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Electrochemical Activation of Galactose Oxidase: Mechanistic Studies and Synthetic Applications

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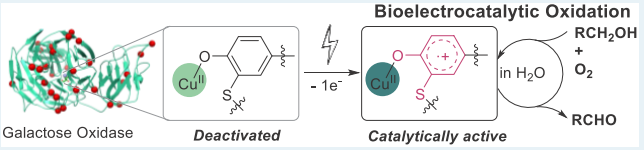
ABSTRACT: The enzyme galactose oxidase (GOase) is a copper radical oxidase that catalyzes the aerobic oxidation of primary alcohols to the aldehydes and has been utilized to that end in large-scale pharmaceutical processes. To maintain its catalytic activity and ensure high substrate conversion, GOase needs to be continuously (re)activated by 1e$^-$ oxidation of the constantly formed out-of-cycle species (GOase^{semi}) to the catalytically active state (GOase^{ox}). In this work, we report an electrochemical activation method for GOase that replaces the previously used expensive horseradish peroxidase activator in a GOase-catalyzed oxidation reaction. First, the formation of GOase^{ox} of a specifically engineered variant via nonenzymatic oxidation of GOase^{semi} was studied by UV−vis spectroscopy. Second, electrochemical oxidation of GOase by mediators was studied using cyclic voltammetry. The electron-transfer rates between GOase and various mediators at different pH values were determined, showing a dependence on both the redox potential of the mediator and the pH. This observation suggests that the oxidation of GOase by mediators at pH 7−9 likely occurs via a concerted proton-coupled electron-transfer (PCET) mechanism under anaerobic conditions. Finally, this electrochemical GOase activation method was successfully applied to the development of a bioelectrocatalytic GOase-mediated aerobic oxidation of benzyl alcohol derivatives, cinnamyl alcohol, and aliphatic polyols, including the desymmetrizing oxidation of 2-ethynylglycerol, a key step in the biocatalytic cascade used to prepare the promising HIV therapeutic islatravir.



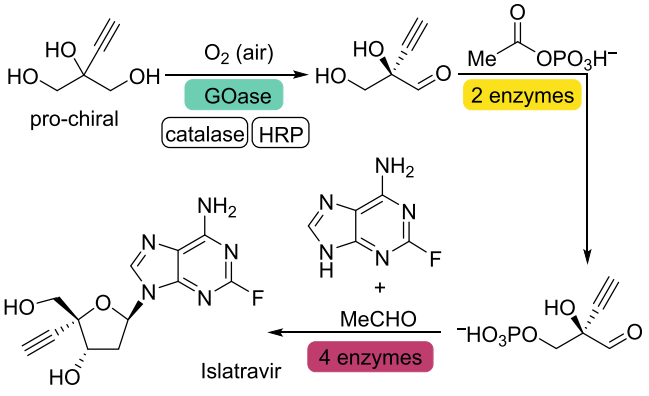
KEYWORDS: *galactose oxidase, biocatalysis, electrocatalysis, alcohol oxidation, proton-coupled electron transfer*

INTRODUCTION

The use of engineered enzymes as catalysts is transforming synthetic organic chemistry and pharmaceutical manufacturing due to the significant opportunities for achieving unparalleled selectivity, increased atom economy, cost savings, improved safety, and applying renewable feedstocks and benign reagents and solvents.[1,2] The recently disclosed fully biocatalytic cascade synthesis of experimental HIV therapeutic islatravir (MK-8591) represents a groundbreaking approach to pharmaceutical manufacturing (Scheme 1).[3] Starting from 2-ethynylglycerol, a three-pot biocatalytic cascade, in a single aqueous stream, forms islatravir in high stereoselectivity and yield. Overall, this process uses nine enzymes, six of which were engineered via directed evolution to achieve catalytic efficiency required by industrial processes.

The most challenging step, which establishes the chirality at the fully substituted carbon atom, is the desymmetrizing oxidation of prochiral 2-ethynylglycerol to form (*R*)-2-ethynylglyceraldehyde, catalyzed by an evolved variant of galactose oxidase (GOase, EC 1.1.3.9). GOase is a metalloenzyme from the copper radical oxidase enzyme family and is one of the most synthetically explored oxidases in manufacturing processes.[4−6] Wild-type and evolved GOase enzymes have been demonstrated to catalyze the irreversible oxidation of primary alcohols to the corresponding aldehydes, with

Scheme 1. Overview of the Biocatalytic Cascade Synthesis of Islatravir



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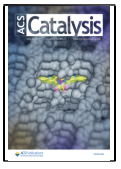

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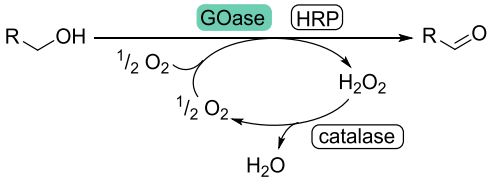
concomitant reduction of O_2 to H_2O_2 (Scheme 2).[7] In GOase-catalyzed reactions, catalase is typically used as an auxiliary enzyme since it catalyzes the disproportionation of the in situ generated H_2O_2, the presence of which can lead to enzyme decomposition or undesired oxidation of the substrate or product.[4,8] Horseradish peroxidase (HRP) is also typically added as an auxiliary enzyme,[4] although its role is less clear and requires additional experimentation and explanation (vide infra).

