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A biosynthetic model of cytochrome c oxidase as an electrocatalyst for oxygen reduction

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Creating an artificial functional mimic of the mitochondrial enzyme cytochrome c oxidase (CcO) has been a long-term goal of the scientific community as such a mimic will not only add to our fundamental understanding of how CcO works but may also pave the way for efficient electrocatalysts for oxygen reduction in hydrogen/oxygen fuel cells. Here we develop an electrocatalyst for reducing oxygen to water under ambient conditions. We use site-directed mutants of myoglobin, where both the distal Cu and the redox-active tyrosine residue present in CcO are modelled. *In situ* Raman spectroscopy shows that this catalyst features very fast electron transfer rates, facile oxygen binding and O-O bond lysis. An electron transfer shunt from the electrode circumvents the slow dissociation of a ferric hydroxide species, which slows down native CcO (bovine 500 s^{-1}), allowing electrocatalytic oxygen reduction rates of $5,000\text{ s}^{-1}$ for these biosynthetic models.

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Mimicking the sophistication of naturally occurring enzymes has been a long-term goal of the scientific community. An artificial analogue that can perform equally well as its natural predecessor will not only provide deeper understanding of the native enzymes, but also enable the development of efficient artificial catalysts. For several decades now chemists have embarked on this daunting pursuit of emulating the efficiency and selectivity of naturally occurring enzymes and several important milestones have been achieved. Efforts from synthetic inorganic chemists have resulted in synthetic models of myoglobin (Mb), galactose oxidase, tyrosinase, cytochrome P450 and cytochrome *c* oxidase (CcO)^{1–5}. Alternatively, there has been fervent pursuit of biochemical constructs inspired by natural metalloenzymes. A series of binuclear non-haem iron, cytochrome *c*, haem oxidases and iron–sulfur enzyme models have resulted from such efforts^{4,6–12}. While none of the synthetic or biochemical models reported so far could match the reactivity exhibited by their natural counterparts, fundamental insights regarding the structure–function correlations of several metalloenzymes have been gained in the process^{7,13–15}. In addition, key information about the secondary coordination sphere interactions present in the protein–active site, which play a dominating role in determining the electronic structure and reactivity of these metalloenzymes, have been identified^{16,17}.

In a biosynthetic approach, stable naturally occurring proteins have been used as scaffolds for creating mimics of several metalloenzymes, such as hydrogenases which are involved in the reversible generation of H₂ from water, haem proteins participating in electron transfer and O₂-binding, non-haem iron and copper enzymes active in small molecule activation, and even novel enzymes containing non-native cofactors^{18–24}. For example, using this approach, biosynthetic models that structurally and functionally mimic CcO and nitric oxide reductase have been reported^{7,25}. Despite decades of focused effort, however, biosynthetic models with catalytic efficiencies approaching those of the naturally occurring metalloenzymes have remained elusive^{26–28}. In this report, we communicate a biosynthetic model of CcO bearing the distal Cu_B and a tyrosine residue that is kinetically more competent in reducing O₂ electrochemically than any known synthetic analogue, as well as native CcO itself.

X-ray crystallography of Mb and its mutant have revealed that its two propionate side chains project out of the protein surface into the solvent (Fig. 1)⁷. Taking advantage of this structural feature, we have previously developed an electrocatalytic O₂

reduction system where the native haem cofactor in Mb is replaced by a modified heme cofactor bearing an alkyne group (Hemin-yne, Fig. 2) so that electrons can be injected directly into the haem from a gold electrode to facilitate O₂ reduction²⁹. This method resulted in a Mb-functionalized electrode bearing 2.15×10^{-12} mol per cm² of protein, which was characterized using several microscopic and spectroscopic techniques²⁹. Over the last few years, a biosynthetic model of CcO has been reported in which two distal residues of Mb (L29 and F43) have been mutated to His, which along with the native His64, form a Cu-binding site, mimicking the distal Cu_B-binding site present in CcO (Cu_BMb)³⁰. Furthermore, in an attempt to mimic the conserved Tyr 244 residue in the CcO-active site, a G65Y mutant of Cu_BMb (G65YCu_BMb) containing redox-active tyrosine residue in the distal site and a variant where a tyrosine residue was crosslinked to the active site histidines were also created (Fig. 1)³¹.

Herein we report the electrocatalytic properties of the G65YCu_BMb (higher synthetic yields than the tyrosine cross-linked variant) immobilized on an Au electrode using the method developed for WT Mb²⁹.

Results

Electrode characterization by SERRS. Surface-enhanced resonance Raman spectroscopy (SERRS) data (Fig. 3a) of the electrodes bearing the G65YCu_BMb protein with and without Cu_B show the oxidation and spin state marker ν_4 , ν_3 , ν_2 and ν_{10} bands at 1,375 cm⁻¹, 1,493 cm⁻¹, 1,585 cm⁻¹ and 1,641 cm⁻¹, respectively. The ν_4 , ν_3 and ν_2 values are consistent with the presence of a five-coordinated high spin haem in the active site on these electrodes bearing the biochemical constructs of CcO³². Also the bands at 1,504 cm⁻¹ and ν_4 at 1,641 cm⁻¹ suggest the presence of a mixture of six-coordinate low spin species, which likely has H₂O as the axial ligand. The positions of these bands in the G65YCu_BMb and their relative intensities are different from Hemin-yne (Supplementary Fig. 1)²⁹.

Electrode characterization by X-ray photoelectron spectroscopy.

