Electronic Supplementary Material (ESI) for ChemComm.

Recombinant oxalate decarboxylase: Enhancement of a hybrid catalytic cascade for the complete electro-oxidation of glycerol

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Materials and methods

Chemicals

4-amino-TEMPO (free radical), glycerol, sodium mesoxalate monohydrate, sodium oxalate, sodium formate, glycerol-$^{13}C_3$, sodium hydroxide and formate dehydrogenase (5.0-15.0 U mg^{-1}, E.C. 1.2.1.2) were purchased from Sigma Aldrich. Oxalate oxidase from barley (45 U/mL, E.C. 1.2.3.4) was supplied from Amano. All chemicals were used as received without further purification.

Cloning, expression and purification of recombinant oxalate decarboxylase from *Bacillus subtilis*

The OxDC gene from *Bacillus subtilis* was amplified with the high-fidelity AccuPrime™ Pfx DNA Polymerase with the plasmid pET-16b-OxDC as the template supplied generously by Professor Steven Ealick from Cornell University (Ithaca, NY). The PCR was performed with T7 promoter primer (5'-TAATACGACTCACTATAGG-3') and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3'). The resulting product was digested by BamH1 and Nde1 and cloned into the pET-9a expression vector (Novagen®, San Diego, CA) previously digested with the same restriction enzymes. The resulting plasmid (pET-9a-oxdc) was sequenced by the Health Science Core facility of the University of Utah and used to transform *E. coli* BL-21(DE3). After selection onto LB-Agar in the presence of 100 $\mu g.ml^{-1}$ kanamycin and chloramphenicol, one positive colony was inoculated into LB broth containing the same quantity of antibiotics and grown overnight at 37° C. Then, a volume of 2 ml was used to inoculate 250 ml of LB broth and the resulting cultures were grown at 37° C until an OD_{600nm} value reached 0.5. At this time, the bacteria were heatshocked at 42° C for 12 min before the addition of 1 mM IPTG and 5 mM $MnCl_2$. The induced cells were then incubated at 30° C for 4 h, with shaking. Cells were then harvested by centrifugation at 5000 g for 15 min at 4° C and resuspended in 50 mM Tris-HCl (pH 7.0) containing 10 µM $MnCl_2$ followed by disruption via the microfluidizer method. After centrifugation at 10000 g for 15 min at 4° C, the soluble fraction of the cell lysate was applied to a DEAE-Sepharose Fast Flow column (2.5 x 25 cm) equilibrated with 50 mM Tris-HCl (pH 7.0) containing 10 µM $MnCl_2$ (buffer A). Elution was performed using a 500 mL linear gradient from 0 to 1 M NaCl. The fractions containing purified OxDC were pooled and concentrated by ultracentrifugation in

Amicon centrifugal filter (Millipore, Billerica, MA) to a final volume of 10 ml. The OxDC solution was then dialyzed overnight against 20mM hexamethylenetetraamine hydrochloride (HMTA) (pH 6.0) containing 300 mM NaCl and stored at -80 °C. A protein concentration of 3 mg/ml was measured with Pierce™ BCA protein assay kit (Life Technologies™, Carlbad,CA).

Enzymatic assays

Enzyme (60 μg) was added to mixtures containing different concentrations of substrate (from 0 to 50 mM of sodium oxalate) in 150 mM citrate-phosphate buffer pH 4.0 in a volume of 500 μL. After 2 min, the reactions were quenched with 0.8 mL of K_2HPO_4 (pH 9.5) followed by the addition of 5 mM NAD^+. The levels of produced formic acid were established in a coupled assay with the addition of 1 U of formate dehydrogenase and the absorbance at 340 nm (ε = 6220 M^{-1} cm^{-1}) was recorded. The formate concentration was quantified by the Michaelis-Menten equation containing K_m and V_m of the formate dehydrogenase obtained in the same condition of this essay.