Scheme 2. Aerobic Oxidation of Alcohols with GOase



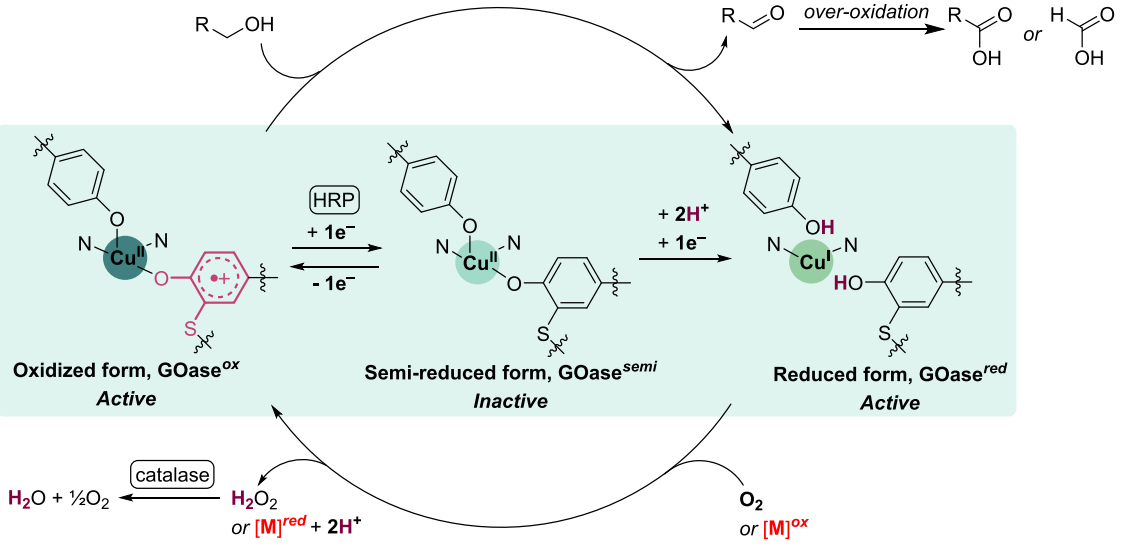
The GOase active site is composed of a single copper center coordinated to two histidine residues, one tyrosine residue, and an unusual cross-linked cysteine−tyrosine unit that can be oxidized to form a stable tyrosyl-based protein radical (Scheme 3).[7] The metalloradical complex combining the Cu(II) center and the tyrosyl radical promotes two one-electron redox steps via the interconversion among three distinct redox forms: a fully reduced Cu(I) species ($GOase^{red}$) responsible for O_2 reduction and a fully oxidized Cu(II) tyrosyl radical species ($GOase^{ox}$) functioning to oxidize alcohols to aldehydes.[7] Additionally, a semireduced nonradical Cu(II) species ($GOase^{semi}$) also exists and is believed to form initially upon reaction of Cu(II) with the *apo*-enzyme or via a $1e^-$ reductive deactivation pathway from $GOase^{ox}$ "falling off" of the catalytic cycle.[9] $GOase^{semi}$ is catalytically inactive because it only undergoes one-electron redox chemistry and is at the incorrect oxidation state to efficiently oxidize alcohol or reduce O_2.[7] Its accumulation during the catalytic cycle correspondingly results in a slower rate of reaction and incomplete substrate conversion.

Peroxidases, such as HRP, have been employed as GOase activators, presumably by recovering the catalytically active $GOase^{ox}$ via $1e^-$ oxidation of $GOase^{semi}$, although the activation mechanism is not well understood.[3,4,7,10] While this three-enzyme system is elegant, the use of HRP introduces significant cost and additional protein burden that complicates downstream purification.[3,5] Activation of GOase by a small-molecule chemical oxidant, such as ferricyanide and hexachloroiridate, has previously been observed spectroscopically.[4,9,11] However, high concentrations of the oxidant were often required, and the effects on substrate conversion were not competitive with activation by peroxidases.

We were interested in developing a nonenzymatic activation strategy for GOase during our process optimization efforts to alleviate the cost and protein burden introduced by HRP. We previously disclosed the use of substoichiometric Mn(III) complexes as a nonenzymatic alternative to HRP to activate GOase.[10] At the same time, we envisioned that the single-electron oxidation activation pathway for GOase could be achieved electrochemically, creating an opportunity to harness electrical energy as a nonenzymatic and sustainable GOase activator.

Electrochemistry is a green and sustainable technology that has been widely applied in bioanalysis, synthetic chemistry, and energy interconversion. Merging biocatalysis with alternative catalytic techniques such as electrocatalysis or photocatalysis further expands the scope of biocatalytic synthesis by accessing higher reactivity and selectivity or unlocking novel enzymatic cascades or un-natural reactivities.[12−16] In many examples of bioelectrocatalytic reactions, terminal oxidants or reductants have been demonstrated to be completely replaceable with electricity, offering enormous potential in the efficient manufacture of pharmaceuticals and fine chemicals.[15,16] Herein, we disclose the development of the first bioelectrocatalytic aerobic oxidation of alcohols via electrochemical activation of GOase.[17,18] We have successfully applied this new synthetic reaction to desymmetrizing oxidation of 2-ethynylglycerol, a key step in biocatalytic cascade synthesis of islatravir, and expanded the synthetic scope to a range of alcohols. Spectroscopic and electrochemical studies have revealed the redox properties of the specifically engineered GALO-104 ($GOase_{Rd10BB}$ in ref 3)[19] as well as the proton-

Scheme 3. Three Oxidation States of the GOase Active Site in the Proposed Catalytic Cycle for Alcohol Oxidation



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coupled electron-transfer (PCET) mechanism of GOase oxidation by the mediator.

RESULTS AND DISCUSSION

First, to understand the active site formation and activation of GOase, we conducted UV−vis spectroscopic experiments with the purified GALO-104 variant.[19] This variant was chosen due to its catalysis, leading to good conversion for the oxidation of 2-ethynylglycerol (~60%), high ee (~85%), and minimal overoxidation using HRP as the activator (see the Supporting Information for conversion and assay yield). The enzyme was expressed as a pro-GOase peptide in the absence of copper to minimize its toxicity to the expression host (*Escherichia coli*). The absorption spectrum of the copper-free GALO-104 protein showed no distinctive spectroscopic features above 300 nm (Figure 1, black dash trace), similar to the reported spectrum of the wild-type GOase from *Fusarium graminearum* (*Fg*GOase).[9] Addition of an excess of $CuSO_4$ to the enzyme led to the rapid growth of a strong absorption peak at 387 nm (Figure 1, black trace), previously assigned to Cu(II)-thiolate ligand-to-metal charge transfer (LMCT) in the cysteine-Cu(II) intermediate formed before the formation of cysteine−tyrosine cross-link.[20] The observation of the Cu(II)-thiolate species is somewhat unexpected, as it was only previously observed under anaerobic conditions for *Fusarium sp.*[21] This intermediate decayed exponentially with $t_{1/2}$ = 15 min into a species exhibiting two weak absorption peaks at 419 and 575 nm (Figure 1, red trace). These features match the ones previously assigned to the semireduced GOase^{semi}, in which the cysteine−tyrosine cross-link necessary for active site maturation was formed.[22] The absorption features for fully oxidized GOase^{ox} were not observed after several hours in air (see the Supporting Information). In contrast, it should be noted that generation of GOase^{ox} was observed upon mixing Cu(II) with copper-free *Fg*GOase in oxygenated buffer, thereby suggesting subtle differences of redox properties between the wild-type *Fg*GOase and GALO-104.[4,21] However, the catalytically competent species GOase^{ox} was obtained from GALO-104 by addition of 10 equiv of sodium persulfate, which is known to produce sulfate radicals ($SO_4^{\bullet-}$), a one-electron oxidant, through electron- or energy-transferring processes.[23] The typical signal for the phenolate to Cu(II) LMCT[24] at 428 nm grew in over a period of 2.5 h in the presence of persulfate (Figure 1, blue trace). To confirm the need to activate GALO-104 to achieve catalysis, the initial enzymatic activity of GALO-104 without and with HRP activation was probed. The GOase activity is 7 times higher in the first 200 min with activation than without for GALO-104 (see Figure S43 in the Supporting Information). Thus, one-electron oxidation of GOase^{semi} is necessary to afford the catalytically active GOase^{ox} in any synthetic application.

