Supporting Information

A Structural Element that Facilitates Proton-Coupled Electron Transfer in Oxalate Decarboxylase

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Overlap extension mutagenesis and plasmid purification. Genes encoding the T615S and T165V OxDC variants were constructed using the overlap extension method (*S1*) with the following primers:

OxDC (NdeI):

Forward: 5′ – GGAGGAAACATCATATGAAAAAACAA – 3′

OxDC (XhoI):

Reverse: 5′ – GTGGTGCTCGAGTTTACTGCATTTC – 3′

T165S:

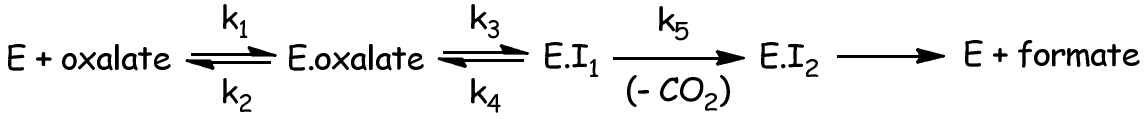
Forward: 5′ – AGCAGTTTCCAGCTGACAGATTGG – 3′
Reverse: 5′ – GAAACTGCTGTTTTCAGAGAATGATCC – 3′

T165V:

Forward: 5′ – AGCGTGTTCCAGCTGACAGATTGG – 3′
Reverse: 5′ – GAACACGCTGTTTTCAGAGAATGATCC – 3′

Briefly, polymerase chain reactions (PCRs) were conducted in solutions containing 40 μL H_2O, 5 μL of *PfuTurbo* AD 10x buffer, 50 ng of template OxDC in pET9a, 0.25 mM dNTP mixture, 0.2 μM of forward primer, 0.2 μM of reverse primer, 1 μL of *PfuTurbo* AD enzyme (50 μL total volume). The first set of PCRs were run for two minutes at 95 ^oC followed by 30 cycles of denaturing at 95 ^oC for 30 s, annealing at 60 ^oC for 30 s, and extension at 72 ^oC for 60 s. After a final extension phase at 72 ^oC for 10 min, the solution was cooled to 4 ^oC. A second set of PCRs to stitch together PCR products from the first round were then performed using solutions containing 40 μL H_2O, 5 μL *PfuTurbo* AD 10x buffer, 1 μL of each of the first round PCR mixtures, 0.2 mM dNTP mixture, 0.2 μM of OxDC forward *NdeI* primer, 0.2 μM OxDC *Xho*I reverse primer, and 1 μL *PfuTurbo* AD enzyme (50 μL total volume). After following a identical set of denaturing, annealing and extension cycles to those used in the first round of PCRs, DNA was purified using a Wizard kit (Promega). The purified DNA was cut at 37 ^oC for 1-2 hours using the *Xho*I and *Nde*I restriction enzymes (New England Biolabs) and ligated into pET32a (Novagen), which had been cut previously with *Nde*I and *Xho*I and treated with calf intestinal alkaline phosphatase. Ligation with T4 DNA ligase (New England Biolabs) was performed at rt for 15 min. JM109 cells were transformed with the ligation mixture (1-5 μL), plated on Luria broth with ampicillin media (LBA) and grown overnight at 37 ^oC. After screening, colonies containing the appropriate insert were grown overnight at 37 ^oC in LBA (50 mL) and plasmids purified using a Wizard kit (Promega).

Detailed analysis of observed kinetic isotope effects for the T165V OxDC variant. Using the following standard minimal kinetic mechanism for the OxDC-catalyzed reaction (*S2*, *S3*):



the apparent IEs were interpreted using the following expression (Eqn. 1):

$$^{x}(V/K) = \frac{^{x}K_{eq3}\,^{x}k_5 + {}^{x}k_3\left(\frac{k_5}{k_4}\right) + \frac{k_3k_5}{k_2k_4}}{1 + \left(\frac{k_5}{k_4}\right)\left(1 + \frac{k_3}{k_2}\right)} \qquad \text{(Eqn. 1)}$$

