REDOX Cycling, pH Dependence, and Ligand Effects of Mn(III) in Oxalate Decarboxylase from *Bacillus subtilis*

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Supplemental Information

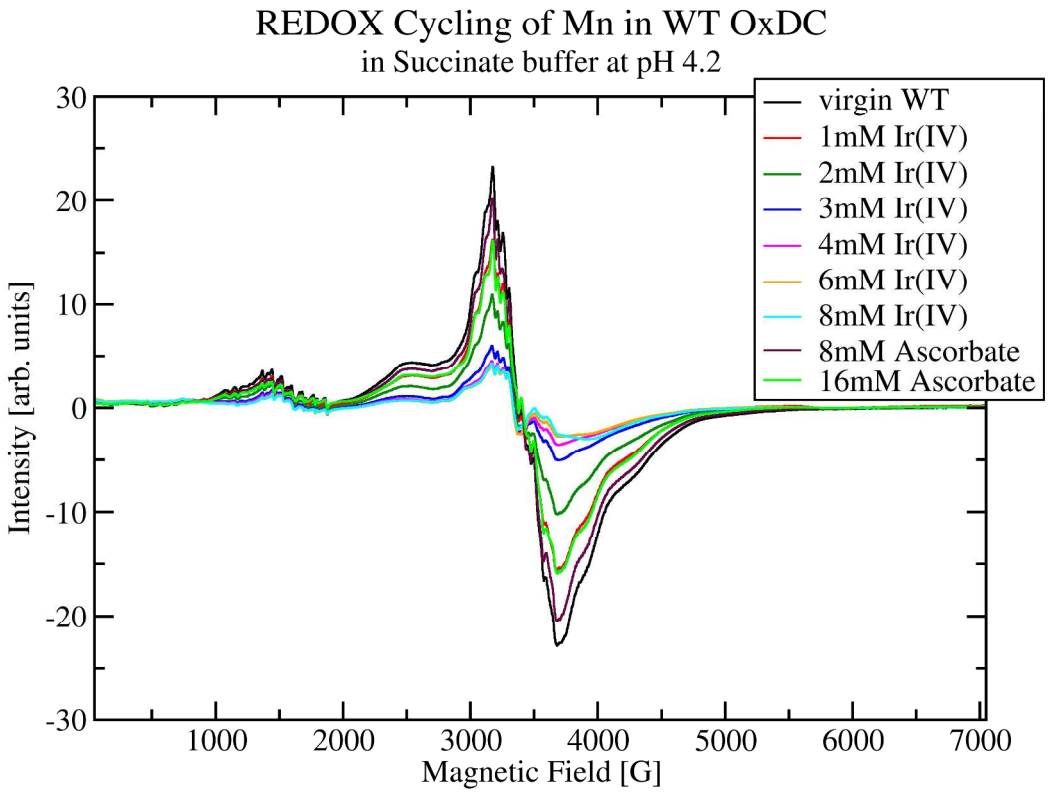

Figure S1: Redox titration of WT OxDC at pH4.2 in 50mM succinate buffer. Spectra were taken with the sample treated with hexachloroiridate(IV) and ascorbate as stated in the legend. Subsequent scans were taken of the same sample which was treated by addition of small aliquots of stock oxidant/reductant solution (100 mM potassium hexachloroiridate and 100 mM ascorbate) to achieve the stated concentrations. The sample was frozen in liquid nitrogen and inserted into the pre-cooled liquid helium cryostat for each EPR scan at 5 K, then thawed and treated with additional oxidant/reductant. EPR parameters: perpendicular mode, microwave frequency of 9.618 GHz, modulation amplitude was 100 kHz, modulation amplitude was 10 G, and microwave power 0.63 mW.

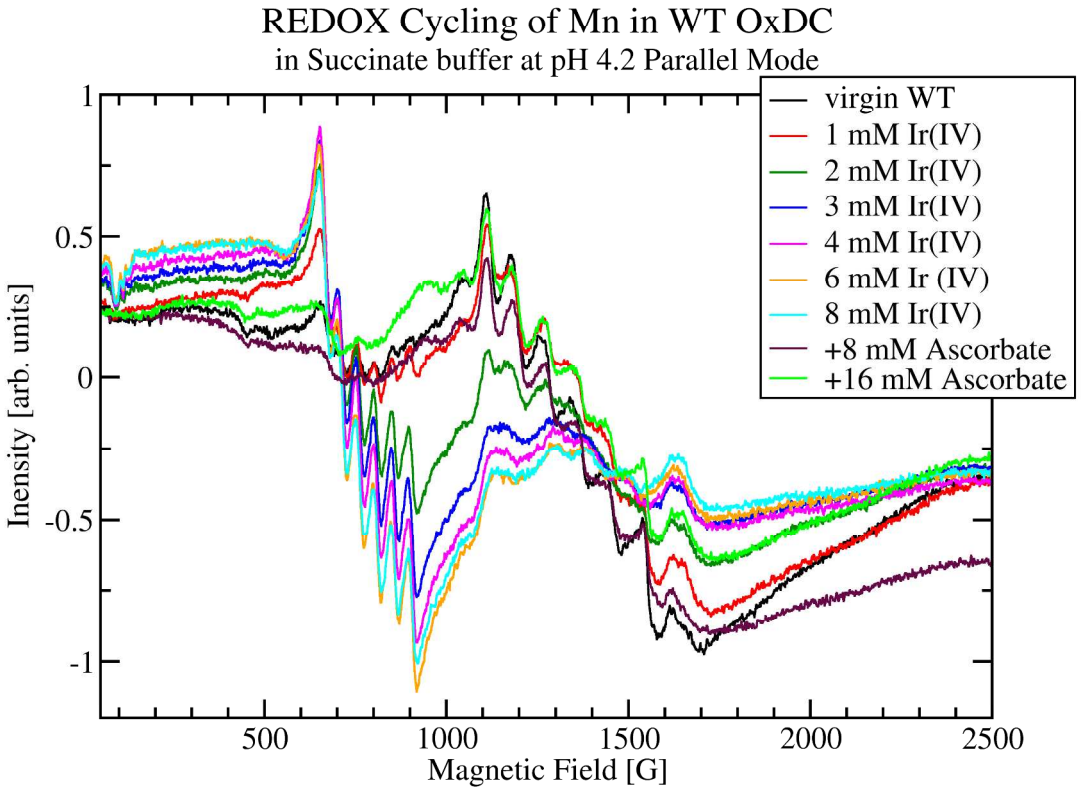

Figure S2: Redox titration of WT OxDC at pH4.2 in 50mM succinate buffer. Spectra were taken with the sample treated with hexachloroiridate(IV) and ascorbate as stated in the legend. Subsequent scans were taken of the same sample which was treated by addition of small aliquots of stock oxidant/reductant solution (100 mM potassium hexachloroiridate and 100 mM ascorbate) to achieve the stated concentrations. The sample was frozen in liquid nitrogen and inserted into the pre-cooled liquid helium cryostat for each EPR scan at 5 K, then thawed and treated with additional oxidant/reductant. EPR parameters: parallel mode, microwave frequency of 9.331 GHz, modulation amplitude was 100 kHz, modulation amplitude was 10 G, and microwave power 0.63 mW.

