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EPR Spin Trapping of an Oxalate-Derived Free Radical in the Oxalate Decarboxylase Reaction

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Abstract

EPR spin trapping experiments on bacterial oxalate decarboxylase from *Bacillus subtilis* under turn-over conditions are described. The use of doubly ^{13}C -labeled oxalate leads to a characteristic splitting of the observed radical adducts using the spin trap *N-tert*-butyl- α -phenylnitrone linking them directly to the substrate. The radical was identified as the carbon dioxide radical anion which is a key intermediate in the hypothetical reaction mechanism of both decarboxylase and oxidase activities. X-ray crystallography had identified a flexible loop, SENS161-4, which acts as a lid to the putative active site. Site directed mutagenesis of the hinge amino acids, S161 and T165 was explored and showed increased radical trapping yields compared to the wild type. In particular, T165V shows approximately ten times higher radical yields while at the same time its decarboxylase activity was reduced by about a factor of ten. This mutant lacks a critical H-bond between T165 and R92 resulting in compromised control over its radical chemistry allowing the radical intermediate to leak into the surrounding solution.

Introduction

Oxalic acid is one of the most common dicarboxylic acids in nature and is considered to be an endproduct of metabolism [1]. It is toxic to animals mainly because of its ability to efficiently chelate Ca^{2+} , Fe^{2+} , and Mg^{2+} , and is the main constituent of urinary tract stones [2]. It accumulates in some dietary plants (e.g., rhubarb, sorrel, spinach) which may lead to hyperoxaluria in animals and humans [3]. There appears to be evidence that certain gut-dwelling oxalate-metabolizing bacteria may protect their hosts from developing hyperoxaluria [4].

Oxalate degrading enzymes have evolved in plants, fungi, and bacteria, and can be classified into three distinct categories that employ different enzymatic strategies albeit using similar structural motifs, i.e., oxalate oxidase (OxOx), oxalate decarboxylase (OxDC), and oxalyl-CoA decarboxylase (OXC) [5]. OxDC has been identified as a promising candidate for diverse biotechnical applications in diagnostics, therapeutics, food processing, wood processing, and agriculture [6], and animal studies have been started to test oral formulations of OxDC designed to degrade dietary oxalate in the stomach [7].

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Our interest in OxDC stems from the desire to understand how the protein can direct its chemistry into two different directions, decarboxylation and oxidation. OxDC catalyzes a remarkable transformation in which the C-C bond of monoprotinated oxalate is *non-oxidatively* cleaved to give carbon dioxide and formate [8–10] (eq. 1).

This reaction requires the presence of Mn(II) in the resting enzyme [11] and is generally accepted to depend on the presence of dioxygen [8,12,13] even though the conversion of oxalate to formate and CO₂ is merely a disproportionation reaction. Kinetic isotope effect measurements are consistent with a catalytic mechanism in which decarboxylation takes place via heterolytic cleavage of the C-C bond in a Mn-stabilized oxalate radical anion **1** [8], which is thought to be formed reversibly by proton-coupled electron transfer involving the side chain of Glu-162 (Scheme 1) [14,15]. On the other hand, no direct evidence has been obtained to demonstrate the participation of the radical anion intermediates **1** and **2** (Scheme 1) under catalytic turnover conditions, presumably because of EPR signal broadening through coupling to the neighboring, paramagnetic metal center. Indirect evidence for the production of the carbon dioxide radical anion has been obtained by spin trapping using α -(4-pyridyl 1-oxide)-N-tert-butyl nitron (POBN) [16], albeit in experiments employing an OxDC mutant that exhibited oxidase rather than decarboxylase activity [15,17].

In an interesting set of observations, Bornemann and co-workers identified a mobile loop segment (Ser-161 to Thr-165), which likely adopts an “open” conformation in the resting enzyme thereby allowing substrate access to the Mn(II)-binding site [18]. Crystallographic studies showed that this loop segment can also adopt a “closed” conformation [19,20], which not only correctly positions the Glu-162 side chain carboxylate for its putative catalytic role [16] but also shields the active site from solvent. Given our interest in understanding how the carboxylate side chain of Glu-162 might modulate OxDC reactivity by controlling proton transfer [15], the crystallographic observation that the hydroxyl groups of Ser-161 and Thr-165 formed hydrogen bonding interactions when the loop adopts a closed conformation was intriguing (Figure 1). The interaction between Thr-165 and Arg-92 was of particular interest because we [14], and others [18], have shown the importance of this arginine residue for catalysis. A series of His₆-tagged OxDC mutants was therefore prepared with which to probe the functional importance of the interactions involving the Ser-161 and Thr-165 side chains (Table 1). We note that recent EPR studies have shown that the presence of the His₆-tag at the C-terminus of the enzyme does not perturb the electronic properties of the Mn(II) centers [21].

