A BIOSYNTHETIC MODEL OF CYTOCHROME C OXIDASE AS AN ELECTROCATALYSTS FOR O₂ REDUCTION
A biosynthetic model of Cytochrome c oxidase (CcO) based on myoglobin scaffold, including both the distal Cu and the redox active tyrosine residue, is used as an electrocatalyst for O₂ reduction. The rate of O₂ reduction by this electrode is 100 times higher than those reported for any synthetic mimic of CcO in spite of having 1000 times lesser catalyst on the electrode. In situ monitoring of the reaction with resonance Raman show that a low spin ferric species and a ferryl species accumulate during steady state catalysis indicating that this catalyst features very fast electron transfer rates to the active site, facile O₂ binding and O-O bond cleavage rates. An electron transfer shunt is utilized to circumvent the kinetic barrier associated with dissociation of a ferric hydroxide species, which slows down the reduction of O₂ at the active site of CcO, resulting in O₂ reduction rate of 5000 s⁻¹.
6.1 INTRODUCTION

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, galactose oxidase, tyrosinase and Cytochrome c oxidase (CcO).[1-6] Alternatively there has been fervent pursuit of biochemical constructs inspired by natural metalloenzymes. A series of binuclear non-heme iron, cytochrome c, heme oxidases and iron-sulfur enzyme models have resulted from such efforts.[1, 7-10] 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, 10-12] In addition, key information about 2nd 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.[13, 14]

In a biosynthetic approach, stable naturally occurring proteins have been used as scaffolds for creating mimics of several metalloenzymes, such as hydrogenases involved in the reversible generation of H₂ from H₂O, heme proteins participating in electron transfer and O₂ binding, non-heme iron and copper enzymes active in small molecule activation, and even novel enzymes containing non-native cofactors.[15-24] For example, using this approach, biosynthetic models that structurally and functionally mimic CcO and nitric oxide reductase have been reported.[10, 25] Despite decades of focused effort, however, biosynthetic models with catalytic efficiencies approaching those of the naturally occurring metalloenzymes have remained elusive. In this chapter we will discuss about a biosynthetic model of CcO bearing the distal Cu₈ and a tyrosine residue that is kinetically more competent in reducing O₂ electrochemically than any known synthetic analogue as well as native CcO itself.
Figure 6.1. Crystal structure of a Mb-based biosynthetic model of CcO (pdb id: 4FWY). The heme cofactor is in a cleft on the molecule protein surface, (color coded according to the charge of the residues), with the propionate groups exposed to the solvent (right). The computer generated active site of G65YCubMb shows the distal CuB bound to histidines.

6.2 MATERIALS AND METHODS

1-Azidoundecane-11-thiol and Hemin-1Fe were synthesized following the reported procedure.[26, 27] 6-Mercaptohexanoic acid was purchased from Sigma Aldrich. Di-sodiumhydrogenphosphate 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 (1000 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.[10, 28]

Several site-directed mutants of a very stable protein like myoglobin were constructed where the distal residues are modified appropriately to construct functional biochemical models of Cytochrome c oxidase (CcO).[10, 20, 28, 29] The copper binding site in CcO has been engineered in sperm whale myoglobin (to mimic CcO active site) by double mutation of two
amino acid residues Leu 29 and Phe 43 present in the distal pocket of Mb to His residues (L29H, F43H, for convenience we will refer to this construct as Cu₈Mb in this chapter).[28] These two histidine residues along with the one already present in the distal pocket of wild type (WT) Mb (His64) create a copper binding site that closely mimic the Cu₈ centre in CcO. Furthermore, in an attempt to mimic the conserved Tyr 244 residue in the CcO active site, mutant of Cu₈Mb (G65YCu₈Mb) containing redox active tyrosine residues in the distal site were also created.[30, 31] This biochemical model reproduced several spectroscopic and coordination properties of native CcO.[31] A resultant heme-Cu₈ Cu₈Mb biochemical model for CcO is reported to catalyze the reduction of O₂ to H₂O with over 1000 turnovers under homogeneous solution.[31]

6.2.1 Formation of mixed Self Assembled Monolayer (SAM) and covalent attachment of Hemin-1Fe on to SAM

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-1Fe was covalently attached using ‘Click’ reaction.[27]

6.2.2 Reconstitution of apoG65YCu₈Mb mutant with Cu₈ to produce the “with Cu G65YCu₈Mb” mutant

1 Equiv. of 1 mM CuSO₄. 5H₂O solution was added to the apoG65YCu₈Mb solution in 100 mM phosphate buffer with pH 7 to produce “with Cu G65YCu₈Mb” mutant. Complete reconstitution of the apoprotein with Cu was further confirmed by the cyclic voltammetric data obtained for the reconstituted protein in solution.

