"Applications of Biomimetic Complexes of Cytochrome c Oxidase in Renewable Energy Research"

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"The best way to have a good idea is to have a lot of ideas."- Linus Pauling

Abstract

The predominant sources of energy used globally today rely on the burning and breakdown of nonrenewable resources, leading to catastrophic effects on life on Earth. Given these detrimental impacts, there has been a surge in renewable energy research in the world of chemistry. Much of this research is based on biological systems that evolved to efficiently and effectively generate clean and renewable energy for their life processes. If researchers can synthesize both structural and functional mimics of the active sites of these enzymes, information may be gained that benefits both the biological and chemical community. One enzyme that is a key target for this type of research is cytochrome *c* oxidase, an oxygen reduction enzyme that is critical for the synthesis of ATP. The reaction synthesized by this enzyme holds promise for fuel cell research, especially in the context of subsequent water splitting reactions. This paper aims to discuss the biochemistry of cytochrome *c* oxidase as well as synthesis and experimentation surrounding key examples of a variety of cytochrome *c* oxidase mimic complexes. These researchers mimic either the function or structure of the cytochrome *c* oxidase active site, unlocking mechanistic and kinetic insight into the rate of oxygen reduction and substrate binding motifs. Serving as valuable precursors for scalable and implementable renewable energy systems moving forward, a review of these binding studies illustrates the importance of continued research in the field. Better understanding these complexes and their scientific merit is crucial for environmental remediation and biochemical synthetic methods.

Keywords: renewable energy, structural/functional mimics, enzymes, cytochrome *c* oxidase, oxygen reduction

Introduction

Fossil fuels are the source of about 80 percent of energy worldwide and currently dominate the energy industry globally. The consumption of this energy source can be detrimental to ecosystems and create harmful environmental impacts.[1] Turning to the biological kingdoms as inspiration for alternative and renewable energy sources holds promise for cleaner energy consumption in the future. A notable approach for alternative energy storage is that of water-splitting reaction, creating hydrogen gas and diatomic oxygen using renewable energy sources such as solar power. Through the synthesis of biomimetic compounds, the fundamental aspects of stoichiometric and catalytic oxygen evolution and reduction reactions may allow for discovery of water-splitting catalysts that can augment knowledge of energy transduction catalysis. Coupling biological and chemical findings increases the foundation of knowledge in the overall energetic processes and systems to be used in the future for improving understanding and preservation of life on earth.

In the world of biology, metalloenzymes are readily used to facilitate a variety of energy-based reactions.[2] In the realm of energy cycling, copper and iron coordination complexes are key to energy storage and transduction pathways.[3] One example of such enzyme class is cytochrome *c* oxidase (C*c*O). Due to the importance of this enzyme in biological systems, C*c*O is a common target for biomimetic chemistry applications. Use of synthetic biomimetic model systems allows more accessible experimentation and can provide means for mechanistic compounds. Previous work, from groups such as those of Karlin and Tolman, has modeled various heme iron and copper oxo complexes to allow for mechanistic and experimental insight into oxidase and oxygen reduction reaction (ORR) chemistry.[4,5] Continued efforts into C*c*O-specific compounds may hold promise for efficient and effective fuel cell research. Within the context of these significant efforts,

this paper aims to investigate the goals and applications of biomimetic chemistry, describe the role of cytochrome *c* oxidase biologically and in mimicry research, and illustrate some findings that may be unlocked from both structural and functional mimics of the cytochrome *c* oxidase enzyme.

Biomimetic Chemistry

In the world of bioinorganic chemistry, there has been a recent surge in drawing inspiration from biological systems to help solve problems related to natural products, renewable energy, polymers, radicals, organic synthesis/techniques, and similar.[6–11] Many researchers have begun working in the world of biomimetic or bioinspired chemistry to elucidate both mechanistic and structural features of these systems.