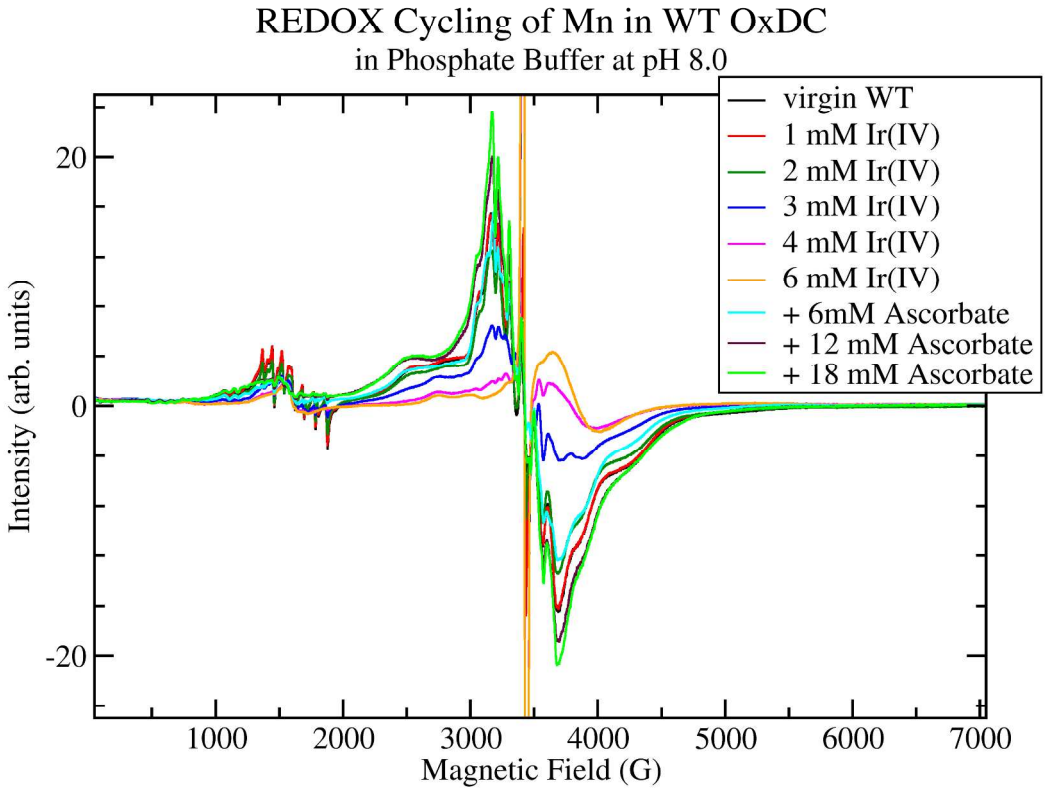

Figure S3: Redox titration of WT OxDC at pH8.0 in 50mM phosphate buffer. Spectra were taken with the sample treated with hexachloroiridate(IV) and ascorbate as stated in the legend. Subsequent scans were taken of the same sample which was treated by addition of small aliquots of stock oxidant/reductant solution (100 mM potassium hexachloroiridate and 100 mM ascorbate) to achieve the stated concentrations. The sample was frozen in liquid nitrogen and inserted into the pre-cooled liquid helium cryostat for each EPR scan at 5 K, then thawed and treated with additional oxidant/reductant. EPR parameters: perpendicular mode, microwave frequency of 9.618 GHz, modulation amplitude was 100 kHz, modulation amplitude was 10 G, and microwave power 0.63 mW.

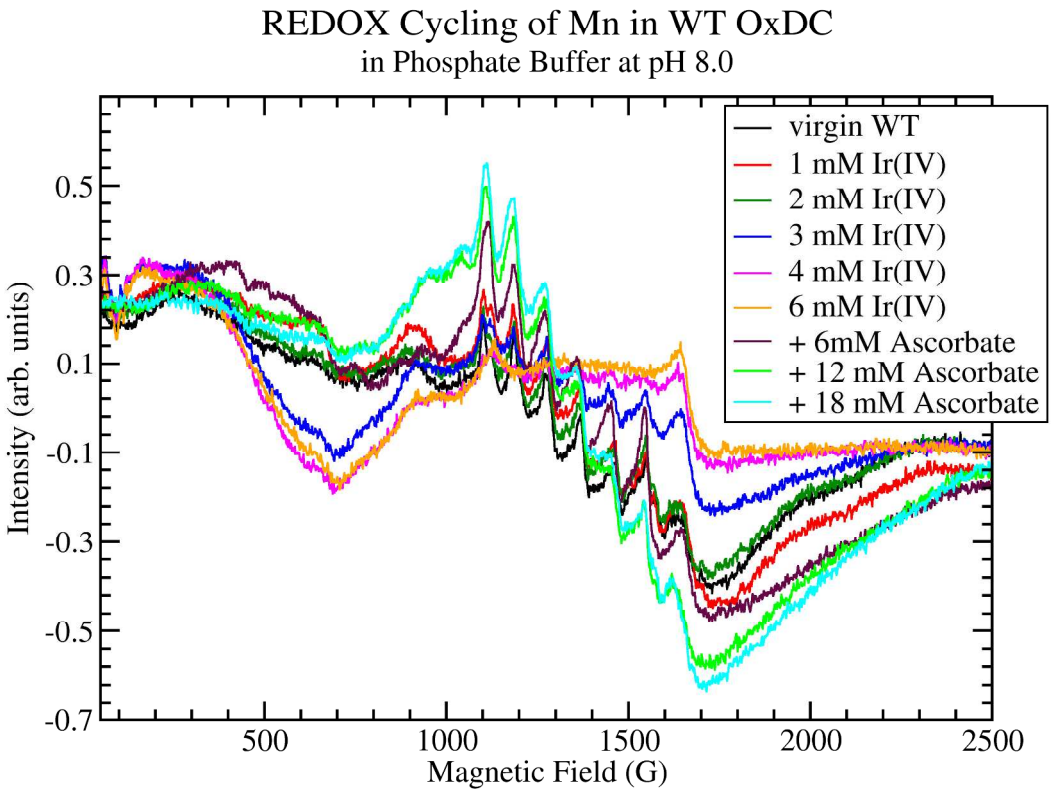

Figure S4: Redox titration of WT OxDC at pH8.0 in 50mM phosphate buffer. Spectra were taken with the sample treated with hexachloroiridate(IV) and ascorbate as stated in the legend. Subsequent scans were taken of the same sample which was treated by addition of small aliquots of stock oxidant/reductant solution (100 mM potassium hexachloroiridate and 100 mM ascorbate) to achieve the stated concentrations. The sample was frozen in liquid nitrogen and inserted into the pre-cooled liquid helium cryostat for each EPR scan at 5 K, then thawed and treated with additional oxidant/reductant. EPR parameters: parallel mode, microwave frequency of 9.331 GHz, modulation amplitude was 100 kHz, modulation amplitude was 10 G, and microwave power 0.63 mW.

