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Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, IRVINE

Roles of Transition Metal Ions in Biological Oxidations:
Mechanistic Insights into Metalloenzymes and Microbial Metabolism

DISSERTATION

submitted in partial satisfaction of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by

Kylie S. Uyeda

Dissertation Committee:

Professor Andrew S. Borovik, Chair

Professor Jennifer A. Prescher

Professor Jenny Y. Yang

2025

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DEDICATION

For my parents.
Just DOne it.

“Science may provide the most useful way to organize empirical reproducible data, but its power to do so is predicated on its inability to grasp the most central aspects of human life:”

So, throughout all the

“fear, love, hate, beauty, envy, honor, weakness, striving, suffering, virtue” ...

–Paul Kalanithi

When Breath Becomes Air

Recall first:

“Your vision is not limited by what your eye can see, but by what your mind can imagine.”

–Ellison Onizuka

Embrace next:

“You miss 100% of the shots you don’t take. –Wayne Gretzky –Michael Scott”

–The Office, Season 5, Episode 23

And remember always:

“Your best is good enough”

–Panda Express fortune cookie

Continue then to *go forth and conquer*,

Even if:

“In the end, it doesn’t even matter”

–Linkin Park

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CURRICULUM VITAE

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Winter Conference Travel Award (UCI Associated Graduate Students)	<i>Winter 2024</i>
Achievement Rewards for College Scientists (ARCS Foundation)	<i>2021 – 2023</i>
Summer Conference Travel Award (UCI Associated Graduate Students)	<i>Summer 2022</i>
Graduate Research Fellowship Program, Honorable Mention (NSF)	<i>2021</i>
Minority Serving Institution Award (UCI Office of Inclusive Excellence)	<i>2019 – 2021</i>
Global Aggie Award for Research Impacting the Global Community (UC Davis)	<i>2018</i>
Dean's Honors Student (Skyline Community College)	<i>2015 – 2016</i>

Presentations

Invited Talks

- K. S. Uyeda, A. S. Borovik. “Deciphering Structure and Function of Quercetin Dioxygenases.” ACS Division of Inorganic Chemistry Jonathan L. Sessler Fellowship for Emerging Leaders in Bioinorganic and Medicinal Inorganic Chemistry Symposium Honoring Marie Heffern. San Francisco, CA. 14 August 2023.

Conference Talks

- K. S. Uyeda, A. S. Borovik. “Deciphering structure and reactivity of quercetin dioxygenases.” Gordon Research Seminar Bioinorganic Chemistry. Ventura, CA. 20 January 2024.
- K. S. Uyeda, A. S. Borovik. “Establishing Structure/Function Relationships of Dioxygenase Enzymes.” Southern California Bioinorganic Meeting. Irvine, CA. 30 September 2023.
- K. S. Uyeda, M. C. Heffern. “Investigating the dependence of oxytocin on divalent metal ions.” Larock Undergraduate Research Conference. Davis, CA. 19 May 2018.

Conference Poster Presentations

- K. S. Uyeda, A. H. Follmer, A. S. Borovik. “Engineered Artificial Metalloproteins: Reactivity Within the Secondary Coordination Sphere.” Gordon Research Conference Metals in Biology. Ventura, CA. 21 January 2024.
- K. S. Uyeda, M. C. Heffern. “Investigating the dependence of oxytocin on divalent metal ions.” UC Davis Undergraduate Research Conference. Davis, CA. 26 April 2018.

Internal/Departmental Presentations

- K. S. Uyeda, A. S. Borovik. “Deciphering Structure and Reactivity of Quercetin Dioxygenases.” Summer Structural Biology Seminar Series. Irvine, CA. 14 August 2023.

- K. S. Uyeda, E. N. Bess. “Ironing out the gut microbiome’s role in Parkinson’s disease.” Systems Microbiology Group. Irvine, CA. 6 May 2021.
- K. S. Uyeda, E. N. Bess. “Ironing out the gut microbiome’s role in Parkinson’s disease.” Graduate Student and Post-Doctoral Colloquium. Irvine, CA. 2 April 2021.

Publications

1. **Uyeda, K. S.;** Follmer, A. H.; Borovik, A. S. Engineered Artificial Metalloproteins to Inform on Native Dioxygenases. *Manuscript in preparation*.
2. **Uyeda, K. S.;** Follmer, A. H.; Borovik, A. S. Selective oxidation of active site aromatic residues in engineered Cu proteins. *RSC Chemical Science*, **2024**, *16*, 98–103.
3. Ortiz de Ora, L.; Balsamo, J. M.; **Uyeda, K. S.;** Bess, E. N. Discovery of a Gut Bacterial Metabolic Pathway that Drives α -Synuclein Aggregation and Neurodegeneration. *ACS Chemical Biology*, **2024**, *19*, 1011–1021.
4. O’Sullivan, J. J.†; **Uyeda, K. S.†;** Stevenson, M. J.; Heffern, M. C. Investigation of metal modulation of oxytocin structure receptor-mediated signaling. *RCS Chemical Biology*, **2023**, *4*, 165–172.
5. Rich, B. E.; Jackson, J. C.; Ortiz de Ora, L.; Long, Z. G.; **Uyeda, K. S.;** Bess, E. N. Alternative Pathway for Dopamine Production by Acetogenic Gut Bacteria that O-Demethylate 3-Methoxytyramine, Metabolite of Catechol O-Methyltransferase. *Journal of Applied Microbiology*, **2022**, *133*, 1697–1708.
6. Stevenson, M. J.; Farran, I. C.; **Uyeda, K. S.;** San Juan, J. A.; Heffern, M. C. Analysis of metal effects on C-peptide structure and internalization. *ChemBioChem*, **2019**, *19*, 2447–2453.
7. Stevenson, M. J.†; **Uyeda, K. S.†;** Harder, N. H. O.; Heffern, M. C. Metal-dependent hormone function: the emerging interdisciplinary field of metalloendocrinology. *Metallomics*, **2018**, *11*, 85–110.

† Denotes equal contributions

Teaching and Service

General Chemistry Lecture and Discussion (Chem 1A, Professor A. S. Borovik)	Fall 2023
Chemical Biology Laboratory (Chem 128L, Dr. Lizett Ortiz de Ora)	Winter 2022
Chemical Biology Laboratory (Chem 128L, Professor Jennifer Prescher)	Fall 2021
Organic Chemistry Laboratory (Chem 51LC remote, Professor Renee Link)	Spring 2021
General Chemistry Laboratory Part II (Chem 1LC, Professor Kimberly Edwards)	Spring 2020
Chemical Biology Lecture and Discussion (Chem 128, Professor Rachel Martin)	Winter 2020
General Chemistry Laboratory Part I (Chem 1LD, Professor Kimberly Edwards)	Fall 2019
Research Mentor for one undergraduate researcher (UCI Minority Science Program)	2023 – 2024
Graduate Chemistry Peer Mentor (UCI Graduate Division)	2020 – 2023
Science Fair Mentor for three high school students (Westminster High School)	2021 – 2023
Graduate Student Mentor for one undergraduate student (Association for Women in Science)	2022
General STEM Mentor for three high school students (Reach Out Teach Out)	2020 – 2021
Chemistry Peer Tutor (Chemistry Peer Mentoring Program UCD)	2017 – 2019
Research Mentor for one undergraduate fellow (Summer Undergraduate Research Fellow)	2018

ABSTRACT OF THE DISSERTATION

Roles of Transition Metal Ions in Biological Oxidations:
Mechanistic Insights into Metalloenzymes and Microbial Metabolism

by

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Doctor of Philosophy in Chemistry

University of California, Irvine, 2025

Professor A. S. Borovik, Chair

The evolution of oxidative metabolism has shaped life on Earth, from ancient anaerobic microorganisms to the complex aerobic metabolism in modern organisms. Chapter 1 explores the emergence of oxygenic photosynthesis, the rise of aerobic respiration, and the pivotal roles of metalloenzymes in oxygen evolution, activation and utilization. Advances in bioinorganic chemistry have provided insight into metalloprotein mechanisms through the development of artificial metalloproteins (ArMs) and bioinspired catalysts. Exploring the interplay between metalloproteins, metabolism, and oxidative stress provides valuable insights into human health and disease. Maintaining systemic metal ion balance is essential for metabolic stability, and the involvement of metal ions in host-microbe interactions further influences physiological and pathological processes. By bridging perspectives from bioinorganic chemistry, enzymology, synthetic biology, microbiome research and biomedicine, this chapter establishes a foundation for understanding metal ion-driven biological transformations and their broader applications.

Chapter 2 describes work towards understanding Cu-containing oxygenase enzymes through the design and interrogation of Cu-ArMs. The influence of secondary coordination sphere interactions—specifically, aromatic residues—on Cu reactivity in engineered ArMs is explored. By introducing aromatic residues in defined spatial orientations, this study reveals their critical role in oxidant

activation and oxidation selectivity. The findings may reveal potential inactivation pathways in Cu-containing enzymes such as lytic polysaccharide monooxygenases (LPMOs) that break down complex polysaccharides, or suggest key primary coordination sphere features in particulate methane monooxygenases (pMMOs). Furthermore, these findings may provide insight into the molecular-level tuning of Cu-based oxidation processes in both natural and synthetic systems.

At the core of oxidative chemistry performed by metalloenzymes are highly reactive metal-oxygen intermediates that drive catalytic transformations essential for biological and synthetic oxidation processes. In comparison to Mn-, Fe- or Cu- containing oxygenase enzymes, Co-containing oxygenase enzymes are rare. Chapter 3 discusses efforts to generate, stabilize, and characterize Co-oxygen species within a protein scaffold, leveraging the protein environment to modulate reactivity through secondary coordination sphere interactions, steric effects, and protonation dynamics. Understanding the nature of these reactive species and the structure-function relationships that govern their behavior provides valuable insights into metal-mediated oxidation reactions, ultimately informing the rational design of novel (bio)catalysts.

Building on some of the design concepts, Chapter 4 explores the preparation and characterization of Co-, Ni-, Cu- and Zn- containing ArMs that mimic naturally occurring dioxygenase enzymes such as quercetin dioxygenases. By systematically comparing metal-substituted ArM variants, this work examines the role of metal ion identity and the local environment surrounding the metal center in modulating substrate binding and reactivity. These studies may provide mechanistic insights into metalloenzyme function and metal-based functional switching.

Beyond the individual metalloproteins, Chapter 5 investigates how transition metal ions and metalloenzymes are more broadly involved in bacterial metabolism and pathological oxidation reactions linked to neurodegenerative diseases. This chapter examines how gut microbiome dysbiosis alters redox conditions to promote dopamine oxidation and α -synuclein aggregation, suggesting a

potential pathway for Parkinson's disease progression. These findings highlight how microbial metabolism may be involved in host-disease mechanisms and further suggests potential strategies to mitigate oxidative stress in the gut-brain axis.

Overall, transition metal ions play critical roles in biological oxidation processes, serving as essential cofactors in enzymatic catalysis while also contributing to oxidative stress in pathological conditions. This dissertation explores the interplay between metal ion coordination, oxidation chemistry, and structure-function relationships in both enzymatic and disease-relevant systems. Using ArMs as biomimetic models, the studies presented herein elucidate key factors governing metal-based reactivity and oxidative transformations. Additionally, the pathological implications of metal-catalyzed oxidation are examined in the context of the gut microbiome and Parkinson's disease. By bridging fundamental enzymatic studies with disease-related oxidative stress, this work provides insight into metal-driven oxidation chemistry with broader implications for catalysis, biomimetic design, and disease mechanisms.

CHAPTER 1

Introduction

The History of Oxygen on Earth and the Evolution of Oxidative Enzymes

Three to four billion years ago, in the primordial oceans of early Earth, life was simple but resilient. The first cells, primitive anaerobic prokaryotes, thrived in an environment devoid of oxygen, instead depending on fermentation and anaerobic respiration to extract energy from simple molecules like dihydrogen, carbon dioxide, and methane. These early life forms utilized metalloenzymes that contained active sites resembling the structures of primordial ocean precipitates, which consisted of transition metal sulfides and oxides such as iron/nickel mackinawite ($\text{Fe}_2/\text{Ni}_2\text{S}_2$), nickel greigite (Fe_3NiS_4), violarite (FeNi_2S_4) or green rust. At the same time, it is hypothesized that peptides were forming in the primordial soup.¹ Thus, arose what are thought to be the earliest metalloenzymes: NiFe and FeFe hydrogenases,^{2,3} carbon monoxide dehydrogenase (CODH)⁴⁻⁷/acetyl-CoA synthase (ACS)⁸, nitrate/sulfite reductases,^{9,10} and methane monooxygenase (MMO)¹¹. These microbes set the foundation for metabolic diversity, proving that life could persist in extreme conditions, while evolving on a grander scale.

Fast forward to 2.5 to 2.3 billion years ago, when a remarkable breakthrough emerged among a new group of bacteria.¹² Cyanobacteria evolved to perform oxygenic photosynthesis, capturing the power of sunlight and storing it in the form of sugars. The Mn_4CaO_5 cluster in the oxygen-evolving complex (OEC) of photosystem II (PSII) catalyzed the oxidation of water, an abundant resource, generating dioxygen as a byproduct (**Figure 1.1**).^{13,14} At first, the dioxygen simply reacted with iron in the oceans, forming vast deposits of banded iron formations—striped layers of iron oxides that persist in ancient rocks today. But as cyanobacteria flourished in shallow waters, dioxygen began to accumulate in the atmosphere, leading to the great oxygenation event. Dioxygen, once a rare and reactive molecule, became a dominant force. This revolution was not welcomed by all. For anaerobic

organisms, dioxygen was toxic—they lacked defenses against ROS, suffered metabolic disruptions and experienced enzyme inactivation due to oxidative damage. Many perished in what would be called the first mass extinction.¹⁵ Others sought refuge in oxygen-free environments, thriving in deep sediments, hydrothermal vents, and anoxic ocean zones. And still, others adapted.

While dioxygen posed a deadly threat to anaerobic life, cells developed metalloenzymes like Mn-, Fe-, or Cu/Zn- containing superoxide dismutase (SOD) and Fe-containing catalase, which neutralized reactive oxygen species (ROS).¹⁶ Some microbes developed metalloenzymes to use dioxygen as a terminal electron acceptor in the electron transport chain: NADH oxidoreductase,^{17,18} succinate dehydrogenase,¹⁹ ubiquinol-cytochrome c reductase,^{20,21} cytochrome c oxidase,²² and adenosine triphosphate (ATP) synthase.²³ Aerobic respiration unlocked massive energy yields—up to 15 times more ATP per molecule of glucose, compared to fermentation.²⁴ This metabolic process allowed cells to grow larger, more complex, and more energy efficient. Meanwhile, another evolutionary leap occurred when an ancestral eukaryote engulfed an aerobic proteobacterium, which later became the mitochondrion, providing cells with a highly efficient energy source.²⁵

By the late Proterozoic Eon, 800–600 million years ago, dioxygen levels had climbed high enough to support multicellular life, setting the stage for the Cambrian Explosion, a rapid burst of evolutionary diversification. Dioxygen-fueled metabolism enabled the rise of larger, more active organisms—early animals with muscles, nervous systems, and complex behaviors. Predation emerged as a driving force of evolution, as creatures with higher energy demands took advantage of dioxygen’s potential. The evolution of circulatory systems in animals, supported by Fe-containing dioxygen transport proteins such as hemoglobin, helped distribute dioxygen efficiently (**Figure 1.1**).²⁶

Today, oxidative metabolism remains central to life, from single-celled microbes to the metabolic engines that drive humans and other animals. Metalloenzymes have driven the evolution of oxidative metabolism, from early anaerobic pathways to the oxygenic photosynthesis that transomed Earth’s

atmosphere, the emergence of aerobic respiration and the metabolic sophistication of multicellular life (**Figure 1.1**). These enzymes demonstrate the indispensable role of transition metal ions in biological energy conversion, oxygen utilization, and oxidative stress protection. However, dioxygen is a double-edged sword: while essential for modern life, it also contributes to aging, oxidative stress, and cellular damage—a relic of its ancient toxicity. As Earth’s environment changes and new challenges arise, life continues to evolve.

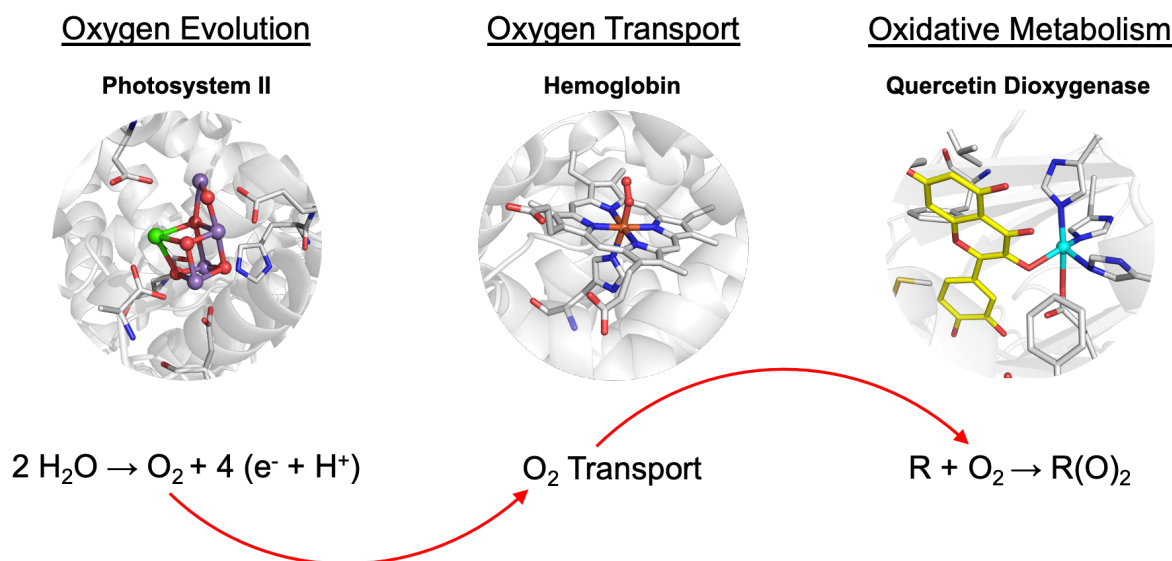


Figure 1.1. Important metalloproteins involved in dioxygen chemistry. **(A)** The active site of the oxygen evolving complex in photosystem II contains a cluster of four manganese centers, one calcium, and five oxygen atoms, shown in purple, green and red spheres, respectively (PDB: 7EDA). The OEC is involved in the photo-oxidation of water, forming dioxygen as a byproduct. **(B)** Dioxygen, shown in red spheres, bound to the heme-iron center of hemoglobin, displayed as sticks (PDB: 1GZX). Hemoglobin reversibly binds dioxygen for transport throughout the body. **(C)** The active site of quercetin dioxygenase, a mononuclear copper-containing dioxygenase, where the copper center is shown in cyan, surrounding amino acid residues as sticks, and the substrate quercetin in yellow (PDB: 1H1L). Dioxygenase enzymes are essential for oxidative metabolism.

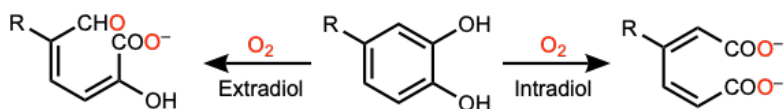
Harnessing the Power of O₂ for Oxidative Transformations

Despite its thermodynamic favorability as an electron acceptor, O₂ is relatively inert towards reactions with organic substrates due to its triplet ground state, which imposes spin restrictions on direct electron transfer to singlet substrates. To overcome this limitation, biological systems utilize transition metal centers contained within monooxygenases and dioxygenases to facilitate biochemical

transformations using O₂. Oxygenases oxidize substrates by catalyzing the transfer of one, in the case of monooxygenases, or both, in the case of dioxygenases, atoms of oxygen from O₂.^{27–29} Metalloenzymes are crucial for oxidation reactions in biological systems because the metal ion(s) within their active sites can act as a redox center, facilitating the exchange of electrons necessary for oxygen atom transfer processes. In native metalloenzymes, activation of O₂ is often mediated by substrate binding to prevent damage to the protein scaffold by the highly oxidizing metal-oxygen intermediates generated during turnover. This reactivity is in part due to the identity of the metal ion(s) within the active site, as well as the influence of the local environments surrounding the metal center(s), which includes the primary coordination sphere—the ligands directly bound to the metal center(s)—and the secondary coordination sphere—the noncovalent interactions that surround the active site.^{30–32} The rest of the protein scaffold can provide solvent accessible pockets or channels to facilitate access to the active site,^{33,34} spatially aligned chains of amino acids to shuttle protons and electrons to and from the active site,^{35–37} global protein motions that can be coupled to reactivity,^{38,39} and interactions with other biomolecules⁴⁰ to further mediate reactivity.

Bioinorganic chemists often focus attention to the metallocofactors at the active site. There are several examples of well-studied oxygenases from a bioinorganic perspective. For example, significant attention has been directed towards understanding of the mechanistic intricacies of catechol dioxygenases, a class of non-heme iron-containing metalloenzymes that play a pivotal role in the microbial degradation of aromatic compounds by facilitating the incorporation of O₂ into catechol substrates.^{41–43} Catechol dioxygenases are primarily classified into two categories based on their mode of ring cleavage: intradiol dioxygenases cleave the aromatic ring between the two hydroxyl groups (*ortho*-cleavage) using an Fe^{III} center, while extradiol dioxygenases cleave the aromatic ring adjacent to the hydroxyl groups (*meta*-cleavage) using an Fe^{II} center (**Scheme 1.1**).^{44,45}

Scheme 1.1. Catechol cleavage reactions catalyzed by extradiol and intradiol dioxygenases

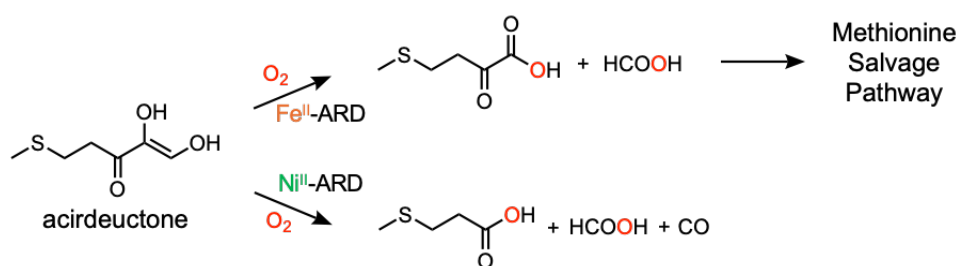


Kinetic,^{46–48} spectroscopic,^{49–60} crystallographic,^{61–65} and computational^{66–68} studies have contributed to understanding of the catalytic mechanisms of both intradiol and extradiol catechol dioxygenases. The catalytic mechanisms of catechol dioxygenases involve several key steps. First the catechol substrate binds to the active site iron through its hydroxyl groups, forming a bidentate complex. Substrate binding to the iron sites differs between the two classes, perturbing the cleavage specificity through the mode of coordination. Molecular oxygen then interacts with the iron-substrate complex. In intradiol dioxygenases, the Fe^{III} center facilitates the formation of a peroxo intermediate. In extradiol dioxygenases, the Fe^{II} center aids in the generation of a superoxo species.⁶⁹ Finally, the activated oxygen species inserts into the aromatic ring, cleaving it and forming *ortho*- or *meta*-cleaved products, respectively.

In most cases, including catechol dioxygenases, oxidative metalloenzymes contain iron in their active sites, which interacts with dioxygen to generate high-valent metal-oxo intermediates that can break strong carbon-hydrogen bonds in organic substrates. From an evolutionary and environmental perspective, iron is the second most abundant transition metal in Earth's crust and was readily available in Earth's early oceans. From a chemical standpoint, iron has the optimal electronic structure and conformational flexibility to generate Fe^{IV}-oxo, a key intermediate for reactivity with C-H bonds.^{22,70–72} Meanwhile, some metalloenzymes utilize other transition metals at their active sites. For example, acireductone dioxygenases (ARDs) are a unique group of enzymes that catalyze different reactions based solely on the identity of the metal within the active site. The Fe^{II} form catalyzes the oxidation of acireductone to formate and 2-keto-4-(thiomethyl)butyrate, a key step in the methionine salvage

pathway (MSP). In contrast, the Ni^{II} form generates carbon monoxide (CO) and 3-methylthiopropionic acid, an off-pathway product (**Scheme 1.2**).^{73,74} Synthetic model complexes and computational studies have resulted in proposals of ARD mechanisms, which have further been likened to the mechanisms of catechol dioxygenases. However, there is no conclusive evidence to support the mechanisms underlying metal-based functional switching.

Scheme 1.2. Oxidation of acireductone catalyzed by ARD. Depending on the metal ion within the active site, distinct products are formed. Fe^{II} -ARD is part of the methionine salvage pathway, while Ni^{II} -ARD forms CO and 3-methylthiopropionic acid.



The off-target pathway of Ni-ARD may have biological implications in mammalian systems. CO is involved in signaling and apoptosis, suggesting a role for Ni-ARD in cellular signaling.^{75,76} Currently, heme oxygenase is the only known CO-generating enzyme in human tissues.^{76,77} Alternatively, Ni-ARD may be involved in pathologies, where it could help prevent apoptosis in tumor cells by producing CO. Upregulation of genes associated with ARD has also been implicated in a variety of carcinomas, and has been suggested to regulate matrix metalloproteinase MT1-MMP which has been associated with tumor metastasis.^{78–82}

Deeper understanding of ARDs and related enzymes has implications in various fields. Further spectroscopic, structural and computational studies can inform on the structural and functional features that govern the unique reactivities of different transition metal complexes. New enzymes can be engineered by substituting nonnative metal ions into metalloprotein active sites, inspired by the metal-based functional switching exhibited by ARD. Small changes in protein structure upon Fe vs

Ni binding to ARD active sites opens questions regarding metal ion sensing in biological systems. On a systemic level, regulation of mammalian ARDs and methionine dependence have been identified in many human cancer cell lines, and identifying the roles of ARDs in disease states could lead to the development of therapeutics. And beyond mammalian ARDs, comparisons across phylogenies of life (i.e. bacterial ARDs) can inform on broader evolutionary perspectives.

An Approach to Understanding Native Metalloenzyme Functions

A core area of bioinorganic chemistry focuses on uncovering the intricate mechanisms and structure/function relationships underlying native metalloenzymes towards development of (semi)synthetic (bio)catalysts. A central theme in the Borovik group is the exploration of how secondary coordination sphere influences metal-mediated dioxygen activation. Previous work from the group has provided insights into how noncovalent interactions, such as hydrogen bonds (H-bonds), can stabilize otherwise elusive reactive intermediates and modulate the reactivity of metal centers in both synthetic systems (**Figure 1.2**) and artificial metalloproteins (ArMs).^{83–91} Moreover, the group has developed several ArMs to model the active sites of native metalloproteins.^{91–94}

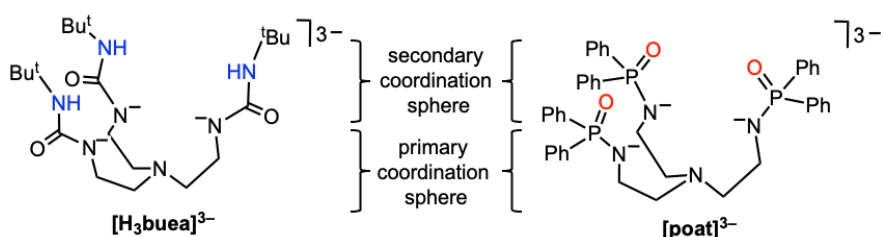


Figure 1.2. Examples of C_3 tripodal ligand frameworks to investigate the role of secondary coordination sphere H-bonding interactions in stabilizing metal-oxygen species developed by the Borovik lab.

To this end, portions of this dissertation will discuss molecular design in ArMs, an approach that harmonizes chemical biology and synthetic chemistry techniques to introduce non-native metallocofactors into naturally occurring or *de novo* designed proteins.⁹⁵ Overarching goals of preparing

ArMs include designing effective biocatalysts,⁹⁶ advancing biotechnology applications,^{97,98} and deepening fundamental understanding of protein structure and assembly.^{99–101} There are several general approaches to designing and preparing ArMs, which have been comprehensively reviewed by the Borovik group in 2022.⁹⁵ Since then, one approach to engineering proteins, the rational design of new proteins from first-principles methods, was awarded the Nobel Prize in 2024.^{101–103} Other approaches include reengineering/repurposing native metalloenzymes. ArMs can be further optimized for desired activities through directed evolution.¹⁰⁴

One method of preparing ArMs is by introducing synthetic metal complexes into proteins that would not otherwise contain metal sites. The first example of this approach was reported by Whitesides, where a biotinylated diphosphinerhodium(I) complex was confined within an avidin protein host to catalyze the asymmetric hydrogenation of α -acetamidoacrylic acid.¹⁰⁵ Subsequent work from the Ward group has utilized this biotin-(strept)avidin construct to develop several ArMs that catalyze a wide variety of reactions.^{106–115} The Borovik group has further developed ArMs using biotin-streptavidin (biot-Sav) to understand the influence of the local environment on the activity of metal sites. Previously, the Borovik group has used biot-Sav to engineer artificial copper proteins that model cupredoxins (**Figure 1.3**)^{90,92} or stabilize Cu^{II} –hydroxoperoxido species,⁸⁹ and artificial iron proteins that model non-heme dioxygenases⁹³ or stabilize a dinuclear iron–hydroxido species.⁹¹

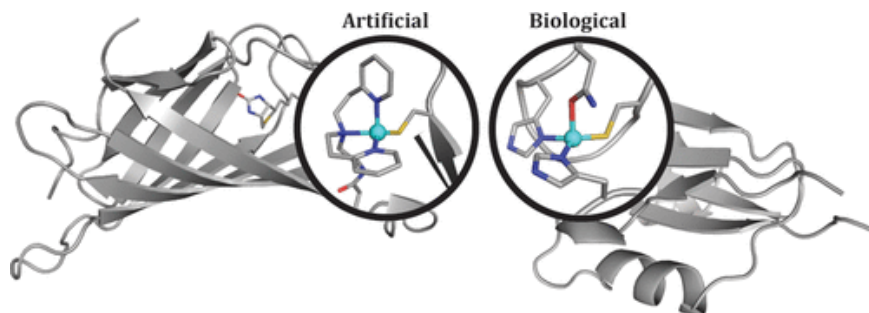


Figure 1.3. Active site of cupredoxins modeled by ArMs developed in the Borovik lab. Mann, S. I.; Heinisch, T.; Weitz, A.; Hendrich, M. P.; Ward, T. R.; Borovik, A. S. J. *Am. Chem. Soc.* 138, 9073–9076. Copyright 2016 American Chemical Society. Reprinted with permission.

In the previous examples, variants of streptavidin (Sav) were used as protein hosts. Sav is a homotetrameric protein that does not contain natural metal cofactors.^{116–119} The function of Sav in nature is unknown; however, its exceptionally high binding affinity for biotin ($K_d=10^{-14}$ M) has been leveraged for many applications.^{120,121} We take advantage of this high binding affinity to anchor synthetic biotinylated metal cofactors within Sav. Because Sav assembles as a dimer of dimers where two adjacent biotin-binding domains create what we refer to as the solvent-accessible vestibule, this approach offers flexibility in the design of the synthetic metal cofactors (**Figure 1.4**). Furthermore, site-directed mutagenesis (SDM) can be used to introduce coordinating ligands as well as manipulate the local environment surrounding the metal centers.

