascorbic acid was used to repeat the hand-quenched and RFQ experiments. A characteristic splitting of the radical signal due to its coupling to the ^{13}C nucleus was observed in both samples (Fig. 4). Lowering the power to 0.3 mW and narrowing the field sweep to 50 G allowed detection of the radical species with minimal contributions from Cu_A^{2+}. The linewidth of the natural abundance ascorbyl radical is remarkably narrow, measuring 9 G, yet subtle hyperfine coupling broaden the edges of the signal (arrows). The hyperfine structure can be fit by adding four protons, expected from the hydrogen-atoms attached at the C_4, C_5, and C_6 positions [49]. Adding the ^{13}C nucleus with an isotropic hyperfine coupling of 6.54 in the C_1 position simulates the splitting seen in the isotopic data, similar to that reported for the ascorbyl radical (5.74 G) in the literature [49,50]. The hyperfine structure was well-fit using isotropic values in both the frozen liquid and RFQ samples, suggesting that the hyperfine anisotropy is too small to significantly influence the quality of the simulations. The simulation parameters are shown in Table 1.

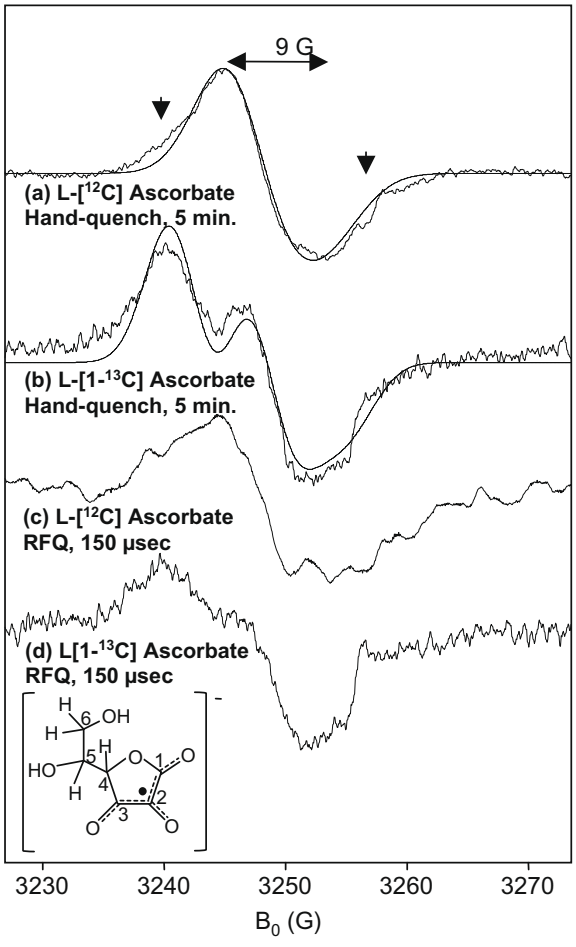

Fig. 4. X-band EPR spectra of C*c*O prepared with isotopically-labeled ascorbate. C*c*O (150 µM) completely reduced by (a) 10 mM natural abundance ascorbate or (b) 10 mM L-[1-^{13}C] ascorbate and catalytic amounts of Cyt *c* under Ar then mixed with O_2-saturated buffer and hand-quenched at 5 min. C*c*O (150 µM) completely reduced by (c) 10 mM natural abundance ascorbate or (d) 10 mM L-[1-^{13}C] ascorbate and catalytic amounts of Cyt *c* under Ar then mixed with O_2-saturated buffer and rapid freeze-quenched at 150 µs. Simulations are shown in the dotted lines with parameters shown in Table 1. EPR conditions were: frequency, 9.1 GHz; power, 0.3 mW; temperature, 77 K.

Table 1
Simulation parameters for the ascorbyl radical.

	L-[^{12}C] ascorbic acid	L-[1-^{13}C] ascorbic acid
g_x	2.0068	2.0068
g_y	2.0066	2.0066
g_z	2.0023	2.0023
a_{H4}	1.76[a]	1.76[a]
a_{H5}	0.07[a]	0.07[a]
$a_{H6,1}$	0.19[a]	0.19[a]
$a_{H6,2}$	0.19[a]	0.19[a]
a_{C1}	–	6.54[a]

[a] Hyperfine couplings are given in units of Gauss.