Next, we explored the feasibility of electrochemical activation to generate the catalytically active GOase^{ox}. Oxidation of *Fg*GOase has been reported for common electrochemical mediators such as ferrocyanide[5,9,11] and ferrocene derivatives.[25,26] We evaluated the electrochemical reaction between GALO-104 and different mediators using cyclic voltammetry (CV), specifically by measuring plateau currents for the oxidation peak of the mediator in the presence of GALO-104 and 2-ethynylglycerol.

GALO-104 alone does not exhibit any peaks between 0 and +1.0 V vs Ag/AgCl in the CV measurements with 2-ethynylglycerol (see Figure S27 in the Supporting Information). The absence of CV response is typical for enzymes, as direct electron transfer between electrodes and enzymes in solution is impeded by the poor electrical connection between the electrode surface and buried active sites.[27−29] Mediators such as (ferrocenylmethyl)trimethylammonium chloride ($FcCH_2NMe_3^+Cl^-$) and ferrocenecarboxylic acid (FcCOOH), however, present a reversible wave even in the presence of the substrate, thereby showing that mediators in this study do not oxidize 2-ethynylglycerol in the absence of the enzyme.[30] The reversible wave of FcCOOH increases in the presence of GALO-104 and no substrate, thereby showing that the mediator reacts with GALO-104 (see Figure S36 in the Supporting Information). Catalytic currents and S-shaped waves were observed in the presence of 2-ethynylglycerol, mediators, and GALO-104 under a nitrogen atmosphere, thereby showing that electron transfer between mediators and GALO-104 is possible in the absence of O_2 (Figure 2). Indeed,

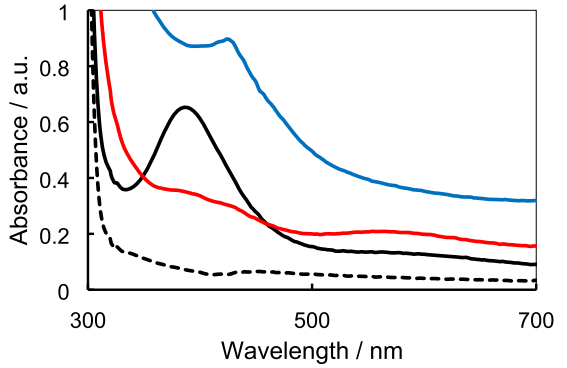

Figure 1. UV−vis spectra of apo-GALO-104 (black dash trace), in situ generated copper-bound GALO-104 upon mixing *apo*-GALO-104 and $CuSO_4$ before forming the C228-Y272 cross-link (black trace), GOase^{semi} after formation of C228-Y272 cross-link (red trace), and GOase^{ox} upon mixing GOase^{semi} and $Na_2S_2O_8$ (blue trace).

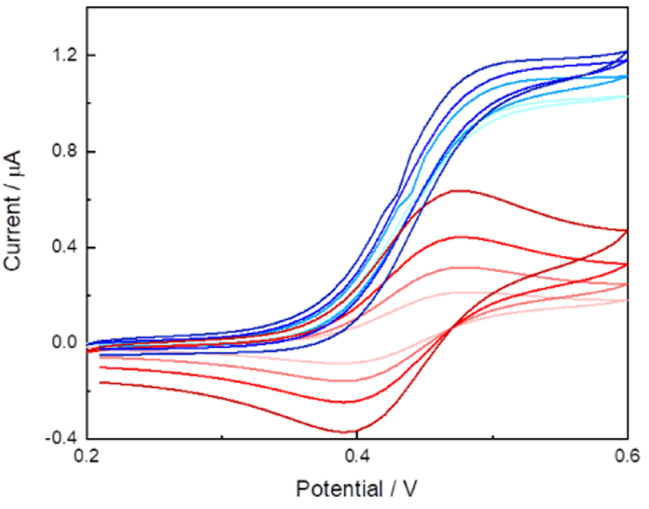

Figure 2. Cyclic voltammetry of 100 μM $FcCH_2NMe_3^+Cl^-$ in the presence of 93 mM 2-ethynylglycerol under a N_2 atmosphere; scan rate: (rose pink wave) 2 mV·s^{-1}, (fuschia wave) 5 mV·s^{-1}, (red wave) 10 mV·s^{-1}, and (maroon wave) 20 mV·s^{-1}. Cyclic voltammetry of 100 μM $FcCH_2NMe_3^+Cl^-$ in the presence of 93 mM 2-ethynylglycerol, 39 μM GALO-104, and 50 μM $CuSO_4$ under a N_2 atmosphere; scan rate: (spring green wave) 2 mV·s^{-1}, (sky blue wave) 5 mV·s^{-1}, (navy blue wave) 10 mV·s^{-1}, and (dark blue wave) 20 mV·s^{-1}.

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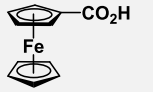
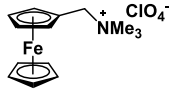
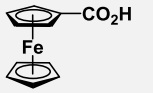
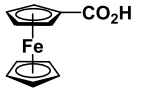
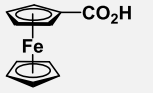
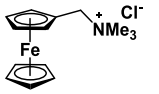
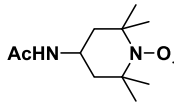
when the mediator oxidizes the enzyme to GOase^{ox}, the in situ generated reduced mediator is readily reoxidized at the anode.[31] As the enzyme turns over, the reaction of GOase^{ox} with the alcohol substrate generates GOase^{red}. In the absence of O_2, GOase^{red} can be reoxidized to GOase^{ox} by the mediator again (see Scheme 3), thereby leading to an increased catalytic current. The shape of the currents seen in the CV experiments holds additional information on the reaction between GALO-104 and the mediators. In the presence of high concentrations of the substrate and low concentrations of the mediator, the catalytic current becomes independent of the scan rate. When the reaction between the mediator and the enzyme is rate limiting, the kinetic formalism introduced by Savéant et al. can be applied, i.e., the rate can be extracted by fitting the ratio of the catalytic current and the reversible current in the function of the square root of the scan rate (see the Supporting Information for details).[32]