6.2.3 Reconstitution of apoG65YCu₈Mb mutants to G65YCu₈Mb with and without Cu₈ on heterogeneous SAM surface

For all the experiments on heterogeneous SAM surfaces the Hemin-1Fe modified – COOH SAM surfaces were incubated with a 20 μM apoprotein (apoG65YCu₈Mb) solution for 2
hours. The supernatant solution was drained and the surface was cleaned with water. The immobilization of the mutant is further confirmed by the absorption spectra of the surface fabricated with those mutants (Fig. S12).

### 6.2.4 Cyclic Voltammetry

All electrochemical experiments were done in pH 7 phosphate buffer containing Potassium hexafluorophosphate (KPF₆). 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.

To ensure that the SAM surface is stable during the electrocatalytic investigations, discs bearing just the SAM was subjected to several rotations (200-1000 rpm) and its capacitive current was found not to change indicating that the SAM is retained on the electrode during these dynamic electrochemistry experiments (Fig. S7, S8). 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-1Fe) decayed there was a steady loss of ORR current indicating degradation of the active site but the capacitive currents of the SAM were unaltered (Fig. S8).

### 6.2.5 Surface Enhanced Resonance Raman Spectroscopy (SERRS) and SERRS-RDE

The excitation wavelength used in the resonance Raman (rR) 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 protocols.[32] The SERRS-RDE set up is described in reference.[33] The data for the oxidized state was obtained by holding the potential of the disc at 0 mV vs NHE and the data during steady state ORR was obtained by holding the disc at -300 mV vs NHE and the disc was rotated at 300 rpm. Normally data were acquired over a period of 300 s.
6.3 RESULTS

6.3.1 Spectroscopic characterization of the bioelectrodes fabricated with the mutants

X-ray crystallography of myoglobin (Mb) and its mutant have revealed that its two propionate side chains project out onto the surface (Fig. 6.1).[10] Taking advantage of this structural feature, we have previously developed an electrocatalytic O$_2$ reduction system where the native heme cofactor in Mb is replaced by a modified hemin cofactor bearing alkyne group (Hemin-1Fe, scheme 6.1) so that electrons can be injected directly into the heme from a gold electrode to facilitate O$_2$ reduction.[27] This method resulted in a Mb-functionalized electrode bearing $3 \times 10^{-13}$ moles of protein, which was characterized using several microscopic and spectroscopic techniques.[27] Over the last few years a biosynthetic model of CcO has been reported in which two distal residues of myoglobin (L29, 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$_B$Mb).[28] Furthermore, in an attempt to mimic the conserved Tyr 244 residue in the CcO active site, a G65Y mutant of Cu$_B$Mb (G65YCu$_B$Mb) containing redox active tyrosine residue in the distal site was also created.[31]

The AFM data of the G65YCu$_B$Mb functionalized electrodes show clear increase in the average height relative to both SAM covered surface (Fig. S1) and a Hemin-1Fe functionalized surface. The presence of 5-7 nm higher features after protein immobilization indicates the attachment of the protein molecules to the surface (size of Mb is 4.5x4.5x2 nm).[34] Surface enhanced resonance Raman spectroscopy (SERRS) data (Fig. 6.2, left) of the electrodes bearing the G65Y-Cu$_B$Mb protein with and without Cu$_B$, show the oxidation and spin state marker $\nu_4$, $\nu_3$ and $\nu_2$ bands at 1375, 1493 and 1585 cm$^{-1}$ respectively. The $\nu_4$, $\nu_3$ and $\nu_2$ values are consistent with the presence of a six coordinated high spin heme in the active site on these electrodes bearing the biochemical constructs of CcO.[35] These signals are a very different from those obtained on electrodes bearing only Hemin-1Fe (Fig. S2).[27]
Scheme 6.1. Reconstitution of apoprotein in-situ with Hemin-1Fe groups that are covalently attached to mixed self-assembled monolayers of thiols on an Au electrode. The modified hemin is indicated as Hemin-1Fe.
Figure 6.2. SERRS spectra of G65YCu₈Mb with (green) and without (red) the distal Cu₈ in air saturated 100 mM phosphate buffer (pH 7) solution.

