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

Sofiene Abdellaoui, David P. Hickey, Andrew R. Stephens and Shelley D. Minteer*

The complete electro-oxidation of glycerol to CO_2 is performed through an oxidation cascade using a hybrid catalytic system combining a recombinant enzyme, oxalate decarboxylase from *Bacillus subtilis*, and an organic oxidation catalyst, 4-amino-TEMPO. This system is capable of electrochemically oxidizing glycerol at a carbon electrode collecting all 14 electrons per molecule.

Carbohydrates such as sugars and alcohols are promising biofuel sources because of their high energy density, low cost, broad availability and low toxicity. In order to exploit one hundred percent of this high energy density, many researchers have focused on the development of fuel cells utilizing electrocatalytic systems with precious metals, organic catalysts and biocatalysts.[1–3] It was shown that precious metal catalysts such as gold and palladium are able to catalyze the oxidation of simple molecules such as hydrogen, methanol and formic acid at low temperature, but lack the ability to efficiently oxidize larger and more energetic compounds such as ethanol, ethylene glycol, glycerol or other more complex polyols.[4–7] Alternatively, many enzymes exist in nature that are able to catalyze the oxidation of a multitude of biofuels with high catalytic rate at ambient temperature and mild aqueous environments. Moreover, these biocatalysts can be combined to shape a multi-enzymatic system able to catalyze the deep oxidation of biofuels *via* a sequence of cascade oxidations. Many researchers have successfully shown the use of multi-enzyme cascades for the complete oxidation of biofuels at bioanodes.[8–14] However, many of the enzymes used in these studies are limited by their stability, high substrate specificity and their optimal physicochemical operating conditions (pH, temperature and electrolytes). On the other hand, small organic oxidation catalysts, such as (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO), are able to oxidize a wide range of oxygen, nitrogen and sulfur-containing functional groups. However, such catalysts often require alkaline conditions and are not able to facilitate the complete oxidation of a substrate as they are not known to break carbon–carbon bonds. Despite their individual limitations, it is possible to merge the advantages of enzymatic and organic oxidation catalysts *via* hybrid systems.

Previously, the complete electrochemical oxidation of glycerol to CO_2 was demonstrated using a hybrid system combining a commercial enzyme, oxalate oxidase (OxOx) from barley, and an organic oxidation catalyst, 4-amino-TEMPO (TEMPO-NH_2).[15] TEMPO is an effective electrochemical alcohol and aldehyde oxidation catalyst that doesn't possess the substrate specificity limitations that are typically exhibited by enzymes.[16–19] This catalyst can be electrochemically oxidized from its nitroxyl radical form to active oxoammonium ion (Fig. S1, ESI†).[20,21] Additionally, recent work has shown that TEMPO-NH_2 has the capacity to operate under acidic conditions that are required for enhanced biocompatibility.[15] It was previously shown that OxOx is able to catalyze the oxidation of simple carboxylic acids such as mesoxalic acid and oxalic acid.[22,23] In this cascade reaction, TEMPO-NH_2 oxidizes glycerol (**1**) to mesoxalic acid (**6**) through 5 steps (Fig. 1). Then, the combination of OxOx and TEMPO-NH_2 can transform mesoxalic acid (**6**) to glyoxylic acid (**7**), oxalic acid (**8**), and finally CO_2. Nevertheless, this system remains hindered by the limited substrate range of OxOx, the low specific activity of OxOx, and the weak compatibility of pH range between TEMPO and OxOx. Moreover, four electrons are lost through the enzymatic oxidation of mesoxalic acid and oxalic acid as OxOx is not capable of mediated electron transfer with known redox mediators (*i.e.* osmium complexes, ferrocene derivatives, or quinone derivatives). In order to improve and increase the efficiency of this hybrid system, it is necessary to focus on the enzyme component.