The rates of electron transfer between the different mediators and GALO-104 presented in Table 1 are within

Table 1. Rates of Electron Transfer (k) between GOase and Selected Mediators

GOase	*Mediator*	*pH*	*E^0 (V)* [a]	*k_{sec} ($\times 10^3$ $M^{-1}s^{-1}$)*
*Fg*GOase		9	0.33	0.25 [b,26]
*Fg*GOase		9	0.44	0.75 [b,26]
*Fg*GOase	$K_3Fe(CN)_6$	7	0.17	0.54 [b,11]
*Fg*GOase	O_2	7	0.35	1,900 [b,11]
GALO-104		7	0.33 [e]	3.2 [e]
GALO-104		8	0.33 [e]	5.3 [e]
GALO-104		9	0.33 [e]	8.6 [e]
GALO-104		7	0.43 [e]	5.1 [e]
GALO-104	ABTS	7	0.51 [c,e] 0.88 [53,d]	9.5 [e]
GALO-104		7	0.65 [e]	33.0 [e]
GALO-104	K_3IrCl_6	7	0.73 [e]	33.0 [e]

[a] E^o vs Ag/AgCl. [b] Converted from the pseudo-first-order rates using mediator concentrations reported in the original literature. [c] $ABTS^0$/$ABTS^{\bullet+}$. [d] $ABTS^{\bullet+}$/$ABTS^{2+}$. [e] Experimentally determined in this work, see the Supporting Information for details; ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

an order of magnitude of previously reported rates between ferrocene derivatives and *Fg*GOase.[11,25,26,33] Interestingly, ln k increases with the redox potential of the mediator, i.e., the driving force of electron transfer, independent of the charge of the mediator.[34] This result likely signifies that the electron transfer between the mediator and GALO-104 is involved in the rate-determining step for the GOase oxidation under anaerobic conditions,[35] as opposed to mediator-enzyme binding[32] or substrate conversion.[36,37] At low electrochemical driving force (the first three data points in Figure 3, top), the

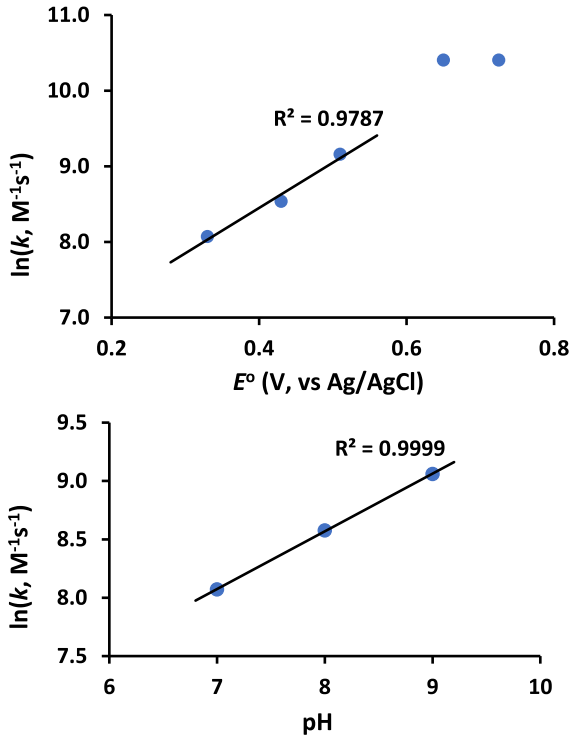


Figure 3. Linear correlation between the rates of electron transfer and redox potential of the mediator (top; at pH 7) or pH (bottom, with $FcCO_2H$) in buffer; $FcCO_2H$ = ferrocenecarboxylic acid.

dependence of ln k on the driving force is linear[38] and $\partial \ln k/\partial(-\Delta G^\circ) = 5.6\ eV^{-1}$,[39] which is smaller than that expected for a PCET reaction with a rate-determining electron transfer followed by a rapid proton transfer (otherwise called ETPT, $\partial \ln k_{(ETPT)}/\partial(-\Delta G^\circ) = 20-45\ eV^{-1}$).[35,38,40]

The mechanism of GOase, like many other redox-active proteins,[41,42] requires the shuttling of both protons and electrons for catalytic turnover. For the same reaction featuring both proton transfer (PT) and electron transfer (ET), concerted proton-coupled electron transfers (PCET) often present the lowest kinetic barrier and, thus, the fastest rate compared to the stepwise pathways kinetically controlled by either PT or ET.[38,40] To determine whether a PCET process is involved in the mechanism of GOase, we investigated the influence of pH on the ET rates, as reported in Figure 3 (bottom) and Table 1.[33,43] As the pH was increased, the electron-transfer rate was demonstrated to increase as well. Similar pH dependence of the electron-transfer rate for Tyr/Tyr• and Trp/Trp• to inorganic complexes has been observed previously and was interpreted as concerted proton-coupled electron transfer (PCET), with the phosphate buffer serving as the proton acceptor at buffer strengths similar to ours.[44,45] The small dependence on the redox driving force and the pH dependence of the oxidation rate therefore suggest that the

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oxidation of GALO-104 by redox mediators at pH 7−9 likely follows a concerted PCET mechanism.[37,46−48]

Encouraged by the results from CV studies, we aimed at developing a synthetically useful bioelectrocatalytic oxidation reaction via electrochemical activation of GOase. In aqueous buffer, proton reduction provides the other half-reaction at the cathode to close the electrochemical cell. Considering the GOase oxidation potential previously reported in the literature for *Fg*GOase (0.2 V vs Ag/AgCl)[49] and the proton reduction potential (−0.6 V vs Ag/AgCl at pH 7) on a Pt electrode, we estimated an operational voltage of >0.8 V between the cathode and the anode.[50] Because hydrogen production is the desired cathodic reaction, electrode materials having low overpotential on hydrogen production are preferred. To facilitate the desired alcohol oxidation on the anode, an oxidizable anode should be avoided. Therefore, platinum was selected for both the anode and the cathode materials. Phosphate buffer was selected because of its stability toward oxidation combined with the compatibility for the enzymatic transformation.