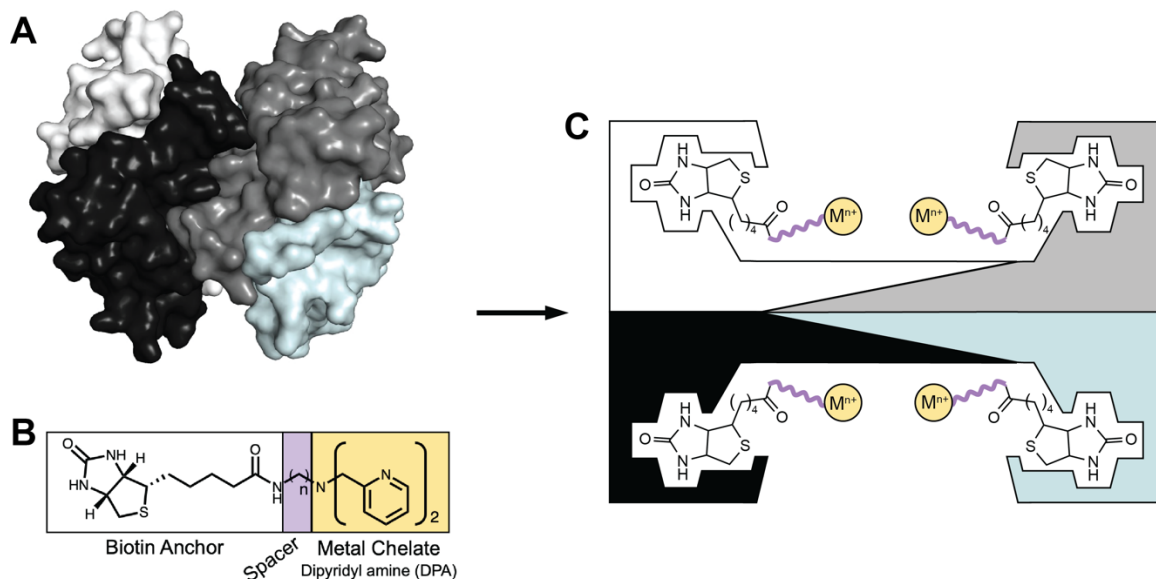


Figure 1.4. Engineered ArMs using a biotin-streptavidin construct. **(A)** Surface representation of tetrameric Sav, with each monomeric subunit shown in a different color. **(B)** A ChemDraw representation of the biotinylated ligands used in this dissertation. The spacer can be varied where n = ethyl, propyl, or butyl to position the metal center at various positions within the vestibule. **(C)** Diagram of the resulting ArM showing the biotinylated metal complexes anchored in the vestibule through biotin binding and coordination to amino acids in the vestibule.

Metalloprotein active sites are finely tuned molecular architectures that leverage metal ions to drive essential biochemical processes. However, the presence of these metal ions in biological systems extends far beyond individual protein function. The delicate balance of metal ion availability, trafficking, and storage is critical, as disruptions can lead to metal-associated disease states such as copper dysregulation in Wilson's and Menkes diseases, and metal dyshomeostasis in neurodegenerative diseases such as Alzheimer's and Parkinson's disease.^{122,123}

Inevitably, oxidative metabolism generates ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$) which can lead to oxidative stress.^{124–126} Oxidative stress results in direct or indirect ROS-mediated damage of important biomolecules including nucleic acids,^{127–129} proteins,^{130–132} and lipids.^{133–136} To mitigate oxidative stress, human cells employ a network of antioxidant enzymes that neutralize ROS and maintain redox homeostasis. SODs convert superoxide into hydrogen peroxide and dioxygen, preventing the accumulation of highly reactive radicals.¹³⁷ Catalase decomposes hydrogen peroxide into water and dioxygen, preventing the generation of hydroxyl radicals via the Fenton reaction.¹³⁸ Glutathione peroxidases reduce hydrogen peroxide and lipid peroxides into water and alcohols using glutathione (GSH) as a cofactor.¹³⁹ Peroxiredoxins reduce hydrogen peroxide, organic hydroperoxides or peroxynitrite, and work in conjunction with thioredoxin (Trx) and thioredoxin reductase (TrxR) which reduce disulfide bonds.^{140–143} Heme oxygenase degrades pro-oxidant heme into biliverdin, iron and carbon monoxide.⁷⁷

To maintain homeostasis, human cells have developed tightly regulated systems for metal ion uptake, transport, storage, and efflux (**Figure 1.5**). These processes involve specialized proteins that facilitate metal ion acquisition while preventing toxicity. Let us again focus on iron. Iron homeostasis begins in the duodenum, where dietary iron is absorbed by enterocytes. Non-heme iron is reduced to Fe^{II} by duodenal cytochrome b (Dcytb)¹⁴⁴ and transported into the cell by divalent metal transporter

1 (DMT1).^{145–148} Heme iron is taken up via heme carrier protein 1 (HCP1)^{149–151} and heme oxygenase releases Fe^{II} into the cell where it can be stored in ferritin.¹⁵² Ferroportin (FPN1) transports Fe^{II} from enterocytes into the bloodstream.^{153–155} Hephaestin (HP)^{156,157} oxidizes Fe^{II} back to Fe^{III} which binds to transferrin (Tf) for systemic circulation.^{158–160} Transferrin receptor 1 (TfR1) binds Tf- Fe^{III} and internalizes it via endocytosis and ferritin stores iron safely inside cells.^{161,162} Once inside the cell, iron is directed towards various functions. Furthermore, the labile iron pool refers to a transient, metabolically active fraction of intracellular iron that is loosely bound, redox-active, and readily chelatable. This pool is essential in cellular iron metabolism, balancing the essential roles of iron with its potential toxicity.¹⁶³

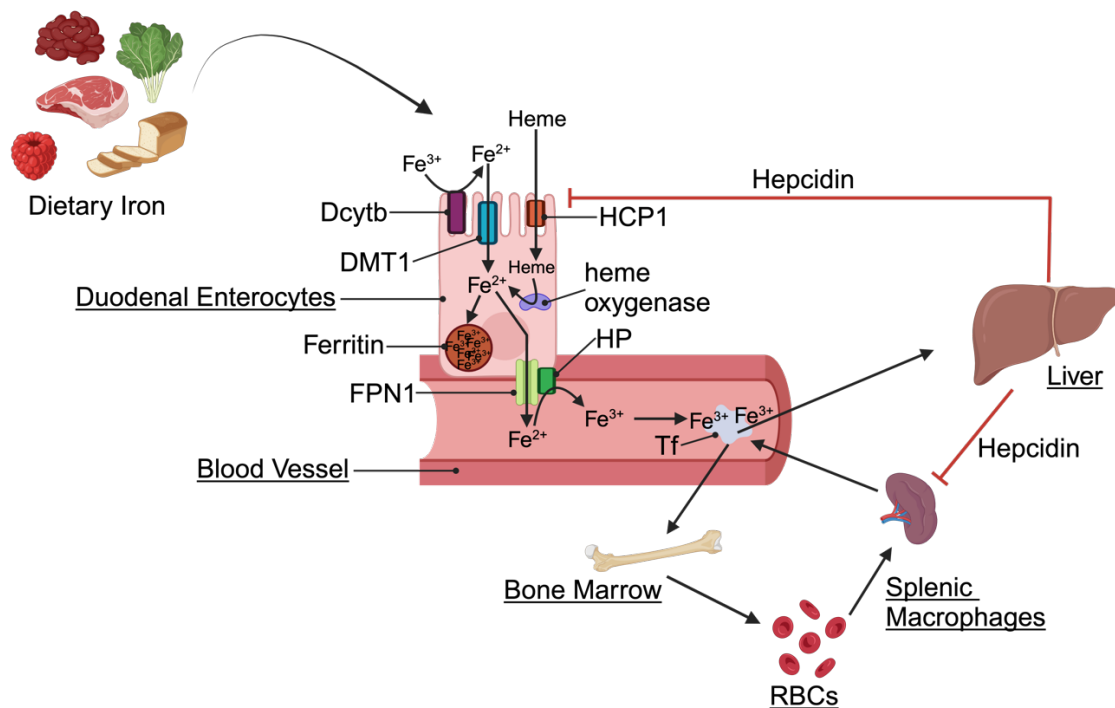


Figure 1.5. Systemic iron homeostasis is maintained in the plasma and tissues by tight regulation of dietary absorption and conservation of stores. Figure created in BioRender.

Cellular iron levels are sensed and controlled by transcription factors and signaling proteins. Hepcidin is upregulated by high iron levels or inflammation to prevent overload, and downregulated by anemia or hypoxia to enhance iron availability for red blood cell (RBC) production.^{164–166} Hepcidin

binds FPN1, triggering its degradation, thus preventing iron export into the bloodstream.^{167,168} Iron can be recycled from aging or damaged RBCs by macrophages which engulf senescent RBCs.¹⁶⁹ Disruptions in these regulatory mechanisms underlie numerous human diseases.

From the above description, it is obvious that iron homeostasis is highly complex and dynamic process, tightly controlled at multiple levels and requiring coordination amongst multi-organ systems. The need for multi-tissue, time-dependent modalities with high sensitivities makes iron metabolism one of the most difficult physiological processes to investigate. Nonetheless, phenotypical studies have linked abnormal metal ion accumulation or deficiency to neurological conditions such as Alzheimer's, Parkinson's (PD) and Huntington's diseases.^{170–173} In PD, disruptions in iron homeostasis, including decreased DMT1 and FPN1 expression, lead to iron accumulation in the substantia nigra. Excess iron in the substantia nigra catalyzes the Fenton reaction leading to the generation of ROS and oxidative stress.¹⁷⁵ ROS can cause detrimental lipid peroxidation, protein aggregation, and mitochondrial dysfunction, all contributing to neuronal death that causes motor deficits. One driver of PD pathogenesis may be iron-mediated oxidation of dopamine, which is involved in the misfolding and aggregation of α -synuclein, a pathological hallmark of PD.^{176–180} These aggregates contribute to the formation of Lewy bodies, leading to neuronal dysfunction.^{181–183}

Given the strong link between iron accumulation and PD pathogenesis, several iron-modulating therapies are being explored. Strategies targeting inhibition of ferroptosis and Fe-induced mitochondrial damages have shown promise as potential avenues for neuroprotection.¹⁸⁴ Iron chelation therapies, such as deferiprone, are being investigated to reduce Fe burden and oxidative stress in PD progression. While iron chelation presents a compelling therapeutic avenue, recent clinical findings underscore the need for caution and the complex balance of iron regulation in the human body.^{185,186}

Beyond their roles in human physiology, metal ions are also at the core of host-microbe interactions, as both pathogenic and commensal microbes have evolved sophisticated mechanisms to acquire, sequester, or resist uptake of metal ions as part of their survival strategies. Throughout evolution, humans and their microbiomes have coevolved in a deeply interdependent relationship, shaping each other's physiology, immunity, and overall survival. Understanding the interplay between metal ion regulation and host-microbe dynamics provides critical insights into both human disease mechanisms and potential therapeutic interventions.

Again, let us focus on iron—commensal gut bacteria play a beneficial role in host iron absorption and homeostasis, modulating bioavailability and influencing dietary metabolism. Microbial fermentation by bacteria such as *Lactobacillus* and *Bifidobacterium* lowers the pH of the gut which improves iron solubility and bioavailability.¹⁸⁷ Some gut microbes secrete iron reductases that contribute to non-heme iron reduction and absorption. Microbial metabolites, such as butyrate and propionate, regulate hepcidin and ferritin expression.^{188,189} Probiotic therapies have been suggested to mitigate iron-induced microbiome imbalances, reducing inflammation and oxidative stress.

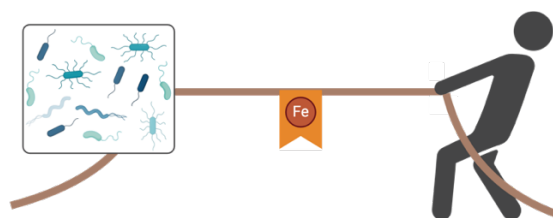


Figure 1.6. Iron is essential for both the human host and gut microbiome survival, yet iron is a limited and tightly regulated resource. This competition plays a key role in nutrition, immunity, and disease. Figure created in BioRender

On the other hand, there is tug-of-war relationship between the human host and certain microbes in the gut microbiome during inflammation (**Figure 1.6**). On one side, we have a healthy human where iron is tightly regulated and sequestered in proteins. To limit microbial growth, the human body can restrict iron availability in a process known as nutritional immunity.¹⁹⁰ Hepcidin is upregulated,

degrading FPN1 and storing iron in transferrin. This process creates an iron-starved environment that prevents bacterial proliferation and influences commensal microbial communities. On the other side, we have microbes which have evolved various mechanisms to scavenge iron from the host environment. Pathogenic bacteria secrete siderophores, small molecules that scavenge iron from host proteins.^{189,191,192} As a response, the host produces siderocalin which can sequester bacterial siderophores and prevent their uptake by bacteria.¹⁹³ So, some bacteria further modify their siderophores to evade host defenses. Many factors can throw an advantage or disadvantage to either side.

Emerging research has highlighted a complex interplay between bacterial siderophores and host mitochondrial function, revealing both detrimental and regulatory effects. One study demonstrated that siderophores from *Klebsiella pneumoniae* can chelate iron within platelets, leading to mitochondrial damage and the induction of mitophagy-dependent apoptosis.¹⁹⁴ Another study indicates that holo-lipocalin-2 (a complex of lipocalin-2, siderophore, and iron) increases mitochondrial ROS production and reduces oxidative phosphorylation in cardiomyocytes. This effect is attributed to the siderophore component, which disrupts mitochondrial bioenergetics, potentially contributing to cardiac dysfunction.¹⁹⁵ Or gut microbiota-derived siderophores can stabilize HIF-1 α signaling, thereby preventing gut dysbiosis through modulation of mitochondrial function.¹⁹⁶

The growing field of metallobiology is now uncovering ways to leverage transition metals for therapeutic purposes. Metal-based antimicrobials are emerging as powerful tools for modulating the gut microbiome and combating antibiotic-resistant infections. Gallium therapy is being explored to exploit bacteria's reliance on iron by using iron-mimicking molecules that disrupt microbial metabolism without harming the host or beneficial gut microbes.¹⁹⁷ Copper and silver nanoparticles are being explored as next-generation antimicrobials, capable of disrupting bacterial membranes and biofilms while minimizing antibiotic resistance development.¹⁹⁸ Additionally, targeted dietary metal

modulation is gaining interest as a way to fine-tune microbiome composition—altering iron, zinc, or manganese intake could shift the balance between beneficial and pathogenic microbes in conditions like inflammatory bowel disease (IBD), metabolic syndrome, and colorectal cancer.¹⁹⁹ Despite their promise, metal-based strategies must be carefully controlled to prevent unintended disruptions to the gut microbiota and host metabolism. Overuse of metals like zinc and copper can alter microbial diversity, potentially leading to gut dysbiosis or the selection of metal-resistant bacterial strains. Furthermore, excessive accumulation of metals in host tissues poses toxicity risks, necessitating precise dosing and targeted delivery approaches. Fine-tuning metal-based interventions, may help to create novel therapeutics that enhance gut health, fight persistent infections, and reduce reliance on traditional antibiotics.

Looking forward, the integration of bioinorganic chemistry, microbiome research, and synthetic biology is poised to drive innovative solutions for antimicrobial strategies, microbiome engineering, and therapeutic metal-based interventions. Advances in spectroscopic, computational, and structural biology techniques will enable deeper mechanistic exploration of metalloenzymes, leading to the development of synthetic metalloproteins and novel metallodrugs. Additionally, leveraging our knowledge of metal-microbe interactions could open new frontiers in precision medicine, gut microbiome modulation, and targeted antimicrobial therapies that exploit microbial metal dependencies. By bridging fundamental enzymatic studies with applied biomedical research, this field holds great promise for transformative breakthroughs in human health, biotechnology, and the fight against antimicrobial resistance.

Overview of the Following Chapters

Oxidation chemistry in biological systems is exceptionally efficient, particularly in copper-containing enzymes such as particulate methane monooxygenases (pMMO) and lytic polysaccharide

monooxygenases (LPMOs). These enzymes outperform synthetic catalysts in C–H bond activation due to unique local environments surrounding their copper cofactors. Recent studies have revealed critical roles for the local environments, such as the newly identified Cu_D site in pMMO and second sphere aromatic residues in LPMOs, though these features are subjects of continued debate. Work discussed in Chapter 3 utilizes the biotin-Sav construct to develop a series of ArMs that mimic active sites of natural copper metalloenzymes. By engineering ArMs with aromatic residues within their secondary coordination spheres, this study systematically examines their influence on Cu reactivity and oxidant activation (**Figure 1.7**). Key findings suggest that the coordination of an active site asparagine, as well as the proximity and orientation of aromatic residues relative to the Cu cofactor critically affect the oxidation outcomes upon exposure to hydrogen peroxide. These findings underscore the importance of the secondary coordination sphere in modulating Cu reactivity, suggest a role for amide coordination in C–H bond activation by pMMOs, and potential inactivation pathways in natural copper enzymes like LPMOs.

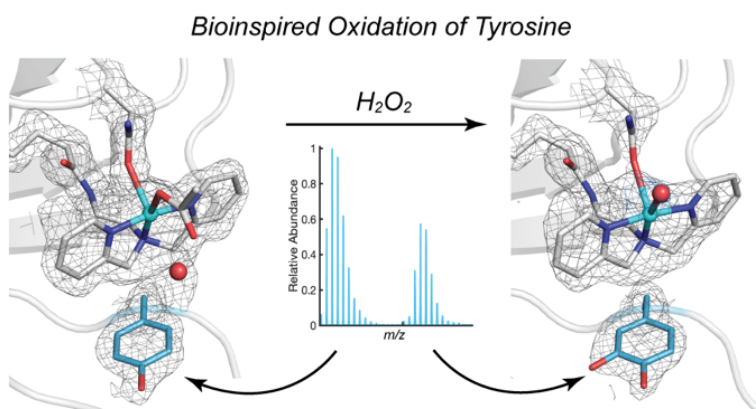


Figure 1.7. Overview of Chapter 2. The Copper’s Entourage: Selective Oxidation of Active Site Aromatic Residues in Engineered Cu Proteins.

Uyeda, K. S.; Follmer, A. H.; Borovik, A. S. *Chem Sci*, 2025, 16, 96–103. Copyright 2015 The Royal Society of Chemistry. Reprinted with permission.

Central to the oxidative chemistry in both biological and synthetic systems is the activation of molecular oxygen or hydrogen peroxide. While Cu- and Fe- based enzymes dominate this field, cobalt-

containing catalysts present a promising alternative due to their redox flexibility and potential for novel reactivity. Chapter 5 explores the development and characterization of cobalt-containing ArMs towards stabilizing and characterizing reactive Co-oxygen adducts (**Figure 1.8**). Spectroscopic and structural analyses reveal that the local coordination environment significantly influences the generation and stability of putative Co-oxygen adducts generated by addition of H₂O₂. These findings can inform future efforts to develop bioinspired cobalt catalysts for selective oxidation reactions and artificial enzyme engineering.

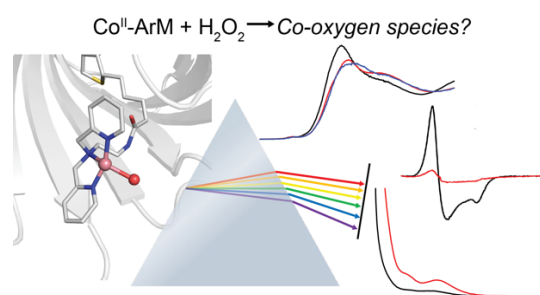


Figure 1.8. Overview of Chapter 3. Towards Stabilizing a Reactive Co-Oxygen Adduct Within a Protein Host.

The stereoselective oxidation of organic substrates represents an important challenge in modern organic chemistry, but understanding of native metalloenzymes can facilitate development of novel (bio)catalysts. We can take inspiration from cambialistic enzymes such as quercetin dioxygenase (QD), which is involved in the regiospecific oxidation of flavonoid compounds by bacteria and fungi. Chapter 4 presents developments in a series of ArMs designed to mimic structural aspects of QD active sites, allowing for stabilization of mechanistically relevant states. Comparative analyses between metal-substituted ArMs and native QDs provide insights into the structure-function relationships governing QD activity (**Figure 1.9**).

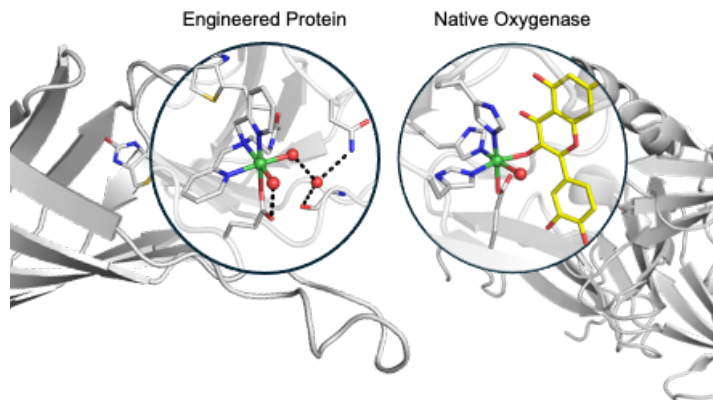


Figure 1.9. Overview of Chapter 4. A Series of Artificial Metalloenzymes to Model Structure and Function of Native Quercetin Dioxygenase Enzymes

One frontier lies in the interplay between microbial metabolism, oxidative stress, and neurodegenerative disease, where dysregulation of metal ion homeostasis plays a critical role. In an oxidative environment, like those of the aging brain, accumulation of iron and oxidation of the neurotransmitter dopamine can induce misfolding and aggregation of a protein called α -synuclein (α -syn). The aberrant aggregation and accumulation of α -syn results in neuronal death and Parkinson's disease (PD). Growing evidence suggests that α -syn aggregation may begin in the gastrointestinal (GI) tract and aggregates may subsequently spread from the gut to the brain via the Vagus nerve. However, the molecular-level mechanisms responsible for initiating this pathogenic process within the context of the GI tract remain ill defined. Despite the presence of significant dopamine and iron stores in the gut, dopamine oxidation is unlikely in a healthy, reducing gut environment. This raises the question of whether gut microbial activity can create conditions that promote dopamine-mediated α -syn aggregation. Chapter 2 investigates a newly identified pathway in which anaerobic metabolism by *Escherichia coli* initiates a cascade of reactions leading to α -syn aggregation (**Figure 1.10**). The results of this work are further discussed in the context of three proposed strategies to manipulate gut microbiome redox potential, mitigate resulting iron speciation/availability, and resulting dopamine oxidation and α -syn aggregation. These findings reveal an opportunity for the development of novel diagnostic and therapeutic interventions for PD.

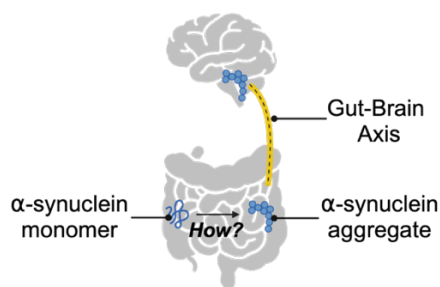


Figure 1.10. Overview of Chapter 5. Ironing out the gut microbiome's role in Parkinson's disease.

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CHAPTER 2

The Copper's Entourage: Selective Oxidation of Active Site Aromatic Residues in Engineered Cu Proteins

Preface

When I joined the Borovik group, my goal was to deepen my understanding of how metal ions influence microbial and host metabolism. The gut microbiome is a complex biochemical environment where metalloenzymes play pivotal roles in driving essential redox transformations. As I explored this area, I gained a deeper appreciation for how fundamental insights into structure/function relationships underlying metalloenzyme mechanisms can guide the development of novel biocatalysts with diverse applications in green chemistry, pharmaceuticals, biofuels and environmental sustainability. Among the diverse array of metalloenzymes, Cu-containing proteins facilitate a wide range of electron transfer processes and oxidation reactions. One example is tyrosinase, which catalyzes the oxidation of phenols to quinones, a key step in melanin biosynthesis. Interestingly, this reaction may represent yet another piece of the cascade of oxidative transformations discussed in Chapter 5. While tyrosinase is a dinuclear Cu-enzyme, ongoing debate surrounds its mechanism—specifically, whether oxidation can be localized at a single Cu-center. The work discussed in this chapter arose from what could be considered a fortunate accident. A simple mix-up, picking up the glycerol stock labeled “2xm-S112F” instead of “2xm-S112E,” unexpectedly led to the discovery of oxidation chemistry within engineered Cu-proteins. The findings discussed herein uncovered a pathway for tyrosinase-like reactivity at a mononuclear Cu-center, as well as implications for enzymes like lytic polysaccharide monooxygenases (LPMOs) or particulate methane monooxygenase (pMMO), offering new insights into Cu-based oxidation mechanisms.

Background

The oxidation of aromatic and (poly)phenolic compounds and the selective functionalization of C–H bonds remain significant challenges in synthetic chemistry.^{1,2} Nature often employs metalloenzymes to facilitate oxidative transformations using O₂ or H₂O₂ as primary oxidants with unmatched efficiency and selectivity. While synthetic systems have successfully emulated the primary coordination sphere, it is becoming increasingly apparent that the local environment, the volume of space surrounding the metallocofactor, is crucial for maintaining function. As such, significant research effort has been devoted towards understanding the mechanisms and structure/function relationships of these naturally occurring catalysts and characterizing the intermediates involved in their reaction mechanisms. For example, mononuclear copper-containing enzymes such as particulate methane monooxygenase (pMMO) and lytic polysaccharide monooxygenases (LPMOs) functionalize strong aliphatic C–H bonds (~100 kcal/mol) under ambient conditions, making them attractive models for developing catalysts for methane-to-methanol conversion and biomass-to-biofuel production.³ Despite their significance, the mechanistic intricacies of pMMO and LPMOs remain uncertain, prompting substantial efforts towards identifying the intermediates involved during turnover.

Insights from structural biology, biophysics, and synthetic model systems have demonstrated that effective C–H bond activation by metalloenzymes requires contributions from many properties associated with the confinement of the metallocofactor(s) within the protein scaffold. In LPMOs, the Cu center is ligated by a conserved set of donors termed the “histidine brace,” while a newly proposed catalytic site in pMMO shows a unique coordination by a nearby asparagine residue (**Figure 2.1**).^{4,5} While the primary coordination sphere has been the focus of many studies, it is now recognized that local environment surrounding the metallocofactor, including the noncovalent interactions within the secondary coordination sphere, is also crucial for function.^{6–8} In particular, the role of a tyrosine or

phenylalanine positioned 3-5 Å from the Cu center in LPMOs is a source of continued debate, where it has been suggested to protect the Cu cofactor from oxidative damage during reactions with H₂O₂.^{9,10} In variants of LPMO where the nearby residue is a tyrosine, hydrogen bonding (H-bonding) interactions with a glutamine in the secondary coordination sphere are crucial for reactivity. Deeper understanding of the role of this aromatic residue is limited, as systematically isolating and manipulating each of these noncovalent interactions within complex protein environments or synthetic model systems remains challenging. There are also phenylalanine residues within 5 Å of the Cu_D site in pMMO, the role(s) of which are even less explored than in LPMOs. The development of artificial metalloproteins (ArMs) offers a controlled and customizable approach to examine the impacts of each individual component on the mechanisms of C–H bond activation.¹¹

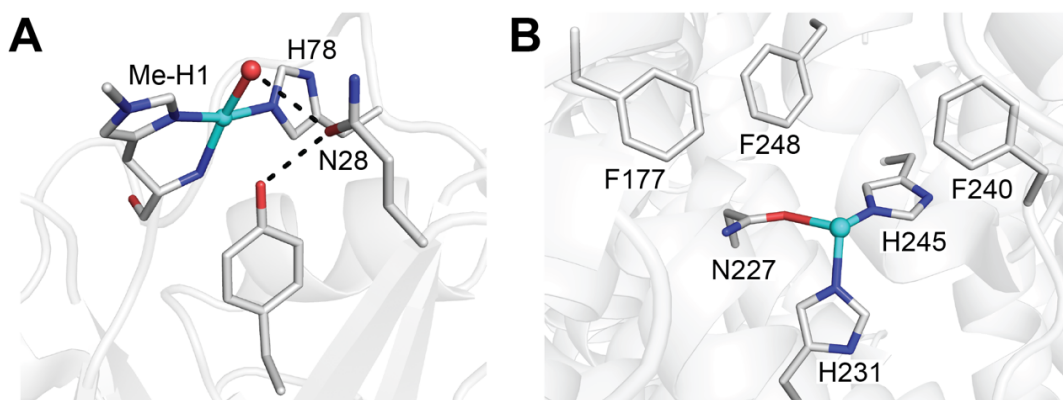


Figure 2.1. Molecular structures of (A) the LPMO active site (PDB: 5ACH) and (B) the Cu_D site of pMMO (PDB: 7S4H). H-bonding interactions are denoted by black dashed lines.

The Borovik group has previously prepared and characterized ArMs as semi-synthetic models of cupredoxins, as well as intermediate states of Cu metalloenzymes by stabilizing Cu^{II}-hydroperoxido (Cu^{II}–OOH) species through the incorporation of H-bond networks within the secondary coordination sphere (**Figure 2.2**).^{12–14} This prompted the preparation of Cu ArMs with aromatic residues near the Cu center to emulate aspects of LPMO active sites and understand the influence of these residues on the activation of oxidants such as H₂O₂. In LPMOs, H₂O₂ has been shown to enable

catalytic activity in place of O₂ and a reductant, although this reaction leads to significant protein degradation.⁹ Regardless of the oxidant, most mechanistic proposals invoke a Cu^{II}–OOH species as a key intermediate.

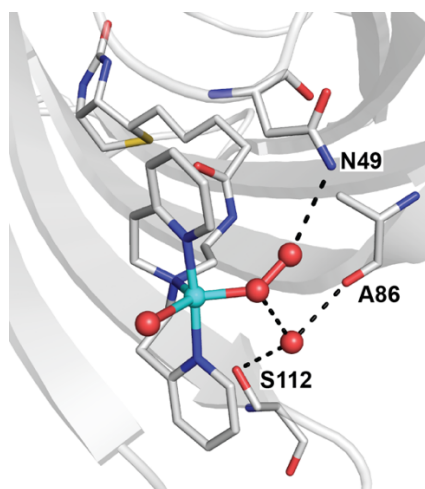


Figure 2.2. Molecular structure of an ArM containing a Cu^{II}–OOH species stabilized by an extended H-bonding network ([Cu^{II}(biot-et-dpa)(H₂O)(OOH)]⁺⊂Sav-WT, PDB: 6ANX).

Results and Discussion

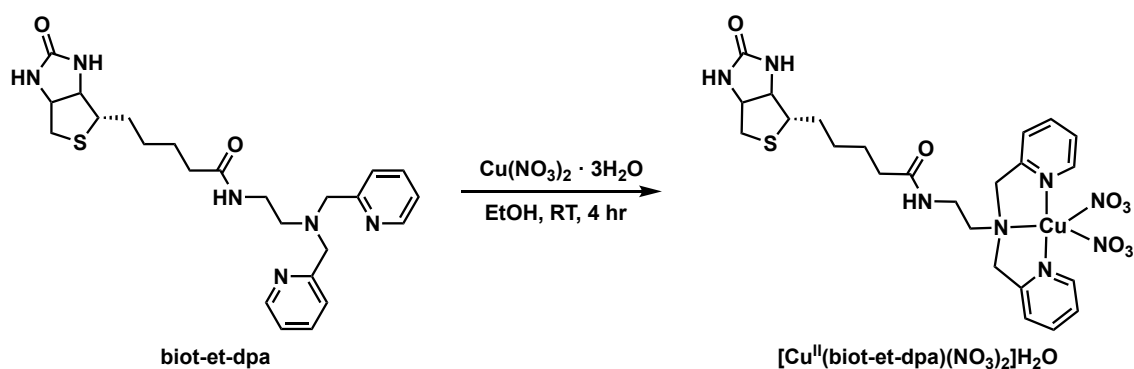
Design and Preparation of ArMs

As discussed in Chapter 1, previous work by Whitesides as well as the Ward group inspired the construction of ArMs by leveraging the strong binding affinity between biotinylated metal complexes and the streptavidin (Sav) protein host to confine synthetic metallocofactors within a protein scaffold.^{15,16} While the Ward group is interested in developing novel catalysts for reactions of interest, the Borovik group uses a similar approach with a semisynthetic biotin-Sav construct to investigate reactive intermediates relevant to biological systems. Previous results from our group led to the incorporation of Cu^{II} ions into the biotinylated bis(2-pyridylmethyl)amine (biot-et-dpa) ligand to position the metal center in close proximity to Sav residue S112, a site that the group commonly manipulates via site-directed mutagenesis (SDM) for engineering ArMs.^{17,18} Preliminary models

suggested that mutating S112 to an aromatic amino acid Y or F would position this residue in proximity to the Cu center, but would not be oriented to participate in direct coordination.¹⁹ To prepare this ArM, a triple mutant variant of Sav, referred to as 2xm-S112Y-Sav was engineered; this variant contains S112Y and two additional mutations, K121A and E101Q, which were found to prevent unwanted interactions with the cofactor.¹⁷ A phenylalanine mutation was also introduced at position 112 to make 2xm-S112F-Sav. Lastly, an additional triple mutant variant of Sav, with a tyrosine mutation at a location predicted to be further from the Cu-center, referred to as 2xm-K121Y-Sav was engineered to further examine the influence of tyrosine positioning and orientation on reactivity.