X-ray photoelectron spectroscopic (XPS) data of a G65YCu_BMb-bound Au electrode clearly indicate the presence of Fe, Cu, C, N and O elements (Supplementary Fig. 2, Supplementary Table 1). The 3p_{3/2}, 2p_{3/2} and 2p_{1/2} binding energy peak for the Fe^{III} of haem group appear at 56.5 eV, 709.4 eV and 722.4 eV, respectively^{33,34}. The 2p_{3/2} and 2p_{1/2} binding energy peak for Cu^{II} in the distal site appear at 931.7 eV and 951.8 eV, respectively³⁵. The N_{1s}

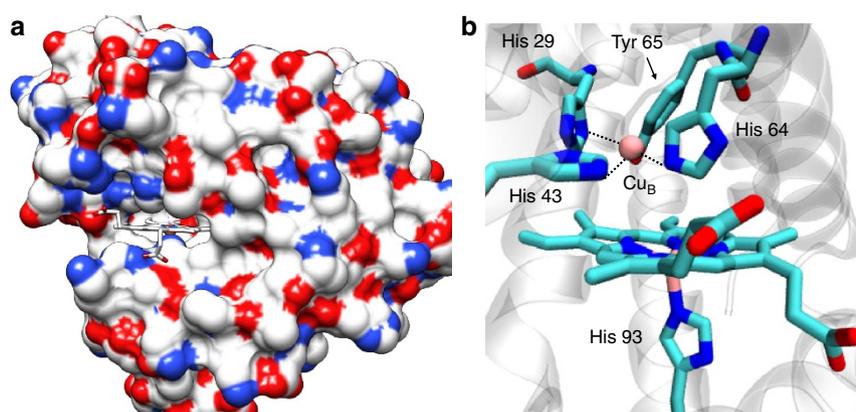


Figure 1 | Crystal structure. Crystal structure of a Mb-based biosynthetic model of CcO, F33Y-Cu_BMb; pdb id: 4FWY. **(a)** The haem cofactor is in a cleft on the molecule protein surface, (colour coded according to the charge of the residues), with the propionate groups exposed to the solvent. **(b)** The computer model of G65YCu_BMb showing its catalytic centre containing the distal Cu_B bound to histidines and a tyrosine 65.

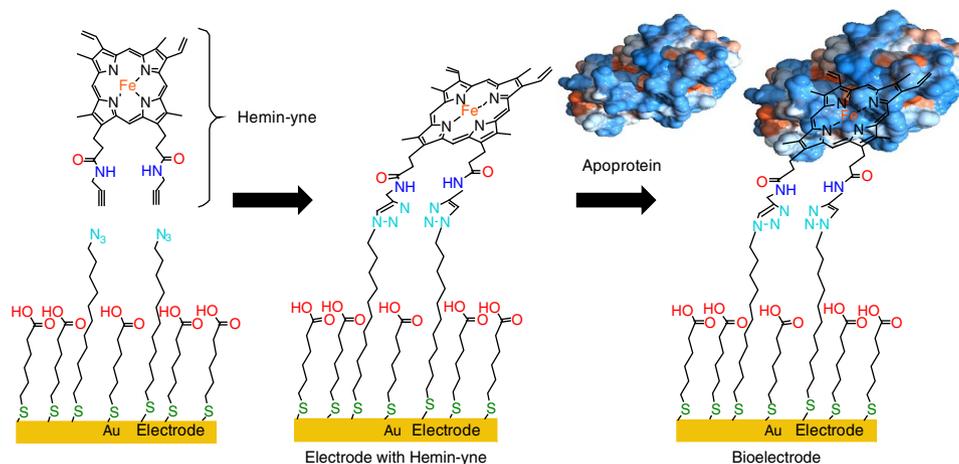


Figure 2 | Construction of the electrode bearing the biosynthetic model. Reconstitution of apoprotein *in situ* with Hemin-yne groups that are covalently attached to mixed self-assembled monolayers of thiols on an Au electrode. The modified hemin is indicated as Hemin-yne.

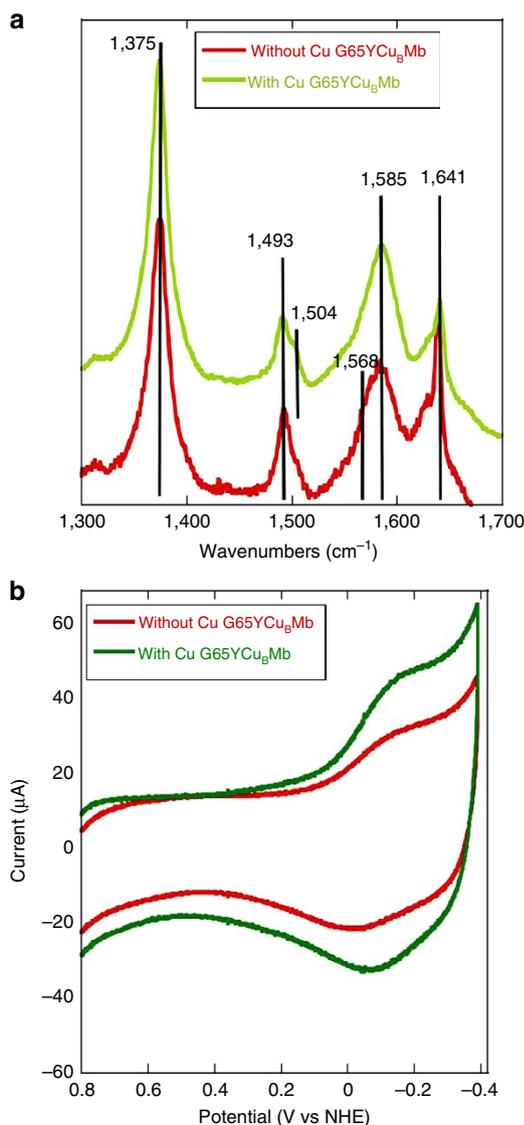


Figure 3 | SERRS and CV data for the electrode fabricated with G65YCu_BMb mutant. (a) SERRS spectra of G65YCu_BMb with (green) and without (red) the distal Cu²⁺ in air-saturated 100 mM phosphate buffer (pH 7) solution. (b) Anaerobic CV of G65YCu_BMb without Cu_B (red) and after Cu_B binding (green). 2 V s⁻¹ scan rate, in degassed, pH 7, 100 mM phosphate buffer using a Pt counter electrode.