We first explored the electrochemical activation of GOase on the electrode surface via direct electron transfer. The reaction mixture of 2-ethynylglycerol (300 mM), $CuSO_4$, GALO-104, and catalase in a sodium phosphate pH 7 buffer was electrolyzed aerobically under a constant current of 0.2 mA for 16 h. The experiment gave low conversion to the product (5%), likely due to slow direct electron transfer between the GOase Cu active site and the electrode, as expected from the CV studies. This result suggests that the mediator is important for GOase activation and O_2 itself does not effectively reactivate the deactivated GOase^{semi}.

The CV studies showed that GOase could be catalytically turned over in the presence of the mediator under anaerobic conditions, resulting in catalytic current. However, bulk electrolysis using mediators [$FcCH_2NMe_3^+Cl^-$, 4-acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl (4-AcNH-TEMPO) or K_3IrCl_6] under a N_2 atmosphere only resulted in 10−15% conversion after 16 h, thereby highlighting the importance of O_2 as the facile terminal oxidant. Even though the mediator is able to shuttle electrons between GOase and the electrode under anaerobic conditions,[51,52] the turnover rate of GOase by the mediator is several orders of magnitude lower than that by O_2, which was deemed not amenable to preparative purposes.

These results prompted us to take advantage of both electrochemical activation of GOase^{semi} by the mediator and rapid regeneration of GOase^{ox} from GOase^{red} by O_2. When bulk electrolysis of the reaction mixture above was conducted at 0.85 V in the presence of both air and $FcCH_2NMe_3^+Cl^-$ as the mediator (15 mM, 5 mol % relative to 2-ethynylglycerol), the desired bioelectrocatalytic oxidation was achieved, affording 2-ethynylglyceraldehyde in 46% yield (67% conversion) and 90% ee favoring the desired (*R*)-enantiomer. The high ee obtained indicates that the substrate underwent oxidation by the enzyme, instead of direct oxidation by the mediator or on the electrode, as both would generate a racemic product. Over the course of the reaction, the concentration of $FcCH_2NMe_3^+Cl^-$ only dropped from 15 to 12 mM, thereby showing its robustness as a mediator. The best nonelectrolytic conditions using 6 wt % of HRP gave 50% yield (70% conversion) and 90% ee, demonstrating the comparability of bioelectrocatalytic oxidation conditions without the cost and protein of HRP. Considering HRP has been widely investigated for biopolymer synthesis and as a co-enzyme in biocatalytic organic synthesis,[4,36] our results indicate that electrocatalysis may serve as an alternative, inexpensive, and sustainable technique to replace peroxidases and achieve the same or even better results in other chemical processes. Additionally, it should be noted that the yield and conversion of the bioelectrocatalytic oxidation of 2-ethynylglycerol are comparable to those for the GOase oxidation using the nonenzymatic manganese(III) activator, though the reaction conditions and scale of the latter were slightly different.[10]

To better understand the mechanism and the factors governing the bioelectrolysis, we conducted a systematic evaluation of the reaction components (Table 2). Electrolysis in the absence of GALO-104 resulted in 6% conversion and 0% ee, suggesting that the alcohol substrate is neither appreciably oxidized directly by the electrode, the copper, nor the mediator. Using GALO-104 cell-free extract (CFE) powder without addition of copper resulted in a low conversion of 13%, likely due to the presence of ppm level exogenous copper in the reaction mixture. Stirring the reaction mixture with the mediator without electrical current gave <5% conversion, demonstrating that the electrochemical driving force is necessary to activate GOase. Without catalase, a lower conversion of 49% and significant formation of formic acid (9% yield) were observed, due to overoxidation and enzyme deactivation by accumulated H_2O_2.[3] Thus, the bioelectrocatalytic oxidation requires all of the following components for efficient catalysis: GOase, Cu, O_2 (air), electrochemical driving force, and the mediator. To test whether the initial and periodic activation of GOase were both required, the reaction mixture was electrolyzed for 1 h and then the electricity was turned off and the reaction mixture was being stirred for another 15 h. Only 16% of conversion was observed, indicating that periodic activation was necessary.

Table 2. Results of Control Experiments for Bioelectrocatalytic Oxidation of 2-Ethynylglycerol[a]

condition	AY (%)	conversion (%)	HCOOH (%)	acid (%)	ee (%)
standard	46	67	3	3	90
no GOase	5	6	1	5	0
no Cu	10	13	2	2	
no catalase	36	49	9	2	
non-electrolysis	1	1	0	0	
no mediator	3	5	0	0	
no air	12	14	1	1	83

[a]Standard reaction condition: 1.0 mmol scale, 300 mM 2-ethynylglycerol, 17 wt % CFE GALO-104, 14 wt % bovine catalase, 5 mol % $FcCH_2NMe_3^+Cl^-$, 200 μM $CuSO_4$, 170 mM NaPi pH 7, Pt‖Pt, undivided cell, 0.85 V, 16 h; mol % and wt % are relative to the alcohol.

Selective synthesis of aldehydes via the oxidation of alcohols has a great synthetic value, especially considering aldehydes are useful building blocks for subsequent enzymatic cascades.[54−56] However, preventing overoxidation to the corresponding carboxylic acids in aqueous media represents a significant synthetic challenge.[57−61] The voltage applied to the two-electrode system had an impact on both the reactivity and the selectivity of the bioelectrocatalytic oxidation (Figure 4). At the same end-of-reaction cutoff time (16 h), applying a voltage below 0.8 V led to a low yield (25%) and conversion (32%); when the voltage applied was increased stepwise from 0.8 to

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ACS Catal. 2021, 11, 7270−7280

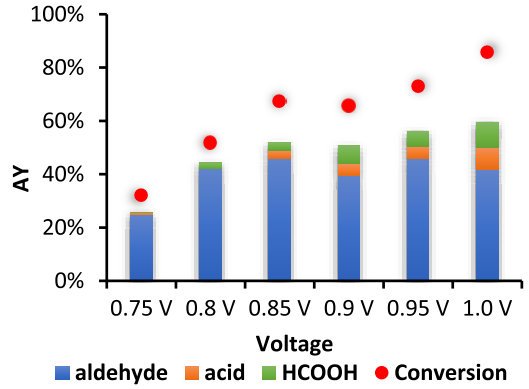
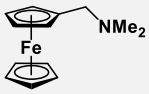
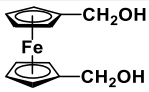
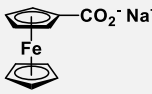
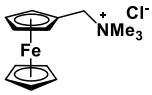
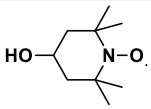
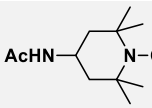
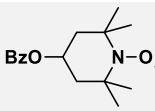


Figure 4. Voltage dependence of bioelectrocatalytic oxidation of 2-ethynylglycerol.