Here x = 13 or 18 for a ^{13}C or ^{18}O IE, respectively, and isotopic substitution is assumed to affect k_3, k_4, and k_5 (*S2*). Proton removal from a carboxylic acid proceeds with a negligible ^{13}C isotope effect (*S4*), and assuming that k_3/k_2 is small at pH 5.7, the values of $^{13}K_{eq3}$ and $^{13}k_3$ can be set to unity. As a result, Eqn. 1 can be re-written as follows, taking 1.04 to be an average value of the ^{13}C IE ($^{13}k_5$) for decarboxylation (*S5*, *S6*):

$$\frac{1.04 + \left(\frac{k_5}{k_4}\right)}{1 + \left(\frac{k_5}{k_4}\right)} = 1.003 \qquad \text{(Eqn. 2)}$$

Solving Eqn. 2 gives a value of $k_5/k_4 = 12.3$ for the T165V OxDC mutant, which is four-fold greater than that determined previously for the wild type enzyme (*S2*).

To interpret the ^{18}O isotope effect at the carbon center that becomes CO_2, we first multiplied the observed $^{18}(V/K)$ by 0.98 to correct for protonation of the carboxylate anion group when the oxalate monoanion (the true substrate (*S2*)) is bound in the Michaelis complex. This step is necessary because (i) oxalate is almost completely in its dianionic form at pH 5.7, and (ii) ^{18}O is enriched in the protonated

carboxylic acid group by 2% (*S7*). The ^{18}O IE effect on the equilibrium constant ($^{18}K_{eq3}$) for reversible formation of the intermediate prior to decarboxylation will therefore be 1.02. Fractionation factor calculations give an estimate of 0.967 for $^{18}K_{eq5}$ and so $^{18}k_5 = 0.983$ assuming it is midway between unity and the value of $^{18}K_{eq5}$. Setting $k_5/k_4 = 12.3$ (from Eqn. 2) and again assuming that k_3/k_2 is small, as before, we obtain the following expression:

$$\frac{0.98[(1.02)(0.983) + 12.3^{18}k_3]}{1 + 12.3} = 0.984 \qquad \text{(Eqn. 3)}$$

Solving this equality gives a value of 1.0042 for $^{18}k_3$.

Turning to the other carbon in the substrate that is ultimately converted to formate, we calculated $^{13}K_{eq3}$ by assuming that $^{13}k_5$ is midway between unity and 1.03, which is a typical value for decarboxylation (*S6*, *S8*). In this case, we can write that:

$$\frac{1.03^{13}K_{eq_3} + 12.3[(^{13}K_{eq_3} + 1)/2]}{12.3 + 1} = 1.009 \qquad \text{(Eqn. 4)}$$

Solving Eqn. 4 gives $^{13}K_{eq3} = 1.0125$. Dividing this value of $^{13}K_{eq3}$ into the ^{13}C fractionation factor of oxalate gives 0.963, yielding a C-O bond order of the oxalate radical of 1.275 (Figure S1). Thus, we conclude that the transition state for the decarboxylation step is not affected by the replacement of Thr-165 by valine. Assuming that $^{18}k_5 = 1.003$, in order to model the loss of C-C-O bending modes in the formate radical anion, the following expression (Eqn. 5) can be written for the ^{18}O IE at this end of the bound substrate:

$$\frac{1.003^{18}K_{eq_3} + 12.3[(^{18}K_{eq_3} + 1)/2]}{12.3 + 1} = 1.006 \qquad \text{(Eqn. 5)}$$

Hence, $^{18}K_{eq3} = 1.0107$ (Eqn. 5), which when divided into the fractionation factor of oxalate gives a value of 0.9524, corresponding to a bond order of 1.25 for the C-O bond at this end of the oxalate

radical anion intermediate (Figure S1). The two estimates of the C-O bond order computed from the ^{13}C and ^{18}O IE measurements are therefore in good agreement.

The minimal kinetic model was validated on the basis of its ability to predict the heavy-atom IEs that were observed for the T165V OxDC mutant at pH 4.2. The smaller IE values determined at the lower pH suggested that k_3/k_2 is no longer negligible under these reaction conditions. In the case of the carboxyl moiety that is converted to CO_2, substitution of the observed ^{18}O IE into Eqn. 1 yields the following expression:

