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Bioelectrochemical Oxidation of Water

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Supporting Information

ABSTRACT: The electrolysis of water provides a link between electrical energy and hydrogen, a high energy density fuel and a versatile energy carrier, but the process is very expensive. Indeed, the main challenge is to reduce energy consumption for large-scale applications using efficient renewable catalysts that can be produced at low cost. Here we present for the first time that laccase can catalyze electrooxidation of H_2O to molecular oxygen. Native and laboratory-evolved laccases immobilized onto electrodes serve as bioelectrocatalytic systems with low overpotential and a high O_2 evolution ratio against H_2O_2 production during H_2O electrolysis. Our results open new research ground on H_2O splitting, as they overcome serious practical limitations associated with artificial electrocatalysts currently used for O_2 evolution.

Water (H_2O) splitting has been a top matter of research for decades.[1−3] In particular, H_2O electrolysis is a very accessible route to synthesize hydrogen (H_2), a high energy density fuel and a versatile energy carrier, while avoiding the use of hydrocarbon sources. H_2O electrolysis requires two electrodes, the cathode for H_2 evolution and the anode for oxygen (O_2) production. The main hindrance to H_2O electrolysis is the need to find appropriate catalysts to perform both redox reactions with high turnover rates at low overpotentials. Enzymatic redox systems can provide those characteristics. O_2-tolerant hydrogenases are considered to be efficient bioelectrocatalysts for H_2 production.[4,5] However, no enzyme with the ability to electrocatalytically oxidize H_2O to O_2 besides photosystem II (PSII) has been identified so far, and the O_2 electrode has a substantial effect on the performance and durability of H_2O electrolyzers because of its intrinsically slow kinetics and poor durability. Indeed, it is possible to link PSII to an electrode and oxidize H_2O to O_2 with sunlight assistance,[6] but its size, lack of stability, and complexity make the task overwhelming. Here we present an enzyme, laccase, that can catalyze O_2 production from H_2O by direct electron transfer of electrons to a polarized electrode. In other words, for the first time, we show O_2 evolution/reduction electrocatalyzed by fungal oxidoreductases at potentials close to the redox equilibrium potential of the O_2/H_2O couple,[7] i.e., 0.98 and 0.79 V vs NHE at pH 4.2 and pH 7.4, respectively.

Fungal laccases are blue multicopper oxidases with an active site populated by four copper cations that catalyze the oxidization of many organic and inorganic compounds using O_2 as the only electron acceptor, generally at acidic pH values (Figure 1).[8] The catalytic mechanism of laccases involves oxidation of the substrate at the T1 Cu site with concomitant reduction of O_2 to H_2O in a trinuclear copper cluster (T2/T3 Cu cluster) placed 12−13 Å away, without releasing H_2O_2.[9] Taking into account the differences in redox potentials between the O_2/H_2O couple and reduced/oxidized substrate couples (phenolic compounds, anilines, metal complexes, etc.), the reverse reaction in a homogeneous system, i.e., O_2 production from H_2O with concomitant reduction of an oxidized substrate, is thermodynamically impeded due to the very positive Gibbs free energy value for this process. However, this limitation can be overcome in heterogeneous bioelectrocatalytic systems since the reaction driving force is regulated by the potential applied externally. Laccase was the very first enzyme for which direct electron-transfer biolectrocatalysis was shown, as early as 1978.[10] However, to the best of our knowledge, there has never been reported any kind of reverse activity for this oxidoreductase, i.e., H_2O oxidation through the Cu cluster, although the enzyme has been broadly used as bioelectrocatalyst for O_2 reduction.[11,12] Taking into account that O_2 production using several Cu complexes as artificial electrocatalysts has already been reported,[13] we have studied the conditions at which the reverse electrochemical behavior of this multicopper oxidase may take place.

Our first strategy focused on bonding a wild-type high redox potential laccase (*Trametes hirsuta* laccase, ThLc) on low-density graphite (LDG) electrodes modified with aminoaryl groups[14,15] (ThLc-LDG, Figure 2A). LDG was chosen as electrode material due to its high overpotential toward O_2 production in an electrolytic experiment, particularly when compared with Pt or even Au. This selection facilitates attributing the results to the presence of laccase on its surface. The first test carried out with ThLc-LDG was to check its standard activity of reducing O_2 at acidic pH by cyclic voltammetry (CV). Under these conditions, a typical bioelectrocatalytic current plateau of ca. −30 μA was obtained (Figure 2B, curve b), indicating that the immobilized laccase was bioelectrocatalytically active toward O_2 electroreduction. Later the ThLc-LDG electrode was placed in phosphate buffer set at pH 7.4, in which the enzyme is inhibited for O_2 reduction,[11,16] but its production should be thermodynamically and kinetically favored. CV measurement showed an anodic wave that started at 0.95 V vs NHE, i.e., quite close to the redox