To determine if the ascorbyl radical originated from the one-electron reduction reaction of Cyt *c*, Cyt *c* and ascorbate were mixed in various ratios and frozen within 5 min. One study on the reduction of single-site metalloenzymes suggested that the dianion acts as the major reductant, forming the anionic ascorbate radical, which decays by a slow disproportionation reaction [22]. However, unlike the situation in the presence of C*c*O, we found that no radicals accumulate at 5 min in preparations of 1:1 or 20:1 Cyt *c* to reductant (Fig. 5a and b). Thus, upon the reduction of Cyt *c* alone, the ascorbyl radical initially formed must undergo further reactions which render it EPR silent. One possibility is that the disproportionation reaction is somehow accelerated under our experimental conditions. Another possibility is that the ascorbyl radical also acts as a reductant for Cyt *c*, producing the diamagnetic product dehydroascorbate in a reaction that is somehow prevented in the presence of C*c*O. Although the chemistry of the process is unclear at this stage, the end result is that ascorbate reduces Cyt *c* alone without the generation of an EPR-detectable concentration of radicals, while reduction in the presence of C*c*O produces ascorbyl radical signals as early as 50 µs which are stable on the timescale of minutes.

A major physiological function of cellular ascorbate is to quench cytotoxic oxoferryl species and protein-based radicals produced on hemoglobin during oxidative stress [51]. Similar reactions have been demonstrated in leghemoglobin [52] and Mb [53]. Thus, a possible mechanism in C*c*O is that ascorbate reacts with an oxoferryl heme or a protein-based radical to generate the observed radical species. We stress that detection of the ascorbyl free radical neither proves nor disproves that C*c*O produces a protein-based radical in the oxygen reaction.

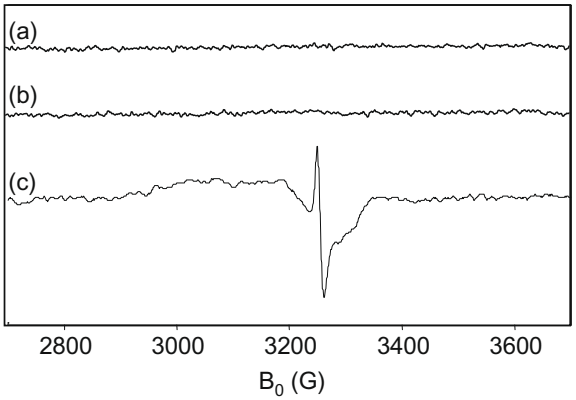

Fig. 5. X-band EPR spectra of Cyt *c* and ascorbate. (a) Cyt *c* (5 mM) is reduced with 5 mM ascorbate and frozen at 77 K at 5 min. (b) Cyt *c* (0.5 mM) is reduced with 10 mM ascorbate and frozen at 77 K at 5 min. (c) C*c*O (150 µM) and 0.5 µM Cyt *c* are reduced with 10 mM ascorbate and frozen at 77 K at 5 min. The pH of all samples is 7.4. EPR conditions as in Fig. 1.

3.3. Previously reported radicals in the reaction of O_2 with ascorbate-reduced CcO

Wilson et al. studied bovine CcO using ascorbate as the reductant to create multiple-turnover conditions and reported a $g \sim 2$ radical with a linewidth of 8 G that amounted to as much as 10% of the enzyme concentration [18]. They noted that when the enzyme is reduced with only ferrocytochrome *c*, which was passed on a column to remove excess ascorbate, the magnitude of the radical signal decreased to ~1% of the enzyme concentration; however, they were unable to determine if the signal resulted from a residual ascorbyl radical or a protein-based radical.

In 2007, Wiertz et al. reported the presence of two radicals in the reaction of ascorbate-reduced *P. denitrificans* aa_3 with oxygen [14]. The 350 μs sample (f) The first species, present from 83 μs to ~1 ms, was assigned as a tryptophanyl radical derivative of W272 (*P. denitrificans* CcO numbering, W236 in bovine) based on simulations of Q-band data. A radical with similar features in X-band spectra was reported in the bo_3 oxidase from *E. coli* [17]. A second unidentified, narrow radical existed from 83 μs through 6 ms. The authors suggested the source to be a "main-chain radical" but concluded that its identity and functionality could not be determined. This second "6 ms" species accounted for ~0.5% of the enzyme concentration and had *g*-values reported as 2.0022, 1.9965, 1.9994, which differ from those reported here for the ascorbyl radical.