The biot-et-dpa ligand was prepared via a three-step route from dpa as previously reported.¹⁸ The Cu^{II}-biot-et-dpa complex was prepared by treating biot-et-dpa with Cu(NO₃)₂•3H₂O in ethanol to afford [Cu^{II}(biot-et-dpa)(NO₃)₂] (**Scheme 2.1**). The Cu ArMs were prepared by addition of a solution of excess Cu^{II}-biot-et-dpa to a solution of Sav in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (50 mM, pH 6). Samples were separated from excess Cu^{II}-biot-et-dpa and concentrated using Amicon Ultra 10K centrifugal filter devices and washed with 5X volume of buffer. To ensure binding of Cu^{II}-biot-et-dpa, a 2-(4'-hydroxyazobenzene)benzoic acid (HABA) displacement assay was performed on each ArM. These studies indicated a 4:1 binding ratio of Cu^{II}-biot-et-dpa to Sav (a homotetramer) for each ArM, confirming the efficient incorporation of the cofactor into the Sav protein host (**Figure 2.3**). The ArMs prepared for this study are listed in **Table 2.1**.

Scheme 2.1. Preparation of Cu^{II}-biot-et-dpa artificial metallocofactor



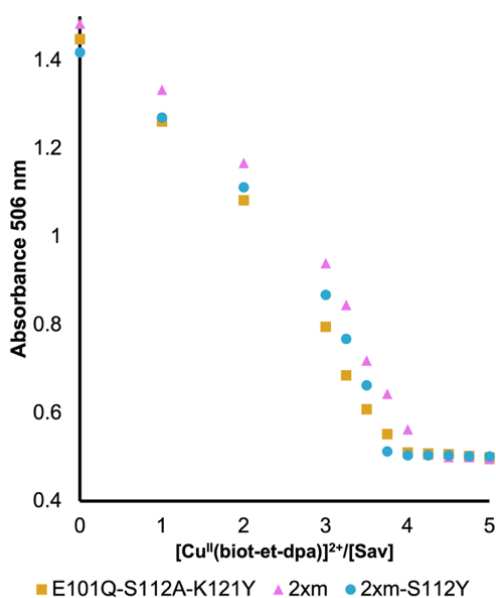


Figure 2.3. HABA titrations of Cu^{II}-biot-et-dpa into Sav variants confirming an average of ~4 biotin-binding sites per Sav molecule for each of the Sav variants used in this study.

Table 2.1. ArMs prepared in this study

	Sav Variant	Cofactor	ArM
1	2xm-S112Y	Cu ^{II} -biot-et-dpa	[Cu ^{II} (biot-et-dpa)⊂2xm-S112Y-Sav]
2	2xm	Cu ^{II} -biot-et-dpa	[Cu ^{II} (biot-et-dpa)⊂2xm-Sav]
3	2xm-K121Y	Cu ^{II} -biot-et-dpa	[Cu ^{II} (biot-et-dpa)⊂2xm-K121Y-Sav]
4	2xm-S112F	Cu ^{II} -biot-et-dpa	[Cu ^{II} (biot-et-dpa)⊂2xm-S112F-Sav]

Spectroscopic Characterization of ArMs

Electronic absorption (UV-vis) and continuous wave (CW) X-band electron paramagnetic resonance (EPR) spectroscopies were used to characterize ArMs in solution. For [Cu^{II}(biot-et-dpa)⊂2xm-S112Y-Sav] (**1**), a feature at $\lambda_{\text{max}} = 15675 \text{ cm}^{-1}$ (638 nm) was observed, attributed to a Cu d-d transition (**Figure 2.4 A**). This feature was slightly red-shifted in the analogous ArM lacking the tyrosine mutation ([Cu^{II}(biot-et-dpa)⊂2xm-Sav], **2**), with $\lambda_{\text{max}} = 15480 \text{ cm}^{-1}$ (646 nm), indicating a

change in the ligand environment of the Cu center (**Figure 2.4 B**). For $[\text{Cu}^{\text{II}}(\text{biot-et-dpa})\text{C}2\text{xm-K121Y-Sav}]$ (**3**), with a tyrosine mutation in a different location, the Cu d-d transition was observed at $\lambda_{\text{max}} = 15723 \text{ cm}^{-1}$ (636 nm) (**Figure 2.4 C**), and for $[\text{Cu}^{\text{II}}(\text{biot-et-dpa})\text{C}2\text{xm-S112F-Sav}]$ (**4**), the Cu d-d transition was observed at $\lambda_{\text{max}} = 15707 \text{ cm}^{-1}$ (637 nm) (**Figure 2.4 D**). The EPR spectrum of **1** was modelled as a single Cu^{II} species (**Figure 2.5**) with $A_{\parallel}$ values given in **Table 2.2**, though these values are an approximation, as they are smaller than the anisotropic broadening for their respective splittings. The EPR spectrum of **2** was modelled as two distinct Cu^{II} species.

Upon addition of 10 equivalents of H_2O_2 to **1**, the Cu d-d transition shifts from $\lambda_{\text{max}} = 15675 \text{ cm}^{-1}$ to 15825 cm^{-1} over 90 minutes, suggesting a conformational change surrounding the Cu center (**Figure 2.4 A inset**). Additionally, the growth of a new shoulder at 24691 cm^{-1} ($\sim 405 \text{ nm}$) is observed, which is reminiscent of previous work suggesting the formation of a $\text{Cu}^{\text{II}}\text{-OOH}$ species (**Figure 2.4 A inset**).²⁰ However, no changes were observed in the EPR spectra of **1** upon addition of peroxide to suggest formation of a $\text{Cu}^{\text{II}}\text{-OOH}$ species (**Figure 2.5** and **Table 2.2**). Upon addition of peroxide to **2**, **3**, or **4** the shift in energy of the Cu d-d band and the growth of the shoulder at 405 nm were not observed (**Figure 3.4 B–D**).

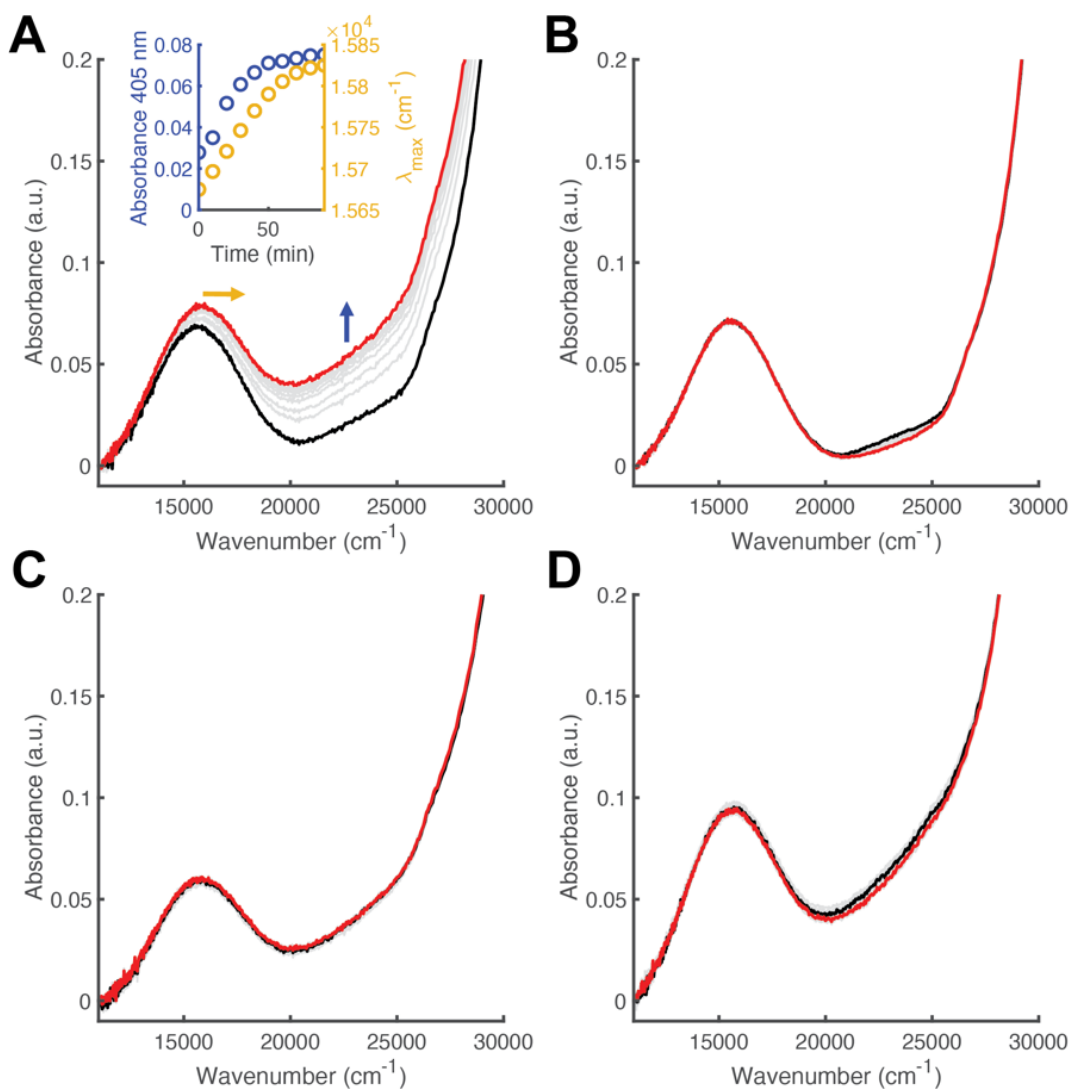


Figure 2.4. UV-vis absorption spectra of (A) Cu-2xm-S112Y-Sav (1, black), Cu-2xm-S112Y-Sav + 10 eq H_2O_2 every 10 min (gray) and after 90 min (red). The shoulder at 24691 cm^{-1} (~ 405 nm) grows in over time (inset, shown in blue) and the Cu d-d band shifts from $\lambda_{\text{max}} = 15675$ cm^{-1} to 15825 cm^{-1} (inset, shown in yellow). (B) Cu-2xm-Sav (2, black), Cu-2xm-Sav + 10 eq H_2O_2 every 10 min (gray) and after 90 min (red). (C) Cu-2xm-K121Y-Sav (3, black), Cu-2xm-K121Y-Sav + 10 eq H_2O_2 every 10 min (gray) and after 90 min (red). (D) Cu-2xm-S112F-Sav (4, black), Cu-2xm-S112F-Sav + 10 eq H_2O_2 every 10 min (gray) and after 90 min (red).

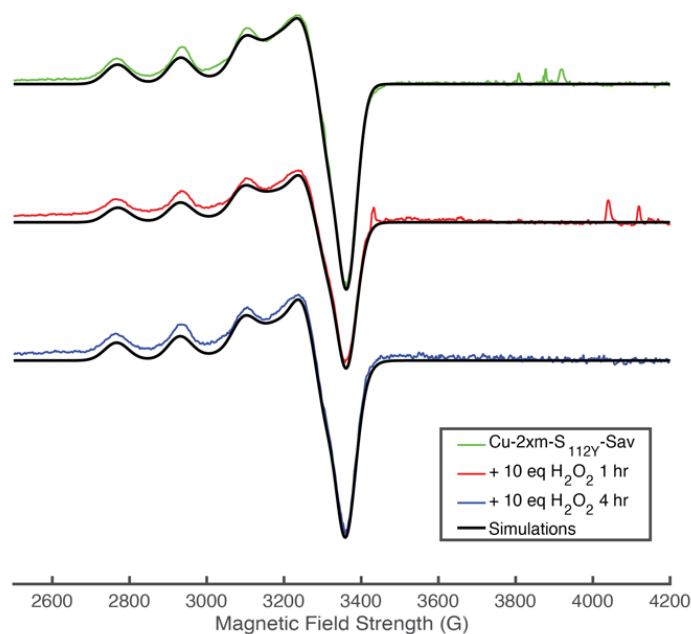


Figure 2.5. X-band EPR spectra of Cu-2xm-S112Y-Sav (green), with 10 eq H₂O₂ after 1 h (red) and after 4 h (blue). Simulations are in black lines. The *g*-values and *A*-values for simulations are given in Table 2.2.

Table 2.2. X-band EPR parameters for simulations shown in Figure 2.5. Due to anisotropic broadening, we cannot confidently assign *A*₂ or *A*₃ values.

		Cu-2xm-S112Y-Sav (1)	Cu-2xm-S112Y-Sav (1) + H ₂ O ₂ 1 hr	Cu-2xm-S112Y-Sav (1) + H ₂ O ₂ 4 hr
	<i>g</i> ₁	2.23	2.23	2.23
	<i>g</i> ₂	2.08	2.08	2.07
	<i>g</i> ₃	2.04	2.04	2.04
	A₁ (MHz)	500	495	501
	A₂	85 ^a	101 ^a	91 ^a
	A₃	65 ^a	62 ^a	57 ^a
Anisotropic Broadening (MHz)	A₁ (MHz)	224	220	217
	A₂	257	303	329
	A₃	143	145	139

^a Values are approximations as they are smaller than the anisotropic broadening for their respective splittings.

Structural Characterization of ArMs

Single crystals of **1** were obtained via a soaking method described previously and diffracted to 1.6 Å resolution (**Figure 3.6 A**, PDB: 9CSU).²⁰ Determination of the molecular structure of **1** revealed a

pentacoordinate, square pyramidal Cu cofactor with the dpa ligand coordinated in a meridional fashion. One of the remaining coordination sites is occupied by an O atom donor from a κ^1 -acetate, which originates from the crystallization buffer. The acetate also participates in H-bonding with a water molecule. The final coordination site is occupied by an O/N atom donor from the N49 sidechain, which undergoes a conformational change to produce a previously unobserved rotamer. Although protein crystallography cannot unambiguously distinguish between O or N of the N49 sidechain, the bound state was modelled as O bound based on the pH of the crystallization conditions (pH 6), which is below the pK_a of the amide. The refined occupancy of the bound conformation of N49 is consistent with the occupancy of the Cu-center ($\sim 65\%$). The centroid distance of S112Y to Cu is 6.5 Å.

Given the positioning of the tyrosine in 2xm-S112Y and the unique amide coordination, the S112F variant was then prepared and characterized (**Figure 2.6 D**, PDB: 9E6Z). The molecular structure of **4** revealed that the orientation F112 in **4** is comparable to that of Y112 in **1**, which allows **4** to maintain support of the N49 coordination to the Cu cofactor (**Figure 2.7**).

Comparison of the structure of **1** and **4** to that of the analogous Cu ArM lacking the S112Y mutation, **2**, shows how the incorporation of the S112Y/F mutation both shifts the position of the Cu center by 0.6 Å towards N49 and changes the orientation preferences of the metallocofactor (**Figure 2.8**). The sterically driven change brings the cofactor in proximity for coordination to N49 in **1** and **4**. Consistent with the observation of two distinct Cu^{II} species in the EPR spectrum of **2**, two distinct copper positions are observed in the anomalous electron density maps of **2** (**Figure 2.9**).and only the dominant conformation of the ligand can be resolved due to the overlap between the two conformations (**Figure 2.6 B**).

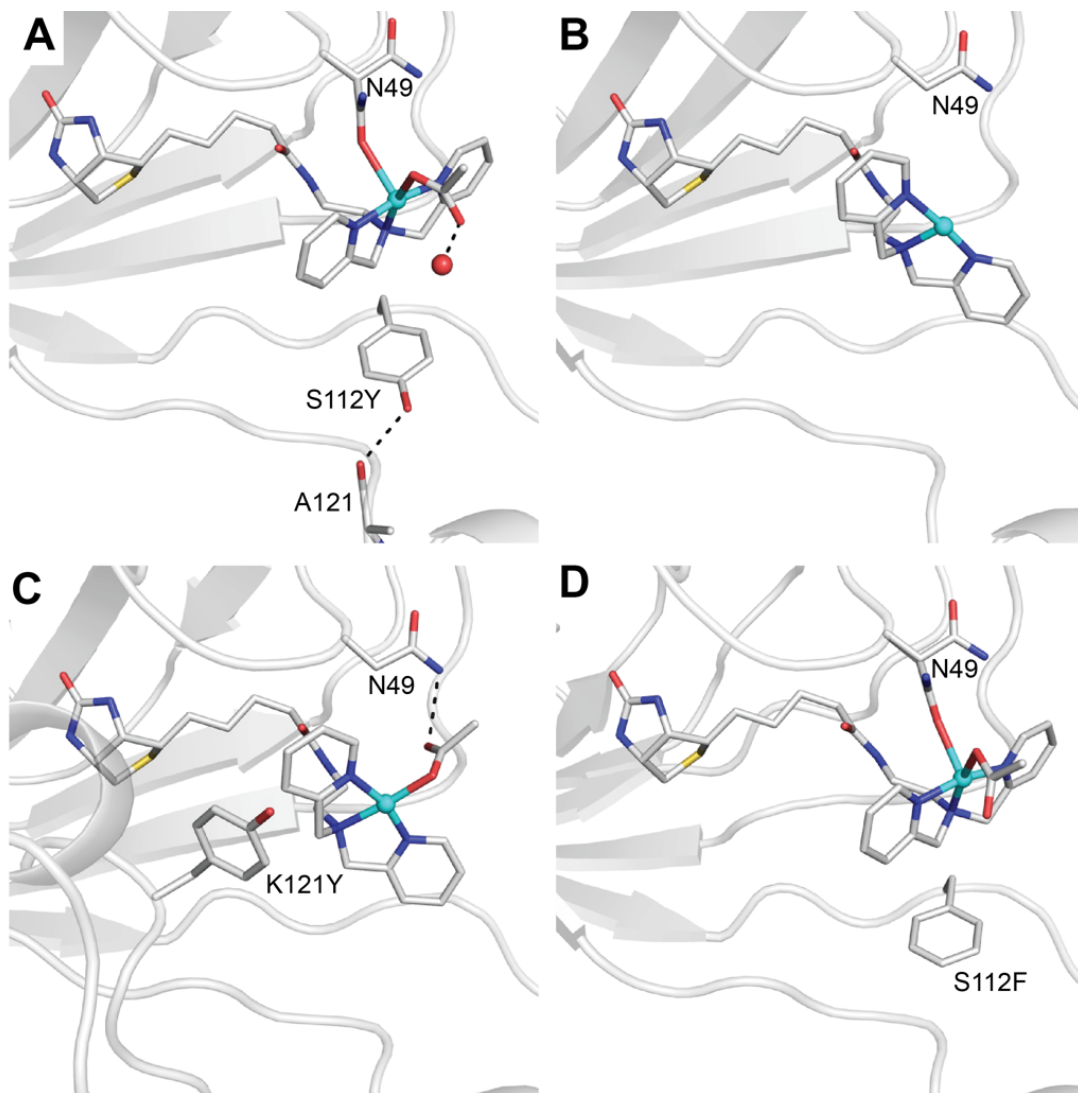


Figure 2.6. Molecular structures of Cu ArMs discussed in this chapter: **(A)** 1 (PDB: 9CSU), **(B)** 2 (PDB: 9CST), **(C)** 3 (PDB: 9CSW), and **(D)** 4 (PDB: 9E6Z).

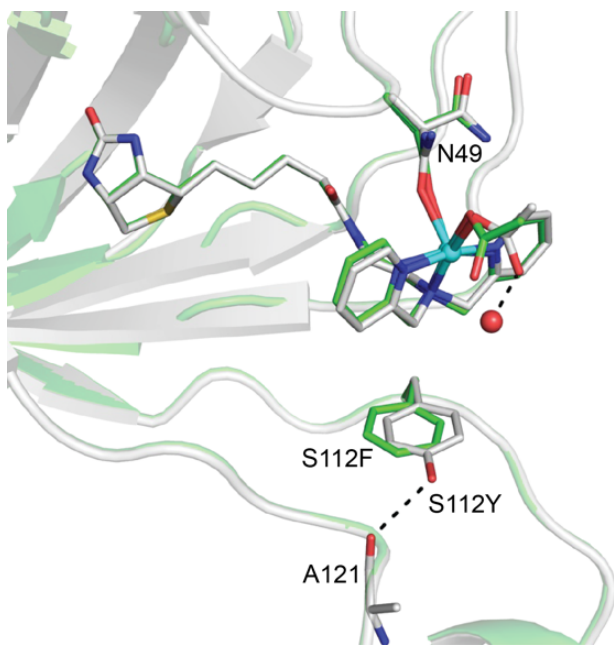


Figure 2.7. Overlay of molecular structures of **1** (PDB: 9CSU, shown in gray) and **4** (PDB: 9E6Z, shown in green). The protein is displayed in cartoon and the Cu complex as well as residues N49 and S112Y/F are displayed in sticks. Copper is colored in cyan and water molecules in red spheres. Hydrogen bonds are suggested in black dashed lines. The orientation of S112Y and S112F relative to the Cu center are comparable, and both mutations support N49 coordination to the Cu cofactor.

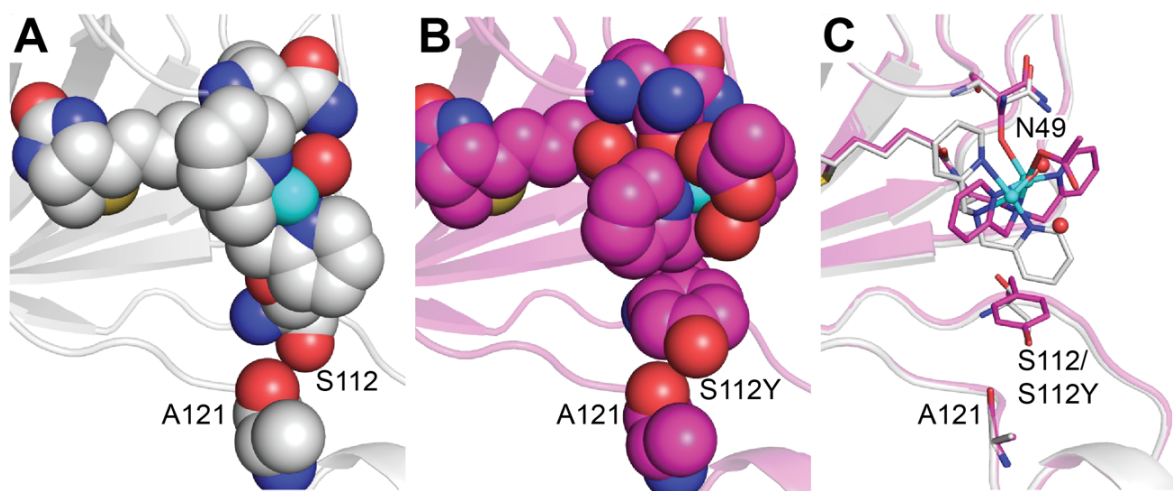


Figure 2.8. Sphere packing models of (A) **2** and (B) **1** suggest incorporation tyrosine at position 112 induces a preferential orientation on the cofactor, as well as shift in the copper position by 0.6 Å towards N49 compared to **2**. (C) Overlay of molecular structures of **2** (grey) and **1** (pink, PDB: 9CSU). The protein is displayed as a cartoon and the Cu complex as well as N49, S/Y112, and A121 as sticks. Copper is colored in cyan and water molecules are colored as red spheres. Hydrogen bonds suggested in black dashed lines.

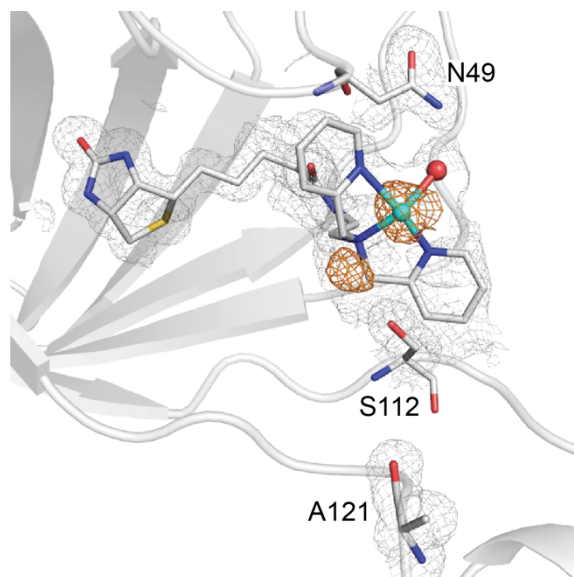


Figure 2.9. Molecular structure of **2** (PDB: 9CST). The protein is displayed as a cartoon and the Cu complex as well as residues N49, S112, and A121 as sticks. Copper is colored in cyan and water molecules are colored as red spheres. The position of the Cu complex as well as residues N49, S112 and A121 are indicated by the $2F_o - F_c$ electron density shown in grey mesh contoured at 1σ , and the Cu positions are indicated by the anomalous electron density shown in orange mesh contoured at 3σ . Only the major conformation of the Cu complex is modeled at 66% occupancy.

We also sought to test whether the incorporation of a tyrosine residue at another location, Sav residue K121, could also position a tyrosine residue within proximity to the Cu center without coordinating. XRD structures of $[\text{Cu}^{\text{II}}(\text{biot-et-dpa})\subset 2\text{XM-K121Y-Sav}]$ (**3**) revealed a 4-coordinate, square planar Cu cofactor with the dpa ligand coordinated in a meridional fashion (**Figure 2.6 C**, and **Figure 2.10**, PDB: 9CSW). The remaining coordination site is occupied by an O atom donor from a κ^1 -acetate. The acetate also participates in H-bonding with the sidechain of N49—this is contrast to the structure of **1**, where the acetate forms an H-bond with a water molecule and N49 coordinates the Cu center. In **3** the tyrosine from the adjacent monomeric subunit of Sav sits 6.7 Å from the copper center, nearly equidistant as in **1**. Despite the similar distance to the tyrosine, the interplay between tyrosine incorporation and N49 coordination is not observed in **3**, likely because Y121 is unable to achieve the steric influence exerted by Y112. Moreover, the positioning of Y121 relative to the Cu-

cofactor, specifically its proximity to the pyridine ring of the dpa ligand and its orientation relative to the Cu center is different than in **1**.

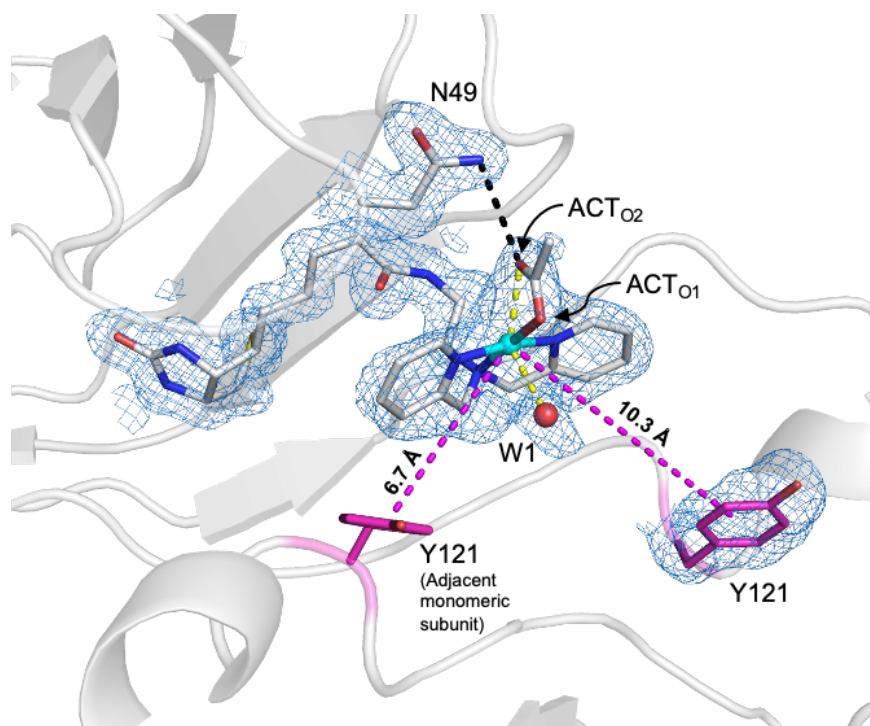


Figure 2.10. Molecular structure of **3** (PDB: 9CSW). The protein is displayed in cartoon and the Cu complex as well as residues N49 and A121Y are displayed in sticks. Copper is colored in cyan and water molecules in red spheres. The position of the Cu-complex as well as residues N49 and Y21 are indicated by the 2F_o-F_c electron density shown in blue mesh contoured at 1σ. Residue Y121 is shown in magenta and is 6.7 Å (adjacent monomeric Sav subunit) or 10.3 Å (same monomeric Sav subunit) from the Cu center where distances are indicated by magenta dashed lines. Hydrogen bonds are suggested in black dashed lines. The Cu-ACT_{O1} and Cu-W1 distances are 2.5 and 2.6 Å, respectively, indicated by yellow dashed lines.

Because our group has shown the ability to stabilize and structurally characterize a Cu^{II}-OOH species in other ArMs, I tried to use a similar soaking method to observe an analogous species in the new Cu ArMs. After several attempts, *in crystallo* reactivity studies of **3** or **4** with H₂O₂ were inconclusive due to heterogeneity and disorder within the crystals. It is possible that the increased disorder of the F112 structures is due to the lack of a stabilizing H-bond exhibited by Y112 in **1** with the backbone carbonyl of A121 (**Figure 2.6**). However, incubation of single crystals of **1** with 1 mM H₂O₂ at pH 6 for ~10 min, revealed a striking result. While the stabilization of peroxide was not

observed, the observation of a small amount of positive difference density in the F_o-F_c electron density map that suggested an additional atom covalently bonded to S112Y (**Figure 2.11 A**). This observation is consistent with the oxidation of S112Y to yield a catechol-like species that forms bifurcated H-bonds with the backbone carbonyl of residue K121 (**Figures 2.11 C, 2.12, and 2.S1, and Tables 2.3 and 2.S2**). Alternatively, the difference density may be explained by the tyrosine residue adopting two conformations (**Figure 2.12 B**). Models of the latter possibility resulted in a structure with a negligible change in crystallographic refinement statistics (**Table 2.3**), suggesting that both molecular models may be plausible, and additional studies are required to distinguish which possibility is correct.

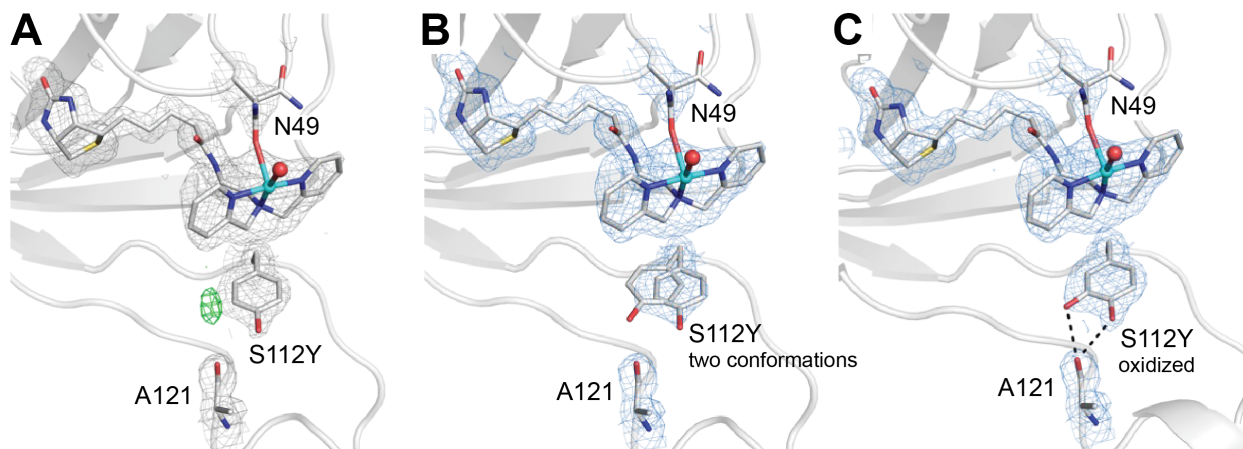


Figure 2.11. (A) Molecular structure of **1** + H₂O₂. Positive difference density in the F_o-F_c map around Y112 contoured at 3σ shown in green mesh. Negative difference density in the F_o-F_c map around Y112 contoured at 3σ shown in red mesh. The position of the Cu complex as well as residues Y112 and A121 are indicated by the $2F_o-F_c$ electron density shown in grey mesh contoured at 1σ . (B) Molecular structure of **1** + H₂O₂ with Y112 modeled in two rotamer conformations (not deposited to PDB), and (C) molecular structure of **1** + H₂O₂ with Y112 modeled as a catechol-like species (PDB: 9CSV). The protein is displayed as a cartoon and the Cu complex as well as N49, Y112 and A121 as sticks. Copper is colored in cyan and water molecules are colored as red spheres with the $2F_o-F_c$ electron density shown in blue mesh contoured at 1σ . Hydrogen bonds are suggested in black dashed lines. Refinement statistics for models represented in B and C are listed in Table 2.3.