peak is broad (Supplementary Fig. 2), as it contains several components due to the presence of amide, haem pyrroles and the triazole groups (resulting from the covalent attachment of Hemin-yne) on the surface³³. Similarly, the C_{1s} peak (Supplementary Fig. 2) contains contributions from different types of C atoms (aromatic, aliphatic, haem and so on) on these protein-modified surfaces³⁶.

Electrode characterization by CV. Cyclic voltammetry (CV) of G65YCu_BMb with and without the distal Cu_B immobilized onto the electrodes in degassed buffer show the haem Fe^{3+/2+} midpoint reduction potential ($E^{1/2}$) at -97 mV and -57.5 mV, respectively (Fig. 3b). The peak separation between the cathodic and the anodic peak for both the cases is ~70 mV (ref. 37). Hemin-yne displays the Fe^{3+/2+} reduction potential at -70.0 mV in the absence of a protein and -135.0 mV when bound to wild-type apo Mb²⁹. In the case of the G65YCu_BMb protein-bound electrodes, the Cu^{2+/+} process overlaps with the Hemin-yne Fe^{3+/2+} process, resulting in approximately twice the area under these CV peaks relative to the G65YCu_BMb-bearing electrodes prior to Cu_B loading. The integrated area under these CV features in the absence of Cu²⁺ indicates that there are $2.55 \pm 0.05 \times 10^{-12}$ mol of protein per cm² of the surface. The ratios of the integrated area under the CV features of G65YCu_BMb functionalized before and after loading the Cu_B is ~1:2 (Table 1, fourth column) which is consistent with the expected 1:1 stoichiometry (that is, every G65YCu_BMb binds one Hemin-yne and one Cu²⁺ ion). Note that the $E^{1/2}$ values of the hemin and Cu_B measured for these electrodes are slightly different from those estimated from potentiometric titration in solution^{7,19}. This is likely due to the interfacial microenvironment of the -COOH-terminated Self Assembled Monolayer (SAM) which is known to shift the apparent formal potentials of redox-active species in its vicinity³⁸. Thus the *in situ* reconstitution of the protein with Hemin-yne on the electrode is evident from the SERRS, XPS (Supplementary Fig. 2) and CV data (Supplementary Fig. 3, Supplementary Fig. 4). The presence of the Cu²⁺ at Cu_B site on the electrode is indicated by XPS and CV data. Taken together, these data indicate the assembly of the G65YCu_BMb, biosynthetic model of CcO covalently attached to the electrode via the linkage between the Hemin-yne and the azide terminated thiols created using click reaction (Fig. 2).

O₂ reduction reactivity of the electrode. In linear sweep voltammetry experiments performed in aerated buffers, large

electrocatalytic O_2 reduction currents are observed by the G65YCu_BMb (with and without Cu²⁺) bearing bio-electrodes at pH 7 at room temperature, as the applied potential is lowered below +100 mV versus NHE (Fig. 4a, Supplementary Fig. 5). Thus as the potential of the electrode is lowered such that the iron in these proteins is reduced to Fe^{II}, an electrocatalytic O_2 reduction current is observed. It is important to note that the potential of O_2 reduction reaction (ORR) (E_{ORR}) is -263 mV, which is more negative than the $E^{1/2}$ (-97 mV), suggesting that the potential determining step of ORR is not the reduction of resting Fe^{III} to Fe^{II} but the reduction of a different species with -166 mV more negative potential (Fig. 4a inset). A more negative E_{ORR} relative to $E^{1/2}$ is mechanistically significant (*vide infra*).

In these active sites, O_2 may be reduced by $4e^-$ and $4H^+$ to H_2O , or by fewer electrons to produce partially reduced oxygen

species (PROS) like O_2^- and H_2O_2 . The extent of $4e^-$ reduction and the second order rate constant (k_{ORR}) of the ORR can be determined using rotating disc electrochemistry (RDE) where the catalytic O_2 reduction current increases with increasing rotation rates (Fig. 4a,b) following the Koutecky–Levich (K–L) equation (equation (1))³⁹.

$$i_{cat}^{-1} = i_K(E)^{-1} + i_L^{-1} \quad (1)$$

where, $i_K(E)$ is the potential-dependent kinetic current and i_L is the Levich current. i_L is expressed as

$$i_L = 0.62nFA[O_2](D_{O_2})^{2/3}\omega^{1/2}\nu^{-1/6} \quad (2)$$

where n is the number of electrons transferred to the substrate, A is the macroscopic area of the disc (0.096 cm²), $[O_2]$ is the concentration of O_2 in an air-saturated buffer (0.26 mM) at 25 °C,

Table 1 | $E^{1/2}$ and coverage.