Oxalate decarboxylase (OxDC), like OxOx, belongs to the cupin superfamily characterized by conserved motifs and a β-barrel domain fold.[24–26] These enzymes use oxalate as a primary substrate and oxygen as either a cofactor for OxDC or an electron acceptor for OxOx.[26] OxDC and OxOx present several similarities such as an active site containing mononuclear manganese ions coordinated by one glutamate and

Departments of Chemistry and Materials Science and Engineering, 315 S 1400 E Room 2020, Salt Lake City, UT 84112, USA. E-mail: minteer@chem.utah.edu

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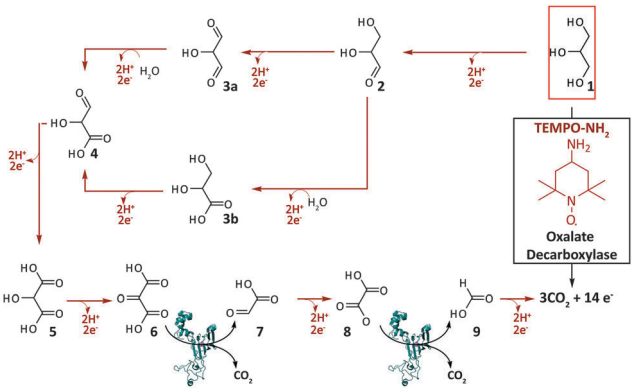

Fig. 1 Electro-oxidation cascade of glycerol by TEMPO-NH_2 and oxalate decarboxylase. The red arrows indicate the reactions with TEMPO-NH_2. The steps in the cascade result in the oxidation of glycerol (**1**) to glyceraldehyde (**2**), and 2-hydroxymalonaldehyde (**3a**) or glyceric acid (**3b**), 2-hydroxy-3-oxopropanoic acid (**4**), tartronic acid (**5**), mesoxalic acid (**6**), glyoxylic acid (**7**), oxalic acid (**8**), and finally formic acid (**9**).

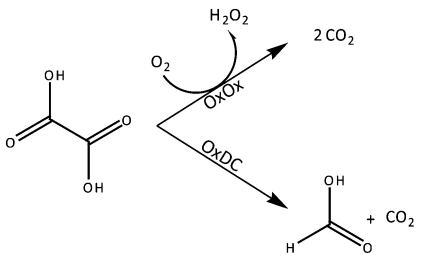

Fig. 2 Enzymatic reaction with oxalic acid by either oxalate oxidase (OxOx) or oxalate decarboxylase (OxDC).

three histidine residues.[24] Unlike OxOx which catalyzes the oxidation of oxalate to CO_2, OxDC catalyzes the hydrolytic C–C bond cleavage of oxalate and forms formic acid and CO_2 (Fig. 2). In order to improve the previous cascade with OxOx, we demonstrate the use of recombinant OxDC from *Bacillus subtilis* with TEMPO-NH_2 for the complete electrocatalytic oxidation of glycerol. It is important to note that it is not common that a non-redox enzyme such as OxDC is used in a bioelectrocatalytic system. However, this enzyme can be easily expressed with *E. coli* with a high specific activity (around 100 U mg^{-1}) (Fig. S2, ESI†) as opposed to OxOx which requires a eukaryote system and commonly suffers from low specific activity (around 1 U mg^{-1}).[27,28] Therefore, the use of OxDC provides advantages in terms of cost and extends the possibilities for enzymatic engineering. Furthermore, we will show that additional electrons can be collected from the oxidation of formic acid to CO_2 by TEMPO-NH_2.

It was shown previously by cyclic voltammetry and HPLC that TEMPO-NH_2 is able to catalyze the first five oxidative steps from glycerol to mesoxalic acid (Fig. 1; 1 to 6).[15] If the OxDC can catalyze the transformation of mesoxalic acid to glyoxylic acid which can be oxidized to oxalic acid by TEMPO-NH_2, the final product will be formic acid. Therefore, it is first necessary to investigate the ability of OxDC to use mesoxalic acid as a substrate and secondly to show the possibility to electro-oxidize formic acid to CO_2 by TEMPO-NH_2 gaining the final electrons for a total of 14 electrons.

