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Research Article

Simple and Directed Immobilization of a Multicopper Oxidase on Flat Bare Gold Electrodes Provides High Catalytic Currents for O_2 Reduction

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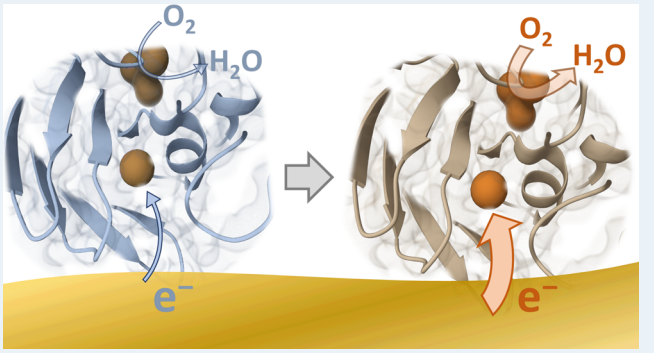
ABSTRACT: The integration of multicopper oxidases with electrodes enables exploitation of the substantial activity of these biocatalysts for the reduction of molecular oxygen. To date, many different strategies have been explored for the fabrication of bioelectrodes, aiming at a stable and favored orientation of immobilized enzymes for ensuring an adequate biocatalytic performance. In this work, we present electrochemical studies of the hyperthermostable *Aquifex aeolicus* multicopper oxidase (McoA) spontaneously adsorbed over gold surfaces. An extremely simple enzyme immobilization strategy is used, taking advantage of the protein structure, which integrates a loop rich in methionine residues (Met-loop) close to the active site. As a result, enzyme orientation for efficient direct electron transfer is ensured without requiring any prior electrode modification. Detailed analysis of the electrochemical responses supports a minimal dispersion of the enzyme orientation over the electrode. Moreover, using an enzyme variant obtained by directed evolution that shows a preferential conformation of the Met-loop over the active site in closed states is shown to improve the interaction with the electrode, resulting in enhanced performance. The obtained results highlight avenues for the use of multicopper oxidases under electrochemical communication with electrode surfaces and showcase the possibility of bioelectrochemical performance improvement, not only through stable and oriented enzyme immobilization but also by tuning the protein structure for improved electron transfer.



KEYWORDS: *multicopper oxidases, direct electron transfer, gold electrodes, oxygen reduction, bioelectrocatalysis*

1. INTRODUCTION

Multicopper oxidases (MCOs) are ubiquitous enzymes in nature that couple the oxidation of diverse substrates with the reduction of molecular oxygen. They incorporate four copper ions in their catalytic centers, three of them constituting a trinuclear cluster where the oxygen reduction reaction (ORR) occurs and a mononuclear Cu site, the so-called type 1 Cu center (T1 Cu), acting as the primary electron acceptor where the oxidation of the enzyme−substrate takes place.[1,2] For several years now, MCOs have gained attention in different research fields. In particular, they have been studied by electrochemical means since the possibility for heterogeneous electron transfer between electrodes and redox proteins was demonstrated.[3,4] Notably, MCOs can be coupled with electrodes so that the latter replaces the substrate as the electron donor.[5,6] Thus, the outstanding catalytic activity of MCOs toward the ORR can be studied in detail, contributing to extensive research in the understanding of electron transport mechanisms in nature, the development of biomimetic electrocatalysts, and the use of enzyme-modified electrodes for practical applications, for example, serving as cathodes for biofuel cells.[7,8]

Bioelectrodes active for the ORR can be obtained after direct adsorption of MCOs over diverse electrode materials.[1,2,9] However, the performance of such electrodes may be limited despite their intrinsically high natural enzymatic activity. The fabrication of efficient enzyme-modified electrodes encompasses several challenges. The approach used during electrode modification must prevent enzyme denaturation and, at the same time, ensure adequate stability of the bioelectrode.[10,11] In addition, optimal electronic communication must be established. As predicted by Marcus' theory, the rate of electron transfer decays exponentially with the donor−acceptor distance.[12] To reach high electron transfer rates in direct electron transfer (DET), an adequate orientation of the enzyme over the electrode is required, ensuring the