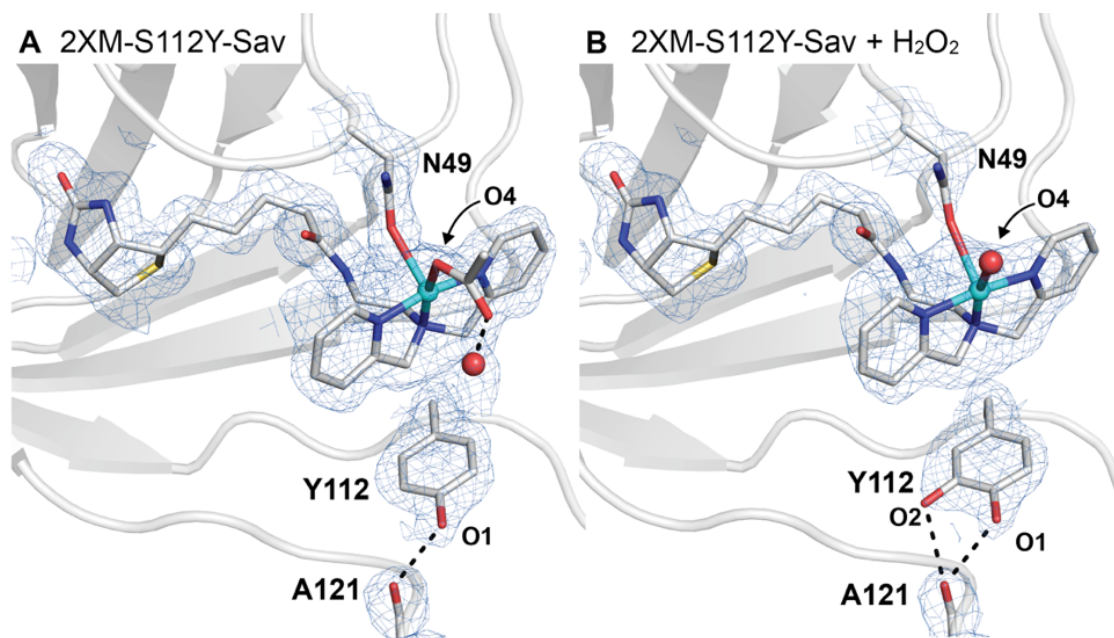


Figure 2.12. Molecular structures of **1** before (**A**, PDB: 9CSU) and after (**B**, PDB: 9CSV) the addition of H₂O₂. The $2F_o - F_c$ electron density map (blue mesh, contoured at 1σ) is highlighted in (**B**) with same labelling as in (**A**). In the molecular models of **1**, N49 was found in two rotameric states, where the occupancy of the bound state is consistent with the Cu occupancy ($\sim 65\%$) in the absence of H₂O₂. In the presence of H₂O₂, the occupancy of this rotamer is reduced to 51%, or roughly two-thirds of the refined Cu occupancy (74%). Only the N49 rotamer nearest to the Cu center is depicted for clarity. Selected bond lengths for **1** (**1** + H₂O₂): Cu–N_{avg} = 2.0 Å (2.1 Å); Cu–O_{N49} = 2.5 Å (2.4 Å); in **1** Cu–O4_{OAc(W)} = 2.3 Å (2.4 Å). The Cu–O_W distance in **1** is 2.9 Å. H-bonds are drawn as black dashed lines. Copper is colored in cyan, oxygen in red, nitrogen in blue, sulfur in yellow, and carbon in grey.

Table 2.3. X-ray crystallography data processing and refinement statistics

Sav Variant	[Cu ^{II} (biot-et-dpa)⊂2XM-Sav] (2)	[Cu ^{II} (biot-et-dpa)⊂2XM-S112Y-Sav] (1)	[Cu ^{II} (biot-et-dpa)⊂2XM-S112Y-Sav] + H ₂ O ₂ (Y112 hydroxylate)	[Cu ^{II} (biot-et-dpa)⊂2XM-S112Y-Sav] + H ₂ O ₂ (2 Y112 rotamers)	[Cu ^{II} (biot-et-dpa)⊂2XM-S112A-K121Y-Sav] (3)	[Cu ^{II} (biot-et-dpa)⊂2XM-S112F-Sav] (4)
PDB Code	9CST	9CSU	9CSV	Not deposited	9CSW	9E6Z
Data Processing						
Unit Cell (a, b, c, α, β, γ)	57.82 Å, 57.82 Å, 184.14 Å, 90°, 90°, 90°	57.54 Å, 57.54 Å, 177.10 Å, 90°, 90°, 90°	57.55 Å, 57.55 Å, 177.73 Å, 90°, 90°, 90°		57.77 Å, 57.77 Å, 184.25 Å, 90°, 90°, 90°	57.54 Å, 57.54 Å, 176.28 Å, 90°, 90°, 90°
Space Group	I4 ₁ 22	I4 ₁ 22	I4 ₁ 22		I4 ₁ 22	I4 ₁ 22
Resolution (Å)	42.09 – 1.13	54.72 – 1.60	27.38 – 1.60		42.08 – 1.302	41.11 – 1.7
Highest resolution shell (Å)	1.17 – 1.13	1.66 – 1.60	1.657 – 1.6		1.349 – 1.302	1.761 – 1.7
R-merge (%)	0.7592 (3.208)	0.7085 (4.619)	0.0964 (1.151)		0.6465 (3.806)	0.2381 (2.023)
No. unique reflections	58555 (5764)	20131 (1966)	20125 (1984)		38754 (3812)	16841 (1647)
Multiplicity	79.7 (79.9)	36.9 (37.1)	6.7 (6.9)		47.5 (48.2)	14.1 (14.5)
Mean I/sig(I)	60.09 (2.15)	13.47 (0.91)	9.00 (1.42)		62.91 (1.26)	8.98 (1.33)
Completeness	99.7 (99.88)	99.49 (97.31)	99.12 (99.90)		99.33 (99.08)	99.92 (100.00)
CC(1/2)	0.946 (0.784)	0.968 (0.309)	0.997 (0.561)		0.957 (0.607)	0.996 (0.524)
Structure Refinement						
R-work	0.1446 (0.2598)	0.1930 (0.3000)	0.2061 (0.3570)	0.2073 (0.3608)	0.1698 (0.3586)	0.2005 (0.3052)
R-free	0.1615 (0.2692)	0.2092 (0.3356)	0.2238 (0.3660)	0.2218 (0.3640)	0.1908 (0.3979)	0.2198 (0.3149)
RMSD bond length (Å)	0.014	0.004	0.007	0.008	0.008	0.014
RMSD bond angle (°)	1.29	0.66	0.88	0.88	1.00	1.27
Avg B-factor (Å ²)	20.10	25.01	30.25	30.24	22.91	20.26
Protein	18.28	23.64	29.25	29.25	21.75	19.50
Cu Complex	25.91	28.11	31.18	33.23	26.56	22.97
Solvent	31.85	39.77	39.99	39.34	32.37	28.46
Occupancy						
Cu	0.69, 0.31	0.65	0.74	0.74	0.63	0.78
Acetate	N/A	0.78	0.71	0.71	0.85	0.80
N49	1.00	0.61 (bound), 0.39	0.51 (bound), 0.49	0.51 (bound), 0.49	1.00	0.58 (bound), 0.42

To further evaluate what caused the change in the electron density maps of **1** upon treatment with H_2O_2 , liquid chromatography high resolution electrospray ionization quadrupole time-of-flight mass spectrometry (LC-HR-ESI-Q-ToF MS) was employed. The deconvoluted ESI mass spectra of **1** after incubation with 10 equivalents of H_2O_2 for one hour revealed significant oxidation of the monomers of 2xm-S112Y-Sav ($16441.4\text{ }m/z$), as evidenced by the growth of two peaks in the peroxide-treated mass spectra at +16 ($16457.8\text{ }m/z$) and +32 m/z ($16473\text{ }m/z$), corresponding to the singly and doubly oxidized species (**Figure 2.14 A**). Notably, after treatment with H_2O_2 , the m/z peak assigned to the singly oxidized species becomes the most abundant, and the m/z peak assigned to the unoxidized species decreases to $\sim 80\%$ relative to the singly oxidized species. Upon reaction of **1** with $\text{H}_2^{18}\text{O}_2$, a +17.6 m/z shift of the monomeric Sav mass was observed, consistent with the incorporation of an ^{18}O atom derived from the 90% isotope enriched solution (**Figure 2.13**).

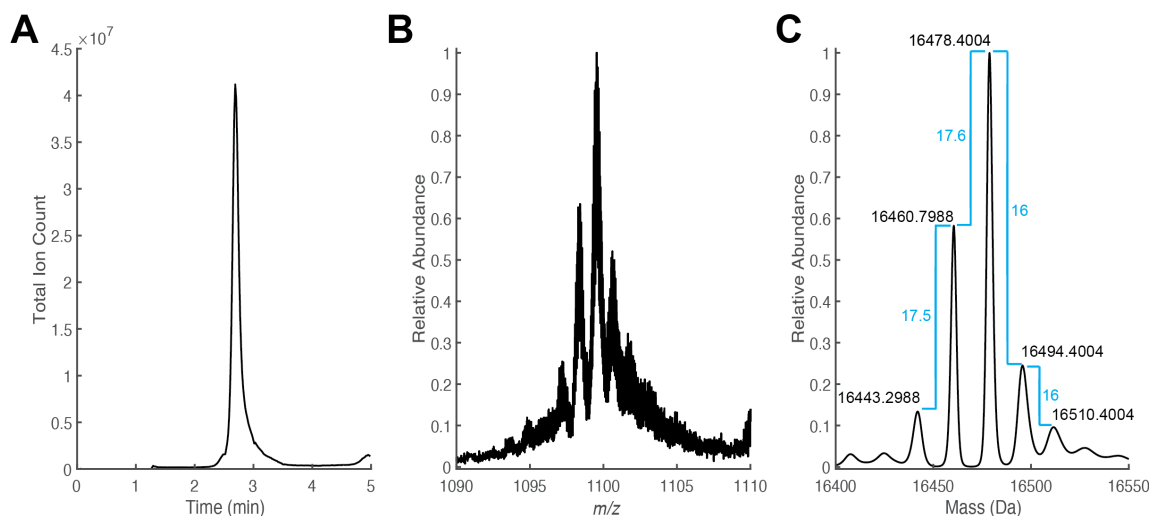


Figure 2.13. (A) Chromatogram of **1** incubated with $^{18}\text{O}_2\text{H}_2$. The major peak was integrated for further processing. (B) ESI QTOF positive ion mode mass spectra showing ions of highest abundance from integration of the major peaks of the chromatogram of **1** incubated with $^{18}\text{O}_2\text{H}_2$. The charge-state ion series containing these ions were used to reconstruct total average mass spectra shown in panel C. (C) Deconvoluted ESI QTOF positive ion mode mass spectrum of **1** incubated with $^{18}\text{O}_2\text{H}_2$. The nominal mass of 2xm-S112Y-Sav monomer is 16443.2998 Da. Two species that are consistent with the incorporation of ^{18}O derived from 90% isotope labeled $^{18}\text{O}_2\text{H}_2$ are observed. There are two more species consistent with nonspecific oxidation derived from ^{16}O .

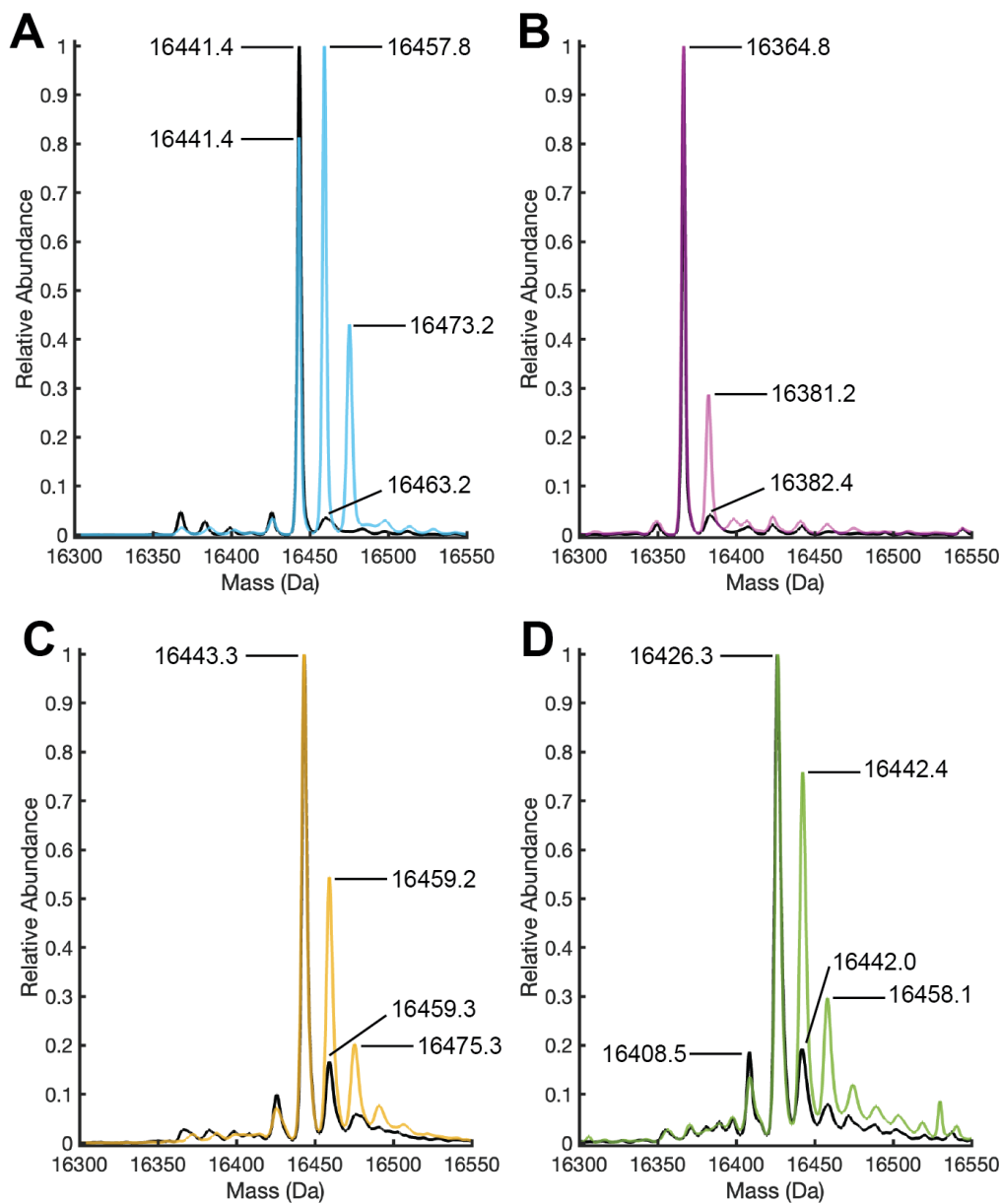


Figure 2.14. Deconvoluted ESI ToF positive ion mode mass spectra of (A) **1** before (black) and after (blue) the addition of 10 equivalents of H₂O₂ and incubation for 1 hour; (B) **2** before (black) and after (pink) the addition of 10 equivalents of H₂O₂ and incubation for 1 hour; and (C) **3** before (black) and after (yellow) the addition of 10 equivalents of H₂O₂ and incubation for 1 hour. (D) **4** before (black) and after (yellow) the addition of 10 equivalents of H₂O₂ and incubation for 1 hour.

In contrast to **1**, incubation of **2**, which lacks the tyrosine mutation, with peroxide reveals only a small amount of oxidation, and the unoxidized peak (16364.8 m/z) remains the most abundant (Figure 2.13 B). Similarly, incubation of **3**, which has a tyrosine mutation at a different location, with

peroxide shows oxidation levels that fall between those observed for **1** and **2** (**Figure 2.13 C and D**). While the peaks corresponding to singly and doubly oxidized species increased in relative abundance compared to those same features in **2**, the most abundant peak still corresponds to the unoxidized protein, indicating that it remained the dominant species in solution. In **3**, Y121 sits 6.7 Å from the copper center as determined by XRD, nearly equidistant to the Cu center as in **1**; however, the interplay between tyrosine incorporation and N49 coordination is not observed in **3** (**Figure 2.10**). This difference is likely because Y121 in **3** is unable to achieve the same steric influence exerted by Y112 in **1**. While peaks corresponding to singly and doubly oxidized species increased in relative abundance compared to those same features in **2**, the base peak at 16443.2 *m/z* still corresponds to the unoxidized protein, indicating that it remained the dominant species in solution.

Based on the observations that positioning of the tyrosine is important for promoting activity and the orientation of F112 in **4** is comparable to that of Y112 in **1**, I reasoned that oxidation of F112 might also be achievable while maintaining the cofactor geometry and orientation. The intact protein HR-ESI-Q-ToF analysis after treatment with H₂O₂ reveals peaks corresponding to the oxidation of **4** upon peroxide treatment, though the most abundant peak still corresponds to the unoxidized protein, indicating that it remained the dominant species in solution (**Figure 2.13 D**).

As a control, 2xm-Sav and 2xm-S112Y-Sav in their apo forms (no biotin nor metallocofactor, **Figure 2.15 A and B**) and when bound to the biotinylated ligand (no Cu, **Figure 2.15 C and D**) were also treated with 10 equivalents of H₂O₂ for 1 hour. Both apoproteins undergo non-specific oxidation, which decreases when the proteins are bound to the biotinylated ligand. These results possibly arise from ligand binding precluding access of H₂O₂ to oxidizable residues.

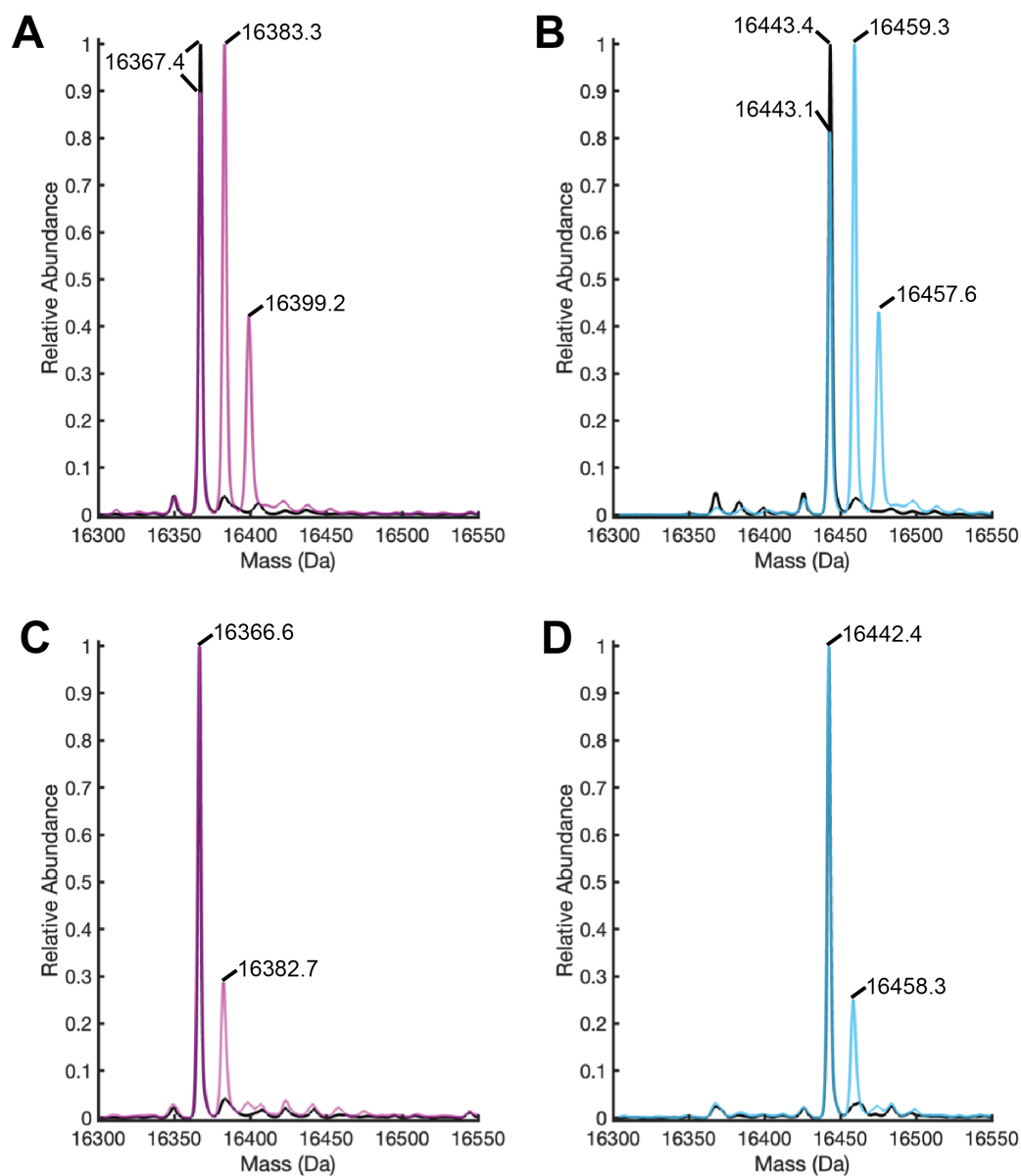


Figure 2.15. Deconvoluted ESI-Q-ToF positive ion mode mass spectra of (A) apo-2xm-Sav, (B) apo-2xm-S112Y-Sav, (C) biot-2xm-Sav, and (D) biot-2xm-S112Y-Sav. Before addition of H_2O_2 shown in black and after 1 h incubation with 10 eq H_2O_2 shown in pink/blue for each panel. Overall oxidation of intact Sav is enhanced in all apo-Sav variants compared to biot-Sav variants, which is possibly due to ligand binding precluding access of H_2O_2 to oxidizable residues

Sites of Oxidation Identified by Trypsin Digest and LC-MS/MS

While whole-protein MS provided support for the oxidation of **1** by H₂O₂, consistent with what is observed in the XRD structures of **1**, I wanted to identify if the site of oxidation was specific to S112Y/F. Enzymatic digestion of **1** by trypsin can result in 44 possible digest fragments (DFs) that can then be analyzed by LC-ESI-MS/MS to identify modified peptide sequences upon incubation with peroxide. Peroxide treatment of **1**, followed by digestion by trypsin and analysis of the resulting DFs (**Table 2.S2**) with LC-ESI-MS/MS revealed the location of oxidation. Among the 44 possible DFs observed by trypsin digestion, the most abundant modified DF (DF31) extends from residue 104 to 132 and contains positions 112 and 121 (**Figure 2.16 A and B**). After incubation with 10 equivalents of H₂O₂ for 1 hour, the mass spectrum of the triply charged ion of DF31 ([DF31]³⁺, 1080.8 *m/z*, retention time 3.07 min) exhibited two additional peaks that are shifted by 5.3 *m/z* (1086.5 *m/z*, retention time 2.87 min) and 10.6 *m/z* (1091.2 *m/z*, retention time 2.97 min) corresponding to the double and single oxidation of DF31 (**Figure 2.16 C**). The chromatogram was integrated over this entire range, from 2.85 to 3.12 min, to capture the relative abundance of both the unoxidized and oxidized DF31 species for further processing (**Figures 2.S2, 2.S10, 2.S11, 2.S12**).

MS/MS secondary fragmentation data were further analyzed for b/y ion fragment identification, [b8]⁺, [b7]⁺, [y20 + 16]²⁺, [y21 + 16]²⁺, and [y22 + 16]²⁺ were identified, confirming the oxidation of Y112 (**Table 2.S3** and **Figure 2.S3**). DF31 also contains other residues known to be susceptible to oxidation by H₂O₂ (H127, W108, and W120), and this analysis of b/y ion fragmentation also indicates oxidation of W120 to some extent, and oxidation of H127 could not be ruled out. However, these residues have centroid distances >9.6 Å from Cu, making them less likely to be modified than Y112, which sits 6.5 Å from Cu, but they may be location of additional oxidation events (**Figure 2.16**). Moreover, the electron density maps of the structures of **1** + H₂O₂ do not exhibit significant difference density to suggest modification of these residues upon exposure to H₂O₂ (**Figure 2.17**).

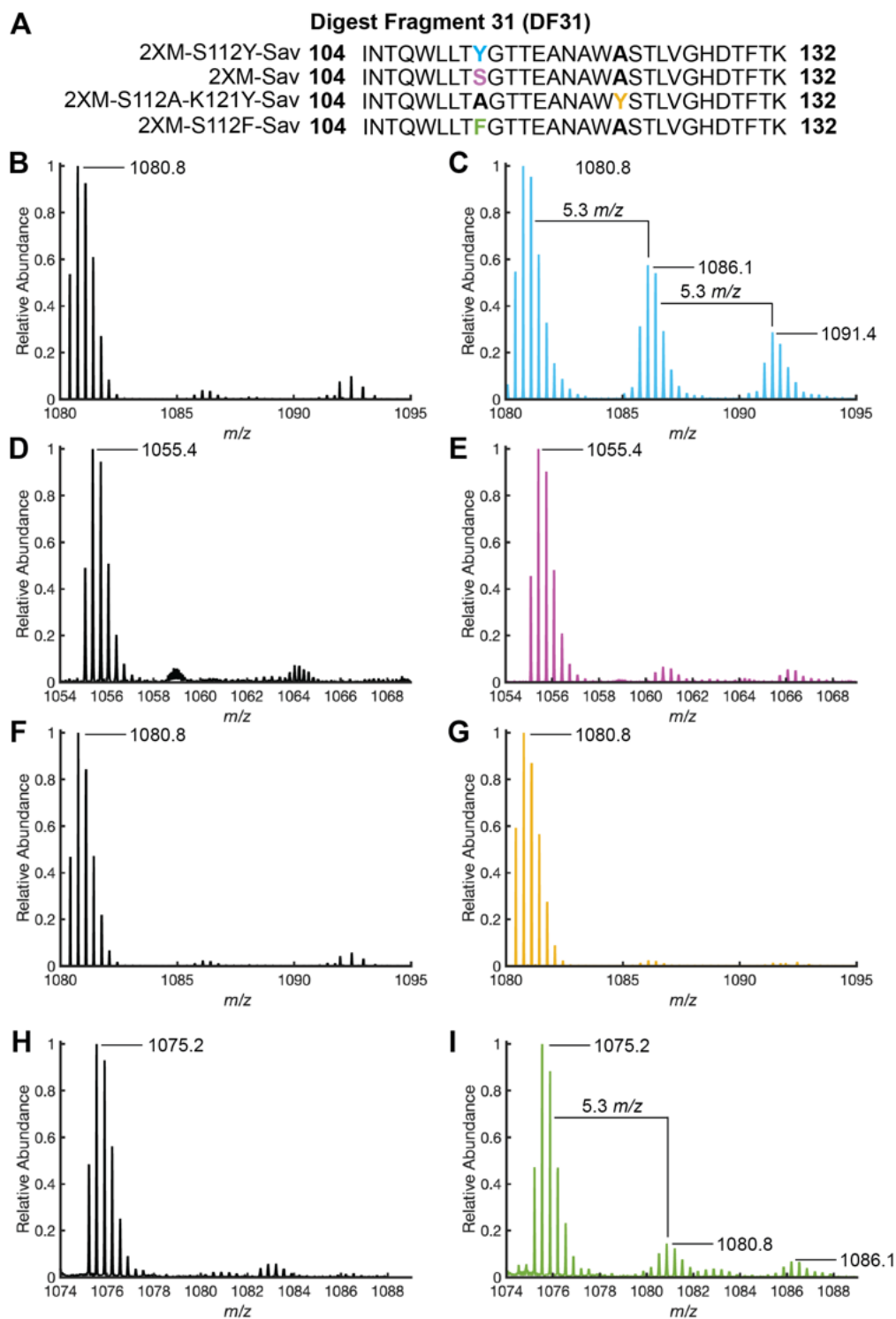


Figure 2.16. (A) Digest fragment 31 sequences of variant **1**, **2**, and **3**. ESI-Q-ToF positive-ion mode mass spectra [DF31]³⁺ resulting from tryptic digests **1** (B) before and (C) after the addition of 10 equivalents of H₂O₂ and incubation for 1 hour. **2** (D) before and (E) after the addition of 10 equivalents of H₂O₂ and incubation for 1 hour. **3** (F) before and (G) after the addition of 10 equivalents of H₂O₂ and incubation for 1 hour. Mass spectra were produced from the integration of LC peaks containing both unoxidized and oxidized DF 31 (retention time 2.85–3.10 min).

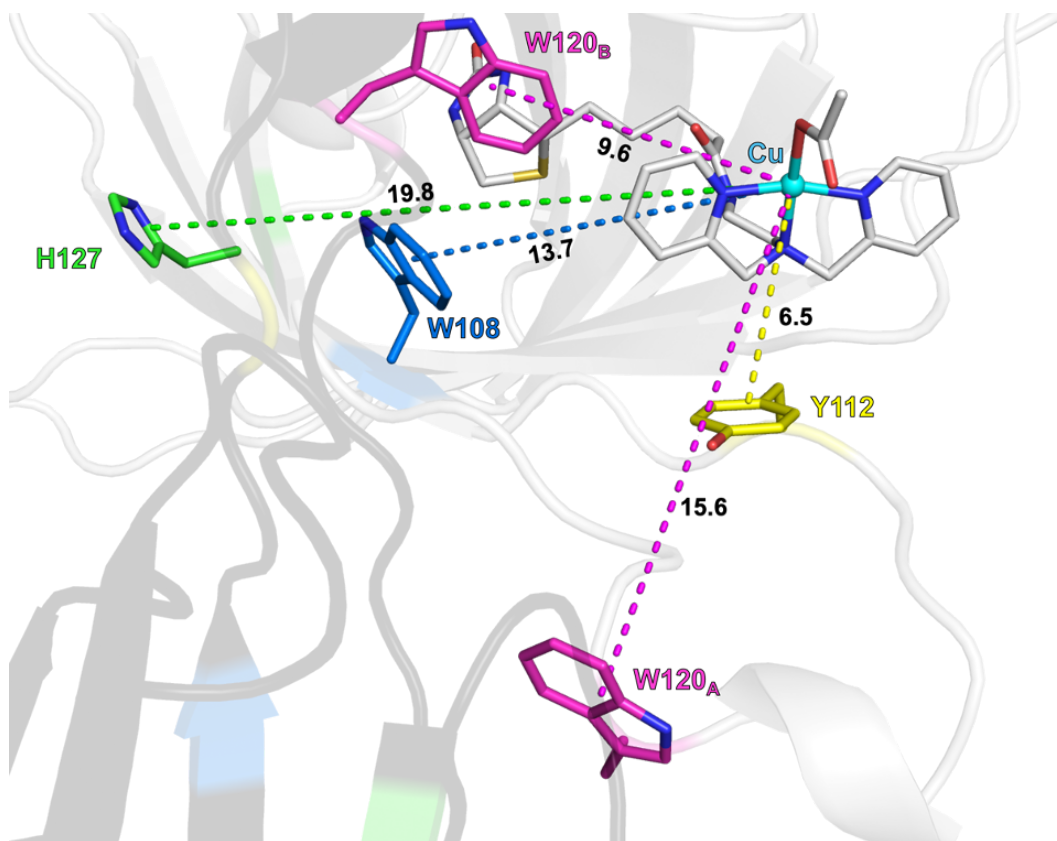


Figure 2.17. Molecular structure of **1** (PDB: 9CSU). The protein is displayed as a cartoon and the Cu complex as well as residues W108, Y112, W120, and H127 are displayed as sticks. One monomeric subunit of Sav is shown in gray (denoted as A) and the adjacent symmetry related subunit (denoted as B) is shown in black. Residues in DF31 known to be susceptible to oxidation are displayed in blue, yellow, pink or green with the corresponding colored dashed lines indicating the distance from the copper center. Residue Y112 of the same subunit is the closest residue to the Cu center, making it most susceptible to Cu-mediated oxidation by H_2O_2 .