For the bovine enzyme, when we subtract the ascorbyl radical and oxidized Cu_A from our data, we are unable to identify a protein-based radical. In addition, no protein-based radical was detected when CcO was reduced with dithionite, which was subsequently removed by a gel-filtration column. The data indicate that protein-based are not observed in bovine CcO when the fully-reduced enzyme is reacted with oxygen; in addition, ascorbate produces an ascorbyl radical in the oxygen reaction of bovine CcO. These data contrast the bacterial system, in which a tryptophanyl radical arises and no ascorbyl radical is reported even when the same concentration (10 mM) is used as a reductant [14].

Either the mechanism of the bovine enzyme or the reagents—specifically, the reductants and mediators—may cause these differences between the bovine and the bacterial systems. The observations of Wilson et al. along with ours are most consistent with differing mechanisms between mammalian and bacterial enzymes. This would suggest that electron donation from an amino acid is unnecessary in the robust mammalian chemistry but occurs in bacterial enzymes. This difference is interesting because in both systems, the metal centers of the fully-reduced enzyme are capable of donating the four electrons necessary to complete the reduction. If radicals were to form in the reaction of bovine CcO, they could be quenched by ascorbate, obscuring the detection of any protein-based radicals. However, it should be noted that in the studies of the bacterial enzymes by de Vries and coworkers, phenazine ethosulfate (PES) was used as the redox mediator in lieu of the physiological reductant, Cyt *c*, used by us and Wilson et al.

3.4. Reaction of O_2 with dithionite-reduced CcO

To avoid the complication from the ascorbyl radical, we examined the CcO reaction by using dithionite as a reductant instead of ascorbate/Cyt *c*. In this series of experiments, the enzyme was exposed to a 10-fold excess of sodium dithionite prior to the reaction with O_2. RFQ samples were prepared as in the ascorbate experiments. X-band CW-EPR measurements show a narrow radical signal with a linewidth of 11 G in intermediates trapped from 50 μs through 6000 μs (Fig. 6a–e). When the narrow radical signal was subtracted from the data, no additional protein-based radicals were detected. The narrow radical disappeared upon annealing

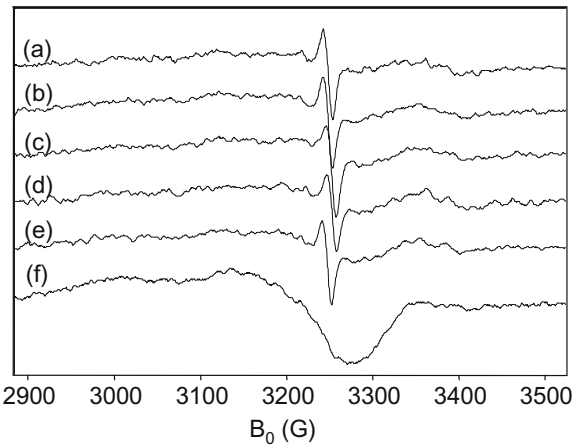

Fig. 6. X-band EPR spectra of CcO prepared with dithionite. CcO (150 μM) completely reduced by 1.5 mM dithionite under Ar then mixed with O_2-saturated buffer and rapid freeze-quenched at (a) 50, (b) 150, (c) 350, (d) 450, and (e) 6000 μs. (f) The 350 μs sample (f) annealed for 1 min at 298 K. EPR conditions as in Fig. 1.

for 1 min, leaving only the Cu_A^{2+} signal (Fig. 6f). In addition, hand-quench samples, prepared by mixing CcO reduced by dithionite and oxygen and quenched at 1 min, showed only cupric Cu_A with no evidence of the narrow radical (data not shown).