Protein	Metal-binding sites	$E^{1/2}$ (mV)	Integrated coverage (mol cm ⁻²)
Hemin-yne reconstituted myoglobin ²⁹	Fe	-135.0 ± 5.0	2.15 ± 0.05 × 10 ⁻¹²
Hemin-yne reconstituted G65YCu _B Mb	Fe	-57.5 ± 5.0	2.55 ± 0.05 × 10 ⁻¹²
	Cu _B , Fe	-97.0 ± 5.0	4.65 ± 0.05 × 10 ⁻¹²

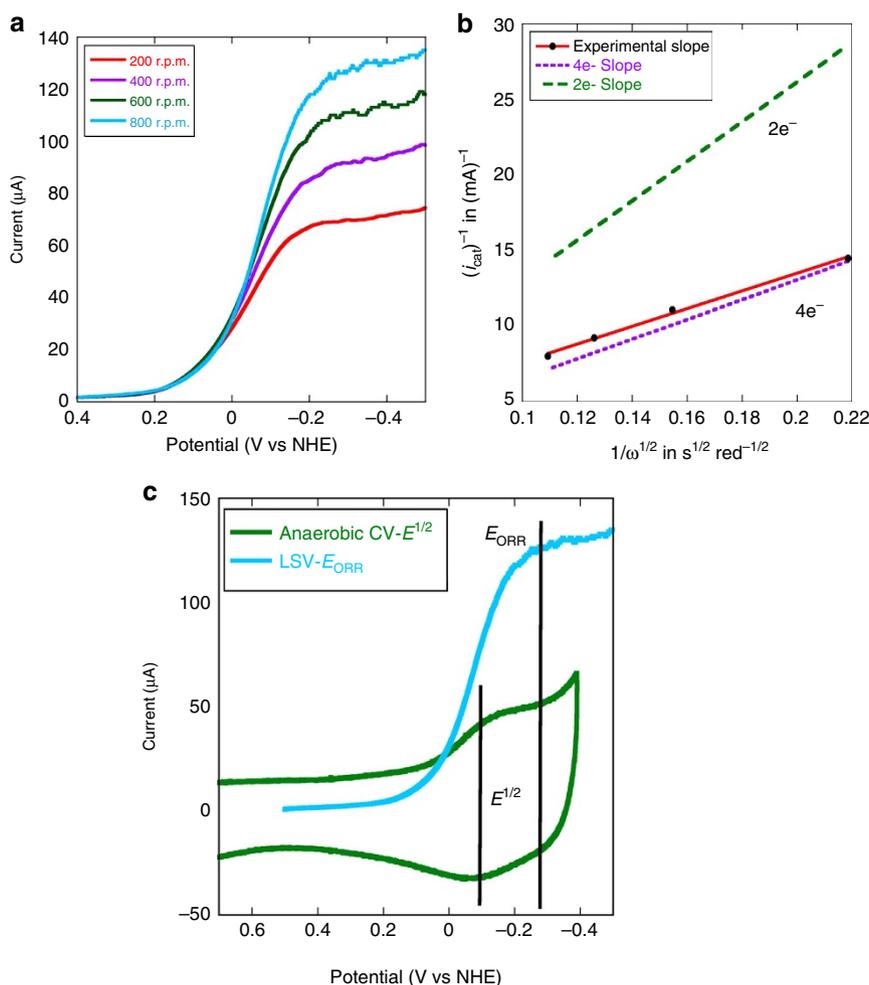


Figure 4 | RDE data for electrode modified with G65YCu_BMb with Cu²⁺. (a) Linear sweep voltammogram of G65YCu_BMb with Cu²⁺ in air-saturated pH 7, 100 mM phosphate buffer solution at 100 mV s⁻¹ scan rate, potentials are scaled relative to NHE and a Pt counter electrode is used. Data are collected at different rotation speeds (200 r.p.m.—red, 400 r.p.m.—purple, 600 r.p.m.—green, 800 r.p.m.—blue). (b) Plot of i_{cat}^{-1} for G65YCu_BMb-bearing bioelectrode at -300 mV potential and at multiple rotation rates, with the inverse square root of the angular rotation rate ($\omega^{-1/2}$) (c) Difference between the potential for O_2 reduction (E_{ORR}) and the midpoint reduction potential of Fe^{3+/2+} redox couple ($E^{1/2}$).

D_{O_2} is the diffusion coefficient of O_2 ($1.8 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$) at 25 °C, ω is the angular velocity of the disc and ν is the kinematic viscosity of the solution ($0.009 \text{ cm}^2 \text{ s}^{-1}$) at 25 °C (ref. 40).

Plot of i_{cat}^{-1} at multiple rotation rates with the inverse square root of the angular rotation rate (i_{cat}^{-1}) for G65YCu_BMb (with Cu²⁺) (Fig. 4b) is linear. The slope of K–L plot is expressed as $1/[n\{0.62FA(D_{O_2})^{2/3}\nu^{-1/6}\}]$, which can be used to experimentally estimate the value of n where n is the number of electrons donated to the substrate, that is, O_2 . The slope obtained from the experimental data for G65YCu_BMb (Fig. 4b) is close to the theoretical slope (Fig. 4b, dotted purple line) expected for a $4e^-$ process and very different from the slope for a $2e^-$ process (Fig. 4b, dotted green line). Thus the G65YCu_BMb bioelectrode predominantly catalyses a $4e^-/4H^+$ reduction of O_2 to H_2O at pH 7.