X-ray photoelectron spectroscopic (XPS) data clearly indicates the presence of Fe, Cu, C, N and O on a G65YCu₈Mb bound surface (S3, Table S1). The 3p₃/₂, 2p₃/₂ and 2p₁/₂ binding energy peak for the Fe of heme group appear at 56.5, 709.4 and 722.4 eV respectively.[36-38] The 2p₃/₂ and 2p₁/₂ binding energy peak for Cu appear at 931.7 and 951.8 eV respectively[39], confirming the presence of both Fe³⁺ and Cu²⁺ on the protein modified surface. The N₁s peak is broad (S3), as it contains several components due to the presence of amide, heme pyrroles and the triazole groups (resulting from the covalent attachment of Hemin-1Fe) on the surface.[36-40] Similarly, the C₁s peak (S3) contains contributions from different types of C atoms (aromatic, aliphatic, heme etc.) on these protein modified surfaces.[41]

Cyclic voltammetry (CV) of G65YCu₈Mb with and without the distal Cu₈ immobilized onto the electrodes, in degassed buffer show the heme Fe³+/²⁺ reduction potential at −97 mV and −57.5 mV, respectively (Fig. 6.3). Hemin-1Fe displays the Fe³+/²⁺ reduction potential at -70.0 mV in the absence of a protein and -135.0 mV when bound to wild type apo Mb.[27] In the case of the G65YCu₈Mb protein bound electrodes, the Cu²⁺/²⁺ process overlaps with the Hemin-1Fe Fe³+/²⁺ process, resulting in approximately twice the area under these CV peaks relative to the G65YCu₈Mb bearing electrodes prior to Cu₈ loading. The integrated area under these CV feature indicates that there are 1.15±0.05×10⁻¹² moles of protein per cm² of the surface. The ratios of the integrated area under the CV features of G65YCu₈Mb functionalized before and after loading the Cu₈ is ~1:2 (Table 6.1, 3rd column) which is consistent with the expected 1:1 stoichiometry (i.e. every G65YCu₈Mb binds one Hemin-1Fe and one Cu²⁺ ion). Note that the E₁/₂ values of the heme and Cu₈ measured for these electrodes are slightly different from those estimated from potentiometric titration in solution.[10, 20] This is likely due to the interfacial
microenvironment of the –COOH terminated SAM which is known to shift the apparent formal potentials of redox active species in its vicinity.[42] The AFM, XPS, CV and SERRS data provide direct evidence for the attachment of Hemin-1Fe on the electrode. The in-situ reconstitution of the protein on the electrode is evident from the AFM and XPS, aerobic CV (Fig. 6.3) data. The presence of the Cu$^{2+}$ at Cu$_B$ site on the electrode is indicated by XPS and CV data. Taken together these data indicate the assembly of the G65YCu$_B$Mb, biosynthetic model of CcO, covalently attached to the electrode via the linkage between the Hemin-1Fe and the azide terminated thiols created using click reaction (scheme 6.1).

![Graph](image-url)

**Figure 6.3.** Anaerobic CV of G65YCu$_B$Mb without Cu$_B$ (red) and after Cu$_B$ binding (green). 2 V/s scan rate, in degassed, pH 7, 100 mM phosphate buffer using a Pt counter electrode.
6.3.2 Reactivity of the bioelectrodes

6.3.2.1 Rotating disk electrochemistry (RDE)

In linear sweep voltammetry (LSV) experiments performed in aerated buffers, large electrocatalytic O\textsubscript{2} reduction currents are observed by the G65YCu\textsubscript{8}Mb bearing bio-electrodes at pH 7 at room temperature, as the applied potential is lowered below +100 mV vs NHE (Fig. 6.5). Thus as the potential of the electrode is lowered such that the iron in these proteins is reduced to Fe\textsuperscript{II}, an electrocatalytic O\textsubscript{2} reduction current is observed. It is important to note that the midpoint potential of ORR (E\textsubscript{ORR}) is -263 mV which is more negative than the E\textsuperscript{1/2} suggesting that the potential determining step of ORR is not the reduction of resting Fe\textsuperscript{III} to Fe\textsuperscript{II} but the reduction of a different Fe\textsuperscript{III} species with -166 mV more negative potential (Fig. 6.5 inset). In these active sites, O\textsubscript{2} may be reduced by 4e\textsuperscript{-} and 4H\textsuperscript{+} to H\textsubscript{2}O or, by 1e\textsuperscript{-} or 2e\textsuperscript{-} or 3e \textsuperscript{-} electrons to produce partially reduced oxygen species (PROS) like O\textsubscript{2}\textsuperscript{-}, H\textsubscript{2}O\textsubscript{2} and OH\textsuperscript{*} respectively.