Electrochemical methods

Electrochemical experiments were performed using a CH Instruments 1033 potentiostat with a standard three electrode cell. Cyclic voltammetry (CV) experiments were performed using a SCE reference electrode, a Pt mesh counter electrode, and a 3 mm glassy carbon as a working electrode. CVs were run at 10 mV s^{-1} and 25° C unless stated otherwise. The complete cascade oxidation of ^{13}C-labeled glycerol were performed using a SCE reference electrode, a Pt mesh auxiliary electrode, and a 1 cm x 1 cm Toray paper as a working electrode; the potential for such experiments was held at 0.80 V vs SCE at 25° C. Toray paper electrodes were prepared by cutting the square electrodes with a lead which was coated with paraffin wax to prevent electrolyte from wicking to the alligator clip.

^{13}C-NMR

^{13}C-NMR analysis was performed on a 400 MHz NMR. Samples were analyzed using D_2O at 25° C.

Catalytic glycerol cascade

The 10 hour glycerol oxidation cascade was performed in a sealed container using 8 mM $^{13}C_3$-glycerol, 5 mM TEMPO-NH_2, in presence and in absence of 1 U/mL OxDC with 50 mM phosphate buffer, pH 5.2, at 25° C. The oxidation cascade was performed with a potential of 0.8 V vs SCE for 10 hours. Prior to the experiment, the buffer solution was purged with N_2 to help remove excess CO_2 in the reaction solution. A small canister of NaOH pellets (~100 mg) was suspended above the solution so that the NaOH was exposed to the atmosphere in the headspace of the reaction container without leaking into the solution. The NaOH was used to capture ^{13}C-enriched CO_2 through the formation of $Na_2CO_3 + H_2O$, which was detected by ^{13}C-NMR (D_2O).

Supplemental figures

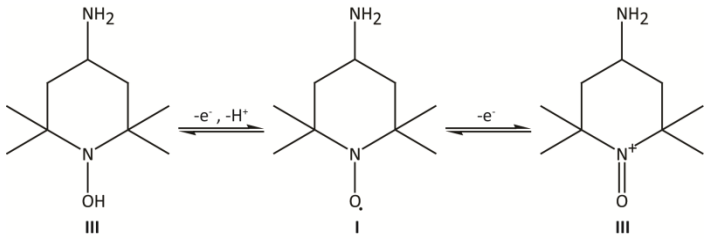

Fig. S1 Redox cycle describing the different oxidation states of TEMPO-NH_2: Nitroxyl radical (I), Oxoammonium ion (II), and Hydroxylamine (III)

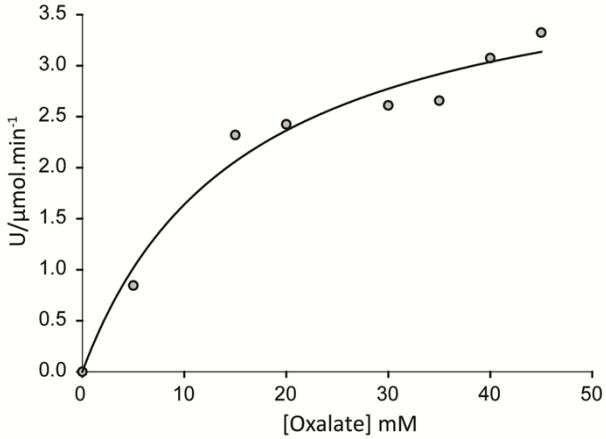

Fig. S2 Michaelis-Menten kinetics of OxDC obtained with FDH-coupled assay.