Broadly speaking, biomimetic and bioinspired chemistry tackle these goals in two similar yet notably different ways. For example, bioinspired chemistry is often regarded as using aspects of the structure/function relationships in biological systems to answer probing questions. Biomimetic chemistry, however, aims to replicate the structure/function relationships as closely as possible to the native environments or enzymes. Biomimetic chemistry may achieve more direct comparative systems if interested in demonstrating relative catalytic efficiency or structural details when compared with the native biological complex. However, bioinspired chemistry may also achieve more accessible synthetic systems that investigate and operate in the same reaction types as the typical costly or sensitive biomimetic materials.[8]

Increasing carbon emissions due to urbanization and technology fuels the necessity of sustainable and renewable energy research. The global demand for solutions to offset these environmental concerns may be informed by nature's ability to adapt and survive over billions of years of habitat and atmospheric shifts. Biomimetic complexes may be used in currently existing

systems to improve their activity and increase catalytic efficiency.[12] For example, one key approach to biomimetic chemistry comes from energy storage applications, such as in batteries. Electrolyte systems using hydroquinone or chloroplast molecules have shown initial promise in terms of energy efficiency.[13] Renewable energy systems may not mean strictly utilizing biological molecules in these frameworks, however. These systems may also come about in engineering applications, including carbon dioxide-recycling polymers, photosynthetic coatings for solar panels, or artificial chloroplasts.[14–16]

As synthetic and characterization methods improve, so do the capabilities of biomimetic complexes. The scope of biomimetic research is vast and continuously expanding. However, there are significant barriers to introduction and investment into renewable energy research. Many countries have not only different priorities, but also different degrees to which they value this type of research. This is quite a significant barrier, as coordinated and universal investment is needed for successful improvements. In addition, these systems require significant research effort as well as administrative approval for implementation. Given this "cost," biomimetic chemistry often faces its most significant stopping point when trying to convince policymakers, corporations, and program officers to fund and implement these findings.[12]

Biochemistry of Cytochrome *c* Oxidase

Cytochrome *c* Oxidase functions as a dioxygen reduction enzyme, utilizing the thermodynamically favorable reduction of molecular oxygen to water to transport free protons across the mitochondrial inner membrane. This proton translocation allows ATP synthase to use transported protons for ATP synthesis.[17] C*c*O exists as a ~400 kDa homodimer with each respective monomer consisting of 13 subunits and four redox centers. Three main components make up the

redox-active centers: a dinuclear Cu_A site, a low spin iron heme *a*, and a binuclear center (BNC) consisting of another iron heme a_3 and Cu_B site (Fig. 1).[18]

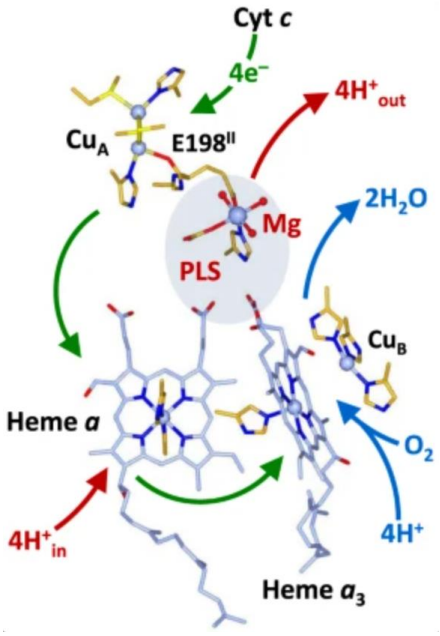

Figure 1- Adapted from (18): *Schematic representation of the electron transfer pathway in the CcO active site. Electron movement shown in green, proton movement shown in red, oxygen/water movement shown in blue.*

Mechanistically, C*c*O functions primarily as the terminal electron transfer enzyme in the mitochondrial electron transport chain. Four electrons are passed sequentially from cytochrome *c*, another electron carrier site, through the dinuclear Cu_A site, to the heme *a*, and terminating at the BNC. Four protons are transported from the negative site of the inner mitochondrial membrane to successfully reduce the O_2 to water, thus driving four final H^+ translocations to fuel ATP synthesis.[19]

Notably, there are two amino acid residues in the inner sphere surrounding the C*c*O active site that help to stabilize the O_2 binding and metal ions. The histidine and tyrosine residues

conserved around the BNC help to support the iron atom within heme a_3 as well as the upper Cu_B site.[20] Given their importance, these residues are typically also conserved in model complexes or across a plethora of organisms.