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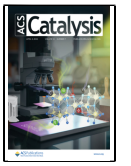

indispensable short distance between the redox center of an immobilized enzyme and the electrode.[13] In the case of MCOs, this implies that their T1 Cu site must be in close proximity to the electrode surface.[14−16]

Several reports have highlighted poor wiring, leading to a negligible current response for MCOs adsorbed over gold surfaces. Particularly, laccases of different origins did not show any catalytic activity after adsorption over bare Au electrodes, which has been attributed to an inappropriate orientation for DET.[8,17] Electrocatalytic activity for O_2 reduction has been observed for bilirubin oxidase (BOD) adsorbed on bare Au, although with very limited stability.[18−22] The loss of activity after enzyme immobilization could be explained by a strong interaction of the proteins with the bare metal surface.[2,21] The integrity of the biomolecules can be preserved by a previous modification of the metal surface with self-assembled monolayers (SAMs) of organic molecules like thiols, minimizing the possibility of denaturation.[15,17,23] The only report to date for an MCO that sustains catalytic activity for ORR on bare Au electrodes is for the MCO from *Pyrobaculum aerophilum* (McoP), which has been attributed to the particularly rigid structure of this enzyme, even though only limited current responses were obtained[24] and the main production of H_2O_2 through a two-electron reaction was reported.[25]

Other studies have shown the development of advanced bioelectrodes using different strategies that allow for controlled enzyme immobilization. For instance, several examples have shown that electrostatic interactions can play a crucial role in ensuring an adequate orientation of enzymes leading to an effective DET.[26−30] Moreover, the orientation of enzymes can be also promoted by the use of adequate electrode modifiers that interact with specific regions in the protein near the redox-active site.[8,31] Other strategies have also shown the use of genetically modified enzymes with specific mutations for site-directed immobilization[32] and the integration of specific binding motifs for directed electrode attachment.[33] This latter strategy can be also exploited taking advantage of the native protein structure. Copper efflux oxidases (CueOs) and other metallo-oxidases are characterized by an additional flexible loop, not found in other MCOs, which is rich in methionine (Met) residues and is located near the T1 Cu site.[34] This Met-rich loop could be used for the directed adsorption of such enzymes over gold electrodes.

In this work, we present the remarkable activity of bioelectrodes based on the hyperthermostable MCO from *Aquifex aeolicus* (McoA)[35−39] adsorbed over bare gold electrodes. The presence of a Met-rich loop adjacent to the T1 Cu site[35] allows enzyme immobilization through interaction with the gold surface promoting an adequate and spontaneous orientation of the protein for DET without requiring any previous modification of the electrode surface. Substitution of the wild-type (WT) enzyme by the variant 2F4 which bears amino acid mutations favoring conformational alterations near the T1 Cu site[36,37] is shown to facilitate electron transfer.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Materials. Gold disk electrodes (AuE, 2 mm diameter) and glassy carbon electrodes (GCEs, 3 mm diameter) were purchased from BAS Inc. The gold disk tip (2 mm diameter) for rotating disk electrode (RDE) measurements was from Bio-Logic Science Instruments. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) was from Sigma-Aldrich. Citric acid monohydrate was acquired from Merck. Disodium hydrogen phosphate dihydrate was from VWR Chemicals. As electrolyte, phosphate−citrate buffer solutions of different pH were used, prepared by mixing 0.2 M Na_2HPO_4 and 0.1 M citric acid in adequate proportions. The pH was measured with a pH meter (Hanna Instruments) and adjusted with 0.1 M NaOH to attain the desired value. All solutions were prepared using ultrapure deionized water (ρ = 18.2 MΩ cm at 25 °C) obtained from a Milli-Q purification system (Millipore). All chemicals were of analytical grade and used as received. The cultivation of recombinant *Escherichia coli* Tuner ΔcueO::kan strains in microaerobic conditions for McoA and 2F4 variant over-production[36] and the protein purification protocol was performed as previously described.[37] Stock solutions of the investigated proteins with a concentration of 3.0 mg mL^{-1} were prepared in deionized water. The activity of the wild-type enzyme and the 2F4 variant was determined before using different substrates.[36] With Cu(I), k_{cat} for McoA WT was (34 ± 7) s^{-1} and for the 2F4 variant (22 ± 6) s^{-1}; with ABTS, k_{cat} for McoA WT was (25 ± 3) s^{-1} and for the 2F4 variant (273 ± 9) s^{-1}. All measurements were performed at room temperature (RT).