1.0 V, the conversion continued to increase (up to 86% at 1.0 V), but the aldehyde yield remained flat (40−50% across all reactions), consistent with competitive byproduct formation. Product analysis by ^{1}H NMR spectroscopy showed a significant formation of 2-ethynylglyceric acid and formic acid (up to 10% each) after electrolysis at 1.0 V. These results suggest that the increased conversion at a higher voltage resulted from unselective oxidation. Correspondingly, careful selection of the operational voltage for this bioelectrocatalytic oxidation is critical for balancing the competing challenges of the desired selectivity and reactivity.

Under aerobic conditions, a wide variety of mediators activated GOase under a constant current of 0.4 mA. This current resulted in an electrochemical driving force at 0.85−1.10 V range, which is capable of readily oxidizing all mediators under consideration. The redox potentials of all relative to the substrate are sufficient for activating GOase and for electrochemical regeneration. For the reactions using ferrocene derivatives, ABTS, or K_3IrCl_6, the yields of aldehyde were in the range of 39−52% (Table 3, entries 1−5 and 8, 52−67% conversion). Implementation of *N*-oxyls led to slightly lower conversion compared to other mediators (entries 6−7 and 9). It should be noted that using a mediator under bioelectrocatalytic conditions did not decrease the ee values, suggesting that the mediator did not impact the stereodetermining step. Unlike the results of electron-transfer rate dependence from the CV studies, the conversions for reactions were not correlated with the redox potential of the mediators. Both the rates of GOase oxidative turnover by O_2 ($\sim 10^6$ to 10^7 $M^{-1}\cdot s^{-1}$)[11,33] and reductive turnover via alcohol oxidation ($\sim 10^4$ $M^{-1}\cdot s^{-1}$)[68,69] are much faster than the rate of GOase turnover by mediators (see Table 1). Therefore, under aerobic conditions, once GOase^{semi} is activated electrochemically by the mediator and enters the catalytic cycle, it undergoes rapid turnover via reaction with O_2 (rather than the mediator) and alcohol until the deactivation occurs again. As a result, the conversion only depends on the overall turnover number for the enzyme, which is constant across all mediators, barring any inhibition or side reactions. The deactivation into wild-type *Fg*GOase^{semi} is reported to occur every few thousand turnovers,[70] such that the redox potential of the mediators has little effect on the substrate conversion, even though the mediator is still necessary to recover the deactivated GOase^{semi}. Considering the results of voltage dependence, the voltage applied to the electrodes has a greater influence on conversion than the nature of the mediator. We therefore suggest that

Table 3. Influence of the Mediator on Bioelectrocatalytic Oxidation of 2-Ethynylglycerol

Entry	Mediator	E^o (V) [a]	AY	Conversion	ee
1		0.20[62]	46%	56%	86%
2		0.26[63]	39%	52%	87%
3		0.33[64,d]	41%	55%	88%
4		0.43[62,d]	46%	67%	90%
5	ABTS	0.51[b,d] 0.88[53,c]	51%	64%	87%
6		0.61[65]	33%	52%	89%
7		0.65[65,d]	30%	43%	86%
8	K_3IrCl_6	0.73[66,d]	52%	62%	88%
9		0.81[67]	21%	32%	N.D.

[a] E^o vs Ag/AgCl. [b] $ABTS^0/ABTS^{\bullet+}$. [c] $ABTS^{\bullet+}/ABTS^{2+}$. [d] Experimentally determined by cyclic voltammetry, see the Supporting Information for details.

when designing a bioelectrocatalytic reaction, choosing a proper range of the voltage should be the major emphasis for achieving chemoselectivity.

A unique feature of electrochemical reactions is the ability to control the generation of oxidant concentration on the electrode surface through the current intensity, avoiding issues that can arise from using high concentrations of stoichiometric oxidants.[71] This benefit is particularly important when stoichiometric oxidants inhibit or deactivate the catalyst. For *N*-oxyl mediators, the electrochemically generated oxoammonium is the intermediate oxidizing GOase; however, using 10 mol % of either Bobbitt's salt (an isolable oxoammonium species) or its reduced form 4-AcNH-TEMPO under nonelectrolytic conditions led to low conversion (<6%). A similar trend was observed for hexachloroiridate(IV/III), where electrolysis using 5 mol % of K_3IrCl_6 resulted in 52% aldehyde product (entry 9, 62% conversion), compared to <10% conversion using 1 or 10 mol % of K_2IrCl_6 under nonelectrolytic conditions. These results highlight the necessity of constant electrochemical regeneration of the redox mediator at low concentrations to achieve higher conversion.

Based on the literature and our experimental results, we propose the following mechanism for the bioelectrocatalytic oxidation (Scheme 4). As suggested by UV−vis spectroscopic studies, the catalytically inactive species GOase^{semi} is formed upon exposing the copper-free GOase to copper(II) salts.

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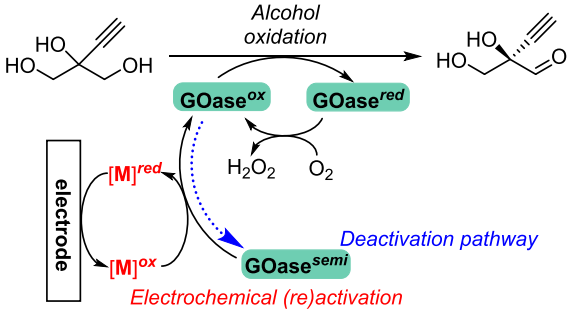
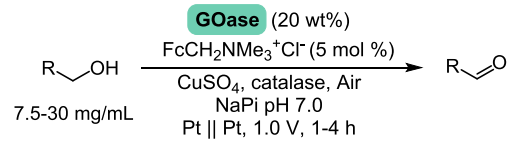
Scheme 4. Proposed Catalytic Cycle for Bioelectrocatalytic Oxidation

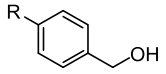
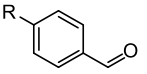
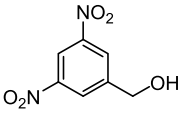
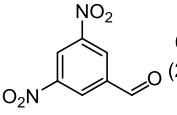
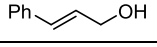
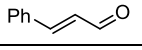