We now report EPR spin trapping experiments using ¹³C-labeled oxalate on these His₆-tagged OxDC mutants, which directly establish that the carbon-based radical observed in previous studies [15] is indeed derived from the oxalate substrate. In addition, we demonstrate that the amount of this species that is trapped is significantly increased by removal of hydrogen bonding interactions involving the Thr-165 side chain, suggesting that it plays a key role in controlling the conformational preferences of the active site loop.

Materials and Methods

Chemicals

The following chemicals were purchased from Fisher: potassium acetate, potassium chloride, and potassium oxalate. N-tert-butyl- α -phenylnitron (PBN) was obtained from Alexis Biochemicals. 1,2-¹³C₂-oxalic acid was purchased from Cambridge Isotope Laboratories. The stock solutions of 1 M potassium oxalate, 2 M potassium acetate buffer

pH 4.1 and 100 mM PBN were prepared in deionized water. The stock solution of 1 M potassium chloride was prepared in 2 M potassium acetate buffer pH 4.1. Due to its low solubility, 1,2-¹³C₂-oxalic acid was dissolved in diluted potassium hydroxide solution. Then the pH of the solution was adjusted to around 4.0 by adding diluted hydrochloric acid solution and its concentration was adjusted to be around 0.4 M.

Preparation of enzymes

Purified pET32a with wild-type or mutant DNA was transformed into competent BL21(DE3) (Novagen) cells and grown on LBA overnight at 37°C. A single colony was grown overnight in 50 mL of LBA at 37°C. Three flasks containing 475 mL of LB were inoculated with 6 mL of overnight culture grown to an OD₆₀₀ of ~0.4. The bacteria were heat shocked at 42°C for 18 min before addition of IPTG and MnCl₂ to final concentrations of 1 and 5 mM, respectively, and grown for 4 hours. The cells were harvested and stored overnight at -20°C. The cells were thawed and resuspended in 60 mL lysis buffer containing 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 10 μM MnCl₂, and 10 mM imidazole and disrupted by sonication on a Sonic Dismembrator 500 (Fisher Scientific). Cellular debris was removed by centrifuging at 13,000 g for 18 min at 4°C. The lysate was loaded onto a Ni-NTA (Qiagen) column previously equilibrated in lysis buffer. The column was washed with 50 mM KPi pH 8.5, 0.5 M NaCl, and 20 mM imidazole. The protein was eluted using 50 mM KPi pH 8.5, 0.5 M NaCl, 250 mM imidazole. Fractions containing the OxDC were pooled and Chelex 100 Resin (Bio-Rad) was added to remove adventitiously bound metals. Metal free storage buffer was prepared using 50 mM Tris-HCl pH 8.5, 0.5 M NaCl, and Chelex 100 resin following the manufacturer's instructions. The protein was buffer exchanged into the metal free buffer using a G-25 column with the resin having been soaked in 1 M EDTA. The fractions containing OxDC were pooled, and if needed concentrated, using an Amicon Centriprep YM-30 (Millipore). All proteins were stored at -80°C until used. Protein samples were submitted for inductively coupled plasma mass spectrometry metal analysis at the University of Wisconsin Soil and Plant Analysis Lab (Verona, WI).

Determination of enzyme activity

Decarboxylase activity assays were run in batches of 100 μL liquid volume containing 50 mM sodium acetate, 0.5 mM *o*-phenylenediamine, 0.2% Triton-X 100, 2.5–100 mM potassium oxalate pH 4.2, H₂O, and 1–3 μg of enzyme to initiate the reaction. After 1–10 min, the reaction was quenched by addition of 10 μL of 1.1 M sodium hydroxide. The formate in these samples was obtained using an end point assay with formate dehydrogenase following standard procedure [8]. The concentration of the protein was determined according to a modified Bradford assay (Pierce, Rockford, IL) for which standard curves were generated with bovine serum albumin [22].