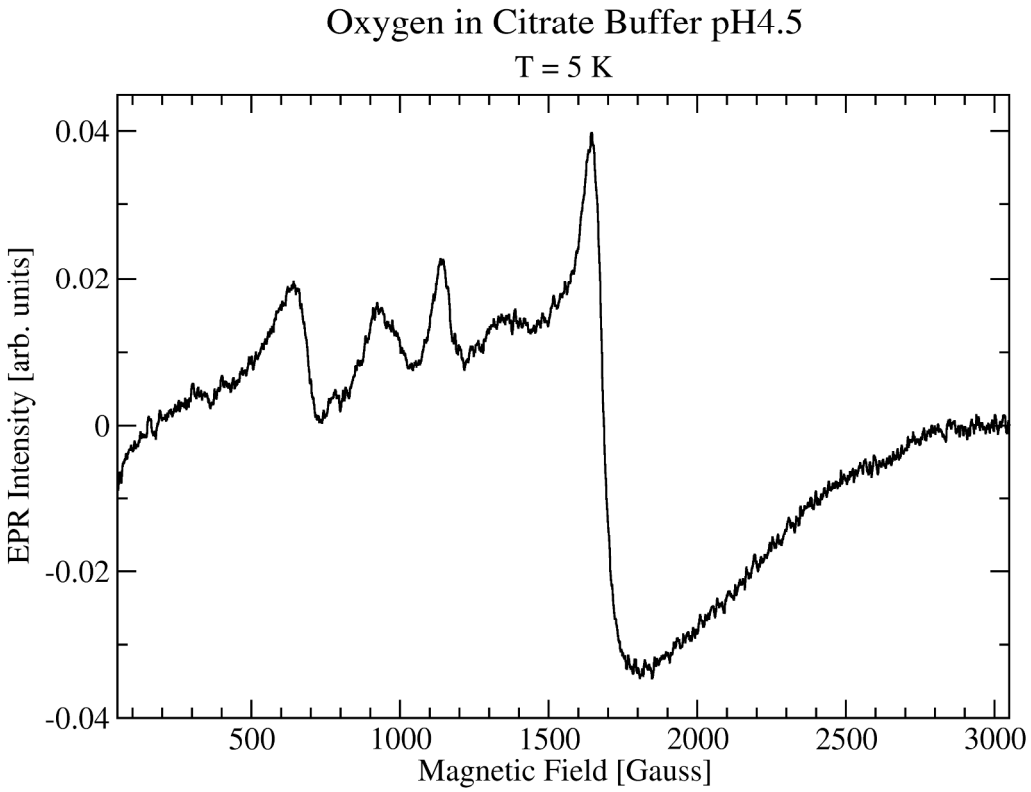

Figure S5: Background spectrum in parallel mode at 5 K. This sample only contained 50 mM citrate buffer at pH4.5 without OxDC. Dissolved dioxygen was present under normal aerobic conditions (approx. 260 μM). Microwave frequency ~9.331 GHz, 5.0 G modulation amplitude, 100 kHz modulation frequency, 60 dB receiver gain, 40 ms conversion time

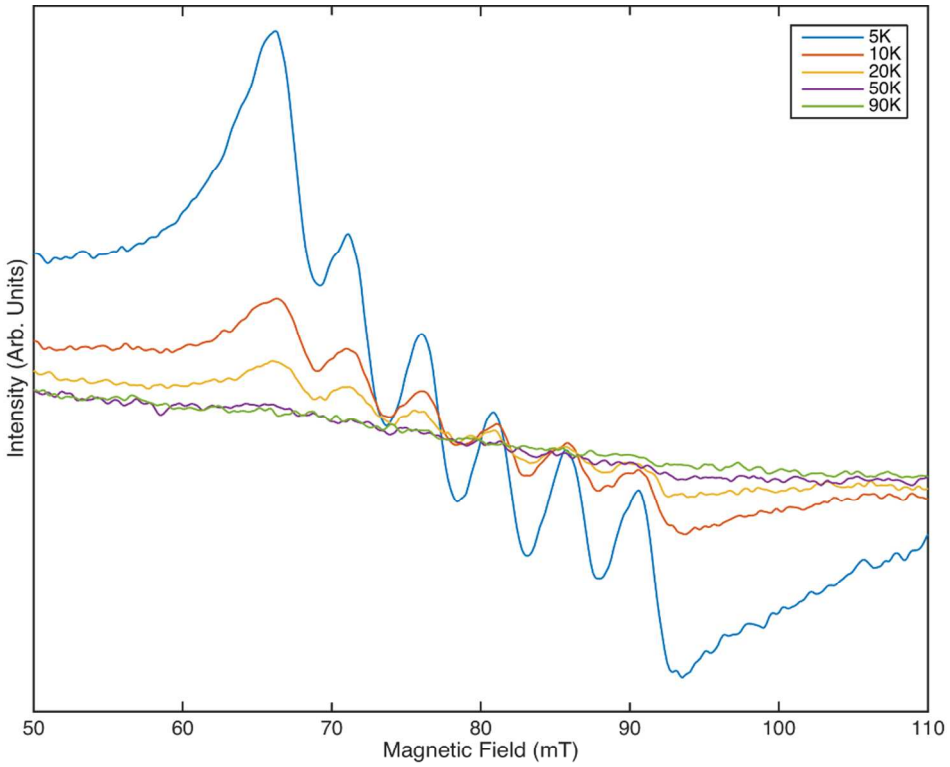

Figure S6: Temperature dependence between 5K and 90K of Mn(III) generated in oxidized wild-type OxDC (25 mg/ml) in 50 mM succinate, 500 mM NaCl posed at pH 4.2 using hexachloroiridate as the oxidizing agent. EPR Parameters: 9.406 GHz microwave frequency, 100 kHz modulation frequency, 10 G modulation amplitude, 2 mW microwave power. Experiments were conducted under non-saturating conditions.