The amount of PROS produced during the ORR, is determined by rotating ring disk electrochemistry (RRDE). In this RRDE experimental set up, the disk electrode at the tip of a rotor is encircled with a Pt ring. Both the disk and the Pt ring are poised at different potential during the experiment. During the reduction of O\textsubscript{2}, the PROS produced at the disk electrode is diffused gradient out from the disk towards the ring and the ring which is poised at a very high oxidizing potential oxidizes H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2}\textsuperscript{-} to O\textsubscript{2} reaching the ring and thereby generates the Pt

<table>
<thead>
<tr>
<th>Protein</th>
<th>Metal binding sites</th>
<th>E\textsuperscript{1/2} (mV)</th>
<th>Integrated Coverage (mol/cm\textsuperscript{2})</th>
</tr>
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<tbody>
<tr>
<td>Hemin-1Fe reconstituted myoglobin[27]</td>
<td>Fe</td>
<td>-135.0±5.0</td>
<td>0.8±0.03\times10\textsuperscript{-12}</td>
</tr>
<tr>
<td>Hemin-1Fe reconstituted G65YCu\textsubscript{8}Mb</td>
<td>Cu\textsubscript{8}, Fe</td>
<td>-97.0±5.0</td>
<td>2.11±0.06\times10\textsuperscript{-12}</td>
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ring current. The ratio of the disk current to the ring current calibrated to the collection efficiency of the ring, directly gives the amount of H₂O₂ produced during O₂ reduction.

**Figure 6.4.** The RRDE set up

![Image of RRDE setup]

**Figure 6.5.** (A) Linear sweep voltammogram of G65YCubMb in air saturated pH 7, 100 mM phosphate buffer solution at 100 mV/s scan rate, potentials are scaled relative to NHE and a Pt counter electrode is used. Data are collected at different rotation speed (200 rpm - red, 400 rpm – purple, 600 rpm – green, 800 rpm - blue). (B) Plot of $i_{\text{cat}}^{-1}$ for G65YCu₈Mb bearing bioelectrode at -300 mV potential and at multiple rotation rates, with the inverse square root of the angular rotation rate ($\omega^{-1/2}$).

The extent of 4e⁻ reduction and the 2nd order rate constant ($k_{\text{ORR}}$) of the O₂ reduction reaction (ORR) can be determined using rotating disc electrochemistry (RDE) where the catalytic
O₂ reduction current increases with increasing rotation rates (Fig. 6.5 A, B) following the Koutecky-Levich equation (Eq. 6.1).[43]

\[ i_{\text{cat}}^{-1} = i_k(E)^{-1} + i_L^{-1} \]  \hspace{1cm} (6.1)

where \( i_k(E) \) is the potential dependent kinetic current and \( i_L \) is the Levich current. \( i_L \) is expressed as

\[ i_L = 0.62nFA[O_2](D_{O_2})^{2/3}\omega^{1/2}v^{-1/6} \]  \hspace{1cm} (6.2)

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 O₂ in an air saturated buffer (0.26 mM) at 25°C, \( D_{O_2} \) is the diffusion coefficient of O₂ (1.8x10⁻⁵ cm²s⁻¹) at 25°C, \( \omega \) is the angular velocity of the disc and \( \nu \) is the kinematic viscosity of the solution (0.009 cm²s⁻¹) at 25°C.[44]

Plot of \( i_{\text{cat}}^{-1} \) at multiple rotation rates with the inverse square root of the angular rotation rate \( (i_{\text{cat}}^{-1}) \) for G65YCu₈Mb (Fig. 6.5 B) is linear. Furthermore, the slope of K-L plot is expressed as \( 1/ \left[ n\{0.62FA(D_{O_2})^{2/3}\nu^{-1/6}\}\right] \) which can be used to experimentally estimate the value of \( n \) where \( n \) is the number of electrons donated to the substrate, i.e. O₂. The slope obtained from the experimental data for G65YCu₈Mb (Fig. 6.5 B) is close to the theoretical slope (Fig. 6.5 B, dotted purple line) expected for a 4e⁻ process and very different from the slope for a 2e⁻ process (Fig. 6.5 B, dotted green line). Thus the G65YCu₈Mb bioelectrode predominantly catalyze a 4e⁻/4H⁺ reduction of O₂ to H₂O, 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 as[45]

