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Multifrequency EPR Studies on the Mn(II) Centers of Oxalate Decarboxylase

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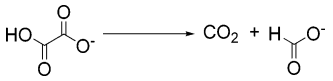
Oxalate decarboxylase from *Bacillus subtilis* is composed of two cupin domains, each of which contains a Mn(II) ion coordinated by four identical conserved residues. The similarity between the two Mn(II) sites has precluded previous attempts to distinguish them spectroscopically and complicated efforts to understand the catalytic mechanism. A multifrequency cw-EPR approach has now enabled us to show that the two Mn ions can be distinguished on the basis of their differing fine structure parameters and to observe that acetate and formate bind to Mn(II) in only one of the two sites. The EPR evidence is consistent with the hypothesis that this Mn-binding site is located in the N-terminal domain, in agreement with predictions based on a recent X-ray structure of the enzyme.

Introduction

Oxalate decarboxylase (E.C. 4.1.1.2) (OxDC)[1] catalyzes the cleavage of the carbon−carbon bond in oxalic acid to yield formic acid and carbon dioxide (Scheme 1). This transformation is chemically interesting because (i) decarboxylation formally yields an acyl anion,[2−4] and (ii) dioxygen is required for an enzyme-catalyzed reaction that involves no net redox change.[5−8] X-ray crystal structures of *Bacillus subtilis* OxDC[9,10] have shown that the monomer is composed of two cupin domains,[11] each containing a single Mn^{2+} ion coordinated by four conserved residues (three histidines and one glutamate), leaving two open coordination sites for water or substrate molecules. The two Mn(II) ions in the resting monomer are in very similar protein environments, which has significantly complicated spectroscopic efforts to establish whether catalysis takes place in only one or both of the two metal sites. In previous X-band studies by our group,[12] a nearly octahedral geometry about Mn(II) in the resting state was inferred from EPR spectra taken in perpendicular and parallel polarizations. X-band EPR spectral perturbations of the Mn centers have also been observed upon the addition of oxalate or formate to the enzyme, but distinguishing which metal signal was perturbed was difficult because the signals were very broad.[12,13]

We now describe the first multifrequency EPR study of OxDC designed to (i) distinguish the two Mn(II) sites and (ii) determine their respective magnetic parameters. Our rationale in using this approach is based on the fact that Mn(II) line widths generally become narrower at higher fields (and thus, higher frequencies), allowing for better spectral resolution of small differences in *g* and *A*. Moreover, effects associated with differing fine structure parameters are more prominent at low and intermediate fields (frequencies). By studying the enzyme at multiple frequencies, we have been able to determine that the two Mn(II) ions have very similar *g* and *A* values but differ in their fine structure parameters *D* and *E*. In addition, we show that the Mn(II) ion with the larger fine structure is solvent-accessible, as demonstrated by a reduction in *D* from 2700 to 2150 MHz and in *E* from 675 to 108 MHz upon addition of acetate or formate to the enzyme.

SCHEME 1: The Reaction Catalyzed by *B. subtilis* OxDC



Materials and Methods

Materials. Protein concentrations were determined using a modified Bradford assay[14] (Pierce, Rockford, IL), for which standard curves were constructed with bovine serum albumin. The metal content of OxDC was quantified at the University of Wisconsin Soil and Plant Analysis Laboratory using inductively coupled plasma mass spectrometry measurements.[15] Samples for EPR were inserted in quartz capillaries (for X-, and W-bands) or Teflon cups, then flash-frozen in liquid nitrogen and inserted into the precooled cryostats.

Expression and Purification of Recombinant, Wild Type OxDC. Recombinant wild type *B. subtilis* OxDC was expressed and purified using a modified literature procedure.[3] To obtain reproducibly high levels of Mn incorporation, protein expression was induced at an optical density (600 nm) of 0.6, and cells were grown at a post induction temperature of 30 °C so as to promote the transport of manganese ions into the bacterial cells.[16,17]

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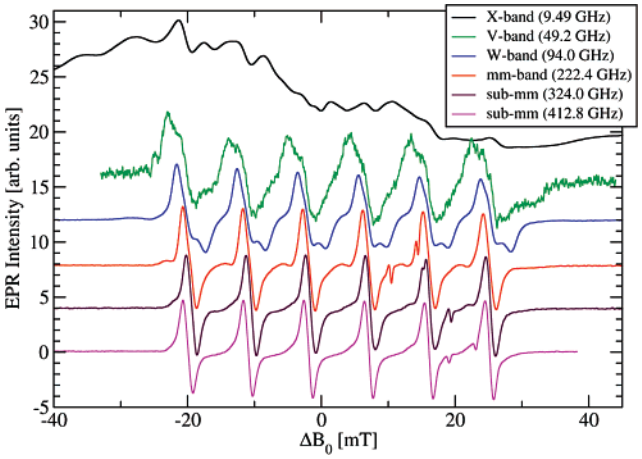