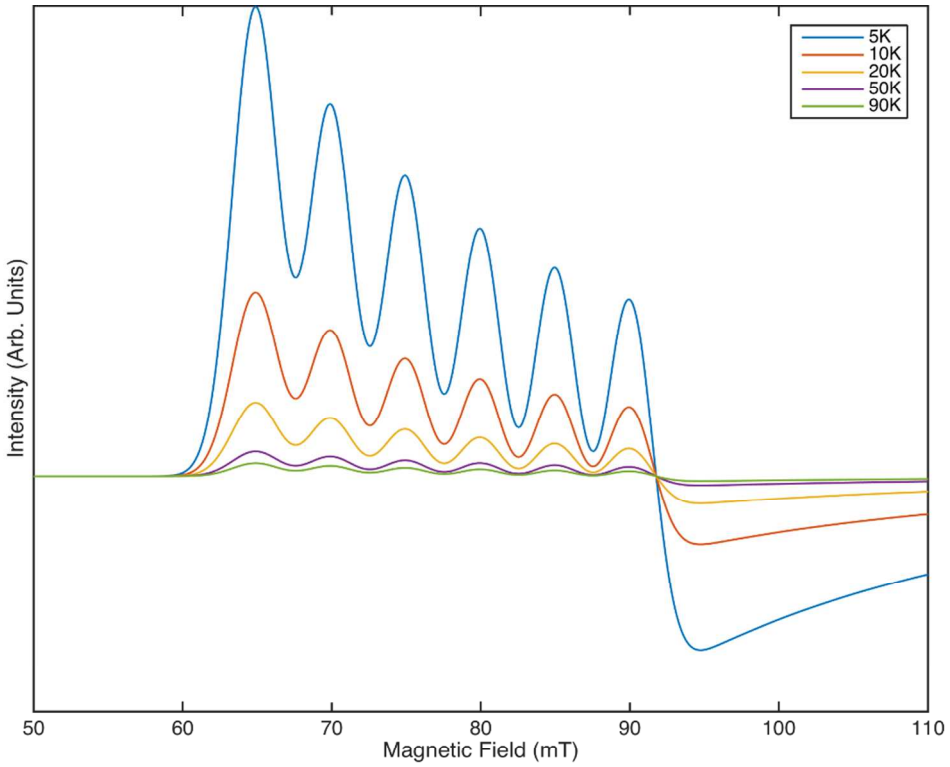

Figure S7: Temperature dependent simulations of Mn(III) generated in oxidized wild-type OxDC between 5K and 90K for confirmation of the sign of D. Simulation parameters: g = 2.00, A = 140 MHz, D = -2.38 cm^{-1}, |E/D| = 0.13.

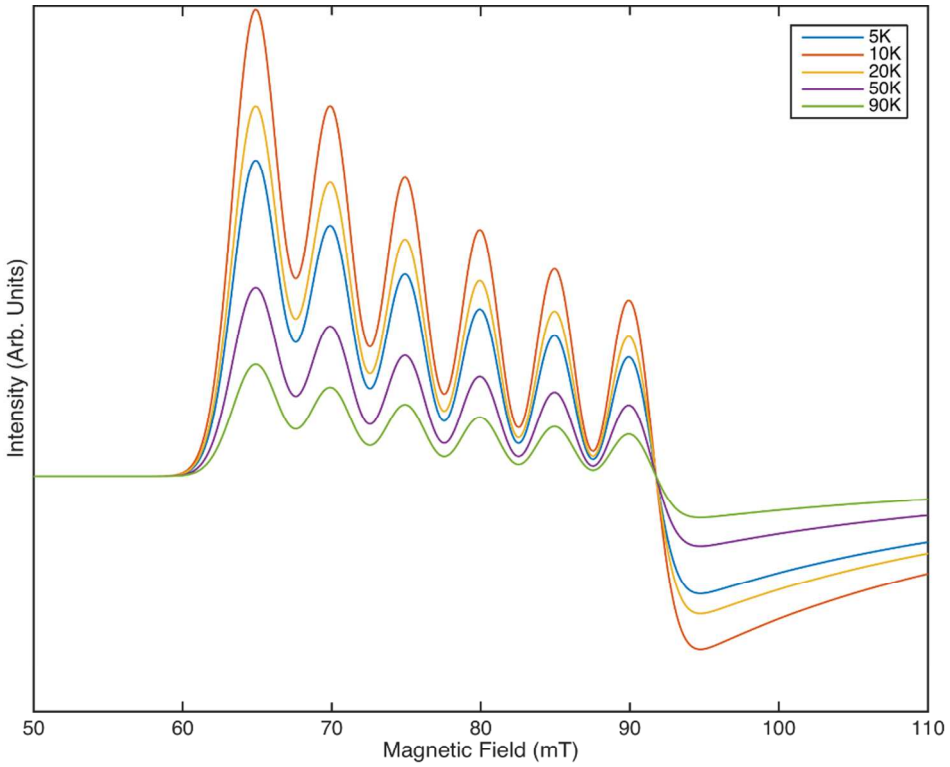

Figure S8: Temperature dependent simulations of Mn(III) generated in oxidized wild-type OxDC between 5K and 90K for confirmation of the sign of D. Simulation parameters: g = 2.00, A = 140 MHz, D = +2.38 cm^{-1}, |E/D| = 0.13.

Redox Cycling of OxDC at High pH(8.0), High Field EPR Spectra

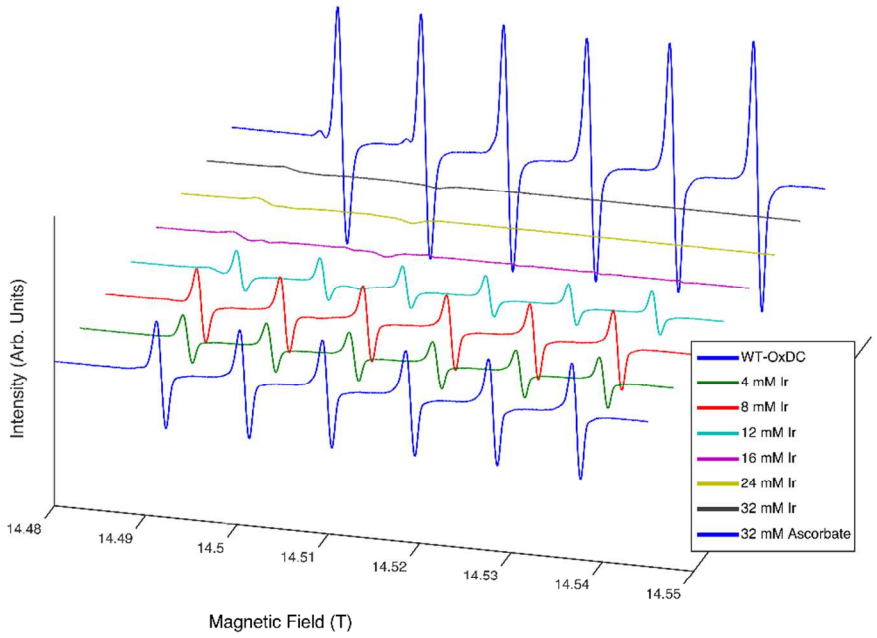

Figure S9: Redox titration of (25 mg/ml) wild-type OxDC in 50 mM phosphate, 500 mM NaCl poised at pH 8.0 using hexachloroiridate and ascorbate as oxidizing and reducing agents, respectively, at 406.4 GHz HF-EPR. Instrumental parameters: 50 kHz modulation frequency, 1 G modulation amplitude, 0.2 mT/s sweep rate and at 20K.