Not only is C*c*O highly conserved, but this enzyme is also needed for all energy-requiring organisms. This enzyme is not only important biologically, but it also facilitates one of the most popular biomimetic reactions sought after in renewable energy chemistry: the oxygen reduction reaction. Given that C*c*O is a four electron reduction process, utilizing artificial C*c*O is a logical choice for fuel cells or metal-air batteries.[21] More specifically, fuel cells hold significant promise for renewable energy storage and subsequent application. If C*c*O biomimetic complexes can be implemented at the cathode of a fuel cell, the anode may be the site of water splitting. The splitting of two moles of water generates one mole of dioxygen and two moles of dihydrogen gas. If a cell has the turnover capability to cycle through both oxygen reduction water splitting, the hydrogen gas produced may be stored for future renewable energy applications.[22]

Functional Cytochrome *c* Oxidase Mimic

While structural mimics of biomimetic energy transduction or storage molecules can provide insight into the oxidation states, binding environment, or broad structural information, the greater goal of biosynthetic mimicry has primarily shifted towards functional mimics of these same complexes. Biosynthetic methods for creation of cytochrome *c* oxidase and similar oxygen reduction metalloenzymes have been sought after in recent decades for their application in the oxygen/hydrogen evolution pathways for use in fuel cells.[19,23–25] Using naturally occurring proteins for scaffolds can help to mimic both the inner and secondary coordination sphere for effective binding and catalytic activity at the metalloenzyme active site.[26,27] One particular report that showed much promise in the 21st century comes from biosynthetic work done by Sohini

Mukherjee, Arnab Mukherjee, Ambika Bhagi-Damodaran, Manjistha Mukherjee, Yi Lu and Abhishek Dey in 2015, demonstrating a functional and structural model of the C*c*O enzyme complete with both the distal Cu_B and tyrosine that has greater kinetic oxygen reduction electrochemical activity than even the native metalloenzyme itself.[20]

In this paper, Mukherjee and collaborators utilize the unique myoglobin structure as the structural backbone for the C*c*O functional mimic. Myoglobin (Mb) contains two propionate side chains that project out into the solvent which can be modified to a hemin cofactor with an alkyne group. This modification allows for direct injection of electrons via a gold electrode, allowing for O_2 reduction directly at the catalytic active site.[26,28] In order to complete the biosynthesis of the complete Mb-mutant based active site for the C*c*O mimic, two distal amino acid residues, namely a lysine and phenylalanine residue in the inner sphere, were mutated to histidine, better mimicking the Cu-binding site. In addition, an inner sphere glycine residue was mutated to a tyrosine for the same purpose as the previous histidine mutations, obtaining the name G65YCu_BMb (Fig. 2).[29,30]