2.2. Electrode Modification. Gold disk electrodes were first polished until mirror finish using microid diamond pastes of decreasing particle size (3, 1, 0.5, and 0.1 μm; Leco Corp.). Before modification with the protein, each electrode was polished using an alumina suspension with a particle size of 0.05 μm (BAS Inc.), rinsed thoroughly with water and ethanol, and dried under a N_2 stream. The electrodes were then modified by drop casting 1.0 μL of protein solution and left to dry at RT (about 20 min). Before measurement, the electrodes were rinsed with buffer.

2.3. Electrochemical Measurements. A standard three-electrode cell configuration was used, with the modified AuE as the working electrode, a Pt-wire as the counter electrode, and $Ag/AgCl/KCl_{sat}$ as the reference electrode (E = 199 mV vs SHE; Tianjin Aida Co.). Bare Au electrodes were subjected to consecutive potentiodynamic cycling between 0.0 and 1.6 V (vs $Ag/AgCl/KCl_{sat}$) at 100 mV s^{-1}, in a solution of 0.5 M H_2SO_4. The real surface area of the electrodes was calculated from the measured charge associated with the reduction of gold oxide and considering the charge of a monatomic layer of oxygen over polycrystalline Au of 390 μC cm^{-2}.[40] A summary of the obtained results is shown in Figure S1. The average surface area was determined to be (0.036 ± 0.002) cm^2 (n = 14; α = 0.05). Electrochemical measurements were performed using an SP-300 potentiostat (Bio-Logic Science Instruments), controlled by EC-Lab software. For RDE measurements, a BluRev rotator system (Bio-Logic Science Instruments) was used. All potentials are indicated versus the standard hydrogen electrode (SHE). Scanning electrochemical microscopy measurements were performed with a PGU 100 bipotentiostat (IPS-Jaissle). A Pt microelectrode (25 μm diameter) was positioned at a tip-to-sample distance of 12.5 μm with a step-motor-driven positioning system (Owis) and polarized at 800 mV versus SHE for H_2O_2 collection, while linear sweep voltammograms were recorded at clean or enzyme-modified gold electrodes. The modified electrodes were characterized under an ambient atmosphere (air-equilibrated solutions) or after purging with Ar (99.999%; Air Liquide) or O_2 (99.995%, Air Liquide), as indicated. The reported current densities were

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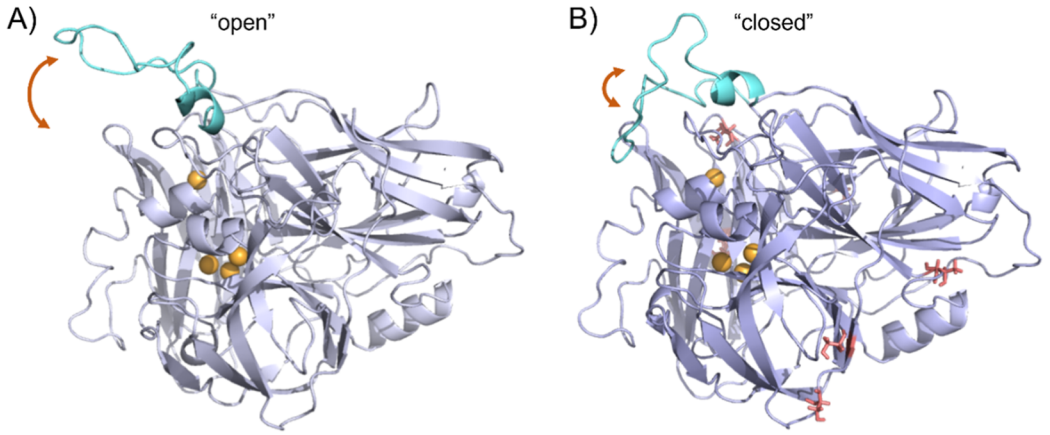