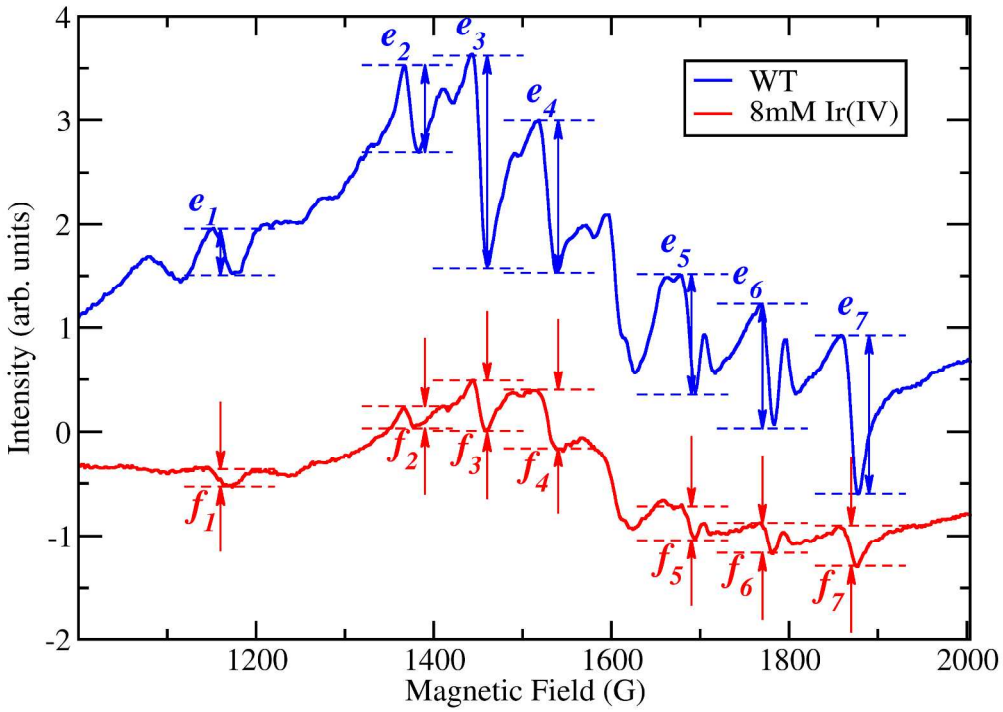

Figure S10: Relative spin quantitation for the Mn(II) signal at g ≈ 4.2 in perpendicular mode for virgin enzyme (blue trace) and the oxidized enzyme (red trace). Spectra are the same as in fig. 1A of the paper.

Quantitative Analysis of the amount of Mn(III) present in virgin enzyme:

For quantitative analysis we consider three different samples, (1) untreated enzyme, (2) fully oxidized enzyme (at 8 mM hexachloroiridate), and (3) fully reduced enzyme (after reduction with excess ascorbate). The perpendicular and parallel mode spectra of virgin and oxidized enzyme at low pH used for this analysis are shown in figs. 1A and 1B and again in figs. 4A and 4B but magnified to the spectral range being evaluated and with arrows indicating the signal intensities used. Fig. S10 shows the $g \approx 4.2$ region of the perpendicular mode spectra for virgin and oxidized enzyme taken from fig. 1A. In addition we wanted to evaluate ascorbate treated enzyme (without prior oxidation). Since it did not show any Mn(III) there was no point in analyzing its parallel mode spectrum. However, the perpendicular mode spectrum of this sample was analyzed for the magnitude of its Mn(II) signals in the $g \approx 2$ and $g \approx 4.2$ region as seen in figs. S11 and S12.

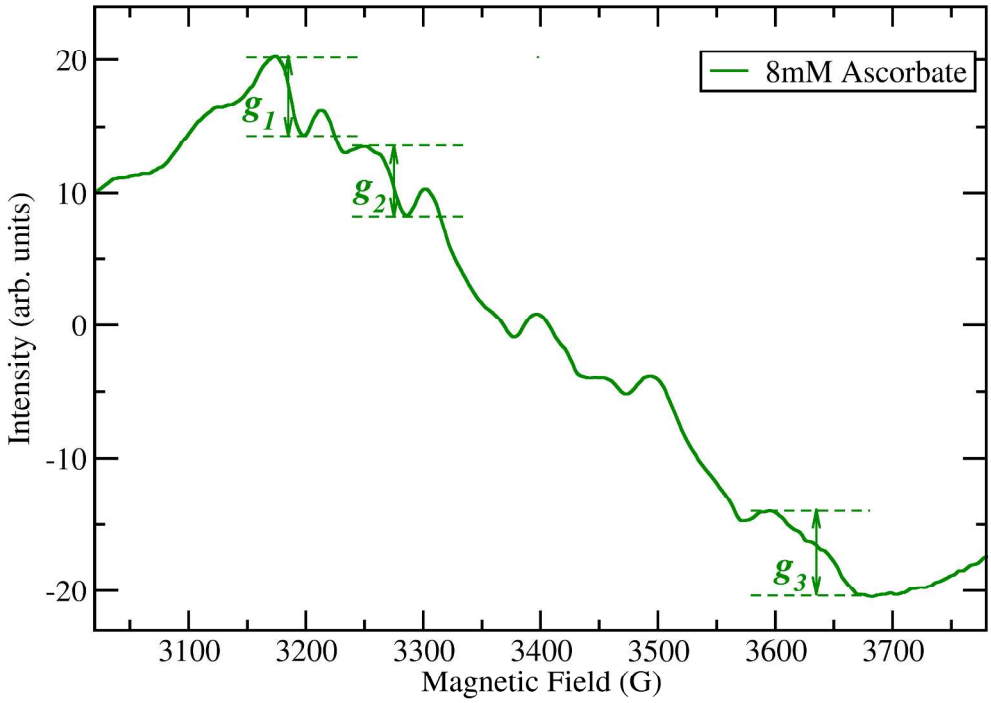

Figure S11: Spin quantitation of the Mn(II) signal at g ≈ 2 in perpendicular mode for reduced enzyme (8 mM ascorbate). The spectral positions at which intensities were taken (labeled g_i) are the same as for the virgin and oxidized samples (see fig. 4A).