The intercept of the K–L plot is the inverse of the kinetic current ($i_K(E)^{-1}$), where $i_K(E)$ is expressed as⁴¹

$$i_K(E) = k_{\text{ORR}} nFA[O_2]\Gamma_{\text{cat}} \quad (3)$$

where, n is the number of electrons, A is the geometric surface area, $[O_2]$ is the bulk concentration of O_2 , Γ_{cat} is the surface coverage of the catalyst (obtained from the integration of the anaerobic CV data) and k_{ORR} is the second order rate constant for O_2 reduction estimated at -300 mV . At this potential, in an oxygenated buffer, the G65YCu_BMb catalyst is involved in substrate diffusion-limited ORR. Using this equation (equation (3)) and the experimentally obtained $i_K(E)$ at -300 mV , the second order rate constant for O_2 reduction G65YCu_BMb is evaluated to be $1.98 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2). The pseudo first order rate can be determined (Table 2) from the second order rate by taking into account the substrate, O_2 concentration under these experimental conditions to be 0.26 mM (Supplementary Fig. 6). The catalytic ORR rate by G65YCu_BMb surpasses those reported for the best artificial synthetic analogues (Table 2).

The G65YCu_BMb biosynthetic Mb scaffold-based bio-electrode for O_2 reduction is remarkably stable. Monolayers bearing covalently attached O_2 -reducing electrocatalysts reported so far have never been stable enough to allow these dynamic electrochemical experiments to determine the kinetic parameters (k_{ORR} , number of electrons and so on). Enzymes like laccases, directly attached to chemically modified graphite electrodes, were found to be stable enough to be investigated with these hydrodynamic techniques²⁷. The failure to perform these experiments has been attributed to degradation of the catalyst during RDE experiment, that is, very small turnover numbers presumably due to the production of PROS during ORR. Rotating ring disc electrochemistry (RRDE) shows formation of only $\sim 6\%$ PROS by the G65YCu_BMb (Supplementary Fig. 7) during ORR, indicating that it reduces 96% of O_2 to H_2O consistent with the RDE data. During the RDE experiments (Fig. 4a), the G65YCu_BMb-functionalized electrodes bearing 10^{-12} mol of the catalyst reduced $1.8 \pm 0.3 \times 10^{-8} \text{ mol}$ of O_2 ($7 \pm 1 \times 10^{-3} \text{ C}$ total charge and $4e^-$ per O_2 molecule) yielding a turnover

number of at least 10^4 . The role of PROS in degrading the catalyst is established by the fact that the electrolytic current (at -0.3 V) remains stable in the presence of $50 \mu\text{M}$ catalase in solution (Supplementary Fig. 8).

Discussion

To understand the facile and selective O_2 reduction catalysed by the G65YCu_BMb biochemical model, the recently developed SERRS-RDE technique is employed⁴². In this technique, the rR spectra of the catalyst (that is, G65YCu_BMb) bearing electrode is collected while the system is involved in steady state O_2 reduction and the species accumulated in the steady state can be identified. For any species to accumulate in steady state, its rate of formation has to be greater than its rate of decay. Thus, while the species preceding the rate-determining step (rds) will accumulate at steady state, the accumulation of a species in steady state does not immediately imply its decay as the rds. In the absence of O_2 , a high spin ferrous species is formed, characterized by a ν_4 and ν_3 vibrations at $1,357 \text{ cm}^{-1}$ and $1,473 \text{ cm}^{-1}$ (Fig. 5a, cyan), respectively, when a cathodic potential of -0.4 V is applied signifying reduction of the resting ferric state (Fig. 5a, red) to the active ferrous state at these potentials. However, when the same reducing potential is applied in an oxygenated buffer the SERRS-RDE data (Supplementary Fig. 9) clearly show the presence of different species during electrocatalytic ORR, which leads to broadening of the ν_4 , ν_3 and ν_2 regions (Fig. 5a, green) relative to the oxidized and reduced states (Fig. 5a, red and cyan). In particular, the ν_3 and ν_2 vibrations discernibly shift to higher energies as indicated by clear increase in intensities at $1,508 \text{ cm}^{-1}$ and $1,591 \text{ cm}^{-1}$ (Fig. 4b), suggesting the accumulation of $\text{Fe}^{\text{IV}}=\text{O}$ species during steady state ORR⁴³. Signals from high spin ferrous, resting high spin ferric, low spin ferric and ferryl species with ν_3 at $1,473 \text{ cm}^{-1}$ (Fig. 5a, cyan; and Fig. 5b, brown), $1,493 \text{ cm}^{-1}$ (Fig. 5a, red and Fig. 5b, dashed green), $1,504 \text{ cm}^{-1}$ (Fig. 5a, cyan and Fig. 5b, cyan) and $1,508 \text{ cm}^{-1}$ (Fig. 5a, green and Fig. 5b, green), respectively, could be convoluted by fitting the ν_3 region of the spectrum. The lack of significant signal from the high spin ferrous species (weak ν_3 at $1,473 \text{ cm}^{-1}$) suggests that O_2 binding to these species is facile in the steady state. These mutants use the basic design of Mb which has a very fast O_2 -binding rate ($10^7 \text{ M}^{-1} \text{ s}^{-1}$) (refs 44,45). This rate is indeed ~ 10 times faster than O_2 binding to the haem a_3 site of CcO (ref. 46). Similarly, the very weak intensity of the high spin (HS) Fe^{III} species indicates that the ET to Fe^{III} resting state is very facile at these potentials as may be expected due to direct attachment of the Hemin- γ ne to the electrode. The significant intensity of ferryl species (ν_2 at $1,591 \text{ cm}^{-1}$ and ν_3 at $1,508 \text{ cm}^{-1}$) entails the O–O bond cleavage leading to its formation to be faster than its decay via reduction under steady state. Thus the reduction of the resting Fe^{III} state, O_2 binding to Fe^{II} are facile in G65YCu_BMb under the reaction conditions. The low spin ferric species accumulated during steady state ORR could be a dioxygen adduct or peroxide adduct similar to those observed in native CcO and its model systems^{46–51}. The low frequency region shows $^{18}\text{O}_2$ -sensitive bands suggestive of the formation of a low spin ferric peroxide and $\text{Fe}(\text{IV})=\text{O}$ (Supplementary Fig. 10).