\[ i_k(E) = k_{\text{ORR}}nFA[O_2]\Gamma_{\text{cat}} \]  \hspace{1cm} (6.3)

where \( n \) is the number of electrons, \( A \) is the geometric surface area, \( [O_2] \) is the bulk concentration of O₂, \( \Gamma_{\text{cat}} \) is the surface coverage of the catalyst (obtained from the integration of the anaerobic CV data) and \( k_{\text{ORR}} \) is the 2nd order rate constant for O₂ reduction estimated at -300 mV potential. Using this equation (Eq. 6.3) and the experimentally obtained \( i_k(E) \) at -300 mV,
the 2\textsuperscript{nd} order rate constant for O\textsubscript{2} reduction G65YCu\textsubscript{b}Mb is evaluated to be 1.98 x10\textsuperscript{7} M\textsuperscript{-1}s\textsuperscript{-1} (Table 6.2). The pseudo 1\textsuperscript{st} order rate can be determined (Table 6.2) from the 2\textsuperscript{nd} order rate by taking into account the substrate, O\textsubscript{2} concentration under these experimental conditions is 0.26 mM. The catalytic ORR rate by G65YCu\textsubscript{b}Mb surpasses those reported for the best artificial synthetic analogues (Table 6.2).

<table>
<thead>
<tr>
<th>ORR Catalysts</th>
<th>Metals</th>
<th>k\textsubscript{ORR}</th>
<th>PROS (%)</th>
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</thead>
<tbody>
<tr>
<td>G65YCu\textsubscript{b}Mb</td>
<td>Cu\textsubscript{b}, Heme</td>
<td>1.98 x 10\textsuperscript{7} M\textsuperscript{-1}s\textsuperscript{-1} or 5148 s\textsuperscript{-1}</td>
<td>~ 6 ± 1</td>
</tr>
<tr>
<td>Synthetic Model\cite{46}</td>
<td>Cu\textsubscript{b}, Heme</td>
<td>1.2 x 10\textsuperscript{5} M\textsuperscript{-1}s\textsuperscript{-1}</td>
<td>~ 10 ± 1</td>
</tr>
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K\textsubscript{ORR} determined at -0.4 V vs NHE, PROS determined at -0.12 V vs NHE