Figure 1. Structures of the multicopper oxidase McoA from *A. aeolicus* wild-type (A) and variant 2F4 (B) obtained by combining X-ray diffraction, small-angle X-ray scattering (SAXS), and Rosetta modeling.[35,36] Copper ions are in yellow, the best average conformation of the reconstructed 29-residue Met-rich loop near T1 Cu is in cyan, and the six mutations in 2F4 (B) are in salmon.

calculated taking into consideration the geometric electrode area.

The bioelectrocatalytic responses for ORR were fitted according to previous theoretical equations for enzyme-modified electrodes presenting variable distances between the enzyme redox center and the electrode surface,[41] adapted to multicopper oxidases[6]

$$i = \frac{i_{lim}}{1 + e^{(F/RT)(E-E^0)}}\left(1 + \frac{1}{\beta d_0}\ln\frac{1 + e^{(F/RT)(E-E^0)} + \frac{k_{cat}}{k_0^{max}} e^{(F/2RT)(E-E^0)}}{1 + e^{(F/RT)(E-E^0)} + \frac{k_{cat}}{k_0^{max}} e^{(F/2RT)(E-E^0)} e^{\beta d_0}}\right) \quad (1)$$

where i is the catalytic current measured, i_{lim} is the enzyme-controlled limiting current, E is the applied potential, E^0 is the potential of the redox center, βd_0 is a dimensionless parameter accounting for the dispersion of enzyme orientations, k_{cat} is the apparent rate constant for enzymatic catalysis, k_0^{max} is the rate constant for interfacial electron transfer at optimal enzyme orientation, F is the Faraday constant, R is the universal gas constant, and T is the absolute temperature.

3. RESULTS AND DISCUSSION

Directed immobilization of redox proteins over electrode surfaces is desired to achieve an adequate disposition of the protein for ensuring optimal interaction for electron transfer. The structure of McoA presents a Met-rich loop near the T1 Cu site (see Figure 1A). The characterization of the conformational landscape of the Met-loop using molecular dynamics simulations showed that this segment is a flexible Ω-loop that preferentially follows open-to-closed transitions exposing or occluding the T1 Cu site.[35] Taking advantage of the enzyme structure (Figure S2), we envisaged the possibility of using this particular feature as an anchor point for the directed immobilization of the enzyme over gold surfaces. This was inspired by a study where the Met-rich region present in *E. coli* CueO was used for enzyme immobilization over carbon nanotubes (CNTs) due to a synergistic effect of $\pi-\pi$ stacking and hydrophobic interactions contributing to an oriented and stable protein attachment.[11] However, this strategy requires prior modification of the electrode surface using CNTs that allow for the specific interaction. In our case, we take advantage of the affinity between sulfur-containing amino acids (i.e., Met) and gold. Therefore, enzyme immobilization does not require any additional modification or functionalization of the electrode surface before protein adsorption. Moreover, gold is an advantageous material serving as an electrode substrate because of its high electronic conductivity, adequate chemical stability under the conditions required for enzymatic catalysis,[2] and high degree of biocompatibility.[25]

Since the Met-rich loop is located close to the access point of electrons to reduce T1 Cu, it was important to verify that DET with the immobilized enzyme is possible and that the Met-loop does not prevent electron transfer by steric hindrance. The electrochemical response for McoA adsorbed over bare Au electrodes (Figures 2A and S3) showed an evident catalytic wave in the presence of O_2, indicating that DET with the immobilized enzyme is possible. As expected, the electrocatalytic response was dependent on the O_2 concentration in the solution, confirming the occurrence of electrocatalytic ORR. To exclude the possibility of the contribution of the electrode material to the catalytic conversion, we also investigated the response of a clean Au electrode in the absence of the enzyme. The response in the latter case showed only a minor contribution within the investigated potential range (Figure 2B), which could be considered negligible in comparison with the responses previously observed in the presence of the enzyme. These results confirmed that McoA undergoes DET with Au electrodes, enabling bioelectrocatalytic O_2 reduction. Note that the catalytic response did not reach a plateau limiting current even when higher electrode rotation rates were used (data not shown), suggesting that heterogeneous electron transfer is the limiting step for the ORR,[15] as discussed below.