Figure 1. Field dependence of the EPR spectra of OxDC in storage buffer (20 mM hexamethylenetetramine HCl, pH 6.0) with 0.5 M NaCl. To facilitate comparison of the $+1/2 \leftrightarrow -1/2$ transitions, spectra are shifted along the B_0 axis. Field positions in *T* at the zero points: 0.3340 (X-band, 9.4873 GHz), 1.7490 (V-band, 49.200 GHz), 3.3545 (W-band, 94.0214 GHz), 7.9427 (222.40 GHz), 11.567 (324.00 GHz), and 14.730 (412.80 GHz). All spectra were taken at temperatures between 5 and 20 K.

EPR Spectroscopy. X-band and W-band spectra were recorded on commercial spectrometers from Bruker Biospin Corp., Elexsys E580 and E680, respectively, which were equipped with their respective standard resonators and cryostats for temperature control. The V-band, mm, and submm band spectra (50, 200−420 GHz) were recorded with a home-built instrument using a 15/17 T superconducting magnet as described by Hassan et al.[18]

Results and Discussion

To avoid complications arising from the binding of ligands other than water to the two free ligand positions on each Mn, our initial experiments used OxDC dissolved in 20 mM hexamethylenetetramine (HMTA) HCl buffer at pH 6.0. HMTA is not expected to bind to Mn(II) because it is positively charged and too bulky to fit into the Mn-binding pockets in the protein.

Figure 1 shows the $g \approx 2$ region of the EPR spectra of OxDC in HMTA buffer pH 6.0 at freqeuncies ranging from X-band (corresponding to 0.34 T) to the submm band (15 T). In this representation, one can clearly see the effect of increasing field on the central $+1/2 \rightarrow -1/2$ sextet of lines. They are substantially broadened at low frequencies due to higher-order contributions of the zero-field splitting (zfs),[19] but broadening is reduced as the sample is moved toward its high-field limit.

No broadening or field-dependent (*g*) or -independent (*A*) splitting of the central lines was observed up to 15 T, where the line width is at its narrowest. This indicates that *g*-anisotropy is $<0.000\ 05$ and that *g* and *A* are, in fact, very similar for the C- and N-terminal Mn(II) ions.

Spectral simulations were performed with the "easyspin" toolbox for Matlab.[20] The main sextet could be simulated considering a Mn(II) center with zfs parameters $D = 1200$ MHz and $E = 250$ MHz (site I in Table 1). However, this did not account for the weaker shoulders on the high- and low-field sides of the lines and a second Mn(II) species was considered (site II in Table 1). Figure 2A shows the simulation, together with the experimental W-band spectrum of OxDC in HMTA buffer. Simulations at the other frequencies with the same parameter set are provided as Supporting Information.

Note that both sites are present in the same proportion, and site II has a considerably higher *D* value (2700 MHz). This explains why its signal intensity is spread out over a broader field range and is seen only in the form of relatively weak shoulders on the narrow and intense lines of the site I signals, even at high frequencies. For this reason, the multifrequency approach was crucial to detect, identify, and characterize the signals from the Mn(II) ion in site II.

The best simulations required substantial *D*- and *E*-strain (20−24%) which is not uncommon for transition metal ions in proteins and, in particular, for Mn(II).[19] The *E*/*D* ratio was found to be ∼21%, indicating considerable rhombicity of the distorted octahedral coordination environment of both Mn(II) ions.

OxDC has maximum activity at a pH value of around 4.0, and it is common practice in the literature to use various types of negatively charged buffer molecules to control pH for spectroscopic and kinetic analysis.[3,9,10,13] Therefore, we investigated the effect of acetate buffer at pH 5.2 on the EPR spectra. At this pH value, the enzyme possesses substantial activity but is also highly soluble. The spectra recorded at various frequencies from X-band to 420 GHz are shown in Figure 3. The differences between this and the previous set of spectra are small and are most obvious in the intermediate to high-frequency ranges (W-band and up). They mainly involve the shoulders associated with site II. Figure 2B shows the experimental W-band signal of OxDC in acetate buffer pH 5.2 with its simulation. The changes in the features of site II are mainly due to a decrease of *D* and *E* from 2700 to 2150 MHz and 675 to 108 MHz, respectively, as well as a small decrease in *g* (see Table 1). The magnetic parameters of site I were unchanged except for an increase in zfs parameter strain, which was also seen for site II. Note that the rather dramatic change in *E* indicates a more axial ligand field environment for site II in the presence of acetate.