6.4 DISCUSSION

The G65YCu\textsubscript{b}Mb biosynthetic Mb scaffold based bio-electrode for O\textsubscript{2} reduction is remarkably stable. Monolayers bearing covalently attached O\textsubscript{2} reducing electrocatalysts reported so far have never been stable enough to allow these dynamic electrochemical experiments to determine the kinetic parameters (k\textsubscript{ORR}, number of electrons etc.). The failure to perform these experiments has been attributed to degradation of the catalyst during RDE experiment i.e. very small turnover numbers (TON) presumably due to the production of reactive oxygen species (ROS) during ORR. Rotating ring disc electrochemistry (RRDE) shows formation of only ~ 4 % ROS by the G65YCu\textsubscript{b}Mb (Fig. S6) during ORR indicating that it reduces 96 % of O\textsubscript{2} to H\textsubscript{2}O consistent with the RDE data. During the RDE experiments (Fig. 6.4 A) the G65YCu\textsubscript{b}Mb functionalized electrodes bearing 10\textsuperscript{-12} moles of the catalyst reduced 1.8±0.3x10\textsuperscript{-8} moles of O\textsubscript{2} (7±1 x 10\textsuperscript{-3} coulombs total charge and 4e\textsuperscript{-} per O\textsubscript{2} molecule) yielding a TON of at least 10\textsuperscript{4}.
To understand the facile and selective O\textsubscript{2} reduction catalyzed by the G65YC\textsubscript{u}bMb biochemical model, the recently developed SERRS-RDE technique is employed. In this technique the rR spectra of the catalyst (i.e. G65YC\textsubscript{u}bMb) bearing electrode is collected while the system is involved in steady state O\textsubscript{2} reduction and the rate determining step (rds) for the process can be identified by characterizing the species accumulated during the steady state turnover i.e. the step removing it in the catalytic cycle is then the rds. In the absence of O\textsubscript{2} a high spin ferrous species is formed, characterized by a \nu\textsubscript{4} and \nu\textsubscript{3} vibrations at 1357 cm\textsuperscript{-1} and 1473 cm\textsuperscript{-1} (Fig. 6.6 A, red and cyan), respectively, when a cathodic potential of -0.4 V w.r.t Ag/AgCl reference electrode, is applied signifying reduction of the resting ferric state to the active ferrous state at these potentials. However when the same reducing potential is applied in an oxygenated buffer the SERRS-RDE data clearly show the presence of different species during electrocatalytic ORR which leads to broadening of the \nu\textsubscript{4}, \nu\textsubscript{3} and \nu\textsubscript{2} region (Fig. 6.6 A, green) relative to the oxidized state (Fig. 6.6 A, red). At these potentials, in an oxygenated buffer the G65YC\textsubscript{u}bMb catalyst is involved in steady state ORR (Fig. 6.6 A). The oxidation and spin state marker peaks generally shift to higher energy with a clear increase in intensity at 1508 cm\textsuperscript{-1} and 1641 cm\textsuperscript{-1} suggesting the accumulation of Fe\textsuperscript{IV}=O species during steady state ORR. Signals from high spin ferrous, resting high spin ferric, low spin ferric and ferryl species with \nu\textsubscript{3} at 1478 cm\textsuperscript{-1} (Fig. 6.6 B, brown), 1494 cm\textsuperscript{-1} (Fig. 6.6 B, dashed green), 1504 (Fig. 6.6 B, cyan) and 1508 cm\textsuperscript{-1} (Fig. 6.6 B, green), respectively, could be convoluted by fitting the \nu\textsubscript{3} region of the spectrum. The dramatic reduction in the signal from the high spin ferric species during ORR (\nu\textsubscript{3} at 1493 cm\textsuperscript{-1} weak) relative to the resting state implies that the electron transfer from the electrode to this high spin species is fast. The lack of significant signal from the high spin ferrous species (\nu\textsubscript{3} at 1473 cm\textsuperscript{-1} weak) suggests that O\textsubscript{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\textsubscript{2} binding rate (10\textsuperscript{7} M\textsuperscript{-1}s\textsuperscript{-1}).\textsuperscript{[47, 48]} This rate is indeed \textasciitilde10 times faster than O\textsubscript{2} binding to the heme a site of CcO.\textsuperscript{[49]} The significant intensity of a ferryl species (\nu\textsubscript{3} at 1508 cm\textsuperscript{-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\textsuperscript{III} state, O\textsubscript{2} binding to Fe\textsuperscript{II} and O-O bond cleavage are very facile in G65YC\textsubscript{u}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.[49-53]

Figure 6.6. (A) SERRS-RDE data on G65YCu8Mb and (B) Components of the rR spectrum.

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 (i.e. ET from Cyt c to CcO is not the rds), the species that would accumulate during turnover, based on the Babkock-Wikström mechanism (Fig. 6.7), are the Fe\textsuperscript{II}-O\textsubscript{2}, Fe\textsuperscript{III}-O\textsubscript{2}\textsuperscript{2-}, Fe\textsuperscript{III}-OOH, Fe\textsuperscript{IV}=O and Fe\textsuperscript{III}-OH species as the rates of formation of these species are greater than their rates of decay.[54, 55] Out of these the Fe\textsuperscript{II}-O\textsubscript{2}, Fe\textsuperscript{III}-O\textsubscript{2}\textsuperscript{-} and Fe\textsuperscript{III}-OOH species will have Raman signatures of low spin heme (Fig. 6.7, shaded yellow), Fe\textsuperscript{III}-OH will have rR signature of high spin heme and the Fe\textsuperscript{IV}=O will have signatures unique to heme ferryl species.[49, 54, 56] The SERRS-RDE data show the presence of species having signatures of low spin Fe\textsuperscript{III} and Fe\textsuperscript{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 the biosynthetic model circumvents accumulation of Fe\textsuperscript{III}-OH and resting Fe\textsuperscript{III} species in the steady state. The overall rate-limiting step of native CcO in solution is the dissociation of hydroxide of the Fe\textsuperscript{III}-OH end product of O\textsubscript{2}
reduction from heme to generate the active ferric resting form and has a 1\textsuperscript{st} order rate constant of 500 s\textsuperscript{-1} [49, 54, 56, 57] This dissociation is required during turnover as the potential of this hydroxide bound form is likely to be more negative that the five coordinate resting oxidized site (which will be regenerated after hydroxide dissociation) and will not be reduced by heme a. While the E\textsuperscript{1/2} of a heme a\textsubscript{3} Fe\textsuperscript{III}-OH species cannot be determined with confidence due to strong interaction potential and cooperativity between the heme a and heme a\textsubscript{3} sites, the potential of a formate bound high spin heme a\textsubscript{3} site (analogous to hydroxide) is ~130 mV more negative than the resting ferric site. [58, 59] The potential determining step of ORR by the G65YCu\textsubscript{b}Mb is 166 mV more negative than the E\textsuperscript{1/2} for the resting high spin ferric state and is likely to be the reduction of the Fe\textsuperscript{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. As a result the rds of ORR is the reduction of the Fe\textsuperscript{IV}=O species which is the next slower step in the catalytic cycle of CcO (Fig. 6.7). Consistent with the above proposal the 1\textsuperscript{st} order rate of ORR catalyzed by G65YCu\textsubscript{b}Mb is 5000 s\textsuperscript{-1} which is in the same order as the rate of reduction of the Fe\textsuperscript{IV}=O species in CcO determined to be 1200 s\textsuperscript{-1}. The five times enhancement of the rate of this step in the biosynthetic model may be due to a combination of better electron transfer pathway to the active site and greater driving force for electron transfer relative to CcO. 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 O\textsubscript{2} activation step of the native enzyme.
Figure 6.7. Comparison between the mechanism of native CcO[49] in solution and the biosynthetic G65YCu₈Mb model on electrode. The parameters of native CcO are indicated in purple and G65YCu₈Mb are indicated in green.