EPR spin trapping

Spin trapping samples contained 100 mM potassium oxalate (or 74 mM ¹³C₂-oxalic acid, 100 mM potassium chloride, 20 mM PBN and were buffered with 0.6 M (0.3 M for ¹³C₂-oxalic acid reactions) potassium acetate buffer pH 4.1. The enzyme was added last to the reaction mixture to have a final concentration of 4.1 mg/mL for WT OxDC, 6 mg/mL for T165S, 7.2 mg/mL for T165V, 4.4 mg/mL for S161T, and 2.8 mg/mL for S161A. The final volume of each solution mixture was 100 μL. Assuming that the observed catalytic activities (see table 1) hold up under these higher enzyme concentrations one expects the available oxalate to be consumed in approximately 2 min. That this is indeed the case was confirmed by the fact that CO₂(g) bubbles were observed in the samples for approximately 1–2 min after oxalate was added to the enzyme solution. The reaction mixture was quickly transferred into a quartz capillary of 1×3 mm ID×OD for EPR measurement. The EPR spectrum was recorded at room temperature, using a commercial Bruker Elexsys E580

spectrometer, equipped with a high-Q cavity (ER 4123SHQE). Instrumental parameters were: 100 kHz modulation frequency, 1 G modulation amplitude, 2 mW microwave power, 9.87 GHz microwave frequency, 20.48 ms time constant, and 81.92 ms conversion time/point. The magnetic field was measured with the calibrated Hall probe that is part of the E580.

Results and Discussion

Burrell et al. [15] published results of spin trapping EPR experiments on their SENS161-4DASN mutant using the spin traps DMPO (dimethylpyrrolidine-*N*-oxide) and POBN. They demonstrated the trapping of a carbon based radical the intensity of which increased with time under turnover conditions. Similar experiments with His-tagged WT OxDc resulted in the observation of only a very weak trapped radical signal when POBN was used and no signal at all when DMPO was used [17]. Based on the observed hyperfine constants it was suggested that the carbon dioxide radical anion had been trapped [15,17].

The SENS161-4DASN mutant possesses significant oxalate oxidase activity. Compared to WT OxDc it shows a specificity switch factor of 225,000, defined as the ratio of ratios of oxidase over decarboxylase activities with WT being normalized to 1 [15]. WT OxDc itself shows only about 1% oxidase activity compared to its decarboxylase activity as determined by the observed k_{cat}/K_M values [15].

A carbon-based DMPO radical adduct with similar hyperfine coupling constants ($a_N = 15.6$ G, $a_H = 18.6$ G), interpreted as the $\text{CO}_2^{\cdot-}$ radical had been observed before in barley oxalate oxidase by Whittaker and Whittaker [23]. Following these observations it is tempting to ascribe the presence of the carbon-based radical in the SENS161-4DASN OxDc mutant to its enhanced oxalate oxidase activity and to assume a similar pathway as in barley oxalate oxidase. However, in oxalate oxidase the radical was trapped in the absence of oxygen also. It was found in substoichiometric quantities, *i.e.*, about 5% of the available protein-bound Mn present. Therefore, it was concluded that the radical was due to an adventitious side reaction involving residual protein-bound Mn(III) and not a direct intermediate in the catalytic cycle. In any case Burrell et al. [15,17] were unable to determine whether the radical in OxDc is kinetically competent or the product of a side reaction related to the enzyme's oxidase activity. We should also note that evidence for the presence of Mn(III) in either WT OxDc or any of the aforementioned OxDc mutants has been lacking.

EPR spin trapping experiments are very useful to demonstrate the presence of short-lived radical species in a given reaction and to distinguish between carbon-, nitrogen-, or oxygen-based radicals. However, it is much more difficult to distinguish and identify different carbon-based radicals by their EPR parameters only. Therefore, we decided to employ ^{13}C -labeled oxalate to investigate the chemical source of the observed radical and to establish a direct link between the trapped radicals and oxalate. If the ^{13}C nuclear spin $\frac{1}{2}$ is present in the observed adduct (see scheme 2), evidenced by a characteristic additional splitting of the 6-line spectrum, it provides clear-cut evidence that the trapped radical is indeed directly derived from the substrate. We have chosen to use the nitron spin trap PBN rather than POBN. POBN is easier to oxidize than PBN which may lead to unwanted side reactions [24,25].