The zfs parameters of Mn(II) have been demonstrated to be sensitive to electrostatic charges in their vicinity.[21] The replacement of one or two water molecules in the coordination sphere of Mn by acetate is certainly expected to change the electrostatic potential around the Mn center and could lead to the observed changes in *D* and *E*. The observation that only site II changes upon exposure to acetate buffer suggests that only site II is solvent-accessible. When formate is added to OxDC in HMTA buffer, the spectral changes observed for site II are very similar to those found for acetate (see Figure 2C). This is not surprising, given that formate and acetate are alike in the polar parts of their structures and are expected to show the same coordination geometries with the metal ion.

The simplest interpretation of these results points to a correlation between the two magnetic parameter sets and the two Mn-binding sites in the protein. The differences in the fine structure are due to subtle differences in the charge distribution in the N- and C-terminal binding sites, whereas the almost identical *g* and *A* are due to similar octahedral coordination in both sites. The fact that both species are reproducibly present in approximately the same concentration in all preparations investigated so far supports this interpretation.

The observation of changes in the fine structure parameters of only site II upon addition of acetate buffer or formate is intriguing and suggests that small molecule binding mainly takes place at site II and not site I under our experimental conditions. Just et al.[10] pointed out a channel leading from the N-terminal Mn binding site to the solvent that may be accessible by the hinge motion of a flexible loop region, but they reported no

TABLE 1: Magnetic Parameters of OxDC Species I and II

	g	A (MHz)	ΔB^a (mT)	D (MHz)	E/D	D- and E-strain (%)
SB,[b] site I	2.000 87	253 ± 2	1.2	1200 ± 50	0.21 ± 0.02	24, 24
SB,[b] site II	2.000 94	250 ± 3	1.2	2700 ± 50	0.25 ± 0.02	20, 20
AB,[c] site I	2.000 86	252 ± 2	1.2	1200 ± 50	0.21 ± 0.02	40, 40
AB,[c] site II	2.000 86	250 ± 3	1.3	2150 ± 50	0.05 ± 0.02	33, 60
SB + formate,[b] I	2.000 87	253 ± 2	1.2	1200 ± 50	0.21 ± 0.02	24, 24
SB + formate,[b] II	2.000 86	250 ± 3	1.2	2150 ± 50	0.05 ± 0.02	33, 60

[a] ΔB: Gaussian line widths. [b] SB: HMTA buffer pH 6.0. [c] AB: acetate buffer pH 5.2.