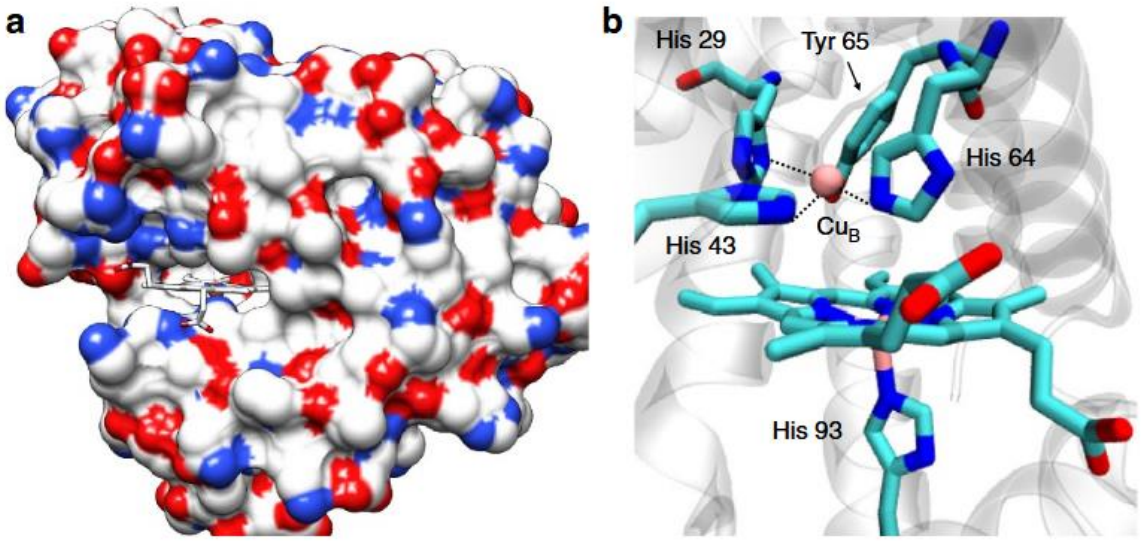

Figure 2: *Crystal structure of the CcO model based in the Mb backbone. (a) Complete color coding of charged amino acid residues depicted the heme site on the surface in the left center. Red represents positively charged residues; blue represents negatively charged residues. (b) Model of the catalytic active site including the iron heme, Cu_B site, and mutated Tyr/His residues.*

Structural characterization was first completed using surface-enhanced Raman spectroscopy, a spectroscopic, two-photon analysis that probes characteristic induced bond vibrations. This technique, similar to FT-IR, provides structural information about the types of bonding and functional groups present in a sample.[31] Raman data of this complex demonstrates vibrational bands consistent with a five-coordinate high spin heme coupled with a six-coordinate low spin species, suggesting a weak, transient bond to water in the active site.[28,32]

X-ray photoelectron spectroscopy (XPS) may also be used, giving basic information about the coordination environment and atom identities in the inner sphere of the direct C*c*O active site.[33] Results from this complex indicate iron, copper, carbon, nitrogen, and oxygen elements in the enzyme active site. Both the nitrogen and carbon peaks are broad due to contributions from amide, heme pyrrole/triazole groups, aromatics, and aliphatic types of each atom.[34,35]

Electrochemical techniques, such as cyclic voltammetry, may also be used to monitor the oxygen reduction catalytic activity of this molecule. Cyclic voltammetry (CV) sweeps a range of voltages, both positive and negative, to monitor the presence and voltage required for reduction and oxidation events in a solution containing the desired catalyst.[36] CV sweeps for the oxygen reduction reaction both in the presence and absence of the C*c*O mimic show a clear increase in the catalytic activity of the oxygen reduction reaction upon addition of the biosynthetic complex (Fig. 3).[20]

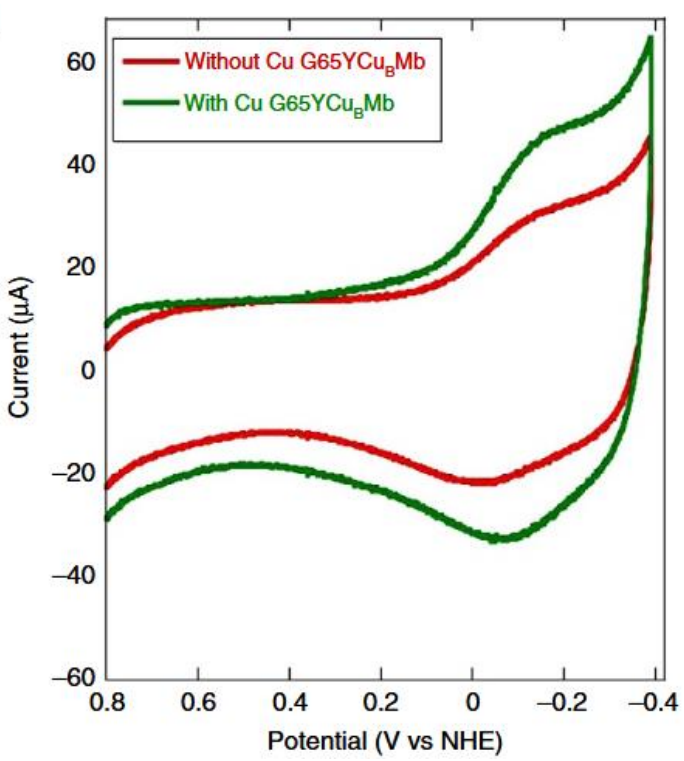

Figure 3: *Air-free CV data of the oxygen reduction reaction comparing Cu$_B$ unbound (red) and bound (green) forms of the CcO mimic. Increase in the magnitude of the reductive wave indicates higher catalytic activity.*

As with all structural analysis techniques, no one analytical method is suited to give a complete picture of the features of a complex. Given the use of this complex as a suitable structural

and functional mimic of C*c*O, the conclusions drawn about the catalytic activity require sufficient overlap with the structural features of the native enzyme. Therefore, this wide array of spectroscopic information is needed for complete and accurate characterization.