Under electrolytic conditions, the mediator is oxidized at the anode and proceeds to oxidize GOase^{semi} to the catalytically active GOase^{ox}. Subsequent alcohol oxidation by GOase^{ox} yields the aldehyde product and GOase^{red}, which is regenerated to GOase^{ox}, along with H_2O_2 as a byproduct via facile PCET with O_2. The in situ generated H_2O_2 is readily consumed by catalase to form water and O_2. During the catalytic process, GOase^{semi}, which is periodically formed via deactivation from GOase^{ox}, is reactivated by the electrochemically generated oxidized mediator, thereby allowing it to reenter the catalytic cycle. Thus, the mediator acts as a redox shuttle that facilitates electron transfer between GOase and the electrode. In fact, at the end of electrocatalytic reactions showing high conversion into aldehyde, only 0.2−0.4 F·mol^{-1} of electrons passed through the reactions, suggesting the "on-demand" nature of the electrochemical activation. The electrons at the anode are transferred to the cathode and are used for proton reduction, thereby closing the circuit in the electrochemical cell. The proposed catalytic cycle indicates that O_2 and the mediator are necessary for efficient generation of GOase^{ox} and high conversion of the substrate by activating GOase^{red} and GOase^{semi}, respectively. Moreover, utilizing the much simpler, well-behaved one-electron redox chemistry of mediators such as ferrocene derivatives, our electrochemical study has shed light on a mechanism of GOase activation by oxidants, which was proposed to undergo a 1e$^-$ oxidation throughout the enzyme−enzyme interaction in the HRP case.[4] Thus, the mechanistic study into the electrochemical activation of GOase should be beneficial to optimizing future GOase-mediated processes.

Having investigated the important factors and identified the best conditions for the bioelectrocatalysis through mechanistic studies, we envisioned that the electrochemical activation strategy for GOase-catalyzed oxidation reactions has broader synthetic applications and is not limited to only the islatravir alcohol substrate or the employed GOase variant. The initial enzymatic activity of several engineered GOase variants [**M$_1$**, **F$_2$**, **M$_{3\text{-}5}$**, and GALO-105 (GOase$_{Rd12BB}$ in ref 3)] has been measured with and without activation. The GOases have an activity between 5.7- and 15.3-fold higher with activation by HRP than without activation (see Figures S44−S47 in the Supporting Information). Therefore, the GOases used in this study all benefit from one-electron activation. To further demonstrate the synthetic value, we expanded the scope of the bioelectrocatalytic oxidation to additional alcohol substrates. We tested the performance of **M$_1$**, **F$_2$**, **M$_{3\text{-}5}$**, and GALO-105 Goases on various alcohol substrates using the optimized reaction conditions, and the best results of "alcohol−enzyme pairs" are presented in Tables 4 and 5 (see Tables S3 and S4 in

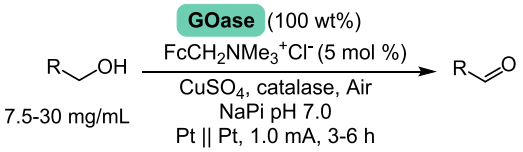
Table 4. Bioelectrocatalytic Oxidation of Benzyl Alcohol Derivatives and Cinnamyl Alcohol

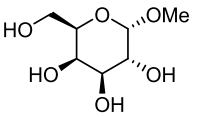
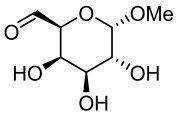
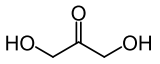
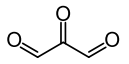
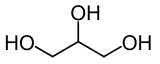
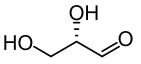
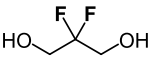
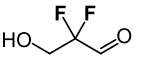
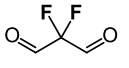
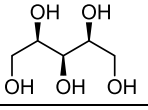
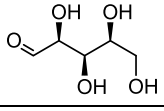


Entry	Alcohol	GOase	Aldehyde	AY	Conversion
1		**M$_{3\text{-}5}$**	 R = H	37% (12% [a])	70% (15% [a])
2		GALO-105	R = OMe	72% (1% [a])	92% (1% [a])
3 [d]		GALO-105	R = CN	85% [b] (1% [a])	99% (1% [a])
4 [d]		**M$_{3\text{-}5}$**	R = CO_2H	57% (5% [a])	66% (5% [a])
5 [d]		**M$_{3\text{-}5}$**		66% [c] (26% [a])	99% (31% [a])
6		**M$_{3\text{-}5}$**		68% (4% [a])	88% (5% [a])

[a]Nonelectrolytic condition. [b]Isolated yield: 82%. [c]Isolated yield: 66%. [d]These products exist as mixtures of aldehydes and hydrates in water.

Table 5. Bioelectrocatalytic Oxidation of Aliphatic Polyols



Alcohol	GOase	Aldehyde [d]	AY	Conversion
	**M$_1$**		74%	91%
	GALO-105		68% (9% [a])	98% (34% [a])
	**M$_1$**		37% [b] (1% [a])	49% (1% [a])
	**F$_2$**		38% (1% [a])	72% (1% [a])
	GALO-105		64% (0% [a])	99% (0% [a])
	GALO-105		76% [c] (5% [a])	92% (6% [a])

[a]Nonelectrolytic condition. [b]ee = 80% (S). [c]ee = 61% (L). [d]These products exist as hydrates in water.

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the Supporting Information for more details). The conversion proved highly dependent on the substrate and GOase variant employed, reinforcing the unique specificity conferred by enzymatic catalysis.

The electro-oxidation of multiple benzyl alcohol derivatives to aromatic aldehydes by either GOase $\mathbf{M}_{3\text{-}5}$ or GALO-105 resulted in the high conversion of the starting material (Table 4, entries 1−5). Specifically, >99% conversion was observed for the oxidation of 4-cyanobenzyl alcohol and 3,5-dinitrobenzyl alcohol (entry 3 and 5). Following organic work-up, 4-cyanobenzaldehyde and 3,5-dinitrobenzaldehyde were isolated at 82 and 66% isolated yields, respectively. The yield for benzaldehyde was only 37% (entry 1), and prolonged reaction led to an even lower yield (15%) despite full conversion (99%), likely because benzaldehyde underwent evaporation due to the vigorous bubbling of air through the reaction mixture. Benzoic acid as the overoxidation product was not observed on ^{1}H NMR spectrum. Oxidation of 4-methoxybenzyl alcohol afforded slightly lower conversion (92%) and 72% yield by ^{1}H NMR spectroscopy (entry 2). Even though the oxidation of benzyl alcohols bearing two electron-withdrawing nitro substituents was achieved within a much shorter time (entry 5, ca. 1 h), *para*-substituted benzyl alcohols did not show a strong correlation between substituent electronic properties and the reaction rate, in good accordance with the reported mechanism of a free radical transition state with no charge buildup on the α-carbon of the alcohols.[7] Furthermore, the reaction was not limited to benzyl alcohol substrates: notably, the bioelectrocatalytic oxidation of cinnamyl alcohol was also achieved, with 10 v/v% DMSO added to improve solubility, providing cinnamaldehyde in 68% yield (entry 6, 88% conversion).