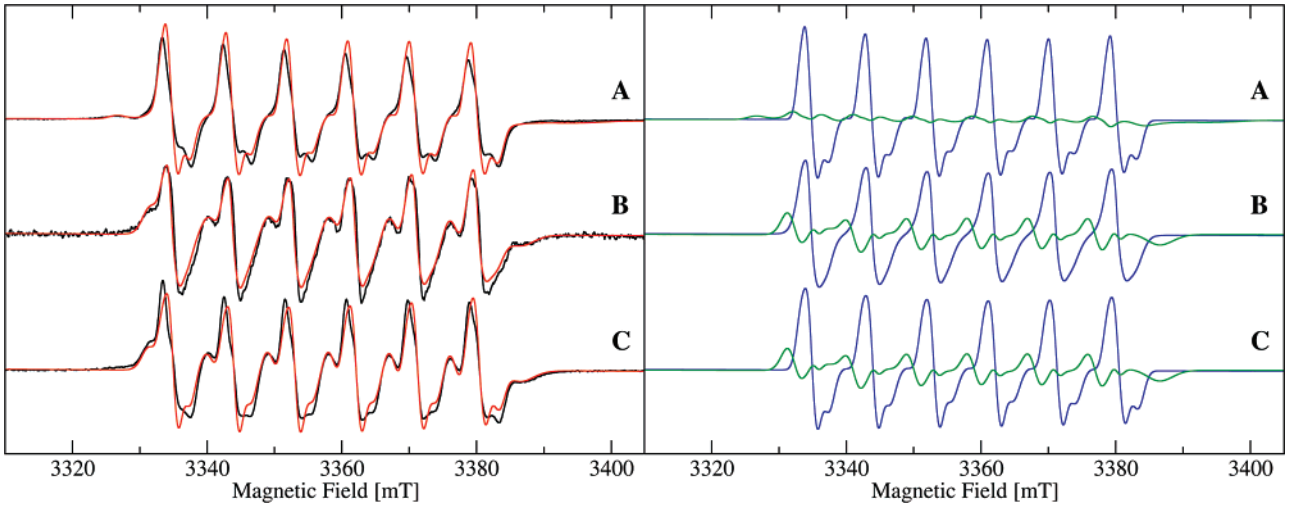

Figure 2. W-Band (94 GHz) EPR spectra of OxDC. (A) HMTA buffer, pH 6.0. (B) Acetate buffer, pH 5.2. (C) HMTA buffer, pH 6.0, and 50 mM formate. The right panel shows the simulation of sites I (blue) and II (green) with the magnetic parameters given in Table 1. The left panel shows the experimental spectra (black) and the sum of the simulations of the two sites in the same proportion.

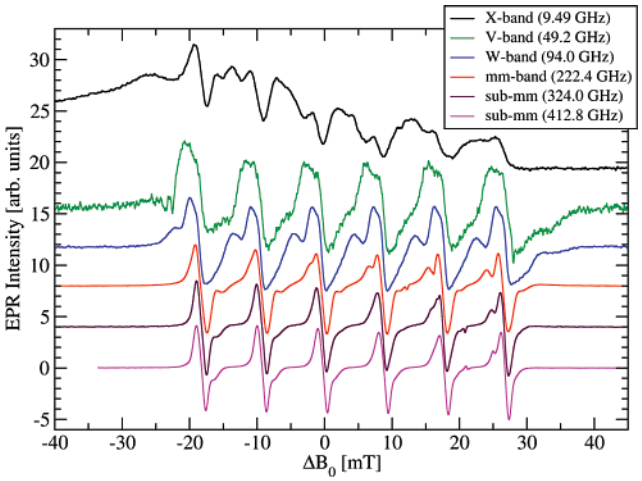

Figure 3. Field dependence of the EPR spectra of OxDC in acetate buffer (50 mM, pH 5.2) with 0.5 M NaCl. The spectra were shifted along the B_0 axis. Field positions in T at the zero points: 0.3339 (X-band, 9.4853 GHz), 1.7470 (V-band, 49.200 GHz), 3.353 (W-band, 94.0206 GHz), 7.9415 (222.40 GHz), 11.563 (324.00 GHz), and 14.7253 (412.80 GHz). Temperatures were set between 5 and 20 K.

obvious solvent channel available for the C-terminal site. Moreover, formate was found coordinated to the N-terminal Mn(II) in the X-ray structure by Anand et al.[9] Therefore, it seems reasonable to identify site II with the N-terminal Mn-binding site and site I with the C-terminal site.

The open and closed conformations[9,10] of OxDC show the C-terminal Mn-binding site in hexa- and penta-coordinated forms, respectively. It is worth noting that D values for penta-coordinated Mn(II) centers in MnSOD have been reported to be 1 order of magnitude higher than what we found for site I.[22] Therefore, our site I magnetic parameters are compatible with the hexa-coordinated Mn(II) ion in the C-terminal open Mn site that is observed in the X-ray structure of OxDC originally published by Anand et al.[9]

Conclusions

A multifrequency EPR approach has allowed us to demonstrate conclusively that two major spectroscopically distinct Mn(II) species are present in equal proportions in the resting state of the enzyme oxalate decarboxylase in HMTA storage buffer. The main difference between these two species is the value of the fine structure parameters with $D_I = 1200$ MHz, $D_{II} = 2700$ MHz, and $E/D = 0.21$. When the enzyme is placed in acetate buffer pH 5.2 or when formate is added, D_{II} is reduced to 2150 MHz and $E_{II}/D_{II} = 0.05$ while D_I and E_I remain the same, indicating that only one Mn(II) is solvent accessible. On the basis of published crystal structure data, we conclude that site I is the C-terminal Mn site and that site II is the solvent-exposed, N-terminal site and, therefore, the site of small molecule (acetate and formate) binding.

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Supporting Information Available: EPR spectra of OxDC in HMTA storage buffer, pH 6.0, with and without formate present and in acetate buffer, pH 5.2, at the various field/frequency combinations and their simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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