First, it was important to verify the ability of OxDC to produce glyoxylic acid from mesoxalic acid. Previously, it was shown that glyoxylic acid can be electro-oxidized to oxalic acid with TEMPO-NH_2 as catalyst.[15] In addition, it was observed that TEMPO-NH_2 is unreactive with mesoxalic acid and oxalic acid (Fig. S3 and S4, ESI†) which is why another catalyst such as OxOx or OXDC is required in this step of the cascade reaction. In order to evaluate the capacity of both enzymes to form glyoxylic acid from mesoxalic acid, solutions of each enzyme were allowed to react with mesoxalic acid for 24 h and the resulting product mixtures were studied by cyclic voltammetry (CV) in the presence of TEMPO-NH_2 to electrochemically oxidize any glyoxylic acid produced. After reaction with OxOx, a catalytic current density of 12 μA cm^{-2} was generated at 0.8 V (*vs.* SCE) in the presence of TEMPO-NH_2 (Fig. 3A) while reaction of mesoxalic acid with OxDC resulted in a current density 8 times higher (92 μA cm^{-2}) (Fig. 4A). The observed catalytic currents were generated by electro-oxidation of the enzymatic product formed from mesoxalic acid with TEMPO-NH_2. From this result, we determined that the product formed with OxDC is glyoxylic acid which can be electro-oxidized by TEMPO-NH_2. In addition, the

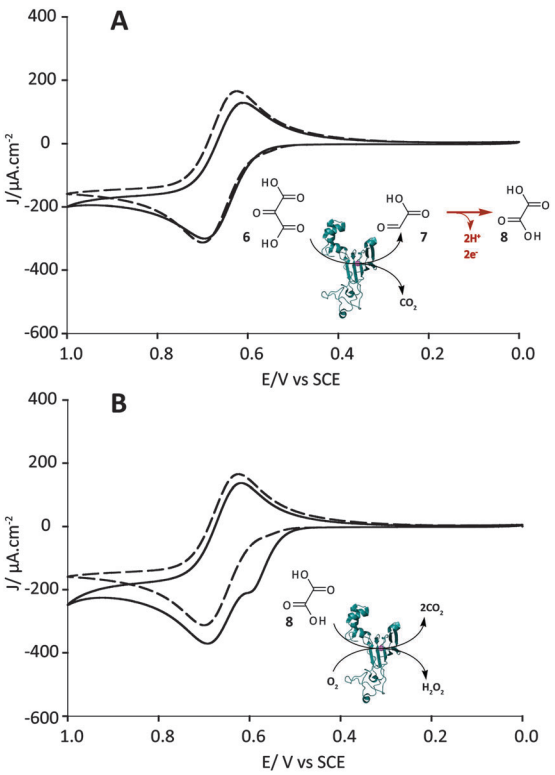

Fig. 3 Catalytic CVs of 5 mM TEMPO-NH_2 in the presence of OxOx. (A) CVs was performed in the presence of 100 mM mesoxalic acid before (dashed line) and after over night enzymatic reaction with OxOx (solid line). (B) CVs obtained in the presence of 50 mM oxalic acid before (dashed line) and after 20 min enzymatic reaction with OxOx (solid line). All of the experiments were performed using a glassy carbon electrode (3 mm diameter) as working electrode with 50 mM of phosphate buffer (pH 5.2), at 10 mV s^{-1} and 25 °C.