The potential of molecular oxygen reduction biologically is -263 mV and is dependent on both the reduction of the Fe^{III}/Fe^{II} iron in the heme as well as the four electron/four proton transfer. The partially reduced oxygen species such as superoxide or peroxide may be monitored via resonance Raman spectroscopy. In this analysis, a resonance Raman spectrum of the C*c*O catalyst mimic is continuously collected as the steady state O_2 reduction is carried out.[37] The Mb scaffold has a second-order O_2-binding rate of 10^7 M^{-1} s^{-1}, more than 10 times faster than the binding affinity to the heme a_3 site of native C*c*O.[38–40] Given this Raman data, comparisons may also be drawn to the next highest reported reduction rate another functional C*c*O analog, also from the work of Boulatov. The comparative reduction rate of 1.2×10^5 M^{-1} s^{-1} is two orders of magnitude lower than the current reported mimic.[41] Experimental data allowed for the suggested comparison of the native C*c*O enzyme when compared with this biosynthetic mimic, giving some context for why this significantly reduced rate of reaction may be achieved (Fig. 4-L).[39]

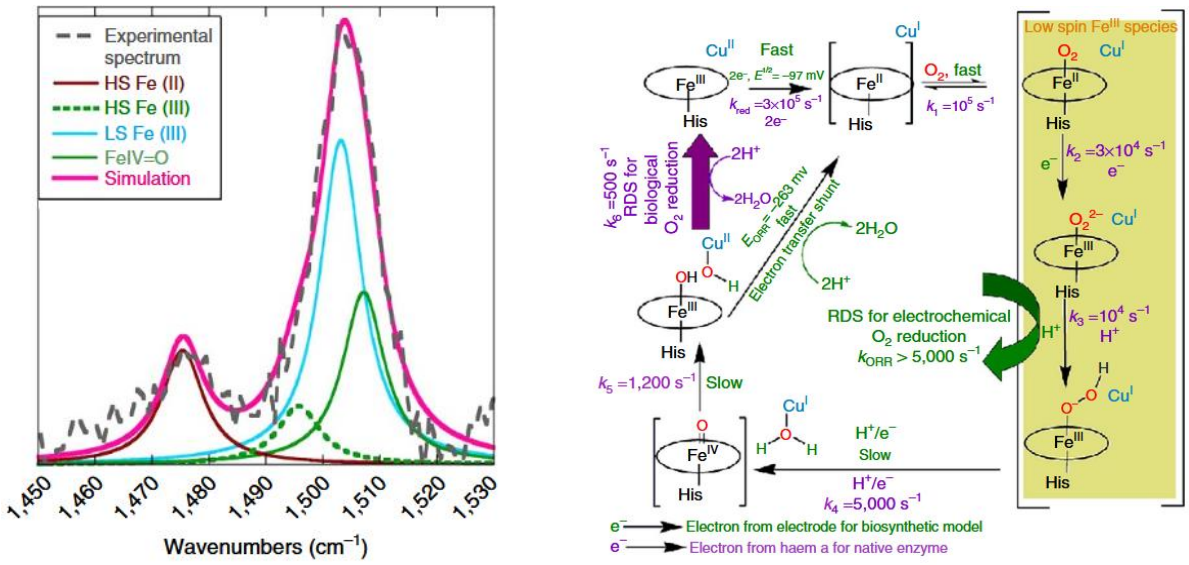

Figure 4: Adapted from Figures 5/6 of (39)- (L) *Iron-oxo binding components of the G65YCu$_B$Mb resonance Raman spectroscopy. Comparison between unbound heme containing high spin (HS) and low spin (LS) Fe$^{II/III}$ and bound Fe^{IV}=O is demonstrated. The shift to higher wavenumber indicated stabilization of the bound oxygen to the iron center.* ***(R)*** *Oxygen reduction mechanistic comparison of native CcO (purple) compared with G65YCu$_B$Mb (green). Native rate constants are listed for each step as a "k" value, mimic information is denoted with electrochemical potentials.*