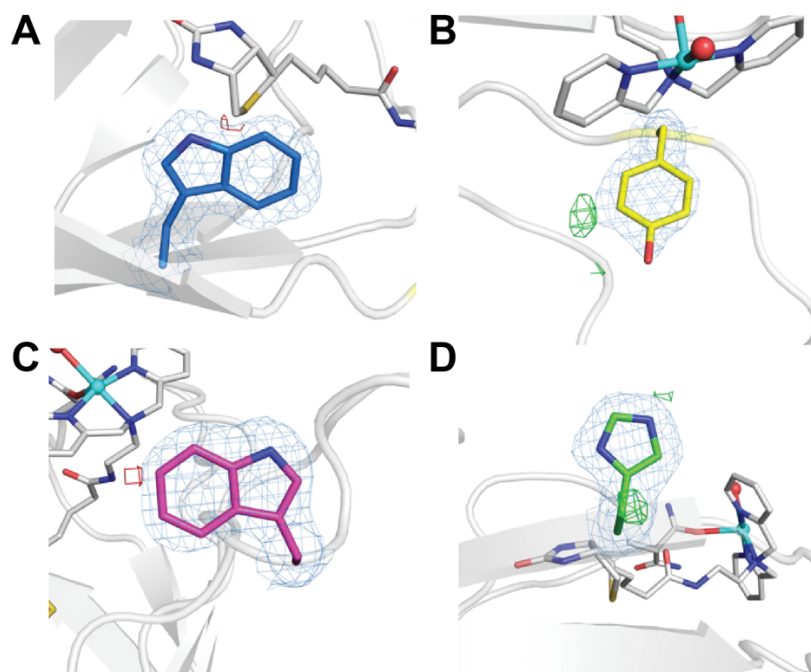


Figure 2.18. Molecular structure of **1** + H₂O₂ highlighting electron density of residues of DF31 that may be susceptible to oxidation: (A) W108, (B) Y112, (C) W120, and (D) H127. Residues of DF31 that may be susceptible to oxidation by H₂O₂ are displayed as sticks. Positive and negative difference density in $F_o - F_c$ map around each residue contoured at 3σ shown in green and red mesh, respectively. The positions of the residues are indicated by the $2F_o - F_c$ electron density shown in blue mesh contoured at 1σ .

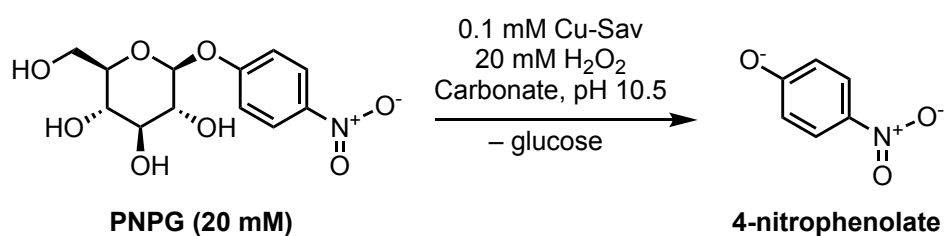
Although I could not directly verify the specific oxidation of *only* Y112 in **1**, comparison to biot-2xm-S112Y-Sav (the analogous biotinylated protein with no Cu) supports the idea that the Cu-cofactor enhances the oxidation of DF31 upon addition of H₂O₂. In this variant, oxidized species of DF31 could not be identified (**Figure 2.S4**). Similarly, in **4**, enzymatic digest analysis reveals peaks corresponding to the specific oxidation of F112 upon peroxide treatment (**Figure 2.16 H and I**). Moreover, in peroxide-treated samples of **2** and **3**, only minor modification of DF31 was observed (**Figure 2.16 D–G**). Surprisingly peroxide-treated samples of **3**, with a tyrosine at 121, showed less modification of DF31 than **2**. Moreover, non-specific oxidation of other residues in **3** is enhanced relative to that in **2**, an observation that is not yet completely understood. Taken together with the structures of **1** and **3**, which reveal differences in their primary coordination spheres, these data suggest

that site-specific oxidation of secondary sphere tyrosines is dependent on 1) the presence of Cu-et-dpa, and 2) the proximity and orientation of the Cu center with respect to tyrosine.

Reactivity with Substrates

The selective oxidation of S112Y in **1** indicated that the confinement and positioning of the Cu-cofactor within this Sav variant may preclude reactions with other substrates. As such, the reactivity of **1**, **2** and **3** towards a commonly utilized external substrate to assay for LPMO-like reactivity, *p*-nitrophenyl- β -D-glucopyranoside (PNPG) was examined (**Scheme 2.2**). The Cu^{II}-biot-et-dpa cofactor alone in solution rapidly reacted with PNPG, as evidenced by the growth of a UV-vis absorbance feature at $\lambda_{\text{max}} = 405$ nm which corresponds to the production of 4-nitrophenolate (**Figure 2.19**). Meanwhile, **1** showed negligible reactivity (**Figure 2.19**). I hypothesized that **2** and **3** would react with PNPG because of the lack of the intramolecular S112Y/F substrate, they also showed negligible reactivity (**Figure 2.19**). These results support the idea that sequestration, confinement, and orientation of the cofactor determines its propensity toward reactivity with the protein host versus external substrates.

Scheme 2.2. Assay for LPMO-like activity



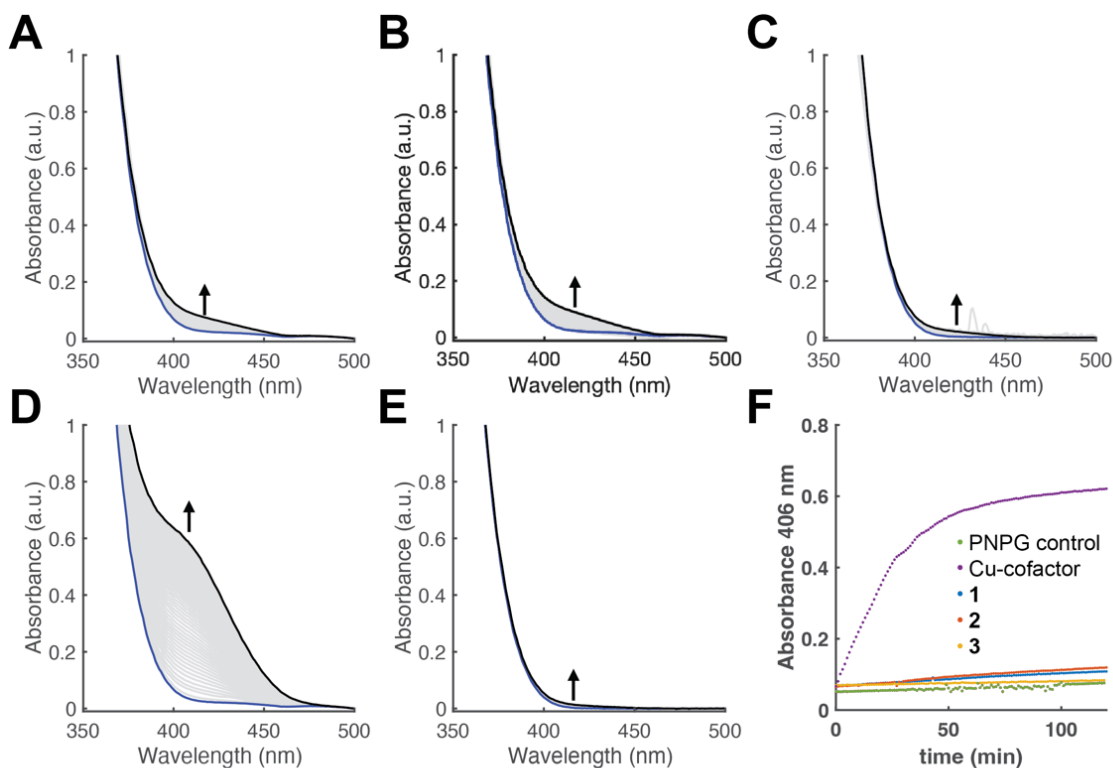


Figure 2.19. Reactivity of (A) **1**, (B) **2**, (C) **3** or (D) Cu cofactor with PNP and H₂O₂ according to Scheme X-2. Spectra of Cu-Sav with 200 equivalents of PNP shown in black and after 120 min reaction with 200 equivalents of H₂O₂ shown in blue. (E) Reactivity of PNP with H₂O₂ as a control. UV-vis absorbance at $\lambda_{\text{max}} = 405$ nm (corresponding to absorbance of 4-nitrophenolate) over 120 min incubation with H₂O₂.

Conclusions

This chapter described the preparation of Cu ArMs with aromatic residues engineered within their secondary coordination spheres and examined their reactivity with H₂O₂. Application of XRD methods suggested their ability to selectively oxidize tyrosine residues within their secondary coordination sphere to catechol/quinone. Additionally, the rotameric change and coordination of N49 in **1** and **4** also suggested its potential role in reactivity. This type of coordination has not been observed in any other ArMs; however, a recent structure of pMMO revealed a new potential catalytic site having a similar coordination to a single Cu center. It is not yet known if this type of coordination is necessary

for function in pMMO, but the lack of coordination in our series of Cu ArMs leads to a decrease in reactivity.

Considering results from our previous work, it is reasonable to propose that a $\text{Cu}^{\text{II}}\text{-OOH}$ species is initially generated upon treatment of our Cu ArMs with H_2O_2 . These earlier Cu ArMs with WT Sav allowed us to pinpoint that stabilization of the $\text{Cu}^{\text{II}}\text{-OOH}$ unit required a H-bond network that included N49 and S112, which are not available for H-bonding in **1** and **4**. The lack of H-bonds to the $\text{Cu}^{\text{II}}\text{-OOH}$ unit produces a more reactive intermediate that is active for oxidation of external substrate. The fact that we observe site-specific oxidation at 112 suggests that reactivity occurs via interaction with an intermediate involving the Cu cofactor which is supported by $\text{H}_2^{18}\text{O}_2$ labeling studies. However, the mechanism(s) followed to produce the oxidized proteins is still uncertain. We also cannot rule out the production of hydroxyl and/or hydroperoxyl radicals during the reaction and as the source of the observed background non-specific oxidation. The reaction was probed using *in situ* EPR spectroscopy at room temperature, but these studies did not reveal the accumulation of an organic radical species.

Employing LC-MS methods in combination with enzymatic digestion of our Cu ArMs further supported the selective oxidation of **1** upon the addition of H_2O_2 . The results demonstrate how the inclusion of aromatic residues within the secondary coordination sphere influences the structure and reactivity of Cu ArMs. Moreover, these results may suggest a potential inactivation pathway for LPMOs and other metalloenzymes upon exposure to H_2O_2 in the absence of external substrates. Importantly, oxidation of tyrosine or phenylalanine residues is dependent on its proximity and orientation relative to the metallocofactor. When the location is appropriate, such as in the case of S112Y and S112F, steric constraints shift the Cu center towards N49, allowing amide coordination and then S112Y and S112F are readily oxidized upon exposure to H_2O_2 . In **3**, the tyrosine residue at 121, while also proximal to the Cu center, does not exert the same steric interactions to position the

Cu center for N49 coordination—and oxidation of 121 was not observed. These results highlight how subtle changes in the local environment surrounding metallocofactors can influence reactivity.

Further studies can probe the role of N49 in these ArMs. To identify if N49 coordinates via the O or N atom of its sidechain, isotopic ^{15}N labelling can be used to improve the sensitivity of EPR spectroscopy for biological applications. In addition to CW X-band EPR spectroscopy, electron-nuclear double resonance (ENDOR) EPR spectroscopy can help elucidate hyperfine interactions of the Cu-center with surrounding N nuclei. Moreover, N49 coordination may be completely ruled out by mutating this residue via SDM.

Experimental Methods and Materials

Preparative Methods

All commercially available reagents were purchased of the highest purity and used as received. Acetonitrile, ethanol, and diethyl ether were degassed with argon and dried by vacuum filtration through activated alumina according to the procedure outlined by Grubbs.²¹ Nanopure water was obtained from Millipore Super-Q Plus water purification system, 120 V, 4-stage purification system with pump. Thin-layer chromatography (TLC) was performed on Whatman 250 μm layer 6 Å aluminum-backed silica plates and visualized using ultraviolet (UV) light. Silica gel chromatography was performed using Fisher reagent silica gel 60 (230-400 mesh) with indicated solvents. Biotin pentafluorophenol ester (biot-PFP)²² and di-(2-picolyl)amine (dpa)²³ were prepared according to literature procedures.

^1H and ^{19}F nuclear magnetic resonance (NMR) spectra were recorded at 500 and 400 MHz respectively. ^1H NMR spectra are reported in ppm on the δ scale and referenced to tetramethylsilane or solvent residual. The data are presented as follows: chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, quin=quintet, m=multiplet, br=broad), and integration.

Preparation of Cu cofactor. The ligand (biot-et-dpa) was prepared as published previously.¹⁹

¹H (500 MHz, DMSO-d₆) δ 8.48 (d, 2H), 7.76 (td, 3H), 7.53 (d, 2H) 7.25 (t, 2H), 6.40 (s, 1H), 6.33 (s, 1H), 4.29 (t, 1H), 4.12 (t, 1H), 3.78 (s, 4H), 3.20 (q, 2H), 3.08 (q, 1 H), 2.81 (dd, 1 H), 2.59 (s, 1H), 2.56 (t, 2H), 2.04 (t, 2H), 1.47 (m, 6H).

The Cu-complex [Cu^{II}(biot-et-dpa)(NO₃)₂] was prepared by addition of a solution of Cu(NO₃)₂•3H₂O (85.2 mg, 0.353 mmol) in 10 mL ethanol to a solution of biot-et-dpa (150.2 mg, 0.324 mmol) in 50 mL ethanol. The pale blue solution was allowed to stir for 4 h before concentrating the solution to ~1 mL followed by precipitation with diethyl ether (**Scheme 2.1**). Blue solid was collected via filtration, washed with diethyl ether and dried under vacuum.

HR-MS (ES, MeOH) *m/z* calcd for C₂₆H₃₂CuN₈O₈S [M – (NO₃)⁺] 621.2, found 621.4.

Preparation of Sav variants. The construction of E101Q-K121A-S112Y-Sav (denoted 2xm-S112Y-Sav), and E101Q-S112A-K121Y-Sav (denoted 2xm-S112A-K121Y-Sav), was achieved by site-directed mutagenesis (SDM) using the codon optimized E101Q-K121A-pET24a-Sav plasmid, the following primers, and Phusion polymerase (New England Biolabs).

S112Y_fwd: 5'-CTGACCTATGGCACCACCGAAGCAAATC-3'

S112Y_rev: 5'-GGTGCCATAGGTCAGCAGCCACTGGGTC-3'

S112A_fwd: 5'-CTGACCGCAGGCACCACCGAAGCAAATC-3'

S112A_rev: 5'-GGTGCCTGCGGTCAGCAGCCACTGGGTC-3'

K121Y_fwd: 5'-GCCTGGTATAGCACCCCTGGT'TGGTCATC-3'

K121Y_rev: 5'-GGTGCTATACCAGGCATT'TGCT'TCGGAC-3'

S112F_fwd: 5'- CTGACCT'TCGGCACCACCGAAGCAAATC-3'

S112F_rev: 5'- GGTGCCT'TCGGTCAGCAGAACTGGGTC-3'

SDM reaction mixtures were transformed into *Escherichia coli* Rosetta or bl21 cells followed by rescue with lysogeny broth (LB) media. The solution was spread aseptically onto LB/kanamycin agar

plates and incubated overnight at 37 °C. Single colonies were used to inoculate 10 mL liquid LB/kanamycin cultures and incubated overnight at 37 °C with shaking at 200 rpm. Of the overnight cultures, 600 µL was saved to prepare glycerol stocks to be stored in 25% glycerol at -80 °C. From the remaining liquid culture, plasmids were isolated using Qiagen Miniprep kit according to manufacturer's instructions. DNA sequencing was performed by Genewiz. Glycerol stocks described above were used to inoculate overnight liquid cultures for expression of Sav mutants. Expression and purification of Sav variants was carried out as reported previously.¹⁸

Physical Methods

Instrumentation. Electronic absorption (UV-vis) spectra were recorded with an Agilent Cary 50 or Cary 60 UV-vis spectrophotometer in a 1 cm quartz cuvette. Electron paramagnetic resonance (EPR) spectra were recorded with a Bruker EMX Series EPR spectrometer equipped with an ER4102ST cavity and ER041XG microwave bridge.

HABA titrations. 4'-hydroxyazobenzene-2-carboxylic acid (HABA) is a dye that absorbs light at 506 nm when bound to Sav. To determine the average number of biotin binding groups per protein molecule, a titration of Cu-complex into HABA-bound Sav was performed and displacement of HABA was monitored by the decrease in 506 nm absorbance. To a 1 cm quartz cuvette, 2.4 mL of 8 µM Sav in 200 mM phosphate buffer (KPi) pH 7 was added. Then 288 µL of a 20 mM (150 equivalents relative to Sav monomer) HABA solution in 200 mM KPi pH 7 was added and allowed to equilibrate for 5 minutes. A 1 mM aqueous solution of Cu-complex was added in 9.6 µL portions until 5 equivalents total were added. Each titration point was spectrophotometrically monitored by following the decrease in absorbance at $\lambda_{\text{max}} = 506$ nm until no further changes in intensity were observed.

General preparation of Cu-Sav in solution. A solution of lyophilized Sav was prepared in nanopure water. This solution was diluted to 150 µM in MES buffer (50 mM, pH 6), and five equivalents of Cu-

complex (750 μ M) were added from 10 mM stock solution in nanopure water. Samples were separated from excess Cu-complex and concentrated using Amicon Ultra 10K centrifugal filter devices and washed with 5X volume of nanopure water or buffer. The concentration of Cu-Sav stock solution was determined based on $\epsilon_M = 167,760$ at $\lambda_{\max} = 280$ nm and diluted to the final working concentration as specifically indicated for each experiment described below.

Electronic absorption spectroscopy. Cu-Sav was diluted to a final concentration of 150 μ M (600 μ M Cu) in 50 mM MES buffer pH 6 in a low-volume 1 cm quartz cuvette. The solution was allowed to equilibrate for 5 min before addition of 10 equivalents of hydrogen peroxide (H_2O_2) relative to the Cu concentration. Spectra were collected every 10 min.

Electron paramagnetic resonance (EPR) spectroscopy. Cu-Sav was diluted to a final concentration of 250 μ M (1 mM Cu) in 50 mM MES buffer pH 6. The solution was allowed to equilibrate for 5 min before addition of 10 equivalents H_2O_2 relative to the Cu concentration. Aliquots of 250 μ L were transferred to an EPR tube and flash-frozen in liquid nitrogen. EPR spectra were collected at 77 K, 2.1 mW, 9.4 GHz and averaged over 10 scans.

Reactivity with PNPG. To assess reactivity with an external substrate, a previously reported assay for LPMO-like activity was used.²⁴ Generally, the assay is carried out at pH 10-11 in the presence of 200 equivalents each of 4-nitrophenyl β -D-glucopyranoside (PNPG) substrate and H_2O_2 . Cu-Sav was diluted to a final concentration of 100 μ M in sodium carbonate buffer (50 mM, pH 10.5). To this solution, 200 equivalents (final concentration 20 mM) PNPG were added and allowed to equilibrate for 5 min. Then 200 equivalents of H_2O_2 (final concentration 20 mM) were added and production of 4-nitrophenolate was monitored by UV-vis at $\lambda_{\max} = 405$ nm.

Protein Crystallization, X-ray Diffraction Data Collection and Processing

Crystallization of Cu-Sav. Apo-Sav protein was crystallized by the sitting drop vapor diffusion method. Diffraction-quality crystals were grown at ambient temperature and pressure by mixing 2.5 μ L protein solution (30 mg/mL for 2xm-S112Y-Sav or 26 mg/mL for 2xm-Sav, 2xm-S112F-Sav, or 2xm-K121Y-Sav lyophilized protein in nanopure water) and 2.5 μ L crystallization buffer (2.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4). The droplet was equilibrated against a reservoir of 100 μ L crystallization buffer solution. Single crystals of Cu-Sav were prepared by soaking apo-Sav crystals soaking buffer (3.0 M ammonium sulfate, 0.1 M sodium acetate, pH 6) for 10 min, followed by a solution of 9 μ L soaking buffer mixed with 1 μ L of Cu-complex (10 mM Cu-complex, 100 mM CuCl₂ in nanopure water) overnight. Upon soaking Sav crystals with Cu-complex, crystals turned from colorless to blue (**Figure 2.S5**). After soaking in Cu-complex, the blue-colored crystals were transferred to soaking buffer for 10 min to remove excess Cu-complex and Cu salts, then to a solution of 9 μ L soaking buffer mixed with 1 μ L H₂O₂ (10 mM) for 5–10 min. Crystals were cryoprotected in Paratone oil (Hampton Research) and shock frozen in liquid nitrogen.

X-ray diffraction data collection and processing. X-ray diffraction data were collected at the Advanced Light Source (BL 5.0.3, 5.0.2 and 5.0.1) or the Stanford Synchrotron Radiation Lightsource (SSRL, BL 12-1 or 12-2). Diffraction images were integrated with iMosflm²⁵ and scaled with AIMLESS (ccp4 Suite).²⁶ For molecular replacement, Phaser-MR (PHENIX Suite)²⁷ was used with PDB: 2QCB with water molecules removed as the input model.

Structural refinement was carried out in PHENIX.REFINE^{27,28} and COOT.²⁹ Restraints for biot-et-dpa (ligand ID: QG7) were created using eLBOW (PHENIX Suite). For water and solvent picking, electron density, and structure visualization, the software COOT²⁹ was used. Figures were drawn with PyMOL (Schrodinger, LLC Version 2.5.2) Crystallographic details, processing, and refinement statistics are given in **Table 2.2**.

Overall structures. Apo-crystals of Sav soaked with Cu-complex constituted space group I4₁22 with unit cell parameters reported in **Table 2.3**. One Sav monomer was obtained per asymmetric unit after molecular replacement. Protein residues 2–11 and 135–159 of the N- and C- termini, respectively, were not resolved in electron density, presumably due to disorder.

General Cu-complex modelling. For all structures of apo-Sav crystals soaked with Cu-complex the following general observations were made: i) residual electron density in the F_o-F_c map in the biotin-binding pocket, and ii) one (Cu-2xm-S112Y-Sav, Cu-2xm-S112F-Sav, and Cu-2xm-S112A-K121Y) or two (Cu-2xm-Sav) peaks in an anomalous density map in the biotin vestibule superimposed with the electron density peak. The residual electron density was fit with the Cu-complex which projected Cu to the position of the strong anomalous density peak. In Cu-2xm-S112Y-Sav, the occupancy of the Cu center was refined to 0.65, and 0.74 with H₂O₂. In Cu-2xm-S112A-K121Y-Sav, the occupancy of the Cu center was refined to 0.63. In Cu-2xm-S112F-Sav, the occupancy of the Cu center was refined to 0.78. In Cu-2xm-Sav, the occupancy of the two copper centers were refined to 0.66 and 0.3. Due to overlap of two cofactor conformations and the uncertainty regarding the geometry of the second conformation coupled to its low occupancy, the deposited structure only contains the model of the major Cu-cofactor conformation (**Figure 2.9**). In Cu-2xm-S112Y-Sav, we also observed residual positive electron density in the F_o-F_c maps surrounding the Cu center. This density was fit to an acetate molecule coordinated to the Cu center in a κ^1 fashion and a water molecule within H-bonding proximity to the acetate molecule. With H₂O₂, this site is occupied by a water molecule. Similarly, in Cu-2xm-S112A-K121Y-Sav, the residual positive electron density in the F_o-F_c map was fit to an acetate molecule coordinated to the Cu center in a κ^1 fashion.

Modeling N49. For structures of Cu-2xm-Sav and Cu-2xm-S112A-K121Y-Sav, residue N49 is modeled as one rotamer. This rotamer is in the same conformation as in our previously reported structures of $[\text{Cu}^{\text{II}}(\text{biot-et-dpea})\text{H}_2\text{O}]_2^{2+} \subset \text{WT-Sav}$ or $[\text{Fe}^{\text{III}}(\text{biot-et-dpa})(\text{OH}_2)_2(\kappa^1\text{-O}_{\text{E112}})] \subset 2\text{xm-Sav}$.

S112E-Sav, where the amide likely participates in H-bonding with R84.^{19,20} For all structures of Cu-2xm-S112Y-Sav, we first modeled N49 in this conformation, but observed negative difference density in the F_o-F_c map surrounding the modelled amide as well as nearby residual positive difference density in the F_o-F_c map suggesting a second conformation of N49. The occupancy of this second N49 conformation was refined to 0.61, consistent with the refined Cu occupancy (0.65). The distance from the Cu center to the heteroatom is 2.4 Å. Due to the inability of the X-ray diffraction experiments to unambiguously determine N vs O coordination, we modeled this as a Cu- κ^1 -O_{N49} based on the Cu-heteroatom bond distance as well as previously published reports of pMMO⁵ and stellacyanin³⁰ active site structures. With H₂O₂ addition, the occupancy drops to 0.51, about two-thirds of the refined Cu occupancy (0.74). Of all Cu-Sav variants reported by our group to date, Cu-2xmS112Y-Sav is the only ArM that exhibits N49 in this second conformation, and the N49 coordination is disrupted to some extent by H₂O₂ addition.

Modeling S112Y. The first 90 degrees in phi rotation for further processing to mitigate possible effects of radiation damage. In this case, the positive density in the F_o-F_c map surrounding S112Y is only observed in Cu-2xm-S112Y-Sav with H₂O₂ addition (**Figure 2.12 A** and **2.18**). This was best modeled as S112Y covalently modified at the *meta* position. Given the proximity and orientation to the backbone carbonyl of A121 suggesting H-bonding interactions (shown in black dashed lines in **Figure 2.11**), the oxidized Y112 may be a catechol or semiquinone species. However, we also modeled S112Y in two rotamer positions of equal occupancies with no meaningful change in crystallographic refinement statistics (**Figure 2.12** and **Table 2.2**). The deposited structure represents a model of the oxidized Y112 corroborated by the LC-MS and trypsin digestion experiments described below.

Adventitious Cu binding. In Cu-2xm-Sav, we observe two additional peaks in the anomalous difference map 2.64 Å from N η _{H87} and 1.9 Å from N η _{H127} which were modeled as Cu atoms. The occupancies were refined to 0.94 and 0.20, respectively. In Cu-2xm-S112A-K121Y-Sav, we observe

one additional peak in the anomalous difference map $2.47 \text{ \AA}$ from $N\eta_{H87}$ which was modeled and refined as a Cu atom with 0.71 occupancy.

Liquid Chromatography/Mass Spectrometry (LC/MS) Methods

Instrumentation. Mass spectra of synthesized small-molecule intermediates were measured with an ACQUITY UPLC H-class system, single quadrupole QDa (Waters Corporation) or orthogonal acceleration time-of-flight (oa-TOF) LCT Premier (Waters Corporation). Mass spectra of intact proteins or peptides were measured with an ACQUITY ultra-performance liquid chromatography (UPLC) H-class system, Xevo G2-XS quadrupole time-of-flight (QTOF) (Waters Corporation).

Sample preparation for mass spectral studies. Cu-Sav was diluted to a final concentration of $150 \mu\text{M}$ in 50 mM MES buffer pH 6. Hydrogen peroxide (100 mM) was added to final concentration of 6 mM (10 equivalents relative to Cu-complex). Samples were allowed to incubate with H_2O_2 for 1 h at room temperature.

Intact Cu-Sav was separated from remaining H_2O_2 and buffer salts in reaction mixtures using Amicon Ultra 10K centrifugal filter devices and washed with 3X volume of nanopure water. The resulting Cu-Sav solution was boiled at 100°C for 15 min to dissociate Cu-complex. Intact apo-Sav was separated from dissociated Cu-complex using Amicon Ultra 10K centrifugal filter devices and washed with 3X volume of nanopure water. Samples were diluted in nanopure water for LCMS.

Isotope labeled H_2O_2 ($^{18}\text{O}_2\text{H}_2$, 90% isotope labeled, 2-2.4% solution in DI water) was obtained from Icon Isotopes. Cu-2xm-S112Y-Sav prepared as described above, incubated with $^{18}\text{O}_2\text{H}_2$ (6 mM , assuming 2% solution in water), and prepared for LCMS as described above.

Analysis of protein modification. Intact apo-Sav sample was analyzed by LC/MS. Proteins were separated away from reaction buffer salts using a phenyl guard column at 45°C (ACQUITY UPLC BEH Phenyl VanGuard Pre-column, $130 \text{ \AA}$, $1.7 \mu\text{m}$, $2.1 \times 5 \text{ mm}$, Waters Corporation). The 5 min LC

method used flow rate of 0.2 mL/min of a gradient of 0.1% formic acid in water (Buffer A), and acetonitrile (Buffer B) according to gradient in **Table 2.S4**.

The Xevo Z-spray ionization source was operated in positive MS resolution mode, 500–4000 Da, with a capillary voltage of 3000 V and cone voltage of 40 V (NaCsI calibration, Leu-enkephalin lock-mass). Nitrogen was used as the desolvation gas at 350 °C and total flow of 800 L/h. Retention times are given in minutes. The major peak of the chromatogram was selected for integration for further analysis (**Figures 2.S6 and 2.S8**). Total average mass spectra were reconstructed from the charge state ion series using the MaxEnt1 algorithm from Waters MassLynx software V4.1 SCN949 according to manufacturer's instructions.³¹ The ions of highest abundance from this charge state ion series are shown in **Figures 2.S7 and 2.S9**.

Enzymatic digestion and LC-MS/MS analysis. Apo-Sav prepared as described above was subjected to solution digestion with trypsin gold (Promega) at 37 °C in ammonium acetate pH 7.8 for 8 hrs, following manufacturer's recommendations. All LC-MS/MS experiments were performed using the same Waters Xevo Q-TOF instrumentation and buffer system as the intact protein analysis listed above. Separation of peptides was performed by reverse-phase chromatography at 45 °C using a reverse-phase C4 column (ACQUITY UPLC Protein BEH C4 column, 300 Å, 1.7 µm, 2.1 x 50 mm, Waters Corporation) and the solvent gradient listed in **Table 2.S5**. A 0.5 min waste-divert method was implemented to remove buffer salts.

The Xevo Z-spray source was operated with a capillary voltage of 3000 V and a cone voltage of 40 V (NaCsI calibration, Leu-enkephalin lock-mass). Nitrogen was used as the desolvation gas at 350 °C and a total flow of 800 L/h. Data was acquired across the 100–2000 Da range in MSe continuum mode with alternating 0.5 sec scans at alternating collision energy: low energy, 0 V, and a high collision energy ramp (15–45 V). The high energy data corresponds to the MS/MS secondary fragmentation of the low energies scans of a similar time.

The data was processed for exact mass peptide matching and *b/y* ion fragmentation confirmation using BioPharmaLynx software (Version 1.3.5, Waters Corporation). The software generates *in silico* peptide digestion data including all potential modifications and maps the real data with a 30-ppm error tolerance to the theoretically calculated masses.