Fig. 2 shows the room temperature EPR spectra of the trapped radicals for WT OxDc, and the four single-site mutants described above using PBN as the spin trap. The spin trap was present when the reaction was initiated by the addition of enzyme to the substrate solution. The spectra shown are the ones with the highest intensity for each time-course. Although quantitative EPR is fraught with substantial uncertainty ($\pm 20\%$ error), it is clear from these

experiments that the WT preparation shows a substantially lower radical yield than the mutant enzyme, T165V. All enzyme preparations yielded a six-line EPR spectrum at $g = 2.006$ with the hyperfine coupling constants $a_N = 15.9$ G, $a_H = 4.5$ G consistent with the PBN- $\text{CO}_2^{\cdot-}$ radical adduct generated from the Fenton-like reaction where formate is converted to the $\text{CO}_2^{\cdot-}$ radical [26]. In the control reactions where either oxalate, enzyme, or spin trap are absent, no signal was observed (data not shown).

When oxalate was substituted by 1,2- $^{13}\text{C}_2$ -oxalate in the reaction mixtures an additional hyperfine splitting of $a(^{13}\text{C}) = 11.6$ G was observed giving rise to a 10-line EPR spectrum (see Fig. 3). Fig. 3 shows the results for the T165V mutant together with its corresponding spectral simulation. All other mutants and the WT OxDC showed identical spectra albeit with different intensities (similar to Fig. 2, data not shown). Two of the observed lines appear with twice the regular intensity indicating that there is accidental spectral overlap of some of the lines. The observation of the ^{13}C -splitting in the EPR spectrum of the trapped radical is clear evidence that it originates from the substrate. These experiments constitute the first direct evidence of the existence and release into solution of the $\text{CO}_2^{\cdot-}$ radical in oxalate decarboxylase under turn-over conditions!

Under the conditions used, the wild type enzyme was estimated to consume the substrate during a period of ~ 120 s. Unfortunately, our experimental dead time, an interval from adding the last reagent of a reaction mixture until completion of the first EPR scan, was around 200 s. Therefore, we could not detect any radical production kinetics during turnover of the wild type enzyme (Fig. 4). We expected and found the same to be true for the mutant enzymes T165S and S161A/T, because of their relatively high decarboxylase activity which is comparable or even higher than that of the wild type (Table 1). Indeed, in most of these cases only the expected slow decomposition of the radical adduct was observed (Fig. 4). On the other hand the time course of the EPR signal intensities for T165V showed a remarkably slow radical production rate. Since this mutant has a decarboxylase activity that is ten times lower than WT it is not surprising that the rate of radical trapping is much slower, too, if radical production is correlated with catalytic turn-over. Based on the kinetic parameters observed for T165V one expects it to take about ten times longer to use up the same substrate concentration. In fact, the EPR intensity starts to saturate after a reaction time of about 1000 s, which is approximately the time it takes T165V to convert the same oxalate concentration. This experiment demonstrates that the trapped radical is a byproduct of the decarboxylation reaction and suggests its kinetic competency, *i.e.*, it appears as an intermediate.

A tyrosyl radical has been identified as a byproduct of catalysis in OxDC [27]. It forms during turn-over but decays quickly after all substrate is used up. It appears to arise in only a small fraction of the enzymes present in a given preparation and appears to serve as a safety mechanism in case the enzymatic reaction was unsuccessful and the active site had been trapped in a high-oxidation state such as Mn(III). Such a situation could occur for example after spontaneous loss of superoxide when only the oxidative half of the decarboxylation reaction was completed, *i.e.*, starting from complex **2**. This reaction would in principle offer an alternative pathway for the recovery of the starting state of the enzyme in case step 3 (backward PCET) could not be carried out after the loss of superoxide. In that case an electron is donated from a nearby tyrosine residue, *e.g.*, Y200 to allow for the reductive half of the decarboxylation reaction to take place. It was therefore of interest to find out whether T165V would exhibit the tyrosyl radical under turn-over conditions as WT did. Fig. 5 shows that T165V produces a carbon-based radical with very similar EPR line shape to the tyrosyl radical observed in WT under the same conditions [27]. Its intensity, observed as a function of the reaction time, confirms its production at the much slower rate with which the mutant

enzyme turns over. This suggests that the catalytic mechanism in the mutant is the same as in WT.