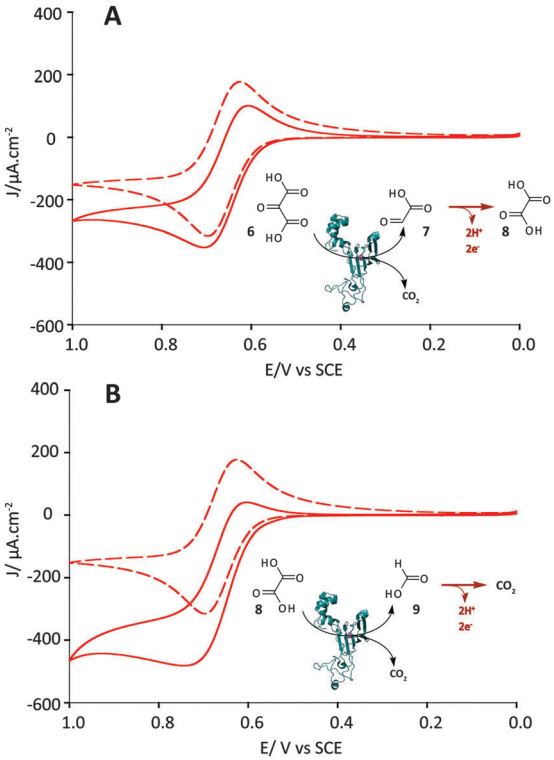

Fig. 4 Catalytic CVs of 5 mM TEMPO-NH_2 in the presence of OxDC. (A) CVs were performed in the presence of 100 mM mesoxalic acid before (dashed line) and after over night enzymatic reaction with OxDC (solid line). (B) CVs obtained in the presence of 50 mM oxalic acid before (dashed line) and after 20 min enzymatic reaction with OxDC (solid line). 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.

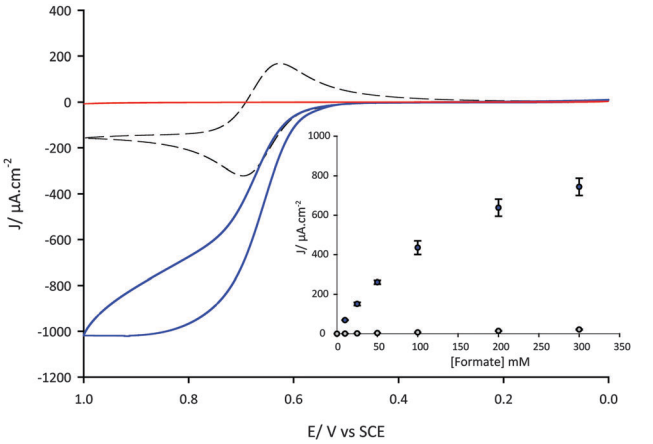

Fig. 5 CVs of 5 mM of TEMPO-NH_2 in the absence (dashed line) and presence (solid line) of 10 mM sodium formate. The red line represents the CV carried out with 10 mM sodium formate without TEMPO-NH_2. Inset: current density at 0.8 V (*vs.* SCE) as a function of formate concentration in the presence (blue circles) and in the absence (gray circles) of 5 mM TEMPO-NH_2. 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.

use of OxDC allows for the generation of higher current densities than the use of OxOx. The same experiment was carried out with oxalate as the substrate in order to show that OxDC facilitates the collection of more electrons through the formation of formic acid (Fig. 4B). After 20 min of reaction with OxDC in 50 mM oxalic acid at pH 5.2 and 25 °C, a catalytic current density of 263 µA cm^{-2} was obtained by electro-oxidation of formic acid with TEMPO-NH_2. A control was carried out with OxOx, which forms hydrogen peroxide (H_2O_2) in the presence of oxalic acid (Fig. 3B). After the enzymatic reaction, a new anodic peak appeared at 0.6 V (*vs.* SCE) indicating that the enzymatically generated H_2O_2 chemically altered TEMPO-NH_2 and irreversibly affected its redox properties (Fig. S5, ESI†).[29] Therefore, these results show that from the mesoxalic acid transformation step, the combination of OxDC with TEMPO-NH_2 is more efficient in terms of coulombic efficiency than the use of OxOx. In addition, we have shown that the H_2O_2 generated by OxOx can alter the redox properties of TEMPO-NH_2 which further advocates the use of OxDC.