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Supplementary Tables and Figures

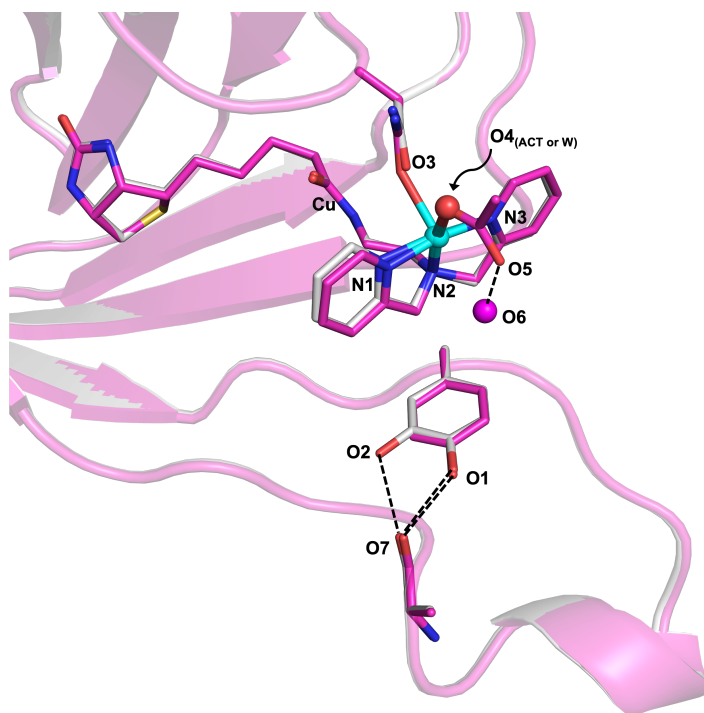


Figure 2.S1. Molecular model of **1** (pink, PDB: 9CSU) and **1** + H₂O₂ (grey, PDB: 9CSV). Atom labeling to indicate select bond lengths and angles listed in Table 2.S1.

Table 2.S1. Select bond lengths and angles according to labeling in Figure 2.S1.

	Cu-2xm-S112Y-Sav (PDB: 9CSU)	Cu-2xm-S112Y-Sav + H ₂ O ₂ (PDB: 9CSV)
Cu–N1	1.9 Å	1.9 Å
Cu–N2	2.0 Å	2.1 Å
Cu–N3	2.1 Å	2.0 Å
Cu–O3	2.5 Å	2.4 Å
Cu–O4 _(ACT or W)	2.4 Å	2.5 Å
Cu–O5	3.2 Å	N/A
Cu–O6	2.9 Å	N/A
N1–Cu–N2	83.2°	84.3°
N2–Cu–N3	82.0°	78.8°
N3–Cu–O4 _(ACT or W)	99.5°	101.2°
O4 _(ACT or W) –Cu–O3	87.6°	84.3°
N1–Cu–O3	94.2°	92.9°
N2–Cu–O3	101.7°	107.9°
N3–Cu–O3	93.1°	90.5°
O1–O7	3.0 Å	3.2 Å
O2–O7	N/A	2.8 Å
O5–O6	2.1 Å	N/A

Table 2.S2. Possible digest fragments of 2xm-S112Y-Sav. Identified ions [DF31]³⁺ and [DF31]²⁺ highlighted in yellow.

DF	mass	m/z +2 charge	m/z +3 charge	m/z +4 charge	m/z +5 charge	position	#MC
1	12686.0661	6344.03305	4229.6887	3172.516525	2538.21322	12-131	5
2	10569.9637	5285.98185	3524.321233	2643.490925	2114.99274	1-102	5
3	9692.771	4847.3855	3231.923667	2424.19275	1939.5542	53-143	5
4	9465.4832	4733.7416	3156.161067	2367.3708	1894.09664	12-102	4
5	9131.4799	4566.73995	3044.826633	2283.869975	1827.29598	59-144	5
6	9003.3849	4502.69245	3002.1283	2251.846225	1801.67698	59-143	4
7	8627.0603	4314.53015	2876.686767	2157.765075	1726.41206	1-83	4
8	8554.1364	4278.0682	2852.3788	2139.5341	1711.82728	53-131	4
9	8374.1476	4188.0738	2792.382533	2094.5369	1675.82952	80-158	5
10	8079.81	4040.905	2694.27	2020.9525	1616.962	1-79	3
11	7864.7504	3933.3752	2622.583467	1967.1876	1573.95008	59-131	3
12	7826.8973	3914.44865	2609.965767	1957.724325	1566.37946	84-158	4
13	7522.5798	3762.2899	2508.5266	1881.64495	1505.51596	12-83	3
14	6996.484	3499.242	2333.161333	1750.121	1400.2968	80-144	4
15	6975.3295	3488.66475	2326.109833	1744.832375	1396.0659	12-79	2
16	6868.389	3435.1945	2290.463	1718.09725	1374.6778	80-143	3
17	6449.2337	3225.61685	2150.744567	1613.308425	1290.84674	84-144	3
18	6321.1387	3161.56935	2108.046233	1581.284675	1265.22774	84-143	2
19	5944.814	2973.407	1982.604667	1487.2035	1189.9628	1-58	2
20	5883.9939	2942.99695	1962.3313	1471.998475	1177.79878	103-158	3
21	5729.7544	2865.8772	1910.918133	1433.4386	1146.95088	80-131	2
22	5333.5535	2667.77675	1778.851167	1334.388375	1067.7107	53-102	3
23	5255.428	2628.714	1752.809333	1314.857	1052.0856	1-52	1
24	5182.5041	2592.25205	1728.501367	1296.626025	1037.50082	84-131	1
25	4840.3336	2421.1668	1614.444533	1211.0834	969.06672	12-58	1
26	4644.1675	2323.08375	1549.055833	1162.041875	929.8335	59-102	2
27	4506.3303	2254.16515	1503.1101	1127.582575	902.26606	103-144	2
28	4378.2353	2190.11765	1460.411767	1095.558825	876.64706	103-143	1
29	4150.9475	2076.47375	1384.649167	1038.736875	831.1895	12-52	0
30	3390.6501	1696.32505	1131.2167	848.662525	679.13002	53-83	2
31	3239.6007	1620.80035	1080.8669	810.900175	648.92014	103-131	0
32	2843.3998	1422.6999	948.7999333	711.84995	569.67996	53-79	1
33	2701.2641	1351.63205	901.4213667	676.316025	541.25282	59-83	1
34	2663.411	1332.7055	888.8036667	666.85275	533.6822	132-158	2
35	2509.1715	1255.58575	837.3905	628.292875	502.8343	80-102	1
36	2154.0138	1078.0069	719.0046	539.50345	431.80276	59-79	0
37	1961.9212	981.9606	654.9737333	491.4803	393.38424	84-102	0
38	1524.7765	763.38825	509.2588333	382.194125	305.9553	144-158	1
39	1396.6815	699.34075	466.5605	350.170375	280.3363	145-158	0
40	1285.7474	643.8737	429.5824667	322.43685	258.14948	132-144	1
41	1157.6524	579.8262	386.8841333	290.4131	232.53048	132-143	0
42	1123.4983	562.74915	375.4994333	281.874575	225.69966	1-11	0
43	708.4039	355.20195	237.1346333	178.100975	142.68078	53-58	0
44	566.2681	284.13405	189.7560333	142.567025	114.25362	80-83	0

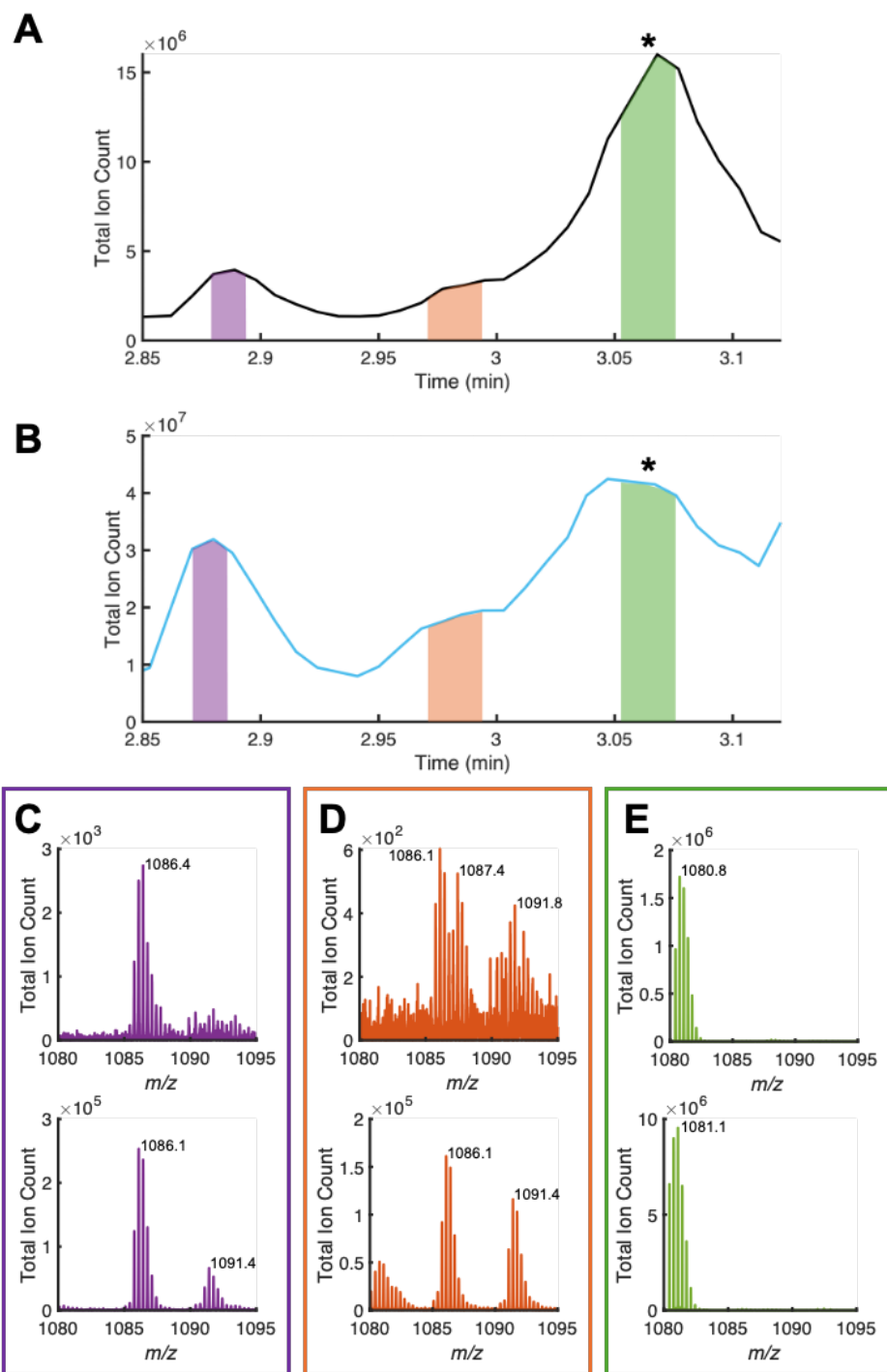


Figure 2.S2. Total area of chromatograms integrated to produce mass spectra in Figure 2.16 for **(A)** **1** and **(B)** **1** + H₂O₂. Integrated area of chromatographic peak eluting at 2.87 min highlighted in purple to produce mass spectra in panel **C** for **1** (**C**, top) and **1** + 10 eq H₂O₂ (**C**, bottom). Integrated area of chromatographic peak eluting at 2.97 min highlighted in orange to produce mass spectra in panel **D** for **1** (**D**, top) and **1** + 10 eq H₂O₂ (**D**, bottom). Integrated area of chromatographic peak eluting at 3.06 min highlighted in green to produce mass spectra in panel **E** for **1** (**E**, top) and **1** + 10 eq H₂O₂ (**E**, bottom). Because of the presence of oxidized DF31 in **1** with and without H₂O₂ incubation (**C** and **D** top), the chromatograms were integrated over this entire range, from 2.85 to 3.12 min, for all samples to capture the relative abundance of both the unoxidized and oxidized DF31.

Table 2.S3. Calculated b/y ion fragments of DF31 in 2xm-S112Y-Sav. Identified unmodified fragments highlighted in yellow. Identified oxidized fragments highlighted in blue.

b⁺⁺	b⁺	b#	Residue	y#	y⁺	y⁺⁺
--	--	1	I	28	--	--
114.5708	228.1343	2	N	27	2998.4217	1563.7620
165.0946	329.1819	3	T	26	2884.3788	1506.7405
229.1239	457.2405	4	Q	25	2783.3311	1456.2167
322.1636	643.3198	5	W	24	2655.2726	1392.1874
378.7056	756.4039	6	L	23	2469.1932	1299.1477
435.2476	869.4880	7	L	22	2356.1092	1242.6057
485.7715	970.5356	8	T	21	2243.0251	1186.0637
567.3031	1133.5990	9	Y	20	2141.9774	1135.5398
595.8139	1190.6204	10	G	19	1978.9141	1054.0082
646.3377	1291.6681	11	T	18	1921.8926	1025.4974
696.8615	1392.7158	12	T	17	1820.8450	974.9736
761.3828	1521.7584	13	E	16	1719.7973	924.4498
796.9014	1592.7955	14	A	15	1590.7547	859.9285
853.9229	1706.8384	15	N	14	1519.7176	824.4099
889.4414	1777.8755	16	A	13	1405.6747	767.3884
982.4811	1963.9549	17	W	12	1334.6375	731.8699
1017.9996	2034.9920	18	A	11	1148.5582	638.8302
1061.5156	2122.0240	19	S	10	1077.5211	603.3117
1112.0395	2223.0717	20	T	9	990.4891	559.7957
1168.5815	2336.1557	21	L	8	889.4414	509.2718
1218.1157	2435.2242	22	V	7	776.3573	452.7298
1246.6264	2492.2456	23	G	6	677.2889	403.1956
1315.1559	2629.3045	24	H	5	620.2675	374.6849
1372.6694	2744.3315	25	D	4	483.2086	306.1554
1423.1932	2845.3791	26	T	3	368.1816	248.6419
1496.7274	2992.4476	27	F	2	267.1339	198.1180
--	--	28	T	1	120.0655	124.5839

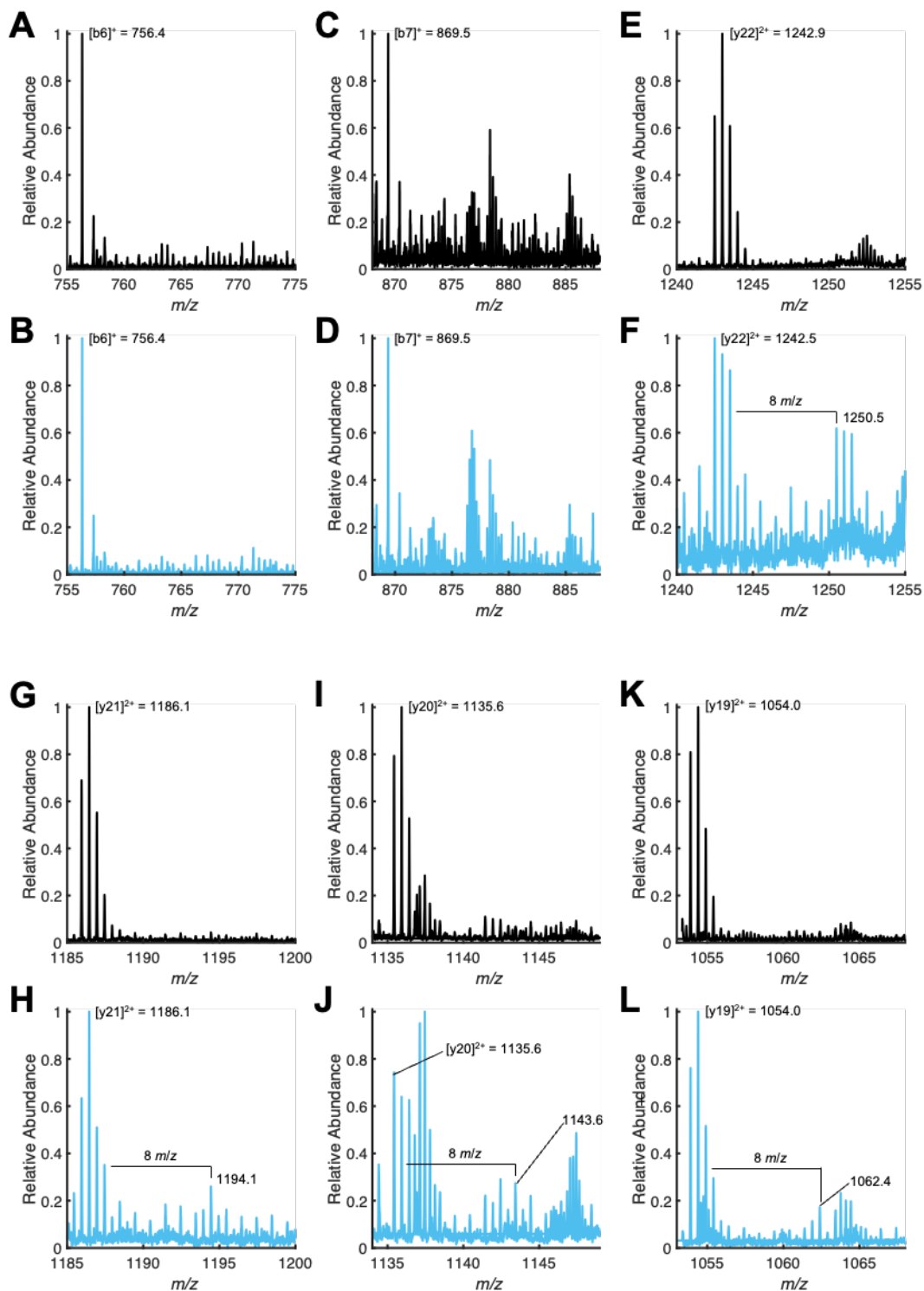


Figure 2.S3. MS/MS analysis of b/y ion fragments of DF31 resulting from tryptic digestion of Cu-2xm-S112Y-Sav indicating oxidation of residue Y112. Fragment b6 (**A**) before and (**B**) after incubation with H_2O_2 , (**C**) and fragment b7 before and (**D**) after incubation with H_2O_2 are not oxidized, ruling out oxidation of residues 104-110. Fragment y22, y21, and y20 (**E**, **G**, **I**) before and (**F**, **H**, **J**) after incubation with H_2O_2 . Oxidized fragments of y22, y21, and y20 appear after incubation with H_2O_2 . (**L**) oxidation of W120 or H127 is also suggested by appearance of oxidized fragment y19 after incubation with H_2O_2 as y19 does not contain Y112.

Table 2.S4. Intact protein LC-MS gradient method

Time (mins)	Flow (mL/min)	% A	%B	Curve
0	0.2	100	0	6
0.5	0.2	100	0	6
2	0.2	10	90	6
2.5	0.2	10	90	6
4	0.2	100	0	6
5	0.2	100	0	6

Table 2.S5. Peptide LC-MS gradient method

Time (mins)	Flow (mL/min)	% A	%B	Curve
0	0.3	97	3	6
0.5	0.3	97	3	6
2	0.3	40	60	6
2.5	0.3	40	60	6
3	0.3	10	90	6
3.5	0.3	10	90	6
4	0.3	97	3	6
5	0.3	97	3	6

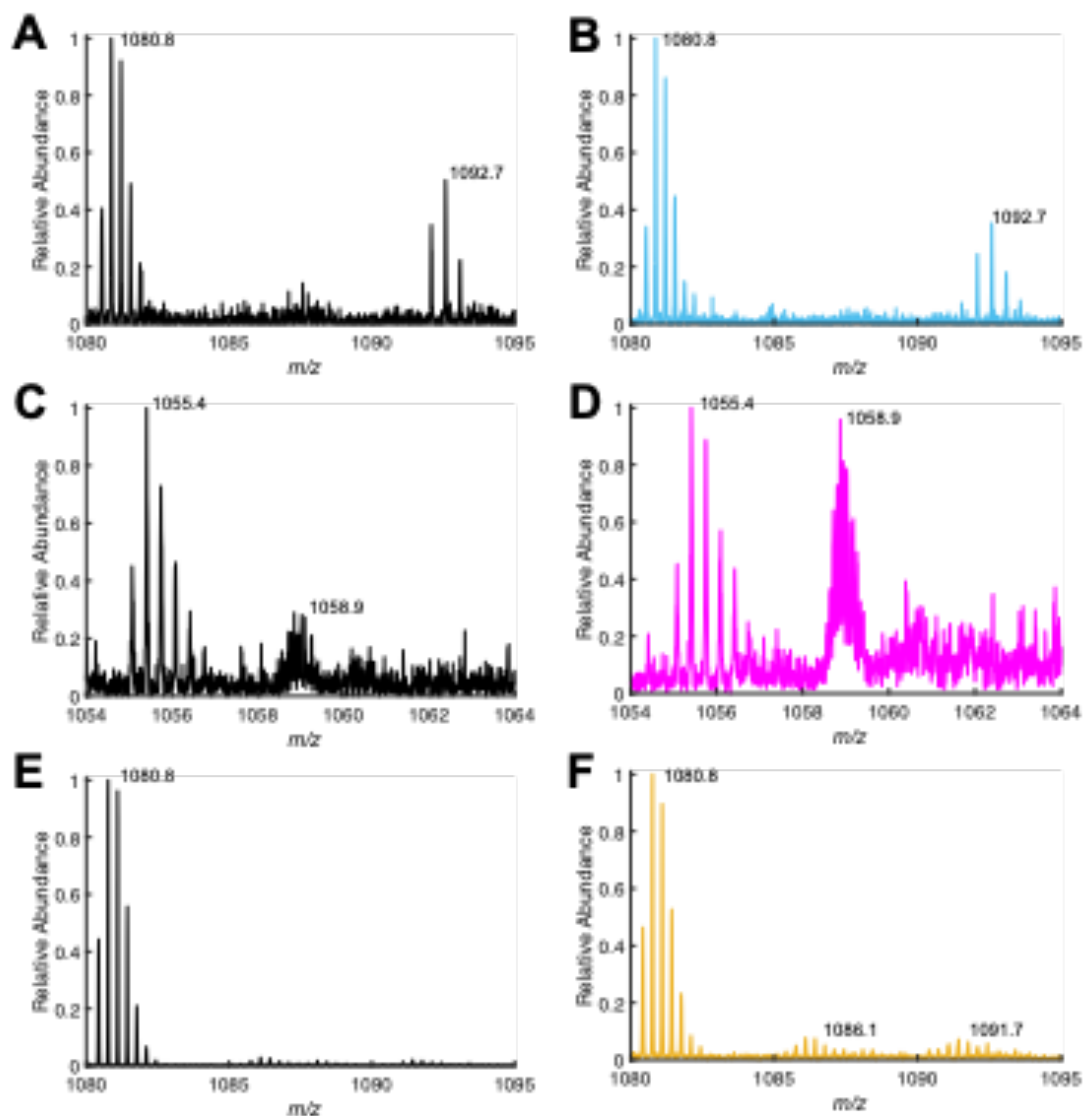


Figure 2.S4. ESI QTOF positive ion mode mass spectra of $[\text{DF31}]^{3+}$ resulting from tryptic digests: (A) biot-2xm-S112Y-Sav before and (B) after the addition of 10 equivalents of H_2O_2 and incubation for 1 h. (C) Biot-2xm-Sav before and (D) after the addition of 10 equivalents of H_2O_2 and incubation for 1 h. (E) Biot-2xm-S112A-K121Y-Sav before and (F) after the addition of 10 equivalents of H_2O_2 and incubation for 1 h.

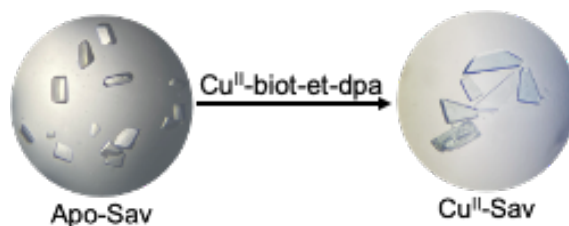


Figure 2.S5. Tetragonal crystals of apo-Sav (left) are colorless. Upon soaking apo-Sav crystals with Cu-complex, crystals turn blue (right).

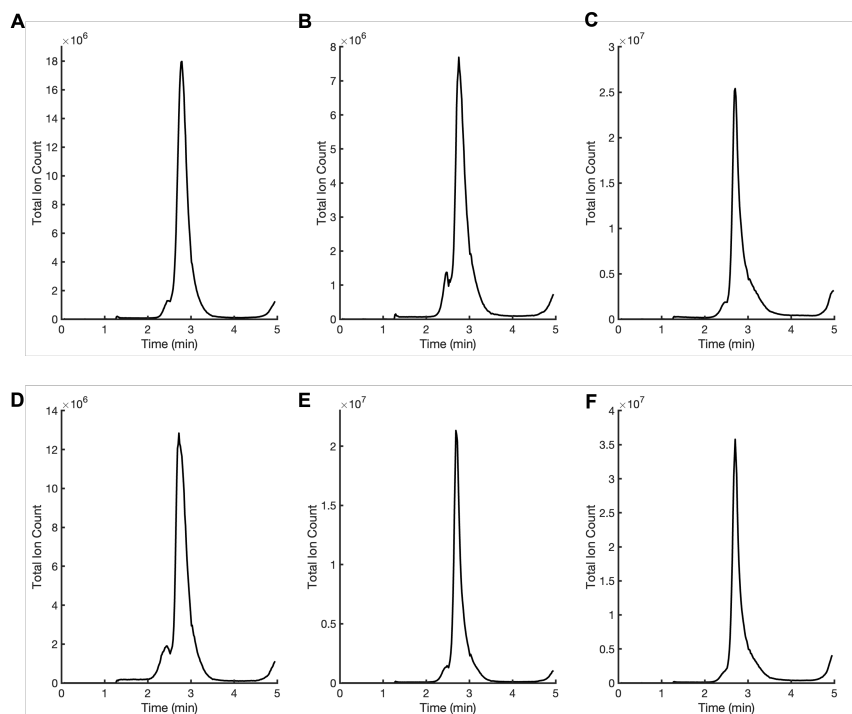


Figure 2.S6. Chromatograms of (A) **2**, (B) **1**, (C) **3**, (D) **2** + H₂O₂, (E) **1** + H₂O₂, and (F) **3** + H₂O₂. The major peak of each chromatogram was integrated at full width/half maximum for further analysis.

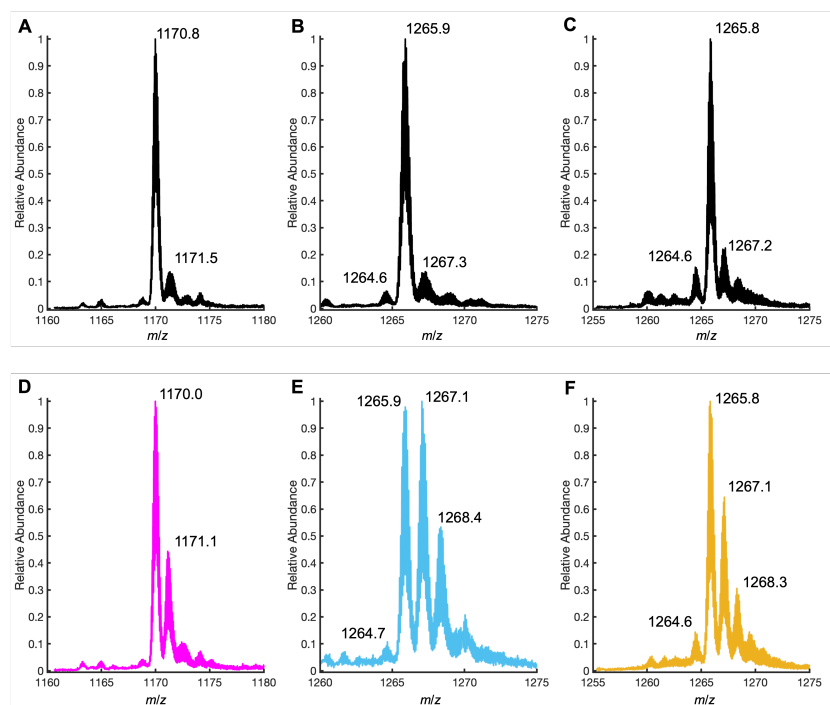


Figure 2.S7. ESI QTOF positive ion mode mass spectra showing ions of highest abundance from integration of the major peaks of the chromatograms of (A) **2**, (B) **1**, (C) **3**, (D) **2** + H₂O₂, (E) **1** + H₂O₂, and (F) **3** + H₂O₂. The charge-state ion series containing these ions were used to reconstruct total average mass spectra shown in Figure 2.14.

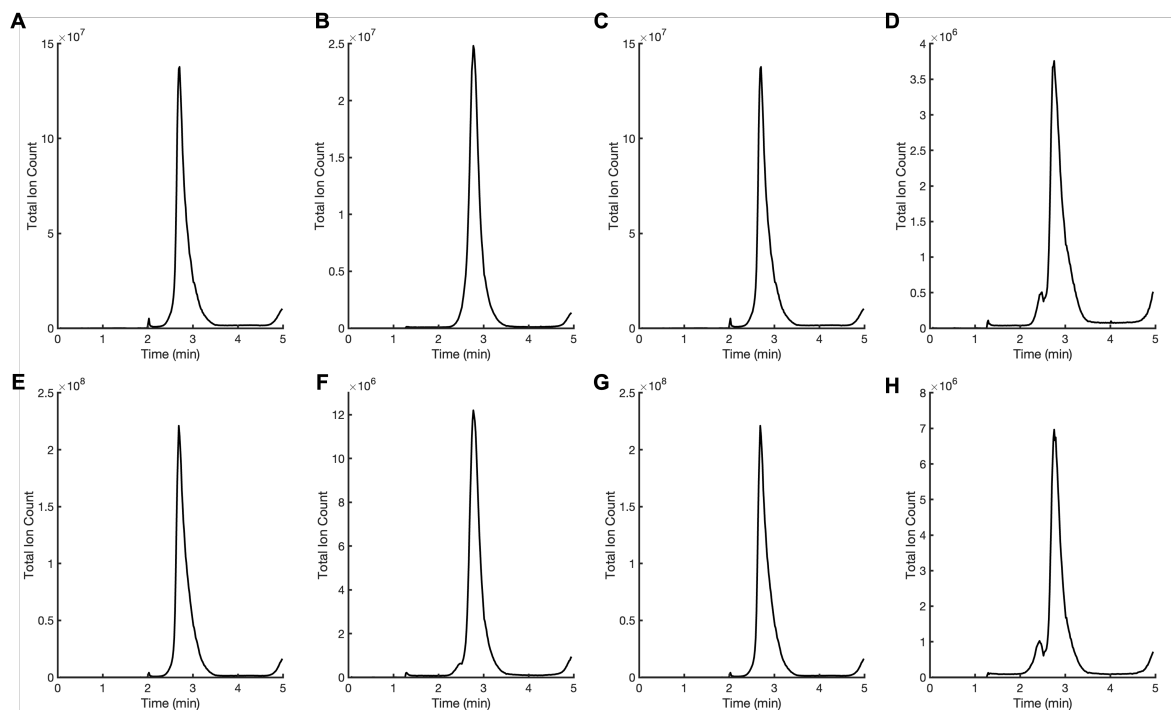


Figure 2.S8. Chromatograms of (A) apo-2xm-Sav, (B) biot-2xm-Sav, (C) apo-2xm-S112Y-Sav, (D) biot-2xm-S112Y-Sav, (E) apo-2xm-Sav + H₂O₂, (F) biot-2xm-Sav + H₂O₂, (G) apo-2xm-S112Y-Sav + H₂O₂, and (H) biot-2xm-S112Y-Sav + H₂O₂. The major peak of each chromatogram was integrated at full width/half maximum for further analysis.