If one were to conceive of a Gedanken steady state turnover experiment with CcO where the electron transfer to the active site is very fast (that is, in the hypothetical situation where ET from Cyt c to CcO is not the rds) as the ET from the electrode to the active site is very fast due to direct attachment of the later to the former, the species that would accumulate during turnover, based on the Babcock–Wikström mechanism (Fig. 6, the parameters of native CcO are indicated in purple and G65YCu_BMb are

Table 2 | k_{ORR} of different ORR catalysts.

ORR catalysts	Metals	k_{ORR}	PROS (%)
G65YCu _B Mb	Cu _B , Haem	$1.98 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ or $5,148 \text{ s}^{-1}$	$\sim 6 \pm 1$
Synthetic model ⁶⁰	Cu _B , Haem	$1.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	$\sim 10 \pm 1$

ORR, O_2 reduction reaction; PROS, partially reduced oxygen species.
 k_{ORR} determined at -0.4 V versus NHE; PROS determined at -0.12 V versus NHE in both cases.

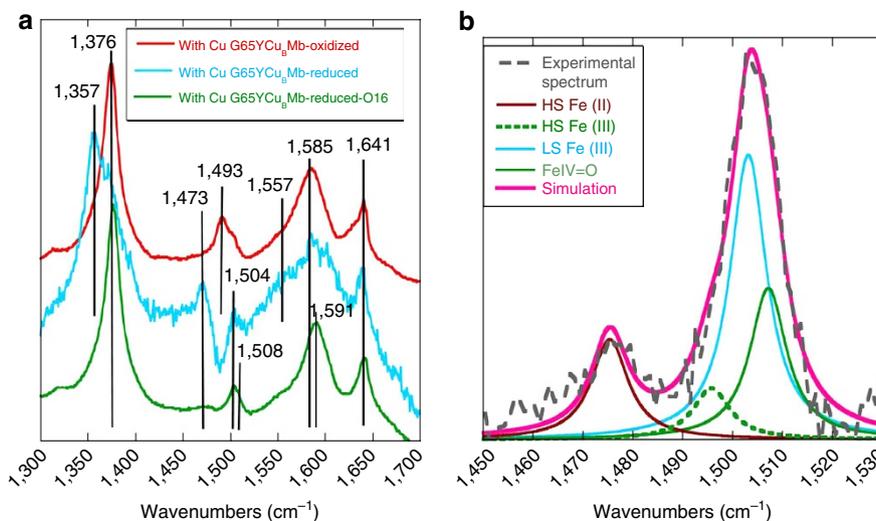


Figure 5 | SERRS-RDE data of G65YCu_BMb-bearing electrode. (a) SERRS-RDE data of G65YCu_BMb-bearing electrode at oxidized (applied potential was 0 V with respect to Ag/AgCl reference electrode), reduced (applied potential was -0.4 V with respect to Ag/AgCl reference electrode) state and in the presence of O₂ (O16) saturated 100 mM pH 7 phosphate buffer and (b) Components of the rR spectrum determined by simulating the spectra of G65YCu_BMb-bearing electrode in the presence of O₂ (O16) saturated 100 mM pH 7 phosphate buffer indicated in green in a.

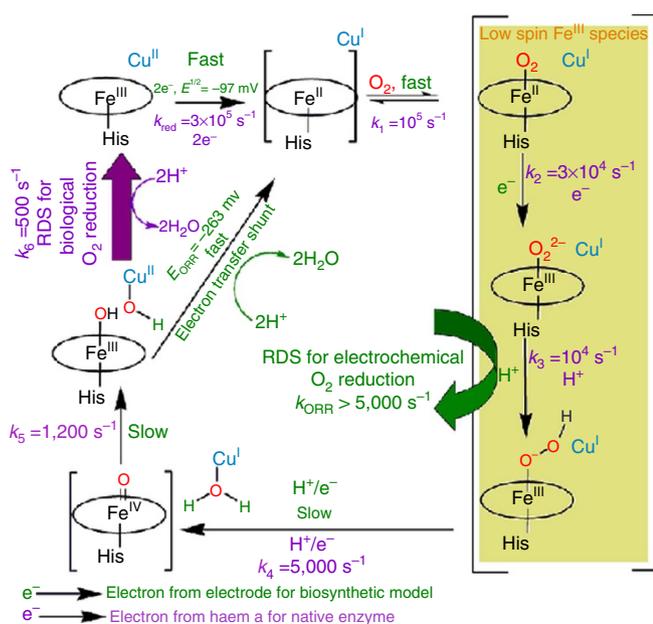


Figure 6 | Mechanisms. Comparison between the mechanism of native CcO (ref. 46) in solution and the biosynthetic G65YCu_BMb model on electrode.