To confirm the oriented immobilization of McoA over Au electrodes through the Met-rich loop, the electrocatalytic response under an O_2 atmosphere was compared using different materials as the electrode substrate, i.e., gold and glassy carbon. As summarized in Figure 3, a higher bioelectrocatalytic activity for the ORR was observed for McoA over Au in comparison to that of the carbon electrode. In addition, the response with the glassy carbon-modified electrode showed a continuous decrease in the catalytic response over consecutive potential cycles. This could be attributed to a weaker interaction of the enzyme with the bare

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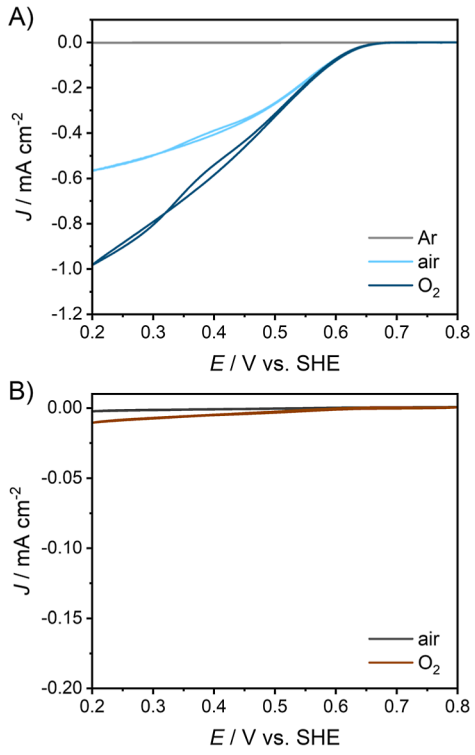

Figure 2. (A) Voltammetric responses for the McoA-modified Au electrode under Ar, air, and O_2 saturated conditions. For clarity, only representative measurements are shown; see Figure S3 for the reproducibility of responses. (B) Responses obtained with a bare Au electrode under air and O_2. Note the different scales for the ordinate axis. For both panels: phosphate−citrate buffer, pH 4.0; scan rate: 10 mV s^{-1}; and electrode rotation rate: 1000 rpm.

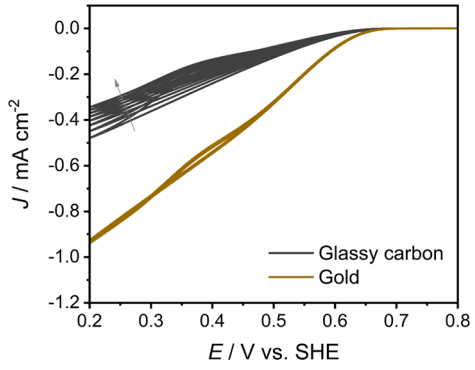

Figure 3. McoA over Au and glassy carbon electrodes under an O_2 atmosphere. Consecutive cycles. The arrow indicates the shift in recorded responses with an increasing number of cycles. Phosphate−citrate buffer, pH 4.0; scan rate: 10 mV s^{-1}; electrode rotation rate: 1000 rpm.

carbon electrode material, leading to enzyme desorption from the electrode surface, as expected since no particular interaction is present favoring a stable immobilization of the protein over the carbon surface. In contrast, practically no differences in response were observed over the same number of consecutive potential cycles with the Au-modified electrode. The increased stability is ascribed to a strong interaction between the Met-rich loop and the gold surface. The Met-loop acts as an anchor for a more stable immobilization and, at the same time, imposes an adequate orientation of the enzyme bringing the T1 Cu center closer to the electrode surface. Furthermore, the flexible nature of this amino acid region seems to ensure an interaction with the bare metal surface that does not compromise the structural integrity of the protein. Indeed, the high and stable biocatalytic signals using McoA over Au electrodes confirmed that the enzyme is not inactivated after adsorption over the bare metal surface. This contrasts with previous reports using other MCOs, as discussed in the Introduction, and can be attributed to the flexible nature of the Met-loop which is used for immobilization of the protein over the electrode. In addition, the hyperthermostable character of the McoA enzyme, with a melting temperature of around 90 °C,[38,39] is associated with an increased protein package density and higher number of ion pairs, which are stabilizing factors when compared with other less thermostable counterparts.[35]