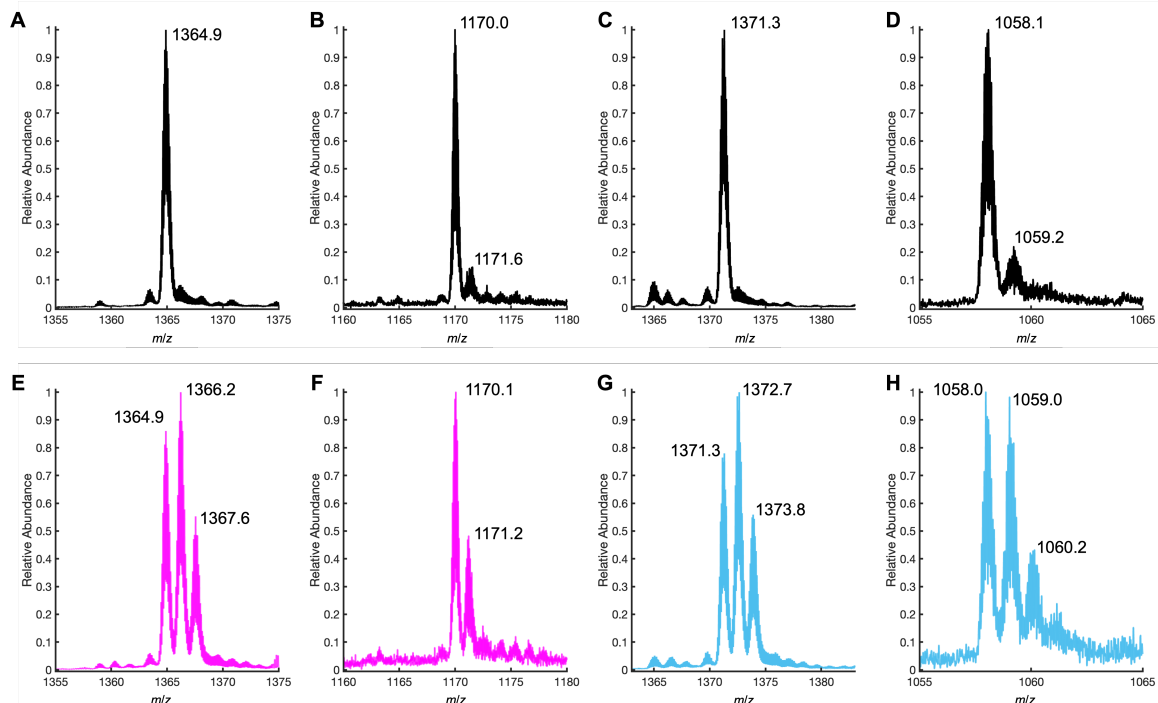


Figure 2.S9. ESI QTOF positive ion mode mass spectra showing ions of highest abundance from integration of major peak of chromatogram of (A) apo-2xm-Sav, (B) biot-2xm-Sav, (C) apo-2xm-S112Y-Sav, (D) biot-2xm-S112Y-Sav, (E) apo-2xm-Sav + H₂O₂, (F) biot-2xm-Sav + H₂O₂, (G) apo-2xm-S112Y-Sav + H₂O₂, and (H) biot-2xm-S112Y-Sav + H₂O₂. The charge-state ion series containing these ions were used to reconstruct total average mass spectra shown in Figure S12.

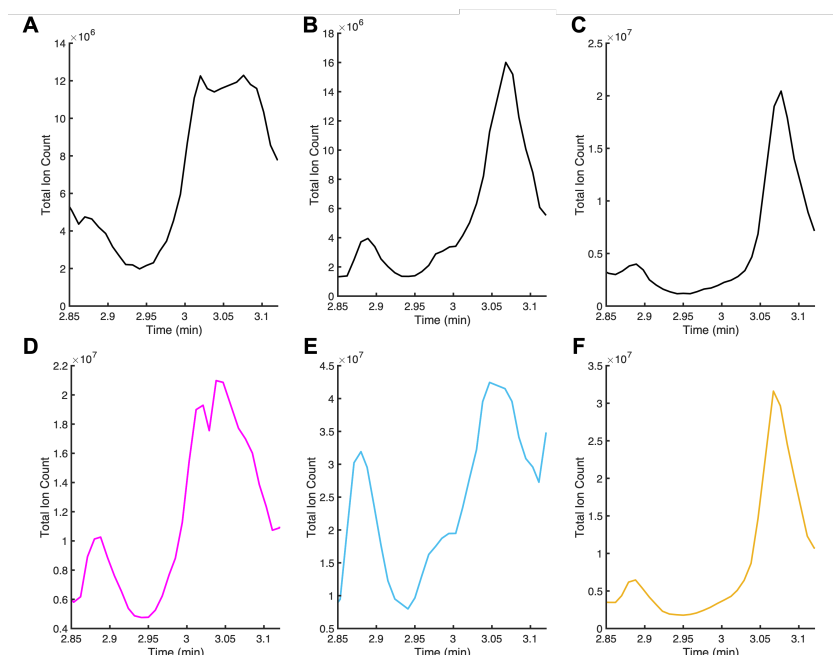


Figure 2.S10. Integrated area of chromatogram after trypsin digestion of (A) 2, (B) 1, (C) 3, (D) 2 + 10 eq H_2O_2 , (E) 1 + 10 eq H_2O_2 , and (F) 3 + 10 eq H_2O_2 . Mass spectra resulting from integration of this area displayed in Figure 2.16.

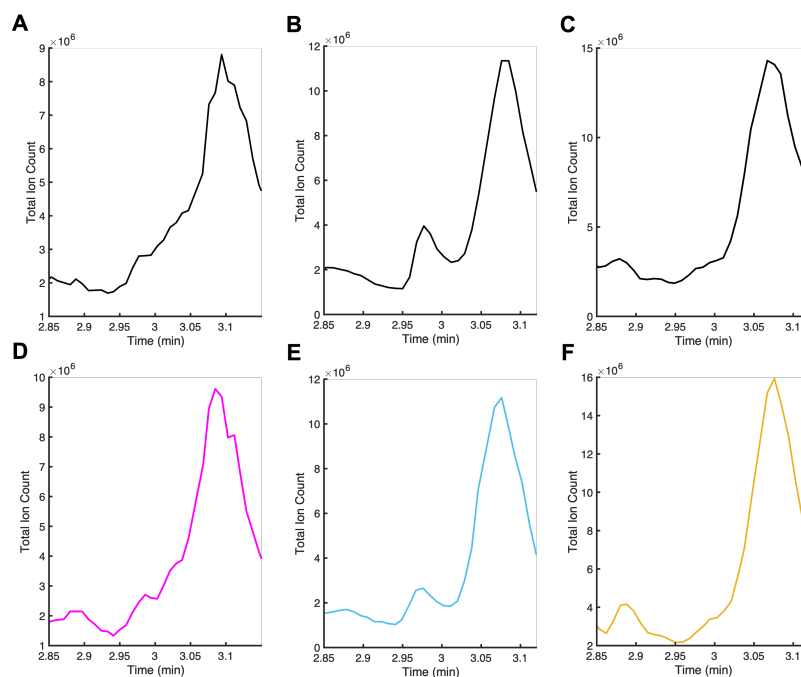


Figure 2.S11. Integrated area of chromatogram after trypsin digestion of (A) biot-2xm-Sav, (B) biot-2xm-S112Y-Sav, (C) biot-2xm-S112Y-K121Y-Sav, (D) biot-2xm-Sav + H_2O_2 , (E) biot-2xm-S112Y-Sav + H_2O_2 , and (F) biot-2xm-S112A-K121Y-Sav + H_2O_2 . The chromatograms were integrated over this entire range, from 2.85 to 3.12 min, for all samples to capture the relative abundance of both the unoxidized and oxidized DF31. Mass spectra resulting from integration of this range are shown in figure 2.S4.

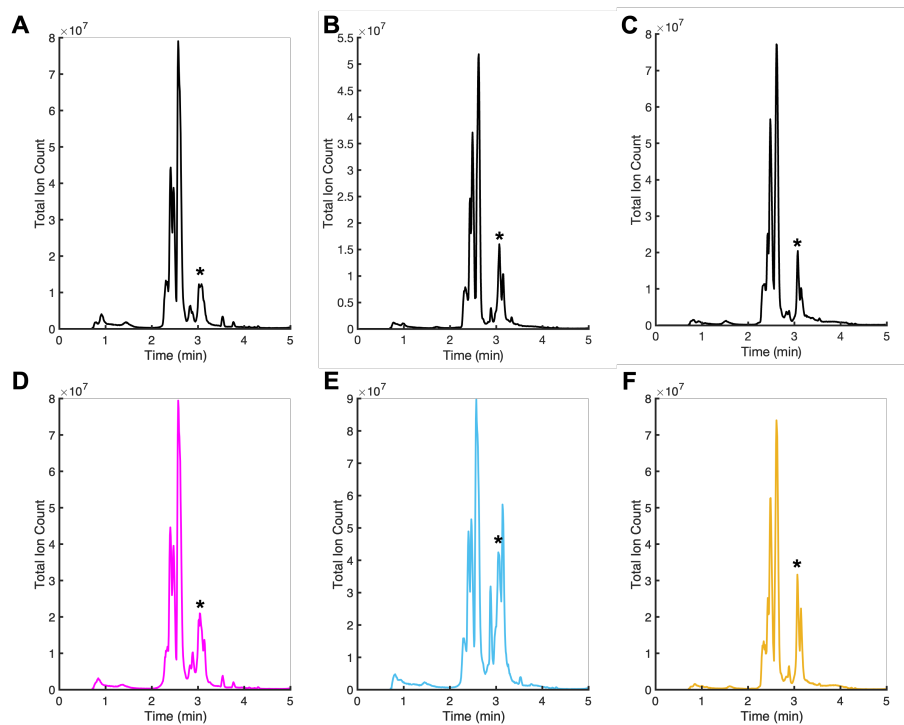


Figure 2.S12. Chromatograms after trypsin digestion of (A) Cu-2xm-Sav, (B) Cu-2xm-S112Y-Sav, (C) Cu-2xm-S112A-K121Y-Sav, (D) Cu-2xm-Sav + H₂O₂, (E) Cu-2xm-S112Y-Sav + H₂O₂, and (F) Cu-2xm-S112AK121Y-Sav + H₂O₂. Asterisks indicate chromatographic peaks containing DF31 in each sample.

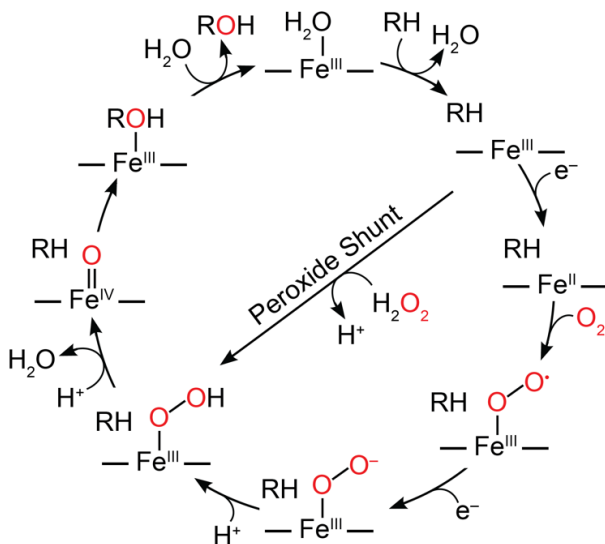
CHAPTER 3

Towards Stabilizing a Reactive Cobalt-Oxygen Adduct Within a Protein Host

Introduction

The activation of molecular oxygen (O_2) for selective oxidative transformations is a reaction of interest in enzymatic catalysis, particularly in metalloenzymes that employ transition metal cofactors to mediate their reactivity. Nature commonly employs iron in O_2 activation reactions due to its abundance and favorable electronic properties, including an accessible $\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$ redox couple (0.77 V vs. SHE) and the ability to access high-valent states like Fe^{IV} . These abilities to undergo multiple redox events and support high-valent oxidation states make it adept for the multielectron chemistry necessary for activation of both O_2 and organic substrates.¹ Additionally, its electronic structure allows it to take on a variety of coordination geometries to promote interactions with dioxygen and form reactive Fe-oxido species.² In particular, Fe^{IV} -oxido species have been implicated as key reactive intermediates in the catalytic cycles of Fe-containing enzymes such as cytochromes P450 monooxygenase (**Scheme 3.1**)³ or α -ketoglutarate (α KG) dependent dioxygenases.⁴

Scheme 3.1. Catalytic cycle of cytochromes P450 monooxygenases. Also highlighted is the “peroxide shunt” pathway, where instead of using dioxygen as an oxidant, hydrogen peroxide initiates the reaction.



Cobalt also offers comparably rich chemistry to iron as demonstrated in a vast number of synthetic systems, and it is somewhat surprising that there is the lack of cobalt-based O_2 activating metalloproteins in biological contexts. Artificial metalloenzymes (ArMs) incorporating cobalt ions offer an opportunity to engineer bioinspired catalysts capable of utilizing O_2 for oxidation chemistry that could lead to new types of reactivities. Furthermore, leveraging protein scaffolds can modulate the coordination environment and electronic structure of cobalt ions, which can provide insight into the fundamental principles governing O_2 activation. This chapter explores the development of cobalt-containing ArMs, focusing on their ability to generate high-valent cobalt-oxygen species to gain insights into the factors that govern cobalt-based oxidation chemistry within a protein.

While O_2 is a powerful oxidant, its activation for productive chemistry often requires specific ligand environments and conditions. As an alternative, hydrogen peroxide (H_2O_2) has been used to generate high-valent Fe-oxido species (**Scheme 3.1**).^{5,6} Reactions of H_2O_2 with Fe^{II} complexes can also result in Fenton chemistry, in which hydroxide ions, hydroxyl radicals, and/or hydroperoxyl radicals are formed.⁷ Co^{II} complexes have also been shown to catalyze H_2O_2 decomposition.^{8–11} However, unlike Fe^{II} systems, in reactions of Co^{II} species and H_2O_2 , it has been reported that the oxidation state of Co does not change and generates distinct ROS including superoxide, singlet oxygen, and hydroxyl radicals.^{12,13} Furthermore, Co^{II}/H_2O_2 -mediated oxidations display maximal efficiency under neutral to basic conditions compared to acidic conditions for analogous Fe^{II} systems.

To gain insight into the mechanisms of Co^{II} -mediated H_2O_2 activation, I wanted to leverage the properties of biotin-Sav ArMs to stabilize intermediates generated upon reaction of Co^{II} -cofactors with H_2O_2 . To this end, the concept of modulating primary and secondary coordination sphere interactions to stabilize normally reactive metal-oxygen species is not new. The Borovik lab has previously stabilized metal-oxygen species through the design of multidentate ligands that regulate both the primary and secondary coordination spheres to promote chemistry.^{14–18} My specific interest

was in applying these design concepts towards stabilizing, characterizing, and tuning the reactivity of a Co-oxygen species within a protein host. This chapter describes initial efforts towards preparing and characterizing a Co-ArM within the biotin-streptavidin construct.

Results and Discussion

Design and Preparation of ArMs

Results presented in previous chapters motivated the design of ArMs used in this study. As discussed in Chapter 2, published work using wild-type (WT) Sav indicated that residues N49, A86, and S112 were involved in hydrogen bonding (H-bonding) interactions that enabled the stabilization of a five-coordinate Cu^{II} -OOH species. Two other mutations, E101Q and K121A (referred to as 2xm), were introduced to increase yields of recombinantly expressed protein and to prevent unwanted interactions with the cofactor. These changes led to the preparation of a double-mutant and three triple-mutant variants of Sav, referred to as 2xm-, 2xm-S112E-, 2xm-S112F-, and 2xm-N49A- Sav. I thought that 2xm-Sav could stabilize a Co-OO(H) species similar to what was observed in the WT Cu ArM. Further comparisons could be made to the 2xm-S112E-Sav variant, where the Co-center is coordinatively saturated (this structure is further discussed in Chapter 4), and 2xm-S112F-Sav or 2xm-N49A-Sav, where key residues implicated in stabilizing the hydroperoxide species are perturbed.

The Co^{II} -cofactor (Co-et-dpa) was prepared as discussed in Chapter 4. Co-ArMs were prepared by addition of a solution of excess Co-et-dpa to a solution of Sav in the appropriate aqueous buffer. Samples were separated from excess Co-et-dpa and concentrated using Amicon Ultra 10K centrifugal filter devices and washed with 5X volume of buffer.

Structural Characterization of ArMs

As discussed in Chapter 4, determination of the molecular structure of Co-2xm-S112E-Sav revealed a six-coordinate, octahedral Co cofactor, where Co is facially coordinated by the dpa ligand, S112E bound in a κ^1 fashion, and two *cis*-O atoms from aqua ligands (**Figure 3.1 A, Tables 3.1 and 3.S1**). Single crystals of Co-2xm-Sav were obtained via the same soaking method and diffracted to 1.5 Å. In Co-2xm-Sav, the Co center is coordinated by three N donors from the dpa ligand, and one O atom from a water molecule in a distorted tetrahedral geometry (**Figure 3.1 B, Tables 3.1 and 3.S1**).

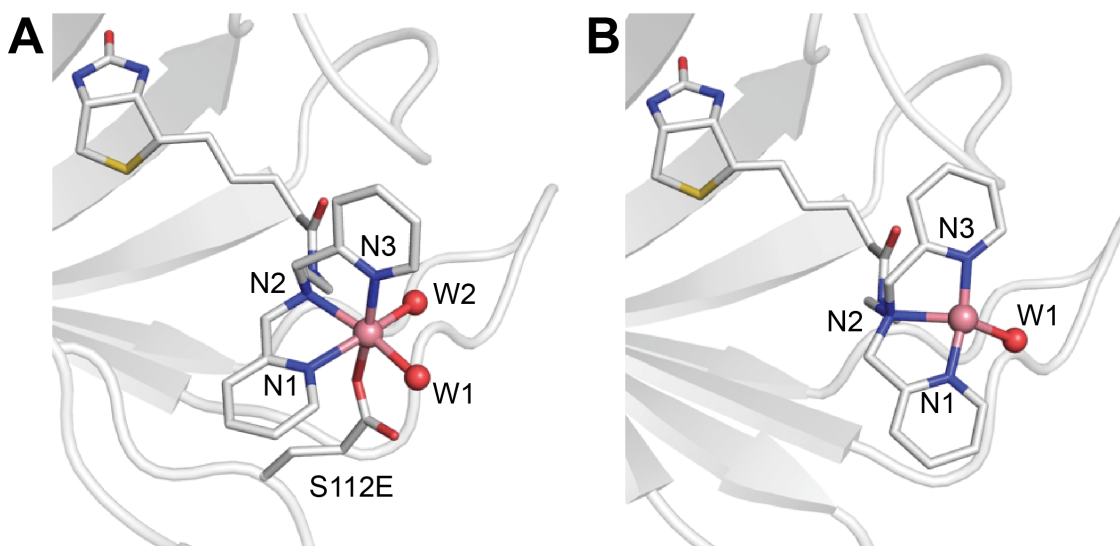


Figure 3.1. Molecular structures of (A) Co-2xm-S112E-Sav and (B) Co-2xm-Sav.

Table 3.1. Select bond lengths (Å) and angles (°) for Co-2xm-S112E-Sav and Co-2xm-Sav according to atom labeling in Figure 3.1

Co-2xm-S112E-Sav				Co-2xm-Sav	
Co–N1	2.2	O1 _{E112} –Co–O _{W1}	92.2	Co–N1	1.8
Co–N2	2.2	O1 _{E112} –Co–O _{W2}	85.5	Co–N2	2.2
Co–N3	2.1	O1 _{E112} –Co–N2	90.5	Co–N3	1.8
Co–O1 _{E112}	2.2	O1 _{E112} –Co–N1	81.8	Co–O _{W1}	2.2
Co–O _{W1}	2.1	N1–Co–N2	73.3	N1–Co–N2	81.7
Co–O _{W2}	2.1	N2–Co–O _{W2}	98.5	N2–Co–N3	82.0
O2 _{E112} –O _{W1}	2.5	O _{W1} –Co–O _{W2}	94.3	N3–Co–O _{W1}	108.5
O2 _{E112} –O _{W2}	4.0	O _{W1} –Co–N1	94.6	O _{W1} –Co–N1	91.1
		N3–Co–N1	101.7	O _{W1} –Co–N2	130.4
		N3–Co–N2	81.5	N3–Co–N1	158.4
		N3–Co–O _{W1}	97.0		

For Co-2xm-Sav, a feature at $\lambda_{\text{max}} = 490$ nm was observed at pH 6 and pH 8, which was putatively assigned to a Co d-d transition. This feature was also observed in Co-2xm-S112F-Sav and Co-2xm-N49A-Sav at pH 6. For Co-2xm-S112E-Sav, this feature was slightly shifted to $\lambda_{\text{max}} = 482$ nm at pH 6 and $\lambda_{\text{max}} = 476$ nm at pH 8, indicating a possible change in the ligand environment of the Co center upon both introduction of a coordinating residue and changes in pH.

Upon addition of H₂O₂ to Co-ArMs, the growth of two new features is observed, but only under certain conditions. In Co-2xm-S112E-Sav, a band at $\lambda_{\text{max}} = 511$ nm and a shoulder at 430 nm arise when 1 eq of H₂O₂ is added at pH 8 (**Figure 3.2**). These features form faster with addition of 10 or 100 eq of H₂O₂. At pH 6, the growth of these features is not observed upon addition of even 100 eq of H₂O₂, but a low-intensity shoulder around 370 nm arises (**Figure 3.2**). Similar changes are observed for Co-2xm-S112F-Sav and Co-2xm-N49A-Sav upon addition of 100 equivalents of H₂O₂ (**Figure 3.4**) and for Co-2xm-Sav upon addition of 1 or 10 equivalents of H₂O₂ at pH 6 (**Figure 3.3**).

For Co-2xm-Sav at pH 6, with addition of 100 eq of H₂O₂, two features at $\lambda_{\text{max}} = 534$ and 430 nm appear over 60 min. Over a longer period of 1000 minutes, the feature at $\lambda_{\text{max}} = 534$ nm shifts to 500 nm, suggesting a structural rearrangement surrounding the Co center (**Figure 3.4**). At pH 8, a band at $\lambda_{\text{max}} = 511$ nm and a shoulder at 430 nm arise over 60 min; however, these features appear to form at different rates, and the feature at $\lambda_{\text{max}} = 511$ nm shifts from 534 nm over this time, suggesting that the reaction may involve two or more distinct steps (**Figure 3.3**). Kinetic analysis of the formation of these absorbance features is at the preliminary stage and results indicate that the reactions deviate from simple first- or second- order kinetics, suggesting a more complex, underlying mechanism.

The growth of new absorption bands upon H₂O₂ addition suggest the potential formation of cobalt-oxygen adducts, where pH and coordination environment are expected to influence reactivity with H₂O₂. Co^{III}-peroxido and -oxido complexes have been reported to exhibit charge transfer bands

reminiscent of the two features observed at 430–540 nm. To gain further insight into what Co-oxygen species is being formed, the reaction of Co-2xm at pH 6 with 100 eq of H_2O_2 was chosen to probe further.

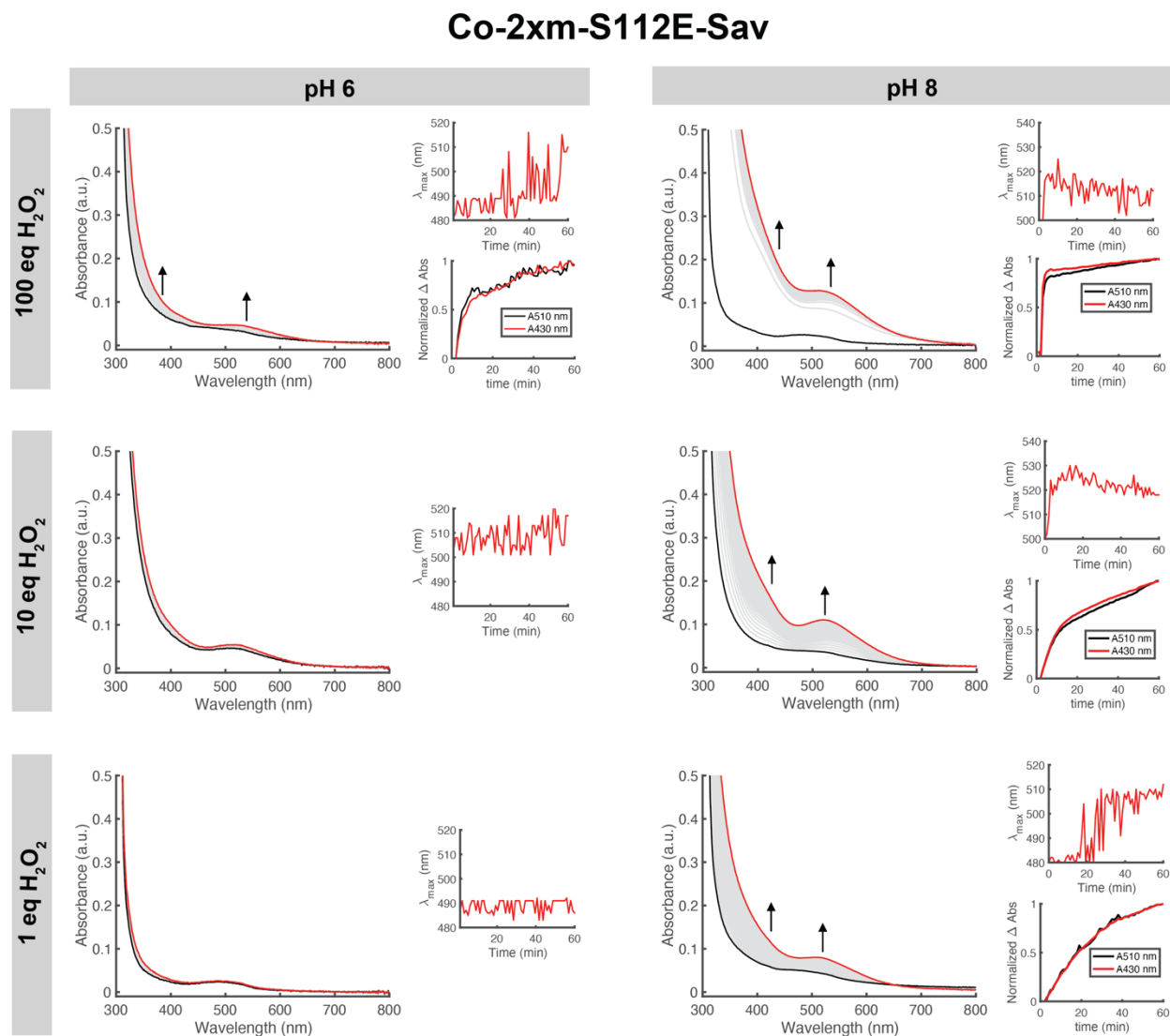


Figure 3.2. UV-vis spectra of Co-2xm-S112E-Sav (black traces) with addition of H_2O_2 monitored over 60 minutes (grey traces and final spectra shown in red). Reactions were monitored at pH 6 or pH 8 with 100, 10, or 1 equivalents of H_2O_2 . For each condition, the corresponding shift in $\lambda_{\text{max}} \approx 500$ nm and growth of absorbance features over 60 minutes is shown to the right of the UV-vis spectra.

Co-2xm-Sav

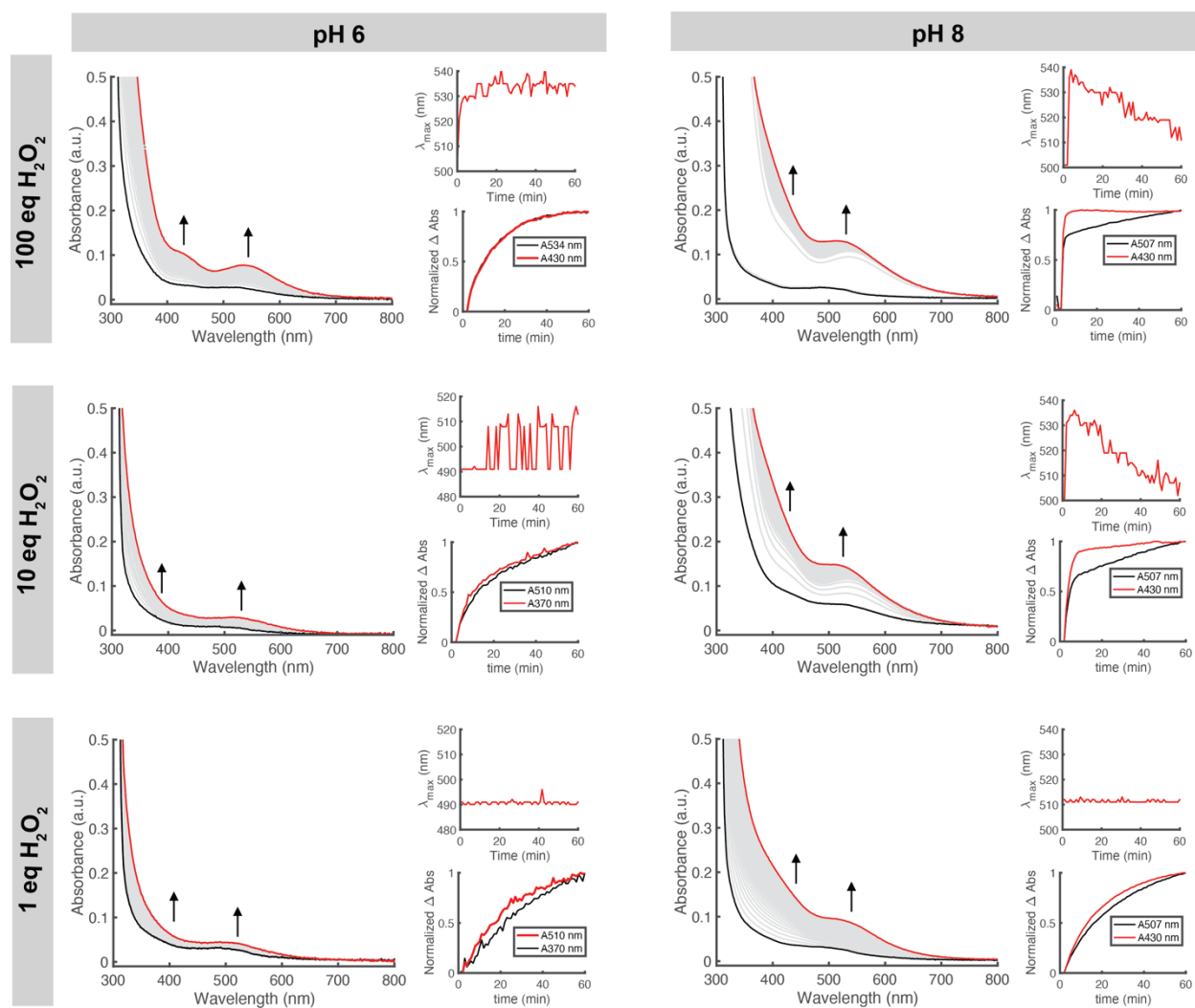


Figure 3.3. UV-vis spectra of Co-2xm-Sav (black traces) with addition of H_2O_2 monitored over 60 minutes (grey traces and final spectra shown in red). Reactions were monitored at pH 6 or pH 8 with 100, 10, or 1 equivalents of H_2O_2 . For each condition, the corresponding shift in $\lambda_{\text{max}} \approx 500$ nm and growth of absorbance features over 60 minutes is shown to the right of the UV-vis spectra.

Co-2xm-Sav

pH 6, 100 eq H_2O_2

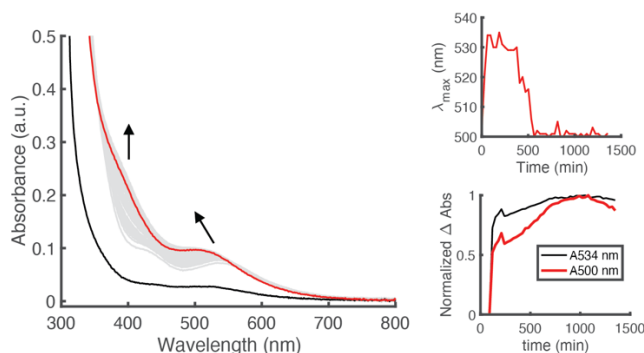


Figure 3.4. UV-vis spectra of Co-2xm-Sav (black) at pH 6 with addition of 100 H_2O_2 monitored over 1300 minutes (grey traces and final spectra shown in red). The corresponding shift in $\lambda_{\text{max}} \approx 500$ nm and growth of absorbance features over 1300 minutes are shown to the right of the UV-vis spectra.

Co-2xm-S112F-Sav

Co-2xm-N49A-Sav

pH 6, 100 eq H_2O_2

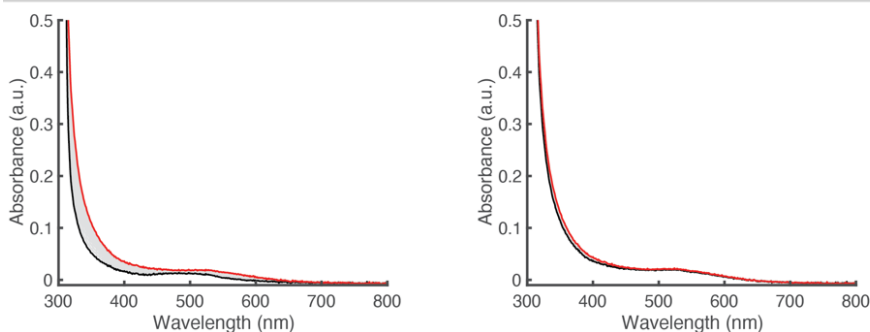


Figure 3.5. UV-vis spectra of Co-2xm-S112F-Sav or Co-2xm-N49A-Sav (black) at pH 6 with addition of 100 H_2O_2 monitored over 60 minutes (grey traces and final spectra shown in red).