The most notable oxygen reduction rate increase arises from the electron transfer shunt tied with the necessary -263 mV electrochemical potential. The increase in O_2-binding capability of this mutant-Mb mimic allows for bypass of the difficult bond cleavage of the Fe^{III}-OH bond cleavage and allows for a more efficient electron/proton transfer step. The Fe^{III}-OH bond has a lower negative reduction potential due to the presence of a strong hydrogen bond between the tightly bound -OH residue on the iron heme with the -OH residue on the Cu$_B$ site in the inner sphere.[42] Given the mechanistic work demonstrated in Fig. 4-R, many of the other ORR steps achieved by the *Cc*O mimic align closely with the native enzymatic pathway. The stabilization of

the Tyr and His residues in the mutant biosynthetic complex allow for additional stabilization of the electron/proton transfer and increased rate of the overall oxygen reduction reaction.[20] Given the efficiency of catalytic activity and the water solubility of this G65YCu_BMb C*c*O mimic, results such as these are primed for future applications in renewable and clean fuel cell use. Not only are the application and synthesis widely accessible, but the electrochemical functionality pairs well with common fuel cell reaction conditions noted to date.

However, no methodology is without faults. While functional mimics of C*c*O provide a plethora of important insights, they also can be quite costly. Materials for these systems may require long syntheses with a mix of enzymes/organic molecules that are difficult to obtain as well as difficult to stabilize. This requires much effort and financial cost. These compounds may also have lower stability and opportunity for catalysis. Given the voltage and storage conditions required for these compounds to undergo the ORR, these compounds are prone to electrochemical damage, often leading to sooner enzymatic breakdown than a native enzyme would experience in a biological system.[9] With the advancements that these models provide to the fields of bioinorganic and renewable energy, these pitfalls are typically endured for the sake of further findings.

Structural Cytochrome *c* Oxidase Mimic

While the previous example demonstrated a functional model of the C*c*O activity, many structural synthetic models may be utilized to further understand these biological systems in a more isolated environment. Structural mimics of metalloenzymes may not elucidate mechanistic or reactivity information, however structural information regarding biochemical ligand design and application may be enabled. Structural analysis information about these C*c*O models may improve biosynthetic methods for organic/inorganic coupled synthesis reactions, helping to improve

synthetic methods, metal insertions, and ligand design. Binding assays may also propose information about competitive binding of common biological small molecules such as NO, CO, or CN^-. Much like structural analysis methods, no singular functional nor structural model will give complete information about the C*c*O enzyme. Some may argue that one method may be more effective than the other, but comparison of the two methods can provide the most complete information about respective enzyme systems. It is often best practice to aim for both structural and functional mimicry to maximize the scope of knowledge learned and applications enabled.

The following synthetic work characterized in this paper comes from a significant name in the realm of cytochrome *c* oxidase chemistry, Dr. James P. Collman. Dr. Collman's work can be found across both native and biosynthetic cytochrome *c* oxidase chemistry. The complete synthetic work on the reported structural model is just one example of his C*c*O work and is a collaboration with Somdatta Ghosh.[43]

The broader scheme for C*c*O model synthesis by Collman and his collaborators may be summarized by three phases: face selection/insertion of the proximal imidazole ligand, attachment of the distal imidazole ligands and introduction of the metals.[44] A summary of the first phase is found in Figure 4. Using metal-induced separation, isolation of the desired stereoisomer may be obtained via bulk adsorbance into alumina. The desired amine groups are protected via CF_3 for further self-assembly. This specific phase successfully creates the iron-free heme and protected substitution on the porphyrin ring.[45] In the second phase, the amine groups are deprotected before introduction of the imidazole groups.[46] Introduction of the phenyl methyl ether may be further converted into the phenol group. The introduction of both the imidazole sidechains as well as the phenol group structurally mimics the necessary inner sphere histidine and tyrosine residues found in the native C*c*O enzyme, priming this synthesis for comparable enzyme structure. The final phase

of the active site synthesis is the introduction of the iron and copper metal centers. The iron bromide salt attaches an iron both in the heme and the Cu_B site.[47] This transformation completes the synthetic model, complete with the iron heme, proximal imidazole, Cu_B site, and substituted phenol ring (Fig. 5). This specific $Fe^{III}Cu^{II}$ArOH synthetic route may also be utilized for other C*c*O enzyme mimics and future studies.