The bioelectrochemical response for McoA over Au electrodes was investigated in the presence of ABTS as a redox mediator in solution. In this case, no increase was observed for the cathodic currents associated with the ORR (Figure S4), suggesting that all immobilized enzymes were effectively wired in DET with the electrode. Scanning electrochemical microscopy was used to investigate the biocatalytic reaction, confirming that H_2O is the product of bioelectrochemical O_2 reduction, without noticeable production of H_2O_2 (Figure S5). Additionally, the enzyme-modified electrodes were also characterized at different pH values in the range between 4.0 and 7.5. As the pH of the electrolyte increased, the start of the biocatalytic wave for the ORR shifted to more negative potentials (Figure S6). The responses were reproducible between different electrodes prepared in the same way and independent of the order of the pH solution measured. The obtained results were consistent with our previous observations over carbon-based electrode surfaces[42] and are also in agreement with similar observations reported before, which have been attributed to the protonation state of amino acid residues near T1 Cu.[43] These observations further confirmed an adequate enzyme orientation imposed through the Met-rich loop interaction with the Au surface, leading to electrochemical communication with the immobilized enzyme through its T1 Cu site.

The obtained results encouraged the investigation of electron transfer for a McoA enzyme variant over Au electrodes. The variant 2F4 has been obtained through directed evolution, showing six mutations scattered across the protein structure (Figure 1B), leading to a 10-fold increased activity for the conversion of bulkier organic substrates, such as phenols and ABTS, in comparison with the WT enzyme.[36,37] A detailed structural analysis[36] has shown that variant 2F4 presents alterations in the conformation of structural loops, including the long Met-rich loop near the T1 Cu site, facilitating interaction and access of bulky substrates. Thus, the idea was to investigate whether these features also have an influence on the electrochemical performance of the protein variant in DET with an electrode. The electrochemical characterization of 2F4-modified electrodes (Figures 4 and S7) showed high electrocatalytic activity for the ORR. In comparison with the response obtained for McoA WT, with current densities of $-(0.95 \pm 0.08)$ mA cm^{-2} at an applied potential of 0.2 V versus SHE under O_2-saturated buffer, the variant 2F4 led to a noticeable increase in bioelectrocatalytic performance with $-(1.31 \pm 0.08)$ mA cm^{-2} under the same conditions. This can be attributed to improved electronic communication as a possible result of alterations in the Met-

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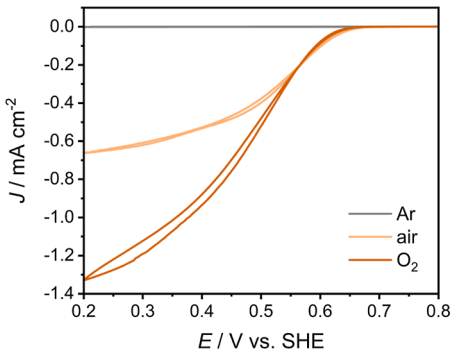

Figure 4. Voltammetric responses for the enzyme variant 2F4 over the Au electrode under Ar, air, and O_2 saturated conditions. Phosphate−citrate buffer, pH 4.0; scan rate: 10 mV s^{-1}; electrode rotation rate: 1000 rpm. Representative measurements are shown; see Figure S7 for the reproducibility of responses.