T165 is at one of the hinge positions of the flexible SENST161–165 loop which plays a role in directing the chemistry between the oxidase and decarboxylase pathways [20]. When T165 is replaced by V the H-bond to R92 is broken (see Fig. 1). This is not the case for the T165S mutant. Loss of this H-bond will affect the stability of the loop in its closed conformation. Following the hypothetical mechanism in scheme 1, destabilization of the closed loop may lead to both a slowing down of decarboxylation by shifting the PCET equilibrium toward reactants in step 1. However, it should also slow down step 3 (backward PCET) by denying the intermediate complex the necessary proton if the protonated E162 is located outside the active site some of the time, thereby potentially extending the lifetime of complex **2**. Assuming that oxalate oxidase activity proceeds along the same pathway but forks at the intermediate complex **2** by extracting the radical electron and producing CO₂ [15], one would expect its final step not to be affected by the loop destabilization allowing for a higher relative activity compared to decarboxylation. This has indeed been observed for the SENS161-4DASN mutant [15]. It also appears to be the case for our T165V mutant, albeit to a much lesser degree, based on preliminary evidence that shows that T165V has the same rate of dissolved O₂ consumption (due to its residual oxidase activity) despite a reduction of its decarboxylase activity by a factor of ten compared to WT (data not shown). Oxidation of the CO₂^{•−} radical could occur either internally directly producing H₂O₂ as proposed in the literature [15], or it could occur externally through the loss of CO₂^{•−} from the active site coupled with the loss of superoxide from the enzyme. One should note that the disproportionation of superoxide to O₂ and H₂O₂ is acid catalyzed and expected to occur quickly with 2nd order rate constants of the order of 10⁷/(Ms) at the pH of our experiment [28]. The fate of the released CO₂^{•−} radical will be oxidation by molecular oxygen resulting in the production of CO₂ and another molecule of superoxide at diffusion-controlled rates [29]. In the first scenario one does not necessarily expect a correlation between oxidase activity and the amount of trapped CO₂^{•−} radicals while such a correlation is expected for the second possibility. Our experiments suggest such a correlation, at least when comparing WT with T165V.

Based on the crystal structure the function of the H-bond between T165 and R92 is to hold R92 in the correct position to stabilize the radical intermediate enough for catalysis to proceed [14,18]. The T165V mutation leaves R92 with more motional flexibility which could potentially slow down the second reaction step (decarboxylation). This should also slow down oxidase activity if the hypothetical reaction mechanism (Scheme 1) applies. However, one would not expect this effect to have a major impact on the competition between oxidase and decarboxylase activities because both reactions likely depend on the second step in the same way. It is therefore easier to explain the large increase in CO₂^{•−} radical yield in T165V by the destabilization of the closed loop or a change in its dynamics rather than by the increased motional flexibility of R92.

Comparable mutations were explored on the other end of the flexible SENS loop, S161. Its replacement with alanine breaks potential H-bonding to Glu-67 and the back-bone near N163 but does not affect enzyme activity in any major way (see table 1) nor does it apparently change the kinetics of the CO₂^{•−} radical anion formation. The yield of the CO₂^{•−} radical adduct is generally higher than in WT but less than in T165V. Since S161 is not the actual hinge site but already part of the flexible loop, it is not clear whether its replacement with alanine would destabilize the closed loop conformation to the same extent as in T165V. It is still possible that the loop dynamics are affected in the S161 mutants as well as in T165S which may lead to the observed increase in the trapping yield of CO₂^{•−}.

At this point a simple picture emerges regarding the function of the flexible SENS161-165 loop. Obviously, the loop has to be open to allow substrate to enter the active site and to allow product to escape into solution. Moreover, the presence of the essential Glu-162 residue on the loop also requires it to be closed during decarboxylase catalysis. This may not be necessary for the enzyme's oxidase activity which could explain how the loop regulates the chemistry of oxalate decomposition. However, the closed loop may also be important to prevent leakage of the $\text{CO}_2^{\cdot-}$ radical out of the protein which could have potential deleterious effects on the enzyme itself as well as other cellular components. This enzyme might thus provide an example where a protein tightly regulates the release of a radical intermediate through the dynamics of a flexible amino acid loop similar to some of the adenosylmethionine radical enzymes [30].

Conclusions

By using ^{13}C -labeled oxalate, we have for the first time demonstrated conclusively the presence of an oxalate-derived $\text{CO}_2^{\cdot-}$ radical under turnover conditions in OxDC. The radical accumulates when substrate is present and decays slowly after oxalate has been used up. The relative yield of the trapped radical is highest in the T165V mutant in which a key H-bond at one of the hinge regions of the flexible SENS161-4 loop is deleted. The loop is critical for controlling the competition between oxidase and decarboxylase activities. Changes in its dynamics and the stability of its closed conformation leads to increased leakage of the intermediate $\text{CO}_2^{\cdot-}$ radical into the solvent suggesting its additional role in controlling the release of a potentially harmful free radical into the intracellular space. With the T165V mutant we have identified an OxDC mutant with compromised control over its radical chemistry.