The bioelectrocatalytic oxidation of aliphatic poly-ols was also studied (Table 5). The constant current was used as overoxidation was not observed with these substrates. Methyl α-D-galactopyranoside was smoothly converted into the corresponding aldehyde in 74% yield using GOase $\mathbf{M}_1$ under the bioelectrocatalytic conditions. Electro-oxidation of dihydroxyacetone and xylitol using GALO-105 each resulted in full conversion, affording 2-oxomalonaldehyde and xylose in 68 and 76% yield, respectively. These results are exciting, since xylitol was previously identified as a poor substrate for Cu-dependent alcohol oxidases CgrAlcOx from *Colletotrichum graminicola* and CglAlcOx from *C. gloeosporioides*. Glycerol has been reported as a poor substrate for *Fg*GOase,[54,72,73] but it was accepted as a substrate for electro-oxidation using GOase_{M1}, affording glyceraldehyde in 37% yield. Interestingly, the electro-oxidation of 2,2-difluoro-1,3-propanediol selectively yielded mono-oxidation or double-oxidation products, depending on the choice of the GOase variant utilized. When GOase $\mathbf{F}_2$ was used, 2,2-difluoro-3-hydroxypropanal was preferentially formed in 38% yield as the mono-oxidation product, and the di-oxidation product, difluoromalonaldehyde, was formed in only 6% yield. Compared with GOase $\mathbf{F}_2$, GALO-105 showed higher reactivity for oxidizing both alcohol moieties, yielding difluoromalonaldehyde cleanly in 64% yield. No hydrodefluorination product was observed by ^{1}H NMR spectroscopy. Difluoromalonaldehyde could represent a valuable fluorinated synthon; however, there is only one prior report on the synthesis of difluoromalonaldehyde, likely due to its instability under the previous workup conditions.[74] Without electrolysis, the conversions for all above-mentioned oxidation reactions were much lower, again showing the necessity of mediated electrochemical activation of GOase for high substrate conversion.

These results collectively show that upon (re)activation of GOase under suitable electrochemical conditions, bioelectrocatalytic oxidation can be applied to a variety of benzyl alcohols, cinnamyl alcohol, and aliphatic poly-ols, demonstrating its synthetic value for industrial biocatalytic processes. Though the GOase $\mathbf{M}_{3\text{-}5}$ or GALO-105 variants demonstrated relatively broad substrate tolerance toward a series of substituted benzyl alcohols, the successful application to aliphatic poly-ols or other alcohols will require further careful identification of the enzyme variant most suitable for each individual substrate for the best performance.

CONCLUSIONS

In summary, we have developed a novel electrochemical activation method for GOase as an attractive alternative to the expensive HRP activator in the GOase-catalyzed oxidation reaction used for the preparation of islatravir. Electrochemistry is an efficient tool that does not introduce additional protein burden as in the case of HRP. GOase activation was determined to be necessary for maintaining the catalytically active Cu(II) tyrosyl radical oxidation state GOase^{ox} in the catalytic redox cycle to ensure high substrate conversion.

We observed the activation process of GALO-104 using spectroscopic techniques. The formation of GOase^{semi} upon mixing Cu and *apo*-enzyme and the generation of GOase^{ox} upon addition of $Na_2S_2O_8$ into GOase^{semi} were both evidenced by UV−vis spectroscopy. The activation of GOase by an electrochemically generated mediator was then demonstrated using cyclic voltammetry, which allowed us to interrogate the PCET mechanism of GOase activation. The electron-transfer rates between GOase and various mediators at different pH values were determined using cyclic voltammetry in the presence of 2-ethynylglycerol. The logarithm of electron-transfer rate ln k increases not only with the redox potential of the mediator but also with pH. The weak dependence on the redox potential of the mediator and pH suggests that the oxidation of GOase by redox mediators under anaerobic conditions at pH 7−9 likely occurs by a concerted PCET process.

Applying this mediated electrochemical GOase activation in a synthetic setting enabled the development of a bioelectrocatalytic aerobic oxidation of alcohols. Both O_2 and the mediator are necessary to achieve high conversions of the alcohol substrates, because O_2 serves to rapidly regenerate the GOase^{ox} from GOase^{red} via a facile PCET process, and the mediator is responsible for reactivating the catalytically inactive GOase^{semi} via $1e^-$ anodic oxidation to form GOase^{ox} and (re)enter the catalytic cycle. The mediator is necessary as an electron relay between GOase and electrodes due to the high kinetic barrier of direct electron transfer. The conversion and selectivity of oxidation were found to be sensitive to the operational voltage, but not correlated with the redox potential of mediators, likely because the mediator-activated GOase underwent rapid turnover via reactions with O_2 and alcohol until the deactivation occurred again. We showed that constant electrochemical generation of the oxidized mediator at low concentrations was far superior to using a larger amount under nonelectrolytic conditions, because it circumvents the inhibition or degradation of the enzyme by the mediator. Compared with the GOase-mediated oxidation using either HRP or a nonenzymatic manganese(III) activator, the

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bioelectrocatalytic oxidation using the electrochemical activation method gave similar yields and conversions; however, because of the tunability of potential, current, and electrode materials, bioelectrocatalysis provides greater control and potential in achieving selective synthesis. Furthermore, electricity is the cheapest and greenest redox source, especially when combined with renewable energy. We therefore believe that electrochemistry provides a powerful and practical way to manipulate the oxidation states of redox enzymes.

The bioelectrocatalytic oxidation of alcohols via electrochemical GOase activation is not limited to a single GOase variant or substrates. The oxidation was successfully demonstrated on a variety of benzyl alcohols, cinnamyl alcohol, and aliphatic poly-ols, including the desymmetrizing oxidation of 2-ethynylglycerol, a key step in biocatalytic cascade synthesis of the HIV therapeutic islatravir. Based on these results and the accompanying mechanistic studies, we believe that merging the power of electrocatalysis and biocatalysis will continue to provide additional opportunities in green and selective synthesis and show an increasing value in the implementation of industrial biocatalytic processes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c01037.

Experimental section; NMR spectra; UV−vis spectra; and electrochemical data (PDF)

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Notes

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