indicated in green⁵² and the arrows in black indicate general route for O₂ reduction by CcO followed in both the native system, as well as in the G65YCu_BMb-immobilized electrode) are the Fe^{II}, Fe^{II}-O₂, Fe^{III}-O₂⁻, Fe^{III}-OOH, Fe^{IV}=O and Fe^{III}-OH species as the rates of formation of these species are greater than their rates of decay^{53,54}. Out of these, the Fe^{II}-O₂, Fe^{III}-O₂⁻ and Fe^{III}-OOH species will have Raman signatures of low spin haem (Fig. 6), Fe^{III}-OH will have rR signature of high spin haem and the Fe^{IV}=O will have signatures unique to haem ferryl species^{46,54,55}. The SERRS-RDE data show the presence of species having signatures of low spin Fe^{III} and Fe^{IV}=O. While the later can originate from only a single species, the former can indicate the presence of any of the three species or a combination of them. The lack of significant high spin signal indicates that the biosynthetic model circumvents accumulation of Fe^{III}-OH and

resting Fe^{III} species in the steady state by facile ET. The overall rate-limiting step of native CcO in solution is the dissociation of hydroxide of the Fe^{III}-OH end product of O₂ reduction from haem to generate the active ferric resting form and has a first order rate constant of 500 s⁻¹ (refs 46,54–56). This dissociation is required during turnover as the potential of this hydroxide-bound form is likely to be more negative than the five-coordinate resting oxidized site (which will be regenerated after hydroxide dissociation) and will not be reduced by haem a. While the E^{1/2} of a haem a₃ Fe^{III}-OH species cannot be determined with confidence due to strong interaction potential and co-operativity between the haem a and haem a₃ sites, the potential of formate and azide-bound high spin haem a₃ site (analogous to hydroxide) is ~ 130 to 200 mV more negative than the resting ferric site^{57,58}. The potential determining step of ORR (defined as the electron transfer (ET) step in catalysis having lowest potential) by the G65YCu_BMb is 166 mV more negative than the E^{1/2} for the resting high spin ferric state and is likely to be the reduction of the Fe^{III}-OH species. Thus the bioelectrode can circumvent the kinetic barrier associated with the dissociation of the hydroxide by directly reducing it to ferrous at 166 mV lower potential. This direct electron transfer to the ferric hydroxide species, which is an intermediate in the catalytic cycle of CcO, circumventing a slow step in catalysis, is an electron transfer shunt analogous to peroxide shunt in cytochrome P450, which overcomes the rate-determining O₂ activation step of the native enzyme⁵⁹. In the mass transfer controlled region of the catalytic current, all ET steps are facile and steps like O₂ binding, protonation and O–O bond cleavage can be rds at these potential. The lack of HS Fe^{II} accumulation in the SERRS-RDE indicates that O₂ binding to Fe^{II} is very fast and not the rds. The O–O bond cleavage in the Babcock–Wikström mechanism involves ET to the active site and not the rds as well. Thus rds of ORR by these biosynthetic models is likely to be the protonation of the Fe^{III}-O₂⁻ species with a first order rate of 5,000 s⁻¹ (Fig. 6).

In an air-saturated buffer (0.26 mM O₂), the pseudo first order rate constant of ORR by G65YCu_BMb is determined to be $\sim 5\text{--}6 \times 10^3$ s⁻¹ (k_{ORR} [O₂]). The highest second order O₂ reduction rate reported for any synthetic mimic of CcO is 1.2×10^5 M⁻¹ s⁻¹; that too on a multilayer having 1,000 times more catalyst than the G65YCu_BMb electrodes⁶⁰. The second order rate

constant of G65YCu_BMb is $10^7 \text{ M}^{-1} \text{ s}^{-1}$ which is, thus, 2 orders of magnitude higher than best synthetic haem/Cu-based O₂ reduction electrocatalyst. Thus the selectivity and kinetic rate of the G65YCu_BMb-bearing electrode surpasses those reported for smaller synthetic analogues and illustrates the advantages of using a biochemical scaffold over a synthetic scaffold. Although the pseudo first order rate constant of the G65YCu_BMb is 10 times faster than the rate of native CcO in solution, such a comparison is vulnerable to differences in reaction conditions (for example, G65YCu_BMb is water soluble but CcO exists in membranes). Alternatively, erstwhile efforts resulting in electrodes bearing native CcO in a manner similar to these bio-electrodes show extremely sluggish O₂ reduction^{60–62}. This is due to improper alignment of this membrane-bound protein on the electrode, which precludes efficient electron transfer to the active site^{63–65}. However, the direct attachment of haem to the electrode utilizing its solvent-exposed propionate groups (that is, a short circuit) enables fast electron transfer to the active site²⁹. This is further supported by the fact that when ethynylferrocene (Fc) is attached to the same surface $\sim 25 \text{ mV}$ peak separation is observed even at 5 V s^{-1} (Supplementary Fig. 11)³⁸, suggesting that the ET is indeed fast. As a result when a CcO-functionalized SAM-covered Au electrode produces $< 1 \mu\text{A}$ electrochemical O₂ reduction current at -300 mV , this bio-electrode produce $\sim 100 \mu\text{A}$ current at similar potentials⁶⁶.

Finally, the G65YCu_BMb mutant has residues in the distal pocket that can help both electron and proton transfer during O₂ reduction (Y65 in G65YCu_BMb). In CcO, the involvement of Tyr 244 residues in proton/electron transfer during O₂ reduction is now widely accepted^{67,68}. Previous biochemical and structural studies on these mutants had indeed indicated the close proximity of this residue to the distal site^{7,19,30}. An analogous biochemical model without the Y65 residue, Cu_BMb, is not as stable as the G65YCu_BMb as the former degrades rapidly during the RDE experiments (Supplementary Fig. 12). In summary, a electron transfer shunt which circumvents the rate-determining dissociation of a ferric hydroxide species by directly reducing it at slightly negative potential, fast O₂ binding, fast electron transfer to the active site and the presence of a protective Y65 residue in a biochemical model of CcO results in O₂ reduction activity 100 times faster than the best synthetic models, order of magnitude faster than CcO immobilized on electrode and follows a mechanism comparable to that of native CcO in solution.