loop conformation state. In 2F4, the loop is more rigid and remains mostly in closed states with minimal open structures, in contrast to wild-type McoA in which the Met-loop is statistically more open (Figure 1). Previous investigations, by means of small-angle X-ray scattering (SAXS), indicated that the Met-loop-T1 Cu distance in the open-state cluster was (26.2 ± 1.4) Å,[35] while in the closed state cluster, the distance was (16.5 ± 1.7) Å,[36] which is approximately 10 Å shorter for 2F4 than for the wild type. The closed conformation of the Met-loop in 2F4, presumably decreases the distance between the electrode and T1 Cu, resulting in a higher beneficial impact on the electrochemical response. Additional evidence for an improved transfer of electrons between the electrode and the enzyme is provided by a comparison of the voltammetric responses. The occurrence of crossing points for McoA WT-modified electrodes (Figure S8) can be explained taking into consideration the accepted mechanism for O_2 reduction by MCOs.[44,45] When electrons cannot be efficiently supplied to T1 Cu, the enzyme slowly decays to the resting oxidized form, which can reintegrate only the catalytic cycle after reduction. Thus, if DET is limited, a higher population of the enzyme in the fully active form for O_2 reduction can only be obtained after the application of highly negative potentials necessary to promote the reduction of the redox centers. Such a limitation was not observed for any of the 2F4-modified electrodes and confirmed a more efficient DET. To support our observations, we investigated electrodes modified with a loop-truncated variant, corresponding to variant 2F4 with a loop of only five amino acid residues instead of a total of 29. The electrochemical response was noticeably lower and more irreproducible in comparison with that obtained for 2F4 with a full-length loop (see Figure S9), providing current densities of only −(0.8 ± 0.2) mA cm^{-2}. Moreover, the reappearance of crossover points in the voltammograms indicated a more restricted electronic communication and confirmed that the Met-rich loop plays a prominent role in enzyme immobilization in an adequate disposition for DET with T1 Cu.

The biocatalytic response for 2F4 over Au electrodes was also investigated using different rotation rates (Figure S10), confirming that under the conditions used the response was not limited by the O_2 mass transport. Taking into consideration that rather flat electrode surfaces have been used in this study, with an average roughness factor of (1.13 ± 0.05), the remarkable biocatalytic responses for ORR are among the highest cathodic currents ever reported for Au electrodes modified with MCOs relative to the real electrode surface area (for a detailed comparison, see Table S1).

The voltammograms recorded for McoA WT and the 2F4 variant did not show a characteristic mass-transport limiting current even under O_2-saturated solutions (see Figures 2A and 4). In contrast, the current responses were kinetically limited by electron transfer. Thus, a theoretical model accounting for the dispersion of interfacial electron transfer rates on the catalytic responses of enzymes over electrodes,[41] later adapted to the specific case of MCO electrocatalysis,[6] was used. The model takes into consideration the orientation dispersion of immobilized enzymes, as given by the dimensionless parameter βd_0, and the ratio between the apparent rate constant for enzymatic catalysis (k_{cat}) and the rate constant for DET to T1 Cu at the optimal enzyme orientation (k_0^{max}). The fitting results with this model are shown in Figure 5 and are summarized in

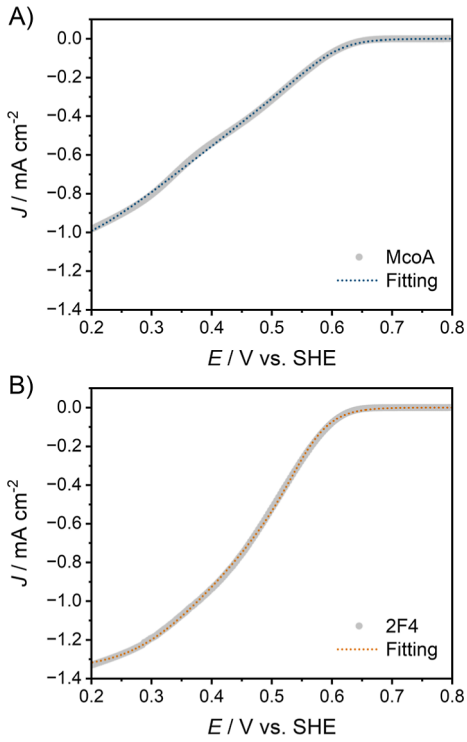

Figure 5. Forward scan of the voltammetric traces recorded for McoA WT (A) and 2F4 variant (B) during electrocatalytic ORR. O_2-saturated phosphate−citrate buffer, pH 4.0; scan rate: 10 mV s^{-1}; electrode rotation rate: 1000 rpm. The dotted lines represent the fitting of the curves to a nonlinear regression analysis as indicated in the main text.