Because previous studies suggested that dithionite spontaneously dissociates into breakdown products such as $SO_2^{-\cdot}$ [34,35], the reductant was tested with both redox and non-redox active proteins. 115 micromolar of CcO, BSA, or Mb were mixed with 50 mM dithionite and freeze-quenched at 50 μs. An intense signal with a linewidth of 11 G was seen in all three enzyme samples (Fig. 7a–c). These observations suggest that the radical arises spontaneously from dithionite and not from interactions with proteins.

To further characterize the radical species, 50 mM dithionite solutions were freeze-quenched with oxygenated or deoxygenated

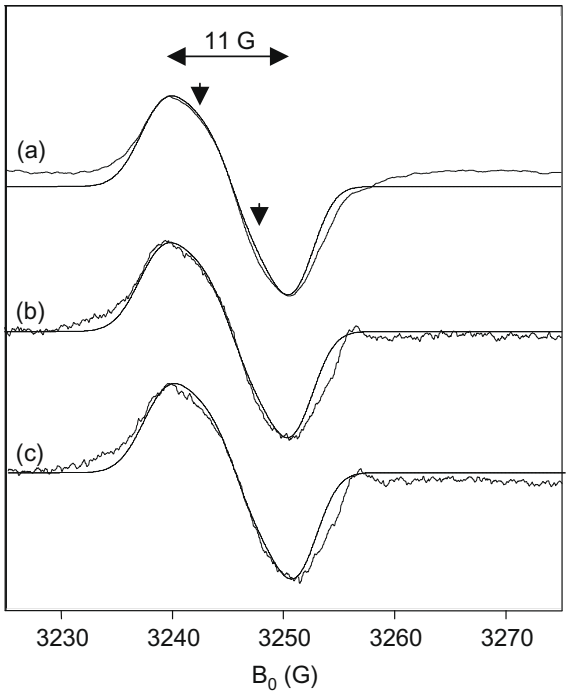

Fig. 7. X-band EPR spectra of various proteins prepared with dithionite. (a) CcO (115 μM) and 50 mM $Na_2S_2O_4$ rapid freeze-quenched at 50 μs. (b) Bovine serum albumin (115 μM) and 50 mM $Na_2S_2O_4$ rapid freeze-quenched at 50 μs. X-band EPR conditions as in Fig. 1.

buffer and the samples were measured by D-band and X-band EPR. High-frequency spectra show perturbations of the g_x and g_z features in the oxygenated sample (Fig. 8b) compared to the deoxygenated sample (Fig. 8a), the origin of which are unknown at this time. However, both of these high-frequency oxygenated and deoxygenated spectra are well-fit by simulations with *g*-tensor values at 2.0089, 2.0052, and 2.0017 (Fig. 8a and b, dotted lines). The isotropic *g*-value is 2.0053, which is in good agreement with the literature value of $SO_2^{-\cdot}$ [54–56]. This study is the first report of the *g*-tensor components of $SO_2^{-\cdot}$. The radical in the samples of 50 mM dithionite with (Fig. 8f) and without oxygen (Fig. 8d) is identical to the radical produced when reacting C*c*O with oxygen (Fig. 8e). Using the well-defined *g*-values, the X-band data can be simulated using parameters in Table 2. The simulations, which are optimized to fit the slight asymmetry of the lineshape (Fig. 7, arrows), include two protons. These protons are likely H-bonding water molecules in the vicinity of $SO_2^{-\cdot}$. In addition, the radical nearly disappears when annealed for 1 min at 180 K (Fig. 8c), which is consistent with our annealing experiments performed at X-band. Unlike the ascorbyl radical, the $SO_2^{-\cdot}$ radical is unstable at room temperature under these experimental conditions.