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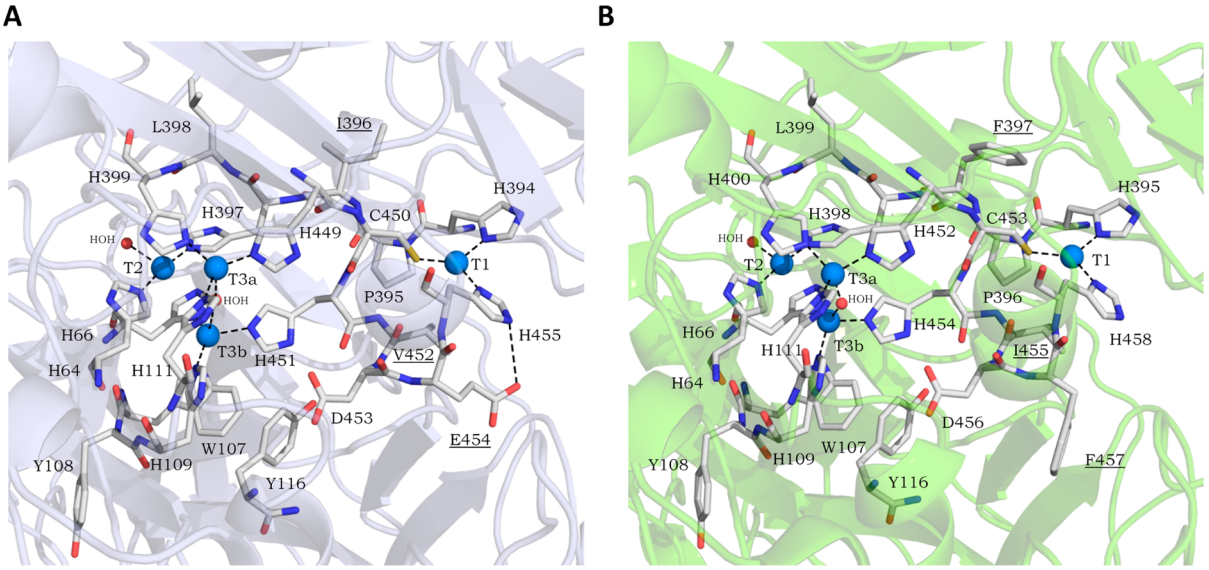

Figure 1. Structural details for the active site of ChU-B mutant (A) and ThLc (B). Amino acid residues are highlighted in stick mode and colored as CPK code. Overall structures are shown as cartoon mode. Copper ions are shown as blue spheres and water molecules as red spheres. Dashed lines indicate interactions between copper ions and amino acids involved in the active site. The mutations introduced in ChU-B are underlined. ChU-B mutant was modeled using as a template the *Trametes trogii* laccase, PDB ID 2HRG (97% of sequence identity with ChU-B). The ThLc crystal structure was from PDB ID 3FPX.