Methods

Materials. 1-Azidoundecane-11-thiol and Hemin-yne were synthesized following the reported procedure^{29,69}. 6-Mercaptohexanoic acid was purchased from Sigma Aldrich. Di-sodium hydrogenphosphate dihydrate (Na₂HPO₄ · 2H₂O) was purchased from Merck. 2, 6-lutidine was purchased from Avra Synthesis Pvt. Ltd. These chemicals were used without further purification. Au wafers were purchased from Platypus Technologies (1,000 Å of Au on 50 Å of Ti adhesion layer on top of a Si(III) surface). Transparent Au wafers (100 Å of Au on 10 Å of Ti) were purchased from Phasis, Switzerland. Au and Ag discs for the RRDE and SERRS experiments, respectively, were purchased from Pine Instruments, USA. The Mb mutants were prepared as reported in the literature^{7,30}. Analysis of the components of the rR spectrum was done by using Lorentzian line shape of peak fit software.

Instrumentation. All electrochemical experiments were performed using a CH Instruments (model CHI710D Electrochemical Analyzer). Bipotentiostat, reference electrode and Teflon plate material evaluating cell (ALS, Japan; <http://www.als-japan.com/1398.html>) were purchased from CH Instruments. The RRDE set-up from Pine Research Instrumentation (E6 series ChangeDisk tips with AFE6M rotor) was used to obtain the RRDE data. The mutant Mb-functionalized or SAM-covered Au surface (disc of 0.1 cm² area for RDE, RRDE and wafer of 0.45 cm² area for CV) was always used as the working electrode. The XPS data were collected in a Omicron (model: 1712-62-11) spectrometer using a high-resolution monochromatic Al-K α source at 1,486.7 eV under 15 kV voltage and 10 mA

current maintaining a base pressure of 5×10^{-10} mbar. The binding energies were calibrated to the Ag 3d_{5/2} peak at 368.2 eV. The resonance Raman experiments were done in the Kr⁺ Laser (Sabre Innova, Model—SBRC-DBW-K) purchased from Coherent, and the data were collected using the Spectrograph (Model—Trivista 555) from Princeton Instruments.

Formation of mixed SAM and covalent attachment of Hemin-yne to it. Mixed self-assembled monolayer of 1-azidoundecan-11-thiol and 6-mercaptohexanoic acid was formed on immersing the properly cleaned Au wafers or disks into the deposition solution containing 1-azidoundecan-11-thiol and 6-mercaptohexanoic acid in 10 ml of ethanol in a desired ratio (typically 1:49). The total thiol concentration of these deposition solutions were always maintained at 1 mM. On this SAM Hemin-yne was covalently attached using 'Click' reaction²⁹.

Reconstitution of Apo-G65YCu_BMb mutants to G65YCu_BMb. For all the experiments on heterogeneous SAM surfaces, the Hemin-yne modified -COOH SAM surfaces were incubated with a 20 μM apoprotein (Apo-G65YCu_BMb) solution for 2 h. The supernatant solution was drained and the surface was cleaned with water. The presence of Cu²⁺ in the non-haem-binding site is confirmed by electron paramagnetic resonance (EPR) spectroscopy (Supplementary Fig. 13). The immobilization of the mutant is further confirmed by the absorption spectra and SERRS of the surface fabricated with those mutants (Supplementary Fig. 14, Supplementary Fig. 15).

Cyclic voltammetry. The CV was performed using Au wafers sandwiched between two Teflon blocks of the Plate material evaluating cell. All electrochemical experiments were done in pH 7 phosphate buffer containing potassium hexafluorophosphate. Anaerobic cyclic voltammetric experiments were done by using degassed buffer (three cycles of freeze-pump-thaw). Ag/AgCl reference electrode and Pt counter electrode were used throughout all the electrochemical experiments except the case of anaerobic experiments where only Ag wire was used as the reference electrode.

The peak areas were estimated by integrating the anodic/cathodic peak of the anaerobic CV of the mutant Mb-functionalized SAM-covered Au surface, using the data acquisition software itself. A line collinear with the background is used to subtract the background. The estimated area has been further confirmed by subtracting the background current of a SAM-functionalized electrode (Supplementary Fig. 11) bearing ferrocene. Both these approaches provide the same estimate.

To ensure that the SAM surface is stable during the electrocatalytic investigations, disc bearing just the SAM was subjected to several rotations (200–1,000 r.p.m.) and its capacitive current was found not to change, indicating that the SAM is retained on the electrode during these dynamic electrochemistry experiments (Supplementary Fig. 16). SAM can also be damaged when the protein atop the SAM degrades during ORR due to the reactive oxygen species produced. When an unstable electrocatalyst (Hemin-yne) decayed there was a steady loss of ORR current, indicating degradation of the active site but the capacitive currents of the SAM were unaltered (Supplementary Fig. 17).

SERRS and SERRS-RDE. The excitation wavelength used in the Resonance Raman experiments was 406.7 nm and the power applied to the sample was 10–15 mW. The spectrograph was calibrated against naphthalene. The Ag surfaces were roughened before SERRS experiments following literature protocols⁷⁰. The SERRS-RDE set-up is described in ref. 42. The data for the oxidized state was obtained by holding the potential of the disc at 0 mV versus NHE, and the data during steady state ORR was obtained by holding the disc at -400 mV versus NHE and the disc was rotated at 300 r.p.m. Normally data were acquired over a period of 300 s.

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Author contributions

S.M. acquired all the data, analysed the data and wrote the paper. A.M. and A.B. purified and provided the mutant proteins and helped write the paper. M.M. helped S.M. in acquiring some data. Y.L. provided the proteins, helped write the paper and developed

the science. A.D. conceived the study, analysed the data, developed the science and wrote the paper

Additional information

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