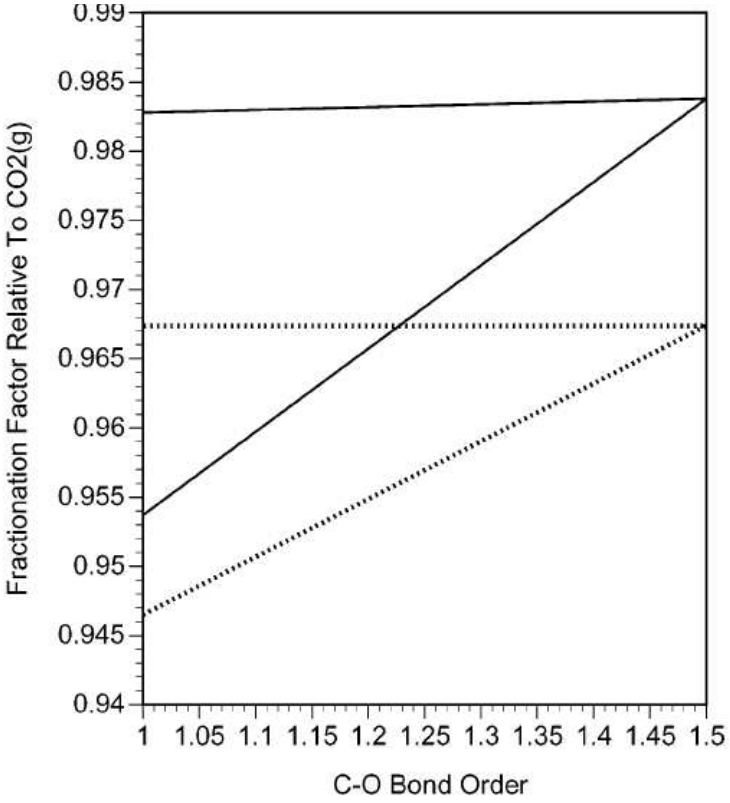
$$\frac{0.99\left[(1.02)(0.983)+(12.3)(1.0042)+12.3\left(\frac{k_3}{k_2}\right)\right]}{1+12.3\left(1+\frac{k_3}{k_2}\right)}=0.991 \qquad \text{(Eqn. 6)}$$

Note that a multiplier of 0.99 is employed here because half of the substrate is monoprotonated at this solution pH (*S9*). Solving this expression gives $k_3/k_2 = 3.2$, meaning that oxalate more often proceeds to products than dissociates from the enzyme *even though the overall turnover number for the T165V OxDC mutant is considerably smaller than that of the wild-type enzyme*. We then employed $k_3/k_2 = 3.2$ in Eqn. 1 to estimate ^{13}(V/K) at pH 4.2 for the oxalate carbon that is converted to that in the CO_2 product. The calculated ^{13}(V/K) value was 1.001, which is good agreement with the observed value of 0.998. Similarly, using the values of $^{13}K_{eq3}$, $^{13}k_3$, $^{13}k_5$, k_3/k_2 and k_5/k_4 obtained using our minimal mechanism, the ^{13}(V/K) value at pH 4.2 is predicted to be 1.002 for the oxalate carbon that is finally released as formate. This value compares favorably with the experimental value of 1.008 for the T165V OxDC variant. In the case of ^{18}(V/K) for formate formation, the overall expression must be multiplied by 1.01 to correct for the half-protonation of the substrate prior to entering the enzyme active site, and so Eqn. 1 takes the form:

$$^{18}(V/K) = \frac{1.01\left[(1.003)(1.0042) + (12.3)(1.0079) + (12.3)(3.2)\right]}{1 + 12.3 + (12.3)(3.2)} = 1.012$$

Experimentally, we find that $^{18}(V/K)$ = 1.004. Although the agreement between the predicted and observed ^{18}O IEs is not perfect, assuming that the Y165V OxDC mutant catalyzes C-C bond cleavage using a two-step minimal mechanism does give a model that is in reasonable agreement with the experimental data.

Figure S1 Fractionation factors of the carbon and oxygen atoms in oxalate as a function of C-O bond order. The sloping lines are for the carboxyl group whose C-O bond order is varied, while the nearly horizontal lines are for atoms in the other carboxyl group in which the C-O bond order is fixed at 1.5. (---) ^{13}C; (···) ^{18}O. This figure has been published previously and is included here for ease of reference. Reprinted with permission from Reinhardt, L. A., Svedruzic, D., Chang, C. H., Cleland, W. W., and Richards, N. G. J. Heavy atom isotope effects on the reaction catalyzed by the oxalate decarboxylase from *Bacillus subtilis*, *J. Am. Chem. Soc. 125*, 1244-1252. Copyright 2003 American Chemical Society.