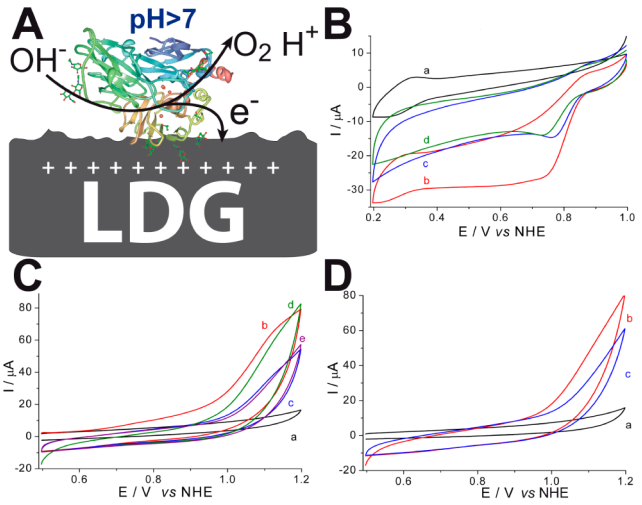

Figure 2. (A) Scheme showing the bioanode prototype based on ThLc. (B) CVs of O_2 reduction: (a) background response of an enzyme-less electrode (NH_2-LDG), (b) initial response of a ThLc-LDG electrode, (c) response after an anodic O_2 production experiment, and (d) response of the electrode after 18h regeneration. (C) CVs of water oxidation: (a) background response of NH_2-LDG electrode, (b) first cycle of a just-prepared ThLc-LDG electrode, (c) second cycle of a just-prepared ThLc-LDG electrode, (d) first cycle of a 18-h-old ThLc-LDG regenerated electrode, an (e) second cycle of a 18-h-old ThLc-LDG regenerated electrode. (D) CVs of water oxidation in the presence of 30 mM NaF: (a) NH_2-LDG electrode, (b) first cycle of ThLc-LDG electrode, and (c) second cycle of ThLc-LDG electrode.

equilibrium potential of O_2/H_2O couple under these conditions (*vide supra*), reaching ca. 80 μA at +1.2 V (Figure 2C, curve b). Subsequent scans caused the signal to decrease when compared to the initial one, although current values of ca. 50 μA at +1.2 V were still measured (Figure 2C, curve c). For a better understanding of the process, some control experiments were performed. It was particularly interesting to check the activity of the electrode in the presence of NaF, a natural inhibitor for the O_2 reduction process catalyzed by ThLc,[11,12,17] proving that under these conditions the O_2 production process was not inhibited. The current intensity under 30 mM NaF was comparable to that of a typical experiment at pH 7.4 in the absence of NaF (cf. curves b in Figure 2C,D).

Despite the promising results obtained with the ThLc-LDG electrode, some drawbacks were clear using this enzyme. Its activity was hampered after several H_2O oxidation cycles and needed a resting period in optimum O_2 reduction conditions, i.e., pH 4.2 acetate buffer, to partially restore the activity (Figure 2C, curve d). As mentioned above, ThLc does not show O_2-reducing catalytic activity at neutral pH because it suffers reversible inhibition by hydroxyl ions. This effect explains the need for the regeneration stage at acidic pH. Therefore, it is feasible that a different high redox potential laccase less optimized for acidic O_2 reduction process but more resistant to hydroxyl inhibition may perform better in O_2 evolution conditions, which is facilitated at neutral or even alkaline pH values. Recently we have reported a high redox potential laccase mutant,[18] ChU-B, specifically designed to avoid anionic inhibitors, i.e., halides and hydroxyls, with sequence identity of 77% and similarity of 83% vs ThLc (Figure 1). The mutant was the genetic product of several rounds of evolution and hybrid approaches to adapt the enzyme to the inclement conditions of human blood (i.e., pH 7.4 with high chloride concentrations) (Figure S1).

Using the same strategy as for ThLc immobilization, ChU-B laccase was covalently bound to the surface of an aminoaryl-modified electrode. The ChU-B-LDG electrode was first tested for O_2 reduction in the same conditions as those used for ThLc-LDG for comparison (Figure S2). The electrochemical results showed that direct electron transfer from the electrode through the T1 site is less favored than that through the T2/T3 site, in agreement with previous results for the ChU-B-LDG electrode.[18] Anodic CV measurements with the ChU-B-LDG electrode at pH 7.4 showed higher anodic currents than with

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the ThLc-LDG electrode (Figure 3A, curve b). Figure 3B shows typical chronoamperometric measurements for ChU-B-

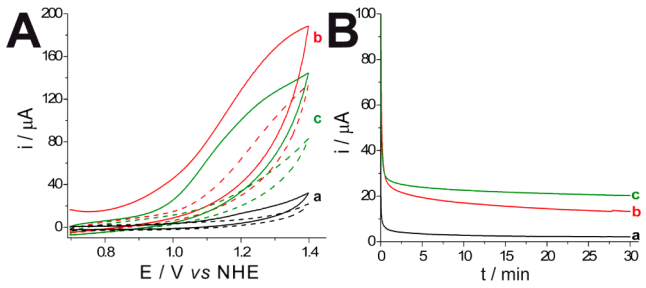

Figure 3. (A) CVs showing the first (solid line) and second (dashed line) scans of (a) NH_2-LDG electrode, (b) fresh ChU-B-LDG electrode, and (c) ChU-B-LDG electrode after a 30 min chronoamperometry at a +1.2 V bias potential. (B) Chronoamperometric measurements for (a) LDG-NH_2 electrode, (b) ThLc-LDG electrode, and (c) ChU-B-LDG electrode.

LDG and ThLc-LDG electrodes, and the result obtained for an aminophenyl-modified LDG (NH_2-LDG) electrode is shown as a control. It can be appreciated that both enzyme-modified electrodes offer a much higher oxidation current than the control electrode.