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Fig. 1.

Cartoon representation of residue side chain distances as observed in the crystal structure of OxDC (1UW8) when the active site loop in the N-terminal cupin domain adopts a “closed” conformation (adapted from [17]) [17,18]. Dashed lines indicate putative hydrogen bonding interactions, and the associated numbers correspond to distances between heavy atoms (Å).

Fig. 2.

X-band EPR spectra of the PBN-radical adducts produced from the reactions between ^{12}C -oxalate and WT OxDC and mutant enzymes in 0.6 M potassium acetate buffer pH 4.1. Reaction mixtures contained 100 mM oxalate, 20 mM PBN, 100 mM KCl, and 2.9 mg/mL for WT OxDC, 6 mg/mL for T165S, 7.2 mg/mL for T165V, 4.4 mg/mL for S161T, and 2.8 mg/mL for S161A. The final pH of each reaction was measured to be around 4.6. The PBN- $\text{CO}_2^{\cdot-}$ radical adduct was produced by a Fenton-like reaction that contained 5 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 5 mM EDTA, 5 mM formic acid, 5 mM PBN, and 5 mM H_2O_2 in phosphate buffer pH 7. All spectra displayed similar hyperfine coupling constants, which are $a_{\text{N}} = 15.9$ and $a_{\text{H}} = 4.5$ (see simulated trace). The EPR spectra reported here were selected from the experiment that yielded the highest trapped radical concentration. The following EPR parameters were used: modulation frequency, 100 kHz; modulation amplitude, 1 G; microwave power, 2 mW; time constant, 20.48 ms; conversion time, 81.92 ms; sweep width, 70 G; microwave frequency, 9.87 GHz.

Fig. 3.

X-band EPR spectra of the PBN-radical adducts produced from the reactions between ^{13}C -oxalic acid and T165V OxDC in 0.4 M potassium acetate buffer pH 4.1. Reaction mixtures contained approximately 74 mM ^{13}C -oxalate, 20 mM PBN, 100 mM KCl, and 7.2 mg/mL enzyme. The final pH of the reaction mixture was measured to be around 4.6. The simulation corresponds to the following set of hyperfine coupling constants: $a_{\text{N}} = 15.9$, $a_{\text{C}} = 11.64$, and $a_{\text{H}} = 4.5$. The following EPR parameters were used: modulation frequency, 100 kHz; modulation amplitude, 1 G; microwave power, 2 mW; time constant, 20.48 ms; conversion time, 81.92 ms; sweep width, 70 G; microwave frequency, 9.87 GHz.

Fig. 4.

Time course experiments of the PBN-radical adduct production obtained from the reactions described in Fig. 2. The height of the first derivative peak was measured to represent the yield of the radical adducts at various incubation times.

Fig. 5.

Intensity of the tyrosyl radical EPR signal (see insert) of T165V as a function of reaction time. A sample was prepared for each time point and contained 2.72 mg/mL T165V, 50 mM oxlate, 50 mM KCl, and 0.2 M acetate buffer pH 4.1. The samples were freeze-quenched in pre-cooled isopentane (close to the freezing point) after the specified reaction times. The signal intensity of the tyrosyl radical was measured from peak to peak height of the first derivative spectrum at $g = 2.006$.

Scheme 1.

Working hypothesis for the putative mechanism of *Bacillus subtilis* OxDC, showing the putative roles of Arg-92 and Glu-162 in catalysis [14]. Note that the structure of the Mn-bound formate radical anion **2** is drawn in a form that is consistent with the C-O bond orders computed on the basis of heavy-atom isotope effect measurements [8]. All metal oxidation states and the role of dioxygen in mediating electron transfer are speculative.

Scheme 2.

Spin trapping of the carboxylate radical derived from oxalate (^{13}C labeled).

Table 1

Mn incorporation and specific activities of WT OxDC and the OxDC mutants used in this study.

Enzyme	Mn ^a	Specific Activity (U/mg)	Specific Activity/[Mn] (U/mg/Mn)
WT OxDC	1.4	40 ± 1	29
S161T	1.5	29 ± 1	19
S161A	1.6	48 ± 3	30
T165S	0.7	22 ± 0.5	32
T165V	1.8	4.5 ± 0.4	2.5

^aNumber of manganese ions/OxDC monomer.