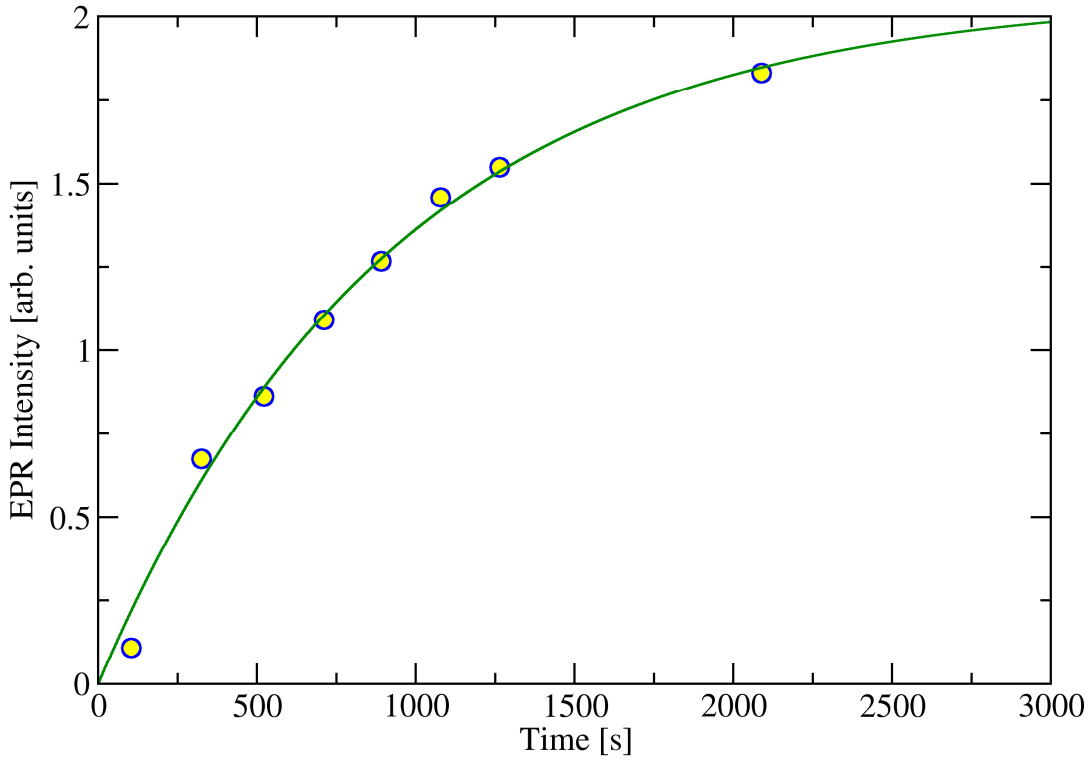

Fractionation Factor Relative To CO2(g)
0.99
0.985
0.98
0.975
0.97
0.965
0.96
0.955
0.95
0.945
0.94
1
1.05
1.1
1.15
1.2
1.25
1.3
1.35
1.4
1.45
1.5
C-O Bond Order

Kinetic analysis of spin trapping and dioxygen consumption first order rate constants for the T165V OxDC variant. The time-dependence of the signal associated with the radical formed by reaction of and was analyzed using the following expression:

$$[\text{adduct}] = [\text{adduct}]_{\infty} \cdot \{1 - \exp(-k_1 t)\}$$

where $[\text{adduct}]_{\infty}$ is the final concentration of trapped radicals using α-phenyl-*t*-butylnitrone (PBN) as the spin trap. Curve fitting (green line in Figure S2) then yielded a value of $k_1 = 1.1 \times 10^{-3}\ s^{-1}$.

Figure S2 Time-dependence of EPR signal intensity for the "spin trapped" adduct obtained by reaction of PBN with formate radical anion. The reaction mixture consisted of the T165V OxDC variant (2 µg), 20 mM PBN, and 100 mM oxalate dissolved in 300 mM NaOAc buffer, pH 4.1, containing 100 mM NaCl. Full experimental details have been published elsewhere (*S10*).



In the case of dioxygen consumption by the T165V OxDC variant, the apparent first order rate constant

was calculated by dividing the initial rate (99 $nM.s^{-1}$) by the concentration of dissolved dioxygen in solution (260 μM) to give a value of $k_1 = 3.8 \times 10^{-4}\ s^{-1}$.

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