Regeneration and durability of the modified electrodes were evaluated. The ThLc-LDG electrode maintained a significant anodic current measured by CV, typically 70 ± 10% of the initial one, over three experiments. After each experiment, regeneration was carried out by $t \geq 1$ h immersion in 100 mM acetate buffer, pH 4.2. ChU-B-LDG electrodes were checked over 3 days, setting them in the same regeneration stage. Additionally, we compared the CVs before and after chronoamperometry, as shown in Figure 3B. These experiments showed a major drawback of ThLc-LDG electrodes, as its activity was decimated even for its standard O_2 electroreduction activity (Figure S3); in contrast, the ChU-B-LDG electrode maintained a high anodic response after chronoamperometry (Figure 3A, curve c).

Table 1. $[O_2]$ and $[H_2O_2]$ Produced by Each Type of Electrode during a Typical Electrochemical Chronoamperometry Measurement, and Efficiency (Coulomb) toward O_2 Formation

sample	$[O_2]$ (μM)	$[H_2O_2]$ (μM)	efficiency (C)
LDG-NH_2	0.6 ± 0.4	0.5 ± 0.3	–
ThLc	3 ± 1	0.5 ± 0.5	0.11
ChU-B	2.9 ± 0.7	0.4 ± 0.5	0.16

Enzyme-based colorimetric detection of possible reaction products (H_2O_2 and O_2) was carried out in triplicate to support the electrochemical results. Quantification of the products was performed inside an anaerobic chamber by running a 30 min chronoamperometry at +1.2 V vs NHE in a 3 mL buffer solution, pH 7.4 (Figure 3B). After each experiment, the cell solution was split into three UV−vis cuvettes for measuring the blank spectrum and the H_2O_2 or the O_2 produced by the enzymatic electrodes (Scheme S1). Approximately 3 μM O_2 was produced by the enzymatic electrodes, whereas 5 times less O_2 was detected, on average, in the control measurements. Additionally, the H_2O_2 detected in all three cases was comparable, so it can be attributed to nonenzymatic production of H_2O_2 by the LDG electrode. The results clearly indicate the ability of laccase-modified graphite electrodes to produce mostly O_2 at neutral pH by applying a potential of +1.2 V vs NHE.

In summary, here we show that it is possible to use laccases together with an electroactive surface as a catalyst for the oxidization of H_2O to produce mostly O_2. An enzyme source designed to increase the tolerance against hydroxyl anions yielded a more stable system, hinting that the configuration of the coordination spheres of both T1 Cu site and T2/T3 Cu cluster influences the durability of the enzymatic electrode. However, the lack of inhibition in the presence of a high concentration of NaF suggests that the intramolecular path of electron transfer between the T1 site and the T2/T3 cluster does not play a major role in the O_2 production mechanism.[19] The orientation of the enzyme on the electrode may play a role, as ChU-B performs better and its O_2 reduction electroactivity suggests it is predominantly oriented with its T2/T3 cluster facing the electrode, in contrast to ThLc. On the other hand, we do not think that the water oxidation activity is caused by a major structural change of the laccases' conformational structure during immobilization, as both enzymes retain O_2 reduction activity. For this bioelectrochemical process, we propose a different mechanism than just reversing the natural one of laccase.[20] Instead, the T2/T3 Cu cluster might behave as a copper complex,[13] taking OH^- anions from the solution and becoming oxidized due to the high potentials applied at the electrode. If this is the case, it could imply the formation of either a μ-peroxide or a bis(μ-oxo) bridge (Cu-OO-Cu) intermediate, as suggested for Cu complexes electrocatalyzing O_2 production.[13] However, this hypothesis remains to be verified. Nevertheless, our results open a way to decrease considerably the overpotentials for electrochemical H_2O splitting.

To conclude, our work shows one of the best solutions, viz. usage of biological catalysts, to a very urgent task: to intensify H_2O electrolysis and reduce energy consumption in order to meet the requirement of sustainable large-scale production of H_2 and O_2. Directed evolution provides the tools to find new laccase mutants that may enhance the enzyme stability and performance for its new-found reversible reaction, allowing for a lower overpotential water-splitting process. The T2/T3 laccase cluster may also inspire efficient biomimetic catalysts for H_2O oxidation. Future work will target unveiling the process's mechanism and testing alternative mutant laccases and other immobilization strategies.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, sequence alignment of ChU-B and ThLc, and CVs for O_2 reduction laccase-modified electrodes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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