In an air saturated buffer (0.26 mM O₂) the pseudo 1st order rate constant of ORR by G65YCu₈Mb is determined to be ~ 5-6 x 10³ s⁻¹ (k_{ORR}[O₂]). The highest 2nd order O₂ reduction rate reported for any synthetic mimic of CcO is 1.2 x 10⁵ M⁻¹s⁻¹, that too on a multilayer having
1000 times more catalyst than the G65YCu₈Mb electrodes.[46] The 2nd order rate constant of G65YCu₈Mb is $10^7 \text{ M}^{-1}\text{s}^{-1}$ which is, thus, two orders of magnitude higher than best synthetic heme/Cu based O₂ reduction electrocatalyst. Thus the selectivity and kinetic rate of the G65YCu₈Mb 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 1st order rate constant of the G65YCu₈Mb is 10 times faster than the rate of native CcO in solution, such a comparison is vulnerable to differences in reaction conditions (e.g. G65YCu₈Mb is water soluble but CcO exists in membranes). Alternatively erstwhile efforts resulting in electrodes bearing native CcO in a fashion similar to these bio-electrodes show extremely sluggish O₂ reduction.[46, 60, 61] This is due to improper alignment of this membrane bound protein on the electrode which precludes efficient electron transfer to the active site.[61-64] However, the direct attachment of heme to the electrode utilizing its solvent exposed propionate groups, (i.e. a short circuit) enables fast electron transfer to the active site.[27] As a result when a CcO functionalized SAM covered Au electrode produces <1 μA electrochemical O₂ reduction current at -300 mV, this bio-electrode produces ~100 μA current at similar potentials.

Finally, the G65YCu₈Mb mutant has residues in the distal pocket that can help both electron and proton transfer during O₂ reduction (Y65 in G65YCu₈Mb). Previous biochemical and structural studies on these mutants had indeed indicated the close proximity of this residue to the distal site.[10, 20, 28] However Mb does not have proton transfer residues in the distal pocket. In CcO, the involvement of Tyr 244 residues in proton/electron transfer during O₂ reduction is now widely accepted.[65, 66] An analogous biochemical model without the Y65 residue, Cu₈Mb, is not as stable as the G65YCu₈Mb as the former degrades rapidly during the RDE experiments (Fig. S10). Thus the efficient ORR kinetics of G65YCu₈Mb suggests that the Y65 residue in the G65YCu₈Mb construct protects the active site from reactive intermediates formed during ORR emulating the role of Y244 residue in CcO and its synthetic analogue.[3, 67]
6.5 CONCLUSION

Due to obvious difficulties associated with linking a heme cofactor buried inside a hydrophobic protein environment to an electrode, these CcO based O₂ reducing electrodes were limited by electron transfer from the electrode to the heme site. However, the solvent exposed heme active site of Mb allowed a direct attachment (i.e. a short circuit) to the electrode. An 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 model, order of magnitude faster than CcO immobilized on electrode and comparable to that of native CcO in solution, a feat not yet achieved in known literature.
REFERENCES