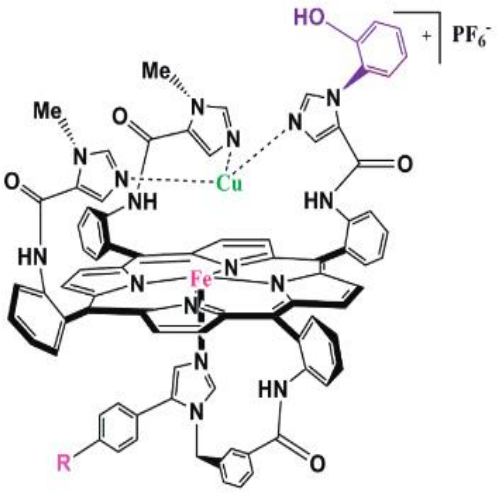

Figure 5- Adapted from (43): *Final structural model of the CcO active site, complete with the Fe and Cu metal centers. Note the PF_6^- anion and variable R group off of the aromatic ring to help stabilize and provide diversity in synthesis. This model includes histidine and tyrosine residues that are relevant to the native CcO structure.*

Primarily, the specific details of the organic reactions used to make this C*c*O structural mimic are useful for a variety of biochemical reasons. Working to synthetically model enzyme systems may give insight into potential biological synthesis by asking questions such as: is this residue important to the active site? How might biological systems bypass this difficult synthetic step? How is this conformation similar to/different from the native enzyme? Biosynthesis, such as this, may also allow for future synthesis of functional enzyme mimics as well as general organic synthesis.

Although this complex may also be utilized as a functional oxygen reduction catalyst, future investigations with the $Fe^{III}Cu^{II}$ArOH complex may elucidate the role of nitric oxide (NO), carbon monoxide (CO), or cyanide (CN^-) on the C*c*O binding site. Nitric oxide is a common biological small molecule used for signaling and respiration control.[48,49] NO is a reversible competitive C*c*O inhibitor due to a binding constant of 10^8 M^{-1} s^{-1} and a K_D of less than 10 nM, making it necessary to investigate the interaction between NO and the C*c*O binding site.[50,51] Given the unpaired electron configuration of the iron in the heme binding site, electron paramagnetic resonance spectroscopy (EPR) may also be utilized. EPR is a spectroscopic method that studies molecules with unpaired electrons, including metal centers and organic radicals in proteins or small molecules. Monitoring these electrons in the presence of a magnetic field provides a spectrum that gives rise to information regarding electron environment, oxidation state, nearby coordinating atoms, and key structural details in the active site. Changes in the EPR spectrum of a material show changes in electron count, pairing, or interactions with local bound materials.[52] In the presence of NO, the $Fe^{III}Cu^{II}$ArOH complex exhibits an EPR active low-spin, six-coordinate nitrosyl species consistent with an Fe-NO binding. Under the presence of O_2 binding, no change in the EPR spectrum is observed, demonstrating no net reaction and displacement of the bound NO. However, when the Cu_B site is introduced, the O_2 is reduced by the copper (I) atom to superoxide, significantly diminishing the Fe-NO binding signal and therefore introducing bound oxygen instead (Figure 6).[43]

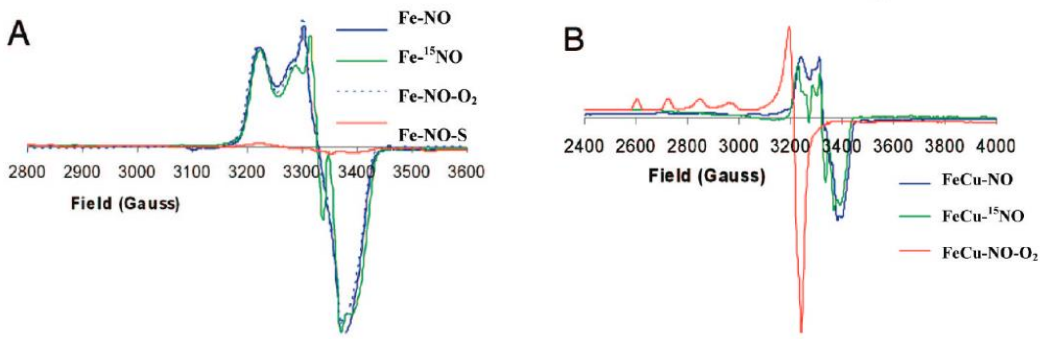

Figure 6: *(a) EPR spectra of NO/O_2 binding in the iron-only active site. Upon addition of excess oxygen, no change in the Fe-NO spectra is observed. (b) EPR spectra of NO/O_2 binding in the iron-copper active site. Significant change in the signal following O_2 addition may be attributed to a copper-superoxide complex formation.*