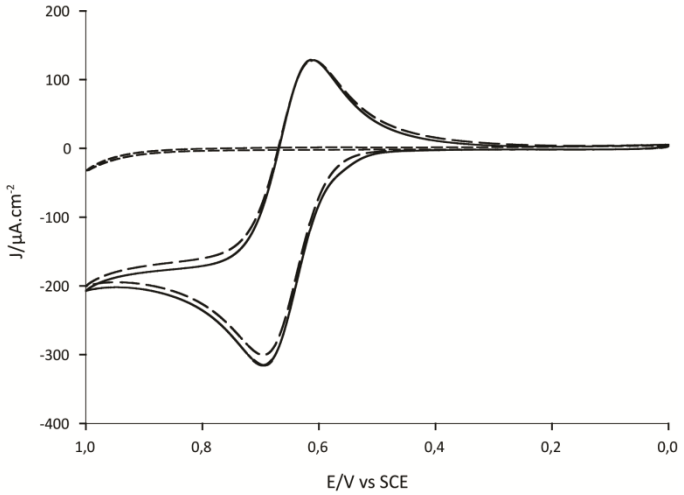

Fig. S3 CVs obtained with 5mM TEMPO-NH_2 in the presence (dashed line) and absence of 100 mM mesoxalate. The short dashed line represents the CV carried out with only 100 mM mesoxalate. All of the experiments were performed using a glassy carbon electrode (3-mm in diameter) as working electrode with 50 mM of phosphate buffer (pH 5.2), at 10 $mV.s^{-1}$ and 25 °C.

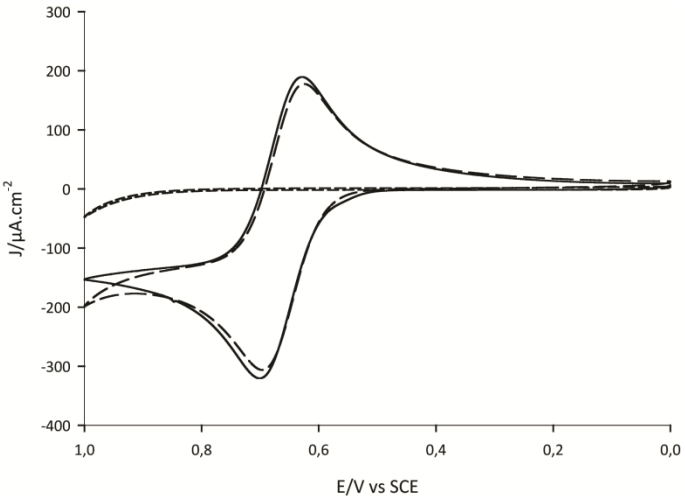

Fig. S4 CVs obtained with 5mM TEMPO-NH_2 in the presence (dashed line) and absence of 50 mM oxalate. The short dashed line represents the CV carried out with only 50 mM oxalate. All of the experiments were performed using a glassy carbon electrode (3-mm in diameter) as working electrode with 50 mM of phosphate buffer (pH 5.2), at 10 $mV.s^{-1}$ and 25 °C.

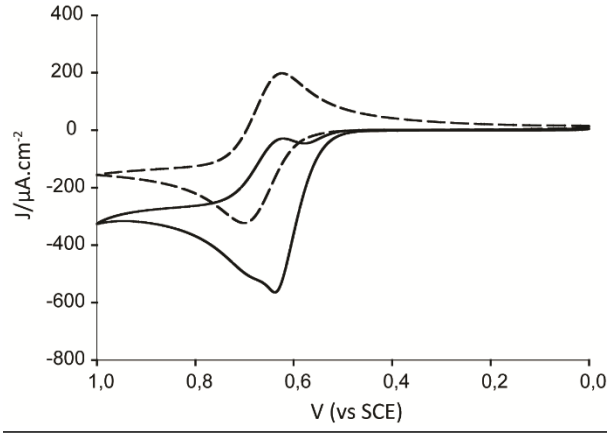

Fig. S5 CVs obtained in the presence of 50 mM oxalic acid before (dashed line) and after 1h enzymatic reaction with OxOx (black solid line). This result shows the alteration of the redox properties of TEMPO-NH_2 by hydrogen peroxide produced by enzymatic oxidation of oxalic acid. All of the experiments were performed using a glassy carbon electrode (3-mm in diameter) as working electrode with 50 mM of phosphate buffer (pH 5.2), at 10 $mV.s^{-1}$ and 25 °C.