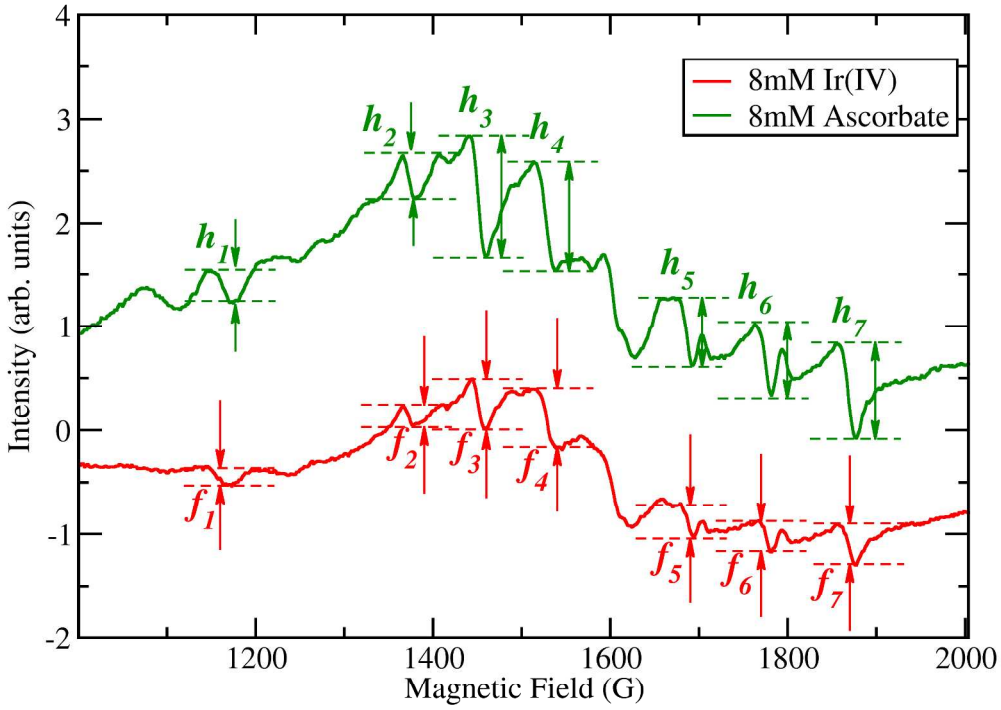

Figure S12: Spin quantitation of the Mn(II) signal at g ≈ 4.2 in perpendicular mode for reduced enzyme (8 mM ascorbate, green trace). The red trace represents the oxidized sample and is the same spectrum as in fig. S10. The spectral positions at which intensities were taken (labeled h_i) are the same as for the virgin and oxidized samples (see fig. S10).

For signal quantitation we focused on three spectral areas in which either Mn(II) or Mn(III) signals were visible. In perpendicular mode spectra the $g \approx 2$ and $g \approx 4.2$ region was analyzed separately. The N-terminal Mn(II) with its lower magnitude of $|D|$ absorbs mainly near $g \approx 2$ while the C-terminal Mn(II) with its larger magnitude of $|D|$ is difficult to pinpoint at X-band. The signal near $g \approx 4.2$ contains intensity from a non-principal axis turning point of Mn(II) species with large zero field splittings and was therefore assumed to contain intensity information primarily about the C-terminal Mn(II). For the intensity of the Mn(III) species the $g \approx 8.8$ region of the parallel mode spectra was used. Table S1 shows a matrix indicating the three different samples and three different spectral regions with the average intensities gathered from the spectra labeled *a* through *h*. This nomenclature will be used in the following discussion of how the quantitation was carried out.

Sample Mn-Species	Virgin enzyme (untreated)	Fully oxidized (8mM iridate)	Fully reduced (8mM ascorbate)
N-terminal Mn(II)	$a = \langle a_i \rangle$ = 16.2204	$b = \langle b_i \rangle$ = 2.9602	$g = \langle g_i \rangle$ = 14.8903
C-terminal Mn(II)	$e = \langle e_j \rangle$ = 1.2406	$f = \langle f_j \rangle$ = 0.3583	$h = \langle h_j \rangle$ = 0.7342
Mn(III)	$d = \langle d_i \rangle$ = 0.1032	$c = \langle c_i \rangle$ = 0.4752	-

Table S1: List of samples used for the quantitative analysis of three different Mn species, the N- and C-terminal Mn(II) and Mn(III). A distinction between N- and C-terminal Mn(III) is not possible at this time and we assume that both have similar spectra. The intensities listed are averages for individual peaks as indicated in figs. 4A, 4B, and S10 – S12. For the N-terminal Mn(II) and Mn(III) three or four spectral lines were used (index i runs from 1 through 3 or 4) while for the C-terminal Mn(II) seven spectral lines were used (index j runs from 1 through 7).

Our problem is to map the eight different EPR intensities on eight different Mn(II) (N- and C-terminal are distinct) and Mn(III) concentrations in order to figure out the desired concentration of Mn(III) in the virgin sample. Note, that the Mn(III) concentration and its EPR intensity in the fully reduced sample are assumed to be zero. This mapping can be done with a system of constraints given by the samples and expressed in terms of linear equations. We assume a linear relationship between the EPR intensity for a given signal and its corresponding species concentration. This linear relationship was enforced experimentally by working in the linear power regime for all signals. Since we have three different Mn species with very different spectra both in parallel and perpendicular modes and since we model the intensity not by spectral simulation but by the average of a reasonable choice of peak-to-trough distances, we expect three distinct scaling factors which enter the system of equations as unknowns. These scaling factors are used to convert EPR intensity into a given concentration and are defined as:

$$\chi_1 = \frac{\left[\mathrm{Mn_{II}^{N}}\right]}{I^{\perp}_{g\approx 2}} \qquad (1)$$

$$\chi_2 = \frac{\left[\mathrm{Mn_{II}^{C}}\right]}{I^{\perp}_{g\approx 4.2}} \qquad (2)$$

$$\chi_3 = \frac{[\mathrm{Mn_{III}}]}{I^{\parallel}_{g\approx 8.8}} \qquad (3)$$

The index i of the scaling factors χ_i (i = 1, 2, 3) refers to the N- and C-terminal Mn(II) signals observed in perpendicular mode (index 1 and 2, respectively), and to Mn(III) observed in parallel mode. The EPR intensities I with superscripts indicating parallel or perpendicular mode spectra and subscript indicating spectral region are the intensities listed in table S1 and are taken from the spectra as described above. We obtain experimental numbers for these EPR intensities from three different samples, untreated, oxidized, and reduced OxDC. They reflect the concentrations of the corresponding Mn species in these different samples. This means that there are 9 different Mn concentrations and 9 different EPR intensities to consider, *i.e.*, three different versions of eqs. (1 – 3) for the three different sample conditions. However, the three scaling factors remain the same from sample to sample since we used identical sample tubes and instrument settings, the only difference between measurements being the addition of a small aliquot of oxidant or reductant. To obtain the three scaling factors we need to set up the following material balance equations that reflect our assumptions of how the different Mn species behave under differing experimental conditions:

$$\left[\mathbf{Mn_{II}}^{\mathbf{N}}\right] + \left[\mathbf{Mn_{II}}^{\mathbf{C}}\right] + [\mathbf{Mn_{III}}] = \gamma\,[\mathbf{E_0}] \qquad (4)$$

$$\left[\mathbf{Mn_{II}}^{\mathbf{N}}\right]_{\mathbf{ox}} + \left[\mathbf{Mn_{II}}^{\mathbf{C}}\right]_{\mathbf{ox}} + [\mathbf{Mn_{III}}]_{\mathbf{ox}} = \eta_{\mathbf{ox}}\gamma\,[\mathbf{E_0}] \qquad (5)$$

$$\left[\mathbf{Mn_{II}}^{\mathbf{N}}\right]_{\mathbf{red}} + \left[\mathbf{Mn_{II}}^{\mathbf{C}}\right]_{\mathbf{red}} + [\mathbf{Mn_{III}}]_{\mathbf{red}} = \eta_{\mathbf{red}}\gamma\,[\mathbf{E_0}] \qquad (6)$$

$$[\mathbf{Mn_{III}}]_{\mathbf{red}} = \mathbf{0} \qquad (7)$$

The subscripts ‘ox’ and ‘red’ represent the concentrations of the corresponding Mn species in the oxidized and reduced samples. No subscript indicates the untreated sample. [E_0] refers to the enzyme concentration in the sample which was determined by the Bradford assay (see Materials and Methods section). The coefficient γ is the Mn content per enzyme subunit which was determined using ICPMS and consistently was around 1.6 for our samples. The coefficients η_{ox} and η_{red} are the dilution factors that arise from the addition of small aliquots of oxidant or reductant to the enzyme sample. They turned out to be 0.926 for the oxidized and 0.917 for the reduced sample. Please note that we didn’t observe a Mn(III) signal in the fully reduced state and therefore assume that its Mn(III) concentration has been reduced to zero. This lowers the number of unknown concentrations to 8 for which 8 different EPR signals were recorded.