CO and CN^- are also inhibitors of the C*c*O active site. When CO and CN^- are bound to the C*c*O active site, both complexes are EPR inactive. However, when treated with equimolar amounts of NO, the EPR spectra shift to match the FeCu-NO spectra, illustrating substitution of both the CO/CN^- with NO. This is due to the higher binding affinity of NO to the iron heme when compared with either CO or CN^-. When treated with Amyl nitrite (AmN) to treat CN^- exposure, the EPR spectrum shifts to that of a Cu-Fe-nitrosyl interaction, not characterized by CN^- binding (Figure 7).

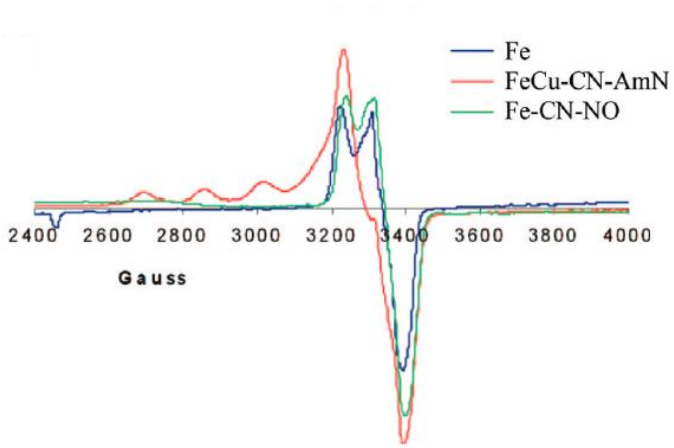

Figure 7: *EPR spectra of CN^-/CO binding upon addition of NO. The similarities between the blue and green spectra show minimal change in the electron configuration following addition of CN in the presence of NO. AmN treatment displaces the CN binding in the FeCu site.*

Given the synthesis of this structurally similar C*c*O active site model complex, further information about binding affinity and inner sphere environments may be deduced. In addition to its structural similarity to the native enzyme, this complex is also a functional mimic as well. In addition to the catalytic activity, however, information about competitive binding may also help inform about relative binding affinities of competitive inhibitors, identities of oxygen intermediates, and similar information. These results provide a more accessible opportunity to learn more about medicinal, biological, and synthetic phenomena outside of the notable biochemical insight.

Conclusions

Cytochrome *c* oxidase is not only a powerhouse in biological energy reactions but also serves as a notable target for renewable energy applications. Discussions and experiments relating to biomimetic chemistry create synthetic handles for modeling biologically relevant energy transduction enzymes, such as C*c*O. As the field of biomimetic chemistry expands, researchers inch closer to scalable enzyme mimics for commercial renewable energy systems. The highly

conserved and energetically important role that C*c*O serves in the electron transport chain is crucial to the generation of ATP and mitochondrial processing, making this a likely candidate for renewable energy applications. Promising results from research done on both structural and functional biomimetic C*c*O complexes not only serve to advance understanding about the enzyme itself but also provides a means to innovate enzyme mimics for future fuel cell use. These synthetic efforts hold great promise for commercial application, reaching reaction rates that are more efficient than the native enzyme itself. Laboratory scale studies of C*c*O also allows for competitive binding, allowing researchers to better identify the role of small molecules that may inhibit catalytic activity of the enzyme mimic system and the native enzyme itself. With implementation of global universal effort and rising concern for environmental health and safety, continued studies into biological metalloenzyme mimics may hold the key to sustainable energy systems. Though much work is still needed to reach the point of industrial application, the progress and promise of the findings within biomimetic C*c*O chemistry cannot be denied and will continue to thrive.

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