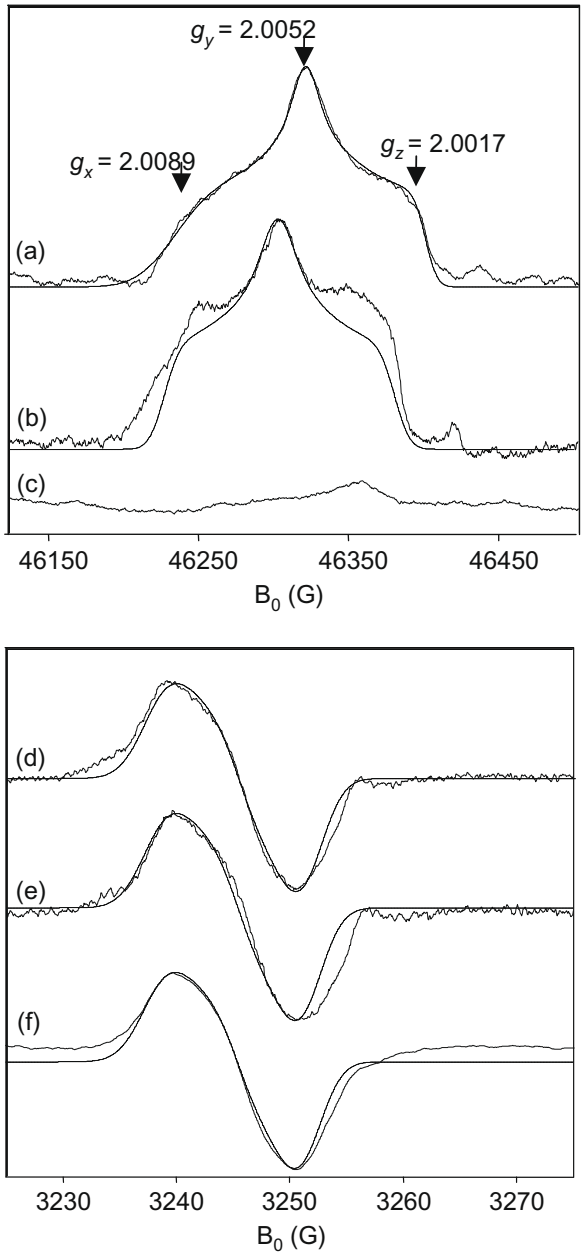

Fig. 8. D-band (top) and X-band (bottom) EPR spectra of dithionite with and without oxygen. (a and d) $Na_2S_2O_4$ (50 mM) in degassed buffer rapid freeze-quenched at 50 μs. (b and e) $Na_2S_2O_4$ (50 mM) with oxygenated buffer and 115 μM C*c*O rapid freeze-quenched at 50 μs. (c) Sample from (b) annealed at 180 K for 1 min. (f) $Na_2S_2O_4$ (50 mM) in oxygenated buffer rapid freeze-quenched at 50 μs. D-band and X-band EPR conditions as in Figs. 2 and 1, respectively.

Table 2
Simulation parameters for the sulfur dioxide anion radical.

	$SO_2^{-\cdot}$
g_x	2.0089
g_y	2.0052
g_z	2.0017
A_{H_2O}	0.5[a]
	2.1[a]
	1.1[a]
A_{H_2O}	0.5[a]
	2.1[a]
	1.1[a]

[a] Hyperfine couplings are given in units of Gauss.

4. Conclusions

This study reports *g*-tensors for the ascorbyl and $SO_2^{-\cdot}$ radicals and establishes that these radicals are formed and can be trapped in the course of C*c*O reacting with oxygen when ascorbate/Cyt *c* or dithionite is used as the reductant. In the frozen solution X-band spectra, the ascorbyl and $SO_2^{-\cdot}$ radicals both appear as relatively featureless single-line spectra with zero crossings at $g \sim 2.005$. The high-frequency spectra provide the resolution of peaks associated with the principal *g*-values and reveal that the ascorbyl radical has near-axial symmetry while the $SO_2^{-\cdot}$ is much more rhombic. This study demonstrates the utility of high-frequency EPR to distinguish between radicals that appear similar at X-band, thereby allowing for unequivocal identification.

Protein-based free radicals play an integral role in the chemistry of life processes and are often studied by EPR spectroscopy. In assessing protein-based radical mechanisms, byproducts from reductants must not be neglected as they may significantly impact the analyses. In bacterial C*c*O, catalytically relevant radicals are formed, but no such radical has yet been unequivocally identified in the mammalian enzyme. Identifying the origin of reducing equivalents used in catalysis is crucial to understanding the link between redox chemistry and proton translocation. However, the question of whether a radical arises in the catalytic reaction of mammalian C*c*O with O_2 remains unclear. Our studies show that ascorbate and dithionite—two universally used reductants in studies of redox proteins—can contribute to EPR spectra. Hence, great care must be taken in the interpretation of data to understand their roles in the reactions studied.

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