Figure S1.1. 3D AFM image of surface modified with (A) SAM only B) Hemin-1Fe functionalized SAM (C) G65YCu₈Mb Hemin-1Fe functionalized SAM.
Figure S1.2. Bearing area plot and 2D Topographic AFM image of G65YCu₈Mb bearing electrode.
S2. SERRS of electrodes bearing Hemin-1Fe and myoglobin reconstituted with Hemin-1Fe

Figure S2. SERRS spectra of Hemin-1Fe and myoglobin reconstituted with Hemin-1Fe in air saturated 100 mM phosphate buffer (pH 7) solution.
Figure S3. High-resolution XP spectra for G65YCu₈Mb (with Cu) mutant immobilized on the SAM covered Au Surface using click reaction. (A) Fe 2p₃/2 and 2p₁/2 (B) Fe 3p₃/2 (C) Cu 2p₃/2 and 2p₁/2 (D) S 2p₃/2 and 2p₁/2 (E) N 1s₁/2 for triazole linkage (F) C 1s₁/2 (G) O 1s₁/2.
Table S1. XPS data of G65YCu₈Mb (with Cu) modified Au electrode

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**S4. O₂ Reduction by the Hemin-1Fe modified surface before and after G65YCu₈Mb (with Cu) protein binding**

![Cyclic voltammogram](image)

**Figure S4:** Cyclic voltammogram for the O₂ reduction by the Hemin-1Fe modified surface before (blue) and after G65YCu₈Mb (with Cu) protein (red) binding, in 100 mM pH 7 phosphate buffer (air saturated) using Ag/AgCl electrode as reference and Pt as counter electrode.
S5. RDE data

Figure S5: Linear sweep voltammogram of G65YC₆Mb (without Cu) modified electrode in air saturated pH 7.0 100 mM phosphate buffer solution at 100 mV/s scan rate and using Ag/AgCl electrode as reference and Pt as counter electrode are plotted at different rotation speed (top). Plot of $(i_{\text{cat}})^{-1}$ at multiple rotation rates with the inverse square root of the angular rotation rate ($\omega^{-1/2}$) is also shown (bottom).
Figure S6: Rotating ring disk electrode (RRDE) data of without Cu, G65YC₈Mb (red) and with Cu, G65YC₈Mb (green) at 10 mV/s and at 300 rpm rotation speed in air saturated pH 7 100 mM phosphate buffer using Ag/AgCl electrode as reference and Pt as counter electrode.
S7. Stability of the mixed SAM of 1-azidoundecan-11-thiol and 6-mercaptohexanoic acid after rotations

**Figure S7:** Cyclic voltammogram of the background current of mixed SAM of 1-azidoundecan-11-thiol and 6-mercaptohexanoic acid, in 100 mM pH 7 phosphate buffer at after rotating the electrode at different rotation rates. (Ag/AgCl reference and Pt counter electrode)
S8. Stability of the G65YCu₈Mb (with Cu) protein modified surface after rotations

Figure S8: Cyclic voltammogram of the G65YCu₈Mb (with Cu) protein modified surface, in 100 mM pH 7 phosphate buffer at different rotation rate using Ag/AgCl electrode as reference and Pt as counter electrode.
Figure S9: SERRS-RDE spectra of G65YCubMb (with Cu) protein modified surface, collected over 500 seconds during a bulk electrolysis experiment at -0.3 V vs NHE during which the electrode is under continuous rotation (300 rpm). Five scans are performed for 100 seconds each (one LSV is typically 20 seconds), in 100 mM pH 7 phosphate buffer saturated with O^{18} isotope, using Ag/AgCl reference electrode and Pt counter electrode.
Figure S10: Decay of O$_2$ reduction current at different angular rotation rate for (A) Hemin-1Fe (B) Hemin-1Fe bound Cu$_8$Mb. Data clearly indicates the rapid decay of these catalysts.
Figure S11: EPR spectra of G65YCubMb (green) with Cu compared with CuSO₄ (black) in 10mM pH 7 phosphate buffer.
**Figure S12:** UV-Vis absorption spectra of G65YCu₈Mb with Cu in 10 mM pH 7 phosphate buffer.
Figure S13: Resonance Raman spectra of the homogenous solution of G65YCu₈Mb (green) without Cu indicating the Fe-His stretching frequency at 220 cm⁻¹.