The material balance equations show the split between N- and C-terminal site only for Mn(II) and not for Mn(III) because we don’t have any experimental spectrum for the latter that would indicate a difference. The C-terminal Mn(III) could potentially be EPR-inactive in X-band parallel mode. In this case we would overestimate the Mn(III) concentration by up to a factor of 2 and it should therefore be kept in mind that we can only obtain an upper limit for the Mn(III) concentration using this approach. The concentration of enzyme, [E_0], doesn’t need to be known as long as we are only interested in the Mn concentrations relative to it.

The system of linear equations represented by the three different versions of eqs. (1 – 3) and eqs. (4 – 7) can be solved using the linear equation solver in Mathematica [1] and restricting the solutions to be real and positive definite. The results for the relative

concentrations of the three different Mn species in the three different samples are listed in table S2.

Sample Mn-Species	Virgin enzyme (untreated)	Fully oxidized (8mM iridate)	Fully reduced (8mM ascorbate)
N-terminal Mn(II)	34%	7%	50%
C-terminal Mn(II)	50%	16%	50%
Mn(III)	16%	78%	-

Table S2: Relative amounts of the N- and C-terminal Mn(II) and Mn(III) in the three samples based on the quantitative EPR results.

The most important result is that Mn(III) in untreated virgin enzyme amounts to about 16% of the total Mn content. This results is based on several assumptions apart from the quantitation of the EPR signals. We assume that initially Mn(III) is located on the N-terminal site since it is easier to oxidize. This is supported by the observation that the C-terminal Mn(II) is not as strongly suppressed as the N-terminal one when the sample is oxidized. We also assume that in the oxidized sample the C-terminal and the N-terminal Mn(III) have the same spectrum and both add up to 78% of the total Mn content. If this is not the case the parallel mode EPR signal at $g \approx 8.8$ would correspond to less than 78% of the overall Mn content. The corresponding signal in the virgin enzyme sample would consequently arise from less than 16% of the total Mn. Therefore, the estimate of 16% has to be considered as an upper limit for the relative amount of Mn(III) in untreated virgin OxDC.

Parallel Mode EPR Spectra of WT OxDC in Citrate Buffer under Turnover Conditions

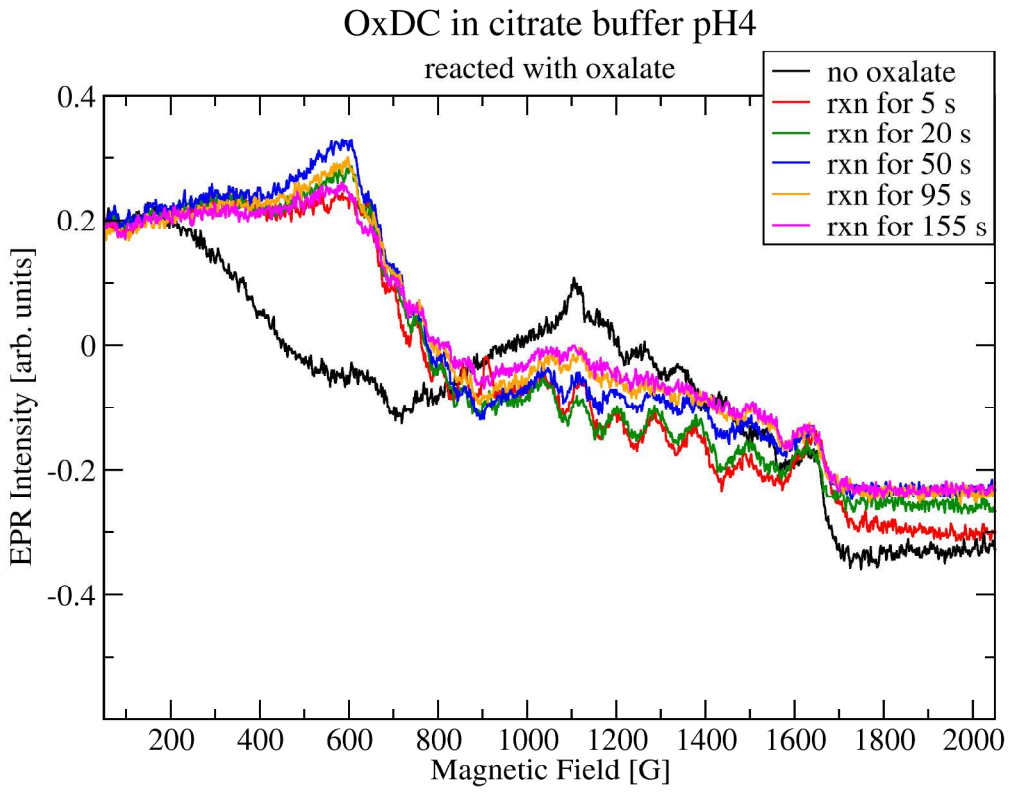

Figure S 13: X-band parallel mode EPR of WT OxDC at pH 4.5 at 5 K at different times after oxalate addition. Black trace: sample in citrate buffer pH4.5 without oxalate, red trace: after addition of 100 mM oxalate pH4.5 and freeze quenched after approximately 5 s of reaction time; green trace: same sample thawed to room temperature and frozen after an additional 15 s of reaction time. Additional traces show the same sample after repeated thawing and refreezing with cumulative total approximated reaction times listed in the legend.

Spectra illustrating the buffer effects on Mn(III):