Table S2. The small values obtained for the orientation dispersion parameter (8.8 for McoA WT and 7.2 for the 2F4 variant) are in line with literature reports highlighting strategies for high and controlled enzyme orientation.[16,30] As a consequence, it can be concluded that a rather narrow orientation dispersion is achieved through the immobilization strategy used, thanks to the Met-rich loop acting as an anchoring point. This was also consistent with the high reproducibility observed between different modified electrodes, which is ascribed to the spontaneously directed immobilization of enzymes during electrode modification. Moreover, values of k_{cat}/k_0^{max} of 0.43 and 0.112 were determined for McoA WT and the 2F4 variant, respectively. Even if an increased k_{cat} could be expected for the 2F4 variant with respect to the WT,[36] the

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obtained results point out a higher rate constant for DET for 2F4-modified electrodes. This confirmed the previous hypothesis that a closed conformation of the Met-loop near T1 Cu in the 2F4 variant facilitates more efficient electron transfer between the electrode and the immobilized enzyme.

Electrodes modified with the McoA WT and the 2F4 variant were also investigated at different pH values. A comparison of the electrochemical performance at pH 7.0 is shown in Figure S11. In both cases, the highest responses were observed at pH 4.0, in agreement with the optimum pH observed for the enzymes.[36,42] Moreover, a comparison of the responses observed at the two pH values showed that the current reached at 0.2 V versus SHE at pH 7.0 was about 31% of that obtained at pH 4.0 for McoA, while for 2F4, this value was 37%, in relation with the improved performance for the bioelectrode constituted by the enzyme variant. A comparison of the performance of both enzymes showed an increased response for the enzyme variant, in agreement with the expected improved performance due to a higher electron transfer rate with the T1 Cu center.

4. CONCLUSIONS

The protein structure of a metallo-oxidase, the MCO from *A. aeolicus* (McoA) integrates a characteristic additional loop close to the T1 Cu site, which is the entry point for electrons for biocatalytic O_2 reduction. This additional amino acid region, rich in methionine residues, can be exploited as an anchoring point for the immobilization of the protein on gold surfaces. As we have shown, this allows for a very simple attachment of the protein to the electrode without requiring any previous electrode modification or functionalization. At the same time, due to the location of the Met-rich loop, the adsorbed enzymes are spontaneously oriented with the T1 Cu site optimally placed for electron transfer with the electrode. The results obtained for an enzyme variant in which the preferential conformation of the Met-loop over T1 Cu is in a closed state translate into an improved electron transfer, leading to an increased response for the ORR with modified bioelectrodes. The analysis of the electrochemical responses was used to investigate the possible dispersion of enzymes over the electrode, resulting in values that indicate a rather homogeneous protein disposition due to directed immobilization. This is also reflected in the high reproducibility of the electrochemical responses. A comparison of the responses obtained with previous reports in the literature clearly indicates that the described approach, in addition to being very simple, leads to substantially high current responses for the biocatalytic ORR, which is remarkable considering that flat bare electrode substrates are used. The obtained results pave the way for the simple fabrication of electrodes that can be used for a detailed characterization of these kinds of enzymes and the potential development of bioelectrodes with substantial O_2 reduction performance.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.4c00516.

Electrochemical surface area characterization, solvent-exposed methionines, reproducibility of McoA-modified electrodes, controls in the presence of ABTS, SECM characterization, catalytic response at different pH values, reproducibility of 2F4-modified electrodes, control with a Met-loop-truncated variant, comparison of rotation rates, comparison of McoA WT and 2F4 variant at pH 7.0, summary of literature reports for MCOs over Au electrodes, and fitting parameters derived from eq 1 (PDF)

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Notes

The authors declare no competing financial interest.

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