The capacity of TEMPO-NH_2 to electro-oxidize formic acid to CO_2 was also characterized by cyclic voltammetry. The CVs were carried out at pH 5.2 in the presence and absence of TEMPO-NH_2. The results shown in Fig. 5 confirm the ability of TEMPO-NH_2 to catalyze the oxidation of formic acid. A maximum catalytic current density (j_{max}) of 970 µA cm^{-2} at 0.8 V (*vs.* SCE) is produced by a solution of TEMPO-NH_2 in the presence of 10 mM formic acid at pH 5.2 and 25 °C. These results show that the electrons from formic acid can be mediated to the electrode by electro-oxidation in the presence of TEMPO-NH_2. A series of CVs was carried out with a range concentration of formic acid at the same pH in the presence of TEMPO-NH_2. The results show the concentration profile for formic acid with TEMPO-NH_2 concentration at 0.8 V (*vs.* SCE) (inset Fig. 5). The signals obtained in the absence of TEMPO-NH_2 are not significant compared to the signals measured in the presence of TEMPO-NH_2 with a j_{max} of 1.57 ± 0.05 mA cm^{-2}.

The study of OxDC by a UV-visible spectrophotometric assay requires the use of a second enzyme to follow the product formation. An NAD-dependent formate dehydrogenase is usually used to follow the production of formic acid from oxalic acid. However, the detection of a new product from a new substrate such as the detection of glyoxylic acid from mesoxalic acid, would require a more complex enzymatic system.[30] In order to show the ability of OxDC coupled with TEMPO-NH_2 to perform the complete oxidation of glycerol, the electro-oxidation of the ^{13}C-labeled glycerol by TEMPO-NH_2/OxDC was carried out in solution and NaOH pellets were used to capture the resulting $^{13}CO_2$ in the form of $Na_2{}^{13}CO_3$. This experiment was performed with TEMPO-NH_2 in the presence and in the absence of OxDC at pH 5.2. The $^{13}CO_2$ from ^{13}C-labeled glycerol generated by the reaction cascade is captured by a small canister of NaOH suspended above the bulk electrolysis solution, thus allowing formation of ^{13}C-labeled sodium carbonate (Na_2CO_3). After 10 h of reaction, the contents of the canister were dissolved in D_2O and analyzed by ^{13}C NMR. The ^{13}C NMR spectra in Fig. 6A show no peak after reaction in the absence of enzyme. A significant peak at 168 ppm corresponding to $^{13}CO_3{}^{2-}$ detected with the combination TEMPO-NH_2/OxDC (Fig. 6B) indicating that CO_2 was formed from

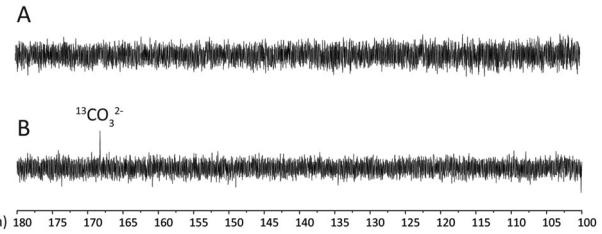

Fig. 6 ^{13}C NMR spectrum of $^{13}CO_2$ trapped in the form of $Na_2{}^{13}CO_3$ from ^{13}C-labeled glycerol after 10 h of oxidation by TEMPO-NH_2 in the absence (A) and in the presence of OxDC (B).

glycerol by enzymatic reaction. Knowing that TEMPO-NH_2 is just able to oxidize glycerol to mesoxalic acid, we can conclude from this result that a decarboxylation of mesoxalic acid was catalysed by OxDC and we have confirmed above that those products are oxidized completely to CO_2.

We have shown that recombinant OxDC from *Bacillus subtilis* can be used as an attractive alternative to commercially available OxOx from barley in combination with TEMPO-NH_2 as part of a hybrid electrocatalytic cascade capable of the complete oxidation of glycerol to CO_2. Use of the non-redox enzyme, OxDC, allows for the collection of additional electrons from the oxidation of formic acid by TEMPO-NH_2 compared to the use of OxOx. The use of OxDC as an alternative to OxOx increases current density 8 times and also avoids the formation of H_2O_2 which can alter the redox properties of TEMPO-NH_2. In addition, OxDC was expressed in *E. coli* which allows for the possibility to modify the enzyme by protein engineering. This advantage could allow for the creation of optimized OxDC with higher activity for mesoxalic acid and/or an enzyme with broader substrate range with the possibility to use other fuels such as carboxylic acids and larger carbohydrates.

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