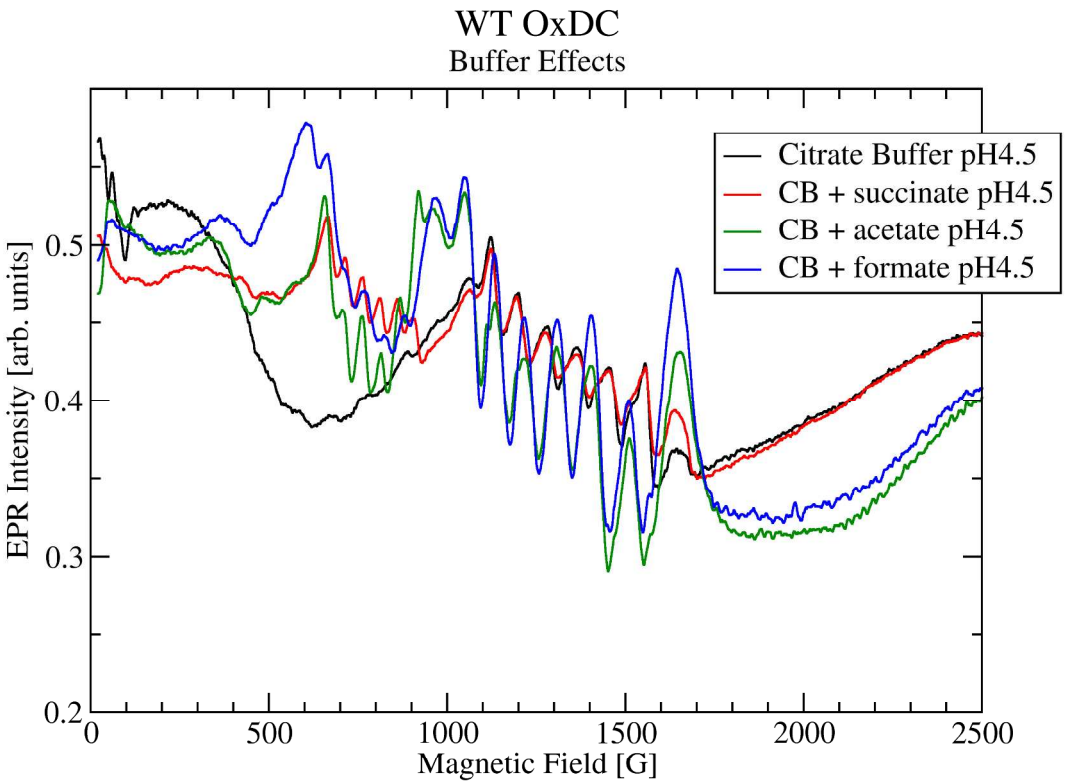

Figure S14: X-band parallel mode EPR of WT OxDC at pH 4.5 at 5 K. Black trace: sample in citrate buffer pH4.5, red trace: after addition of 100 mM succinate pH4.5, green trace: after addition of 100 mM acetate pH4.5 to the original citrate-buffered sample, blue trace: after addition of 100 mM formate pH4.5 to the original citrate-buffered sample.

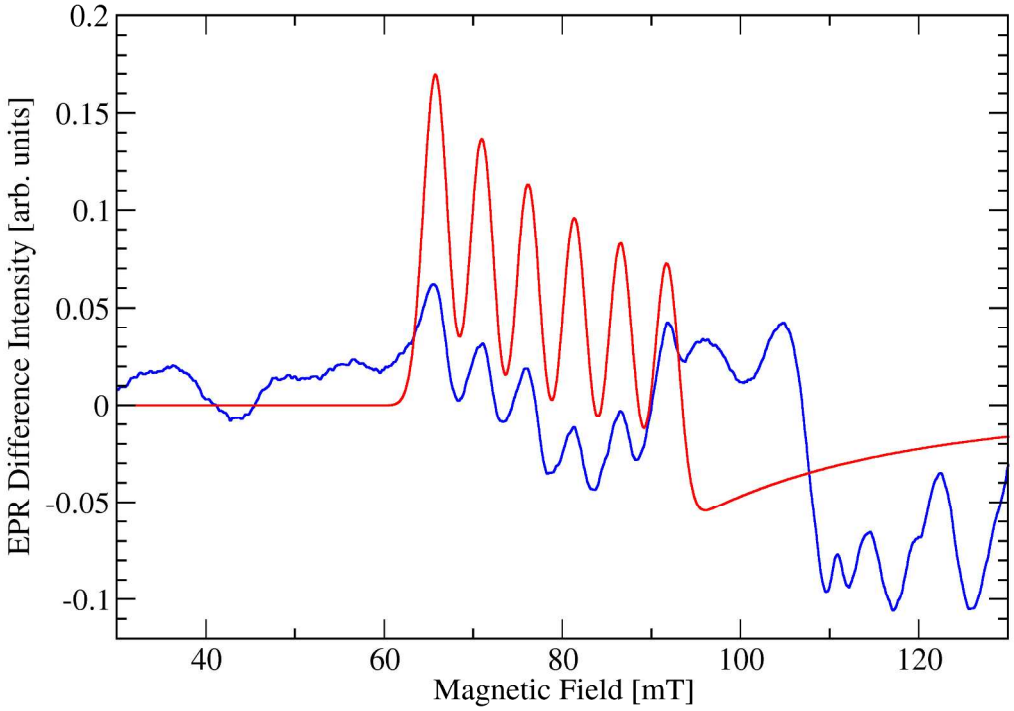

Figure S15: Simulation (red trace) of the Mn(III) signal in OxDC in citrate buffer pH4.5 with 100 mM acetate present. Blue trace: Difference spectrum of sample minus background.

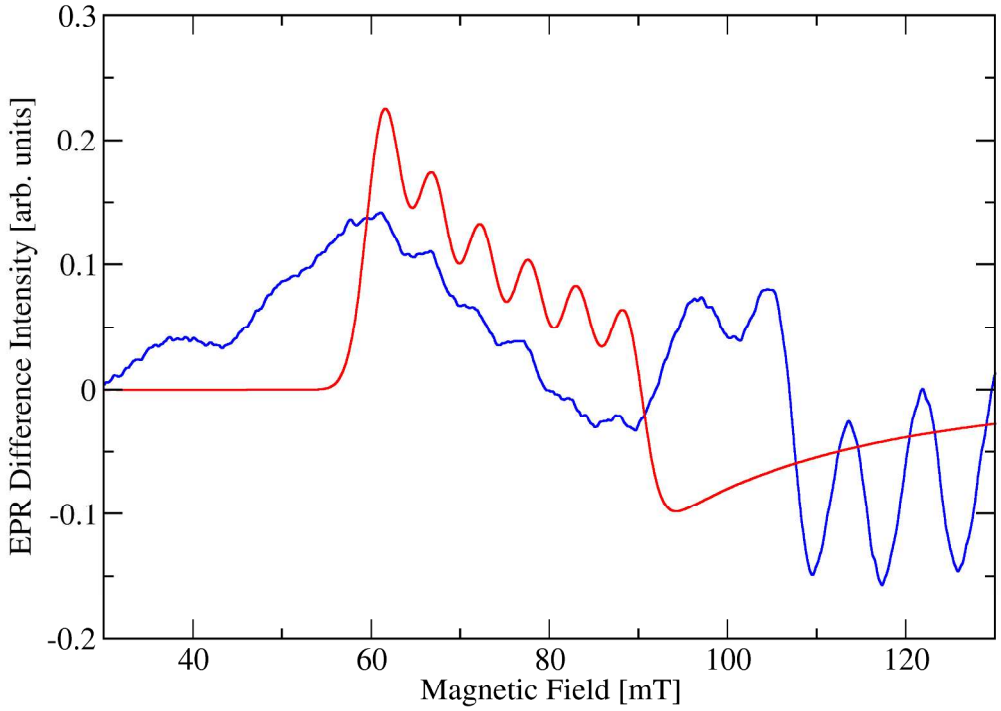

Figure S16: Simulation (red trace) of the Mn(III) signal in OxDC in citrate buffer pH4.5 with 100 mM formate present. Blue trace: Difference spectrum of sample minus background.

Reference:

[1] I. Wolfram Research, Mathematica, Wolfram Research, Inc., Champaign, Illinois.