Electron Paramagnetic Resonance Spectroscopy

Co^{II} centers have d^7 electron configurations allowing them to be probed by perpendicular mode X-band EPR spectroscopy. On the other hand, in typical biological and synthetic systems, Co^{III} is in a low spin d^6 electronic configuration, making it EPR silent. For Co-2xm-Sav at pH 6, an axial high-spin Co^{II} species with g -values = 4.10 and 1.98 is observed (**Figure 3.6**). Upon incubation of the samples with 100 eq H_2O_2 for 60 min, the signal intensity reduces to roughly 10% of the original

intensity. Presumably, addition of peroxide results in the oxidation of Co^{II} to an EPR-silent Co^{III} species.

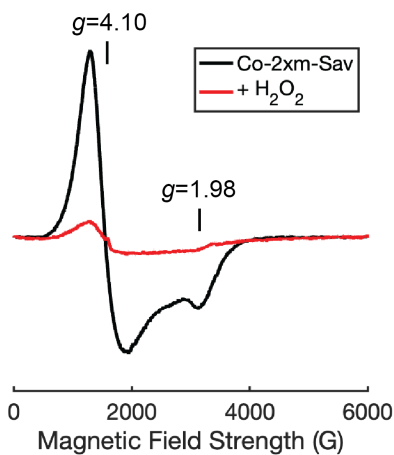


Figure 3.6. Continuous wave X-band EPR spectra of Co-2xm-Sav at pH 6 (black) and upon incubation with 100 eq H_2O_2 for 60 min (red).

X-ray Absorption Spectroscopy

Cobalt K-edge X-ray absorption spectroscopy (XAS) is a powerful technique for studying Co oxidation states, coordination environment, and electronic structure. The X-ray Absorption Near Edge Structure (XANES) is highly sensitive to changes in oxidation state, where higher oxidation states generally lead to a shift in the K-edge toward higher energy reflecting an increased binding energy of the 1s electrons. For Co-2xm-Sav at pH 6, the edge jump is observed at ~ 7719 eV, consistent with a Co^{II} species.¹⁹ Upon addition of 100 eq of H_2O_2 and incubation for 60 min, the edge shifts to ~ 7721 eV, consistent with oxidation of the Co center to form Co^{III} (**Figure 3.7**). After 15 hours incubation with 100 eq H_2O_2 , the Co appears to remain in the Co^{III} oxidation state. In the pre-edge region (7704–7712 eV), a slight shift to higher energy with addition of H_2O_2 is observed, consistent with oxidation of Co^{II} to Co^{III} and may also suggest a change in coordination geometry of the Co center (**Figure 3.7 inset**). Analysis of the Extended X-ray Absorption Fine Structure (EXAFS) region beyond 7730 eV will aid in this determination and has not yet been completed. However, qualitative

changes in amplitude and oscillation frequency upon incubation with H_2O_2 can be observed upon visual inspection of the data (7730–7760 eV) are consistent with differences in ligand identity, the strengths of metal-ligands, or overall coordination geometry between the oxidized and unoxidized samples.

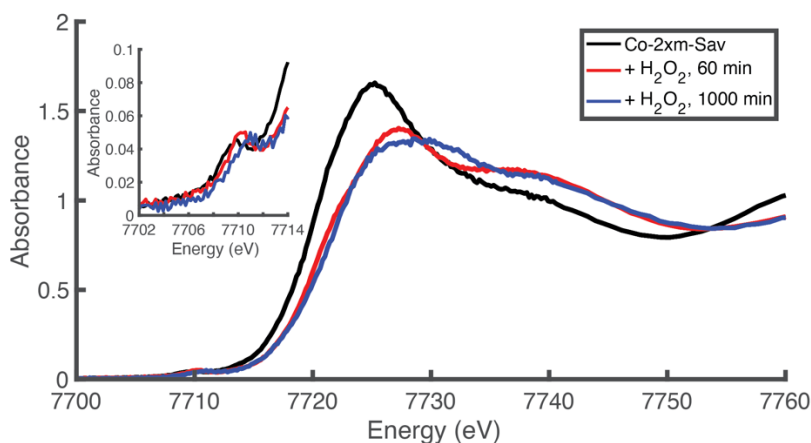


Figure 3.7. Normalized X-ray absorption spectra of Co-2xm-Sav at pH 6 with 100 eq. of H_2O_2 . The pre-edge region is emphasized in the inset.

Conclusions and Future Directions

This chapter describes progress towards preparing and characterizing a Co-oxygen adduct generated upon addition of H_2O_2 to engineered Co-containing proteins. Varying the pH as well as the primary and secondary coordination spheres surrounding the Co-cofactor influences the species formed and their rates of formation upon addition of H_2O_2 as an oxidant. Ongoing kinetic analysis on the formation of these absorbance features suggest that the reactions do not follow straightforward first- or second-order kinetics, indicating a more complex mechanism and is the subject of future investigations. Nevertheless, in Co-2xm-Sav, the species that is formed at pH 6 with 100 eq of H_2O_2 has absorbance features in the UV-vis region that are reminiscent of Co- O_2 adducts with ligand-to-metal charge transfer (LMCT) bands suggesting formation of a peroxide bound intermediate. These

features form and persist over 60 minutes, shifting to higher energies over 1300 minutes, reasonable time scales to allow for further interrogation by EPR and X-ray absorption spectroscopies. In Co-2xm-Sav, EPR spectroscopy reveals a high-spin Co^{II} signal that decreases to roughly 10% intensity upon addition of 100 eq H_2O_2 at pH 6, suggesting oxidation of Co^{II} to Co^{III} . Oxidation to Co^{III} was further corroborated by Co K-edge XAS where a shift both in the pre-edge and edge features to higher energy upon addition of H_2O_2 suggest structural changes upon oxidation. Ongoing analysis of the EXAFS region can provide information about local structure, including bond lengths, coordination number, or types of atoms involved in bonding. Further experimental studies are required to fully characterize the nature of the Co-oxygen species formed, including electronic structure and reactivity.

Resonance Raman (RR) is a powerful tool for probing the nature of Co species formed upon reaction with peroxide, as it selectively enhances vibrational modes associated with chromophoric metal-ligand interactions. When Co reacts with H_2O_2 , it can generate reactive intermediates such as $\text{Co}^{\text{II}}\text{-O}_2^{2-}$, $\text{Co}^{\text{III}}\text{-O}^\bullet$, or $\text{Co}^{\text{IV}}\text{=O}$, each of which exhibits characteristic vibrational signatures in the 700–900 cm^{-1} range. Additionally, these features shift to lower energy when ^{18}O isotopic substitution is used. In combination with experimental evidence, Density Functional Theory (DFT) and time-dependent DFT (TD-DFT) calculations can also provide detailed insights into the electronic structure, spin state, and bonding interactions of the Co-oxygen species.

Materials and Methods

Preparative Methods

All commercially available reagents were purchased of the highest purity and used as received. Acetonitrile, ethanol, and diethyl ether were degassed with argon and dried by vacuum filtration through activated alumina according to the procedure outlined by Grubbs.²⁰ Nanopure water was obtained from Millipore Super-Q Plus water purification system, 120 V, 4-stage purification system

with pump. Thin-layer chromatography (TLC) was performed on Whatman 250 μm layer 6 Å aluminum-backed silica plates and visualized using ultraviolet (UV) light. Silica gel chromatography was performed using Fisher reagent silica gel 60 (230-400 mesh) with indicated solvents. Biotin pentafluorophenol ester (biot-PFP)²¹ and di-(2-picolyl)amine (dpa)²² were prepared according to literature procedures.

¹H and ¹⁹F nuclear magnetic resonance (NMR) spectra were recorded at 500 and 400 MHz respectively. ¹H NMR spectra are reported in ppm on the δ scale and referenced to tetramethylsilane or solvent residual. The data are presented as follows: chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, quin=quintet, m=multiplet, br=broad), and integration.

Elemental analyses (carbon, hydrogen and nitrogen) were carried out using a Thermo Scientific FlashSart CHNS/O Elemental Analyzer (UC Irvine Materials Research Institute).

Preparation of cofactors. The ligand (biot-et-dpa) was prepared as published previously.²³ ¹H (500 mHz, DMSO-d₆) δ 8.48 (d, 2H), 7.76 (td, 3H), 7.53 (d, 2H) 7.25 (t, 2H), 6.40 (s, 1H), 6.33 (s, 1H), 4.29 (t, 1H), 4.12 (t, 1H), 3.78 (s, 4H), 3.20 (q, 2H), 3.08 (q, 1 H), 2.81 (dd, 1 H), 2.59 (s, 1H), 2.56 (t, 2H), 2.04 (t, 2H), 1.47 (m, 6H).

The Co-complex [Co^{II}(biot-et-dpa)(OH₂)₃]Cl₂ was prepared by addition of a solution of CoCl₂ (9.72 mg, 0.0749 mmol) in 10 mL ethanol to a solution of biot-et-dpa (31.9 mg, 0.0656 mmol) in 50 mL ethanol. The purple solution was allowed to stir for 6 h before concentrating the solution to ~1 mL followed by precipitation with diethyl ether. Purple solid was collected via filtration, washed with diethyl ether and dried under vacuum.

HR-MS (ESI, MeOH) m/z calcd for C₂₄H₃₂Cl₂CoN₆O₂S [M – (Cl)]⁺ 562.65. Found: 562.1.

Elemental Analysis for C₂₄H₃₈Cl₂CoN₆O₅S: C 44.18, H 5.87, N 12.88. Found: C 43.397, H 5.288, N 12.572.

Preparation of streptavidin variants. The construction of E101Q-K121A-S112E-Sav (denoted 2xm-S112E-Sav), E101Q-K121A-S112F-Sav (denoted 2xm-S112F-Sav), and E101Q-K121A-N49A-Sav (denoted 2xm-N49A-Sav) was achieved by site-directed mutagenesis (SDM) using the codon optimized E101Q-K121A-pET24a-Sav plasmid, the following primers, and Phusion polymerase (New England Biolabs).

S112E_fwd: 5'- CTGACCGAAGGCACCACCGAAGCAAATC-3'

S112E_rev: 5'- GGTGCCTTCGGTCAGCAGCCACTGGGTC-3'

S112F_fwd: 5'- CTGACCTTCGGCACCACCGAAGCAAATC-3'

S112F_rev: 5'- GGTGCCTTCGGTCAGCAGAACTGGGTC-3'

N49A_fwd: 5'- GTTGGTGCTGCAGAAAGCCGTTATGTTC-3'

N49A_rev: 5'- TTCTGCAGCACCAACTGCGCTTTCATAC-3'

SDM reaction mixtures were transformed into *Escherichia coli* bl21 cells followed by rescue with lysogeny broth (LB) media. The solution was spread aseptically onto LB/kanamycin agar plates and incubated overnight at 38 °C with shaking at 200 rpm. Of the overnight cultures, 600 µL was saved to prepare glycerol stocks to be stored in 25% glycerol at -80 °C. From the remaining liquid culture, plasmids were isolated using Qiagen Miniprep kit according to manufacturer's instructions. DNA sequencing was performed by Genewiz. Glycerol stocks described above were used to inoculate overnight liquid cultures for expression of Sav variants. Expression and purification of Sav variants was carried out as reported previously.²⁴

Protein Crystallization, X-ray Diffraction Data Collection and Processing

Crystallization of Co-Sav. Apo-Sav protein was crystallized by the sitting-drop vapor diffusion method. Diffraction-quality crystals were grown at ambient temperature and pressure by mixing 2.5 µL protein solution (26 mg/mL lyophilized 2xm-Sav in nanopure water) and 2.5 µL crystallization

buffer (2.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4). The droplet was equilibrated against a reservoir of 100 μ L crystallization buffer solution. Single crystals of Co-Sav were prepared by soaking apo-Sav crystals in a solution of 9 μ L soaking buffer (3.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4) mixed with 1 μ L of Co-complex (10 mM Co-complex, 100 mM CoCl_2 in nanopure water) overnight.

X-ray diffraction data collection and processing. X-ray diffraction data were collected at the Advanced Light Source (BL 8.2.1) or Stanford Synchrotron Radiation Lightsource (BL 9-2) at a wavelength of 1 Å. Diffraction images were integrated with software XDS²⁵ or iMOSFLM²⁶ and scaled with AIMLESS (CCP4 Suite).²⁷ For molecular replacement, Phaser-MR (PHENIX Suite)²⁸ was used with PDB: 2QCB with water molecules removed as the input model. Structural refinement was carried out in PHENIX.REFINE^{28,29} and COOT.³⁰ Restraints for biot-et-dpa (ligand ID: QG7) were created using eLBOW (PHENIX Suite). For water and solvent picking, electron density and structure visualization, the software COOT was used. Figures were drawn with PyMOL (Schrodinger, LLC Version 2.5.5). Crystallographic details, processing and refinement statistics are given in **Table 3.S1**.

Overall structures. Sav crystals soaked in metal complex solution constituted space group $I4_122$ with unit cell parameters reported in **Table 3S.1**. One Sav monomer was obtained per asymmetric unit after molecular replacement. Protein residues 2–11 and 135–159 of the N- and C- termini, respectively, were not resolved in electron density, presumably due to disorder.

General M-complex modelling. For all structures of Sav crystals soaked with M-complex, the following general observations were made: i) residual electron density in the $F_o - F_c$ map in the biotin-binding pocket, and ii) one peak in the anomalous density map in the biotin vestibule superimposed with the electron density peak. The residual electron density was fit with the M-complex which projected Co to the position of the strong anomalous density peak. The occupancy of Co was refined to 1.00 for Co-2xm-S112E-Sav or 0.88 for Co-2xm-Sav.

Physical Methods

Electronic Absorbance Spectroscopy. Electronic absorbance (UV-vis) spectra were recorded with an Agilent Cary 50 or Cary 60 UV-vis spectrophotometer in a 1 cm quartz cuvette. A solution of lyophilized Sav was prepared in nanopure water. This solution was diluted to 250 μM in MES buffer (50 mM, pH 6) or HEPES buffer (50 mM, pH 8), and five equivalents of Co-complex (1.25 mM) were added from 10 mM stock solution in nanopure water. Samples were separated from excess Co-complex and concentrated using Amicon Ultra 10K centrifugal filter devices and washed with 5X volume of nanopure water or buffer. The concentration of Co-Sav stock solution was determined based on $\epsilon_{\text{M}} = 167,760$ at $\lambda_{\text{max}} = 280$ nm and diluted to 250 μM in respective buffers.

Electron Paramagnetic Resonance Spectroscopy. Co-2xm-Sav was diluted to a final concentration of 250 μM (1 mM Co) in 100 mM MES buffer pH 6. The solution was aliquoted into two EPR tubes and either nanopure water or 100X stock solution of H_2O_2 was added. Solution was mixed and allowed to equilibrate for 60 minutes then flash-frozen in liquid nitrogen. Continuous wave (CW) X-band EPR spectra were recorded by Dr. Alec Follmer with assistance from Dr. Paul Oyala (CalTech EPR Facility Manager) on a Bruker EMX spectrometer equipped with a 4119HS-W1 cavity. Spectra were collected at 5 K, 2.1 mW, 9.5 GHz and averaged over 5 scans.

X-ray Absorption Spectroscopy. Co-2xm-Sav was diluted to a final concentration of 250 μM (1 mM Co) in 100 mM MES buffer pH 6 with 30% glycerol. The solution was aliquoted into three 500 μL portions and either nanopure water or 100X stock solution of H_2O_2 was added. Solution was mixed and allowed to incubate for indicated time then flash frozen in liquid nitrogen. Co K-edge X-ray absorption spectra were collected by Dr. Alec Follmer on SSRL beam line 9-3 using a 100-element Ge monolith solid-state detector (Canberra) with a SPEAR3 storage ring current of ~ 500 mA at an energy of 3.0 GeV. The beam line 9-3 optics consist of a flat, bent, harmonic rejection vertically collimating Rh-coated Si M0 mirror as well as a cylindrical, bent, Rh-coated focusing M1 mirror. Co

K-edge scans were taken between 7600 and 8500 eV at ~10 K using an Oxford Instruments CF1208 continuous flow liquid helium cryostat using an open-cycle liquid He dewar (BL 9-3). A Co foil was placed in the beam pathway prior to the ionization chamber I_0 and scanned concomitantly for an energy calibration. A Soller slit with a 3 μ m Fe filter was used to increase the signal to noise ratio of the spectra.

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Supplementary Information

Table 3.S1. X-ray crystallography data processing and refinement statistics

Sav Variant	2xm-S112E	2xm
Cofactor	Co-et-dpa	Co-et-dpa
PDB Code	Pending Submission	Pending Submission
Data Processing		
Unit Cell (a, b, c, α, β, γ)	57.69 Å, 57.69 Å, 183.402 Å, 90°, 90°, 90°	57.57 Å, 57.57 Å, 183.77 Å, 90°, 90°, 90°
Space Group	I4 ₁ 22	I4 ₁ 22
Resolution (Å)	41.96 – 1.9	41.95 – 1.5
Highest resolution shell (Å)	1.968 – 1.9	1.55 – 1.5
R-merge (%)	0.07 (0.60)	0.80 (4.69)
No. unique reflections	12673 (1191)	25345 (2486)
Multiplicity	11.5 (7.4)	199.0 (185.0)
Mean I/sig(I)	23.68 (4.08)	29.61 (2.26)
Completeness	99.58 (96.11)	99.76 (100.00)
CC(1/2)	0.999 (0.894)	0.989 (0.671)
Structure Refinement		
R-work	0.1691 (0.3174)	0.1833 (0.2432)
R-free	0.1924 (0.3030)	0.2131 (0.2641)
RMSD bond length (Å)	0.017	0.012
RMSD bond angle (°)	1.34	1.21
Avg B-factor (Å²)	27.99	17.95
Macromolecules	26.77	16.46
Ligands	29.95	33.34
Solvent	36.75	26.37
Occupancy of Metal Ion	1.00	0.87

CHAPTER 4

A Series of Engineered Artificial Metalloproteins to Model Structure and Function of Native Quercetin Dioxygenase Enzymes

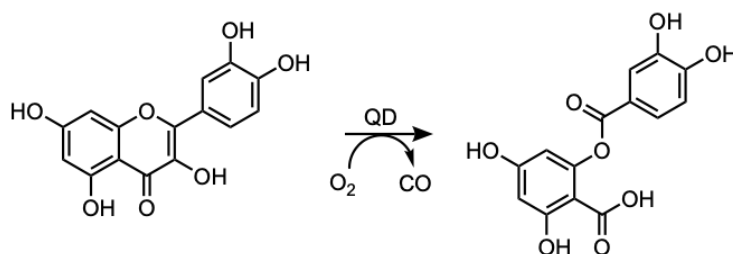
Preface

I learned from previous work in the group that our Fe-ArMs posed many challenges which had been the focus of multiple PhD projects before me. When I joined the Borovik group in the late stages of my third year, I was eager to build on past work while finding ways to connect it with my new research interests. My background had led me to a strong curiosity about oxidative transformations of (poly)aromatic compounds in biological systems, particularly the role of dopamine oxidation in Parkinson's disease—a topic that will be explored further in Chapter 5. I was drawn to the idea of engineering ArMs capable of catalyzing dopamine oxidation, or as Andy succinctly put it: “make stuff that does stuff.” The journey that shaped this chapter began unexpectedly, with a striking lime-green protein crystal that resulted from soaking an Fe-ArM in dopamine solution. Despite my best efforts, I was unable to solve its structure, forcing me to reconsider my approach. This setback led me to explore catechol dioxygenase enzymes, which naturally catalyze similar oxidative reactions. As I delved deeper, I encountered acireductone dioxygenases (ARD)—first introduced in Chapter 1. However, accessing the ARD substrate proved to be a major challenge due to its synthetic and commercial inaccessibility. This difficulty prompted yet another pivot in my research, leading me to investigate quercetin dioxygenases (QD). What excited me most about QD was their dual significance: not only do they provide a model system for understanding metal-ion-dependent dioxygen activation, but they also play a role in microbial defenses against antimicrobial flavonoids. This connection between enzymatic mechanism and microbial survival aligned with my interests and the detailed mechanistic studies for which the Borovik group is well known. Thus, my work evolved from an initial interest in Fe-ArMs to a broader investigation that will be discussed in this chapter.

Introduction

Flavonoids constitute a diverse class of polyphenolic compounds that have broad bioreactivity and implications for human health. For example, quercetin is a plant flavonoid that has recently gained attention for its antioxidant and anti-inflammatory properties.^{1,2} It has also demonstrated antimicrobial activity via various mechanisms including cell membrane damage, inhibition of synthesis of nucleic acids and proteins, disruption of mitochondrial function, and preventing formation of biofilms.^{3–5} As such, several microbes have evolved mechanisms of degrading quercetin.

Scheme 4.1. Reaction catalyzed by Cu-QD: dioxygenation of quercetin substrate to yield 2-protocatechuoyl-phloroglucinol carboxylic acid and carbon monoxide



Quercetin degradation is catalyzed by bacterial and fungal flavonol 2,4-dioxygenases (quercetin dioxygenases, QD) that activate dioxygen for the oxidative cleavage of quercetin to form 2-protocatechuoyl-phloroglucinol carboxylic acid with concomitant release of carbon monoxide (**Scheme 4.1**).^{6–11} The underlying mechanisms by which QD enzymes activate dioxygen remain largely unresolved. Most of the well-studied metalloenzymes that activate dioxygen utilize redox-active iron or copper ions to form reactive metal-superoxo and -peroxo intermediates for the oxidation of their organic substrates.^{12–14} However, dioxygen-activating enzymes like QD fall into a class of cambialistic metalloenzymes, where there is still significant debate around the nature of the intermediates that form during reactivity.^{15–17} This debate is, in part, due to the fact that despite sharing a conserved protein host, cambialistic enzymes can incorporate different metal ions to perform their selective chemistry and display altered levels of activity depending on the metal ion identity, suggesting multiple potential

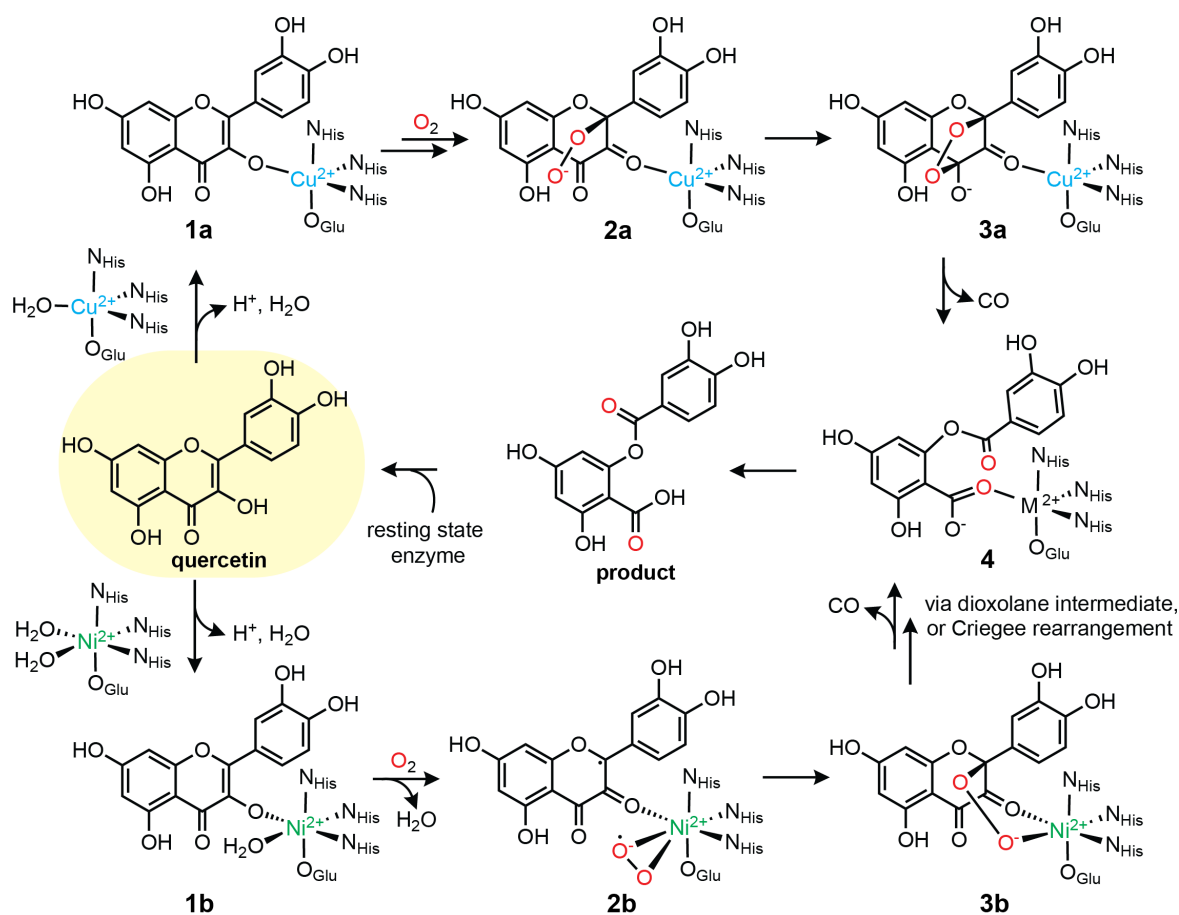
catalytic mechanisms. Furthermore, while comparison of kinetic parameters of recombinantly expressed metal-substituted QD variants have informed on the catalytic efficiencies of different metal ions, the naturally occurring metallocofactors are yet to be identified.¹⁸ Together these ambiguities make mechanistic consensus challenging to achieve. Across all known QD enzymes, however, there are unifying similarities. Specifically, the structures of several QD proteins show that, regardless of the identity of the metal ion within the active site, it is facially coordinated by three histidine residues and one carboxylate residue.^{19–23} The remaining coordination site(s) are occupied by water molecule(s).

QD isolated from filamentous fungi *Aspergillus* contains a type-2 Cu^{II} center.^{19,24–26} In contrast, related bacterial enzymes have also been identified in *Bacillus subtilis*^{10,27} and *Streptomyces* sp. FLA.¹¹ Recombinantly expressed bacterial QDs from *B. subtilis* or *Streptomyces* have been reconstituted to contain several different divalent transition metal centers, where Mn^{II} and Co^{II}, or Ni^{II} and Co^{II} metallocofactors were found to be the most active, respectively.^{28–32} With respect to their mechanisms, the catalytic cycle of fungal Cu-QD is initiated by binding of flavonolate substrate. The Cu-flavonolate complex is suggested to then undergo a valence tautomerism to form a Cu–flavonoxy radical, where nucleophilic attack by dioxygen occurs at the flavonoxy radical (**Scheme 4.2**, 2a).^{23,33–39} Finally, the oxidized depside product, 2-(3,4-dihydroxybenzoyloxy)-4,6-dihydroxybenzoate, is formed with concomitant release of carbon monoxide (CO). In bacterial QD systems, on the other hand, it was suggested that substrate binding is followed by dioxygen binding directly to the metal center to form a M²⁺–superoxide species (**Scheme 4.2**, 2b).^{40–51} Here, the metal center acts as a conduit for electron transfer from substrate to dioxygen, retaining a formal M²⁺ oxidation state.

Mechanistic comparisons between these enzymes are complicated by the fact that, despite their common function and conserved primary coordination sphere, QD enzymes exhibit some degree of selectivity with respect to their metal ion selectivity and activity. For example, Cu^{II} substitution in *Streptomyces* QD does not yield functional enzyme, as is the case for Fe^{II} or Zn^{II} substitution into QD

from *B. subtilis* or *Streptomyces*, suggesting an additional role for metal ion selectivity between enzymes.^{28,29,32} Of the active variants of bacterial QD, metal-substitution is not reported to change the final products generated from reaction with quercetin, but there is significant debate regarding the roles of these metal ions, where the mechanism may be dependent on the specific redox properties of the metal. Mechanistic studies are further challenged due to the transient nature of intermediate species and varying levels of metal ion incorporation into native enzymes.^{18,29}

Scheme 4.2. Proposed mechanisms for Cu-QD (top) and Ni-QD (bottom). Intermediates 2a and 2b highlight the differences in mechanisms depending on the metal center, where O₂ binds at the flavonoxyl radical or the metal center, respectively.



One strategy to gain insight into the mechanistic underpinnings of dioxygen-activating enzymes is through the design and interrogation of synthetic metal complexes with similar reactivity to

metalloproteins. While these studies have provided some understanding of native QDs, synthetic model complexes often lack several key structural features present in proteins and are not compatible with aqueous environments.^{38,39,43,44,46–50,52–57} To address this gap, this chapter describes the development a series of artificial metalloproteins (ArMs) to mimic the active sites of QD enzymes. Using a biotin-streptavidin (biot-Sav) construct, a synthetic biotinylated metallocofactor was prepared and then introduced to Sav, leveraging the high binding affinity of Sav for biotin. This construct allows for systematic incorporation of select metal ions, as well as precise positioning of metal centers within the protein host. This construct provides the opportunity for direct comparisons to be made between the structures and activities of different metal ions in a single engineered protein host designed to emulate specific aspects of native QD active sites and improve our understanding the mechanisms of O₂ activation and substrate oxidation in biology.

Results and Discussion

Design and Preparation of ArMs

As discussed in Chapter 1, the biot-Sav construct serves as an effective platform for the development of ArMs. Our group has previously reported the preparation and structure of an Fe^{II}-containing ArM with three point mutations made to the Sav protein, [Fe^{II}(biot-et-dpa(OH₂)₂(κ^1 -O_{E112}))C2xm-S112E-Sav]. To increase protein expression yields and prevent unwanted interactions with the cofactor, the mutations E101Q and K121A (referred to as 2xm) were made. An additional mutation S112E was made to provide an endogenous carboxylate residue to coordinate the metal center. This ArM will be referred to as Fe^{II}-2xm-S112E-Sav (**Figure 4.1**). This work motivated the development of new ArMs that incorporate Co^{II}, Ni^{II}, Cu^{II}, or Zn^{II} ions to model the active sites of QDs. The metal ions were incorporated into the biotinylated bis(2-pyridylmethyl)amine (biot-et-dpa) ligand to position the metal center in close proximity to Sav residue S112E.

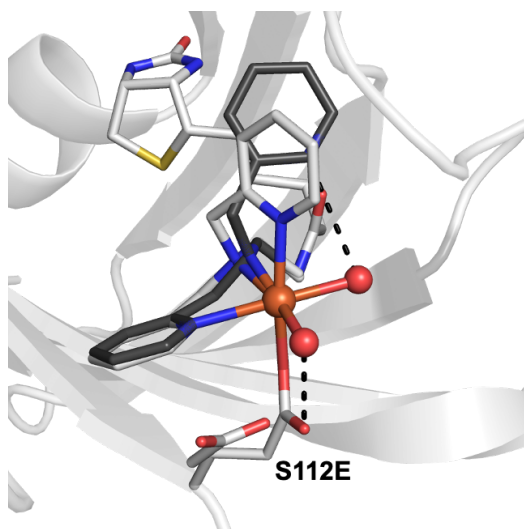


Figure 4.1. Molecular structure of $[\text{Fe}^{\text{II}}(\text{biot-et-dpa}(\text{OH}_2)_2(\kappa^1\text{-O}_{\text{E112}}))\subset 2\text{xm-S112E-Sav}]$, referred to as $\text{Fe}^{\text{II}}\text{-2xm-S112E-Sav}$ (PDB: 6UIY). The protein is displayed as a cartoon and the cofactor and S112E as sticks. The dpa ligand is modelled in two conformations. Fe is colored in orange and water molecules are represented as red spheres. Hydrogen bonds are suggested by black dashed lines.

The biot-et-dpa ligand was prepared via a three-step route from dpa as previously reported.⁵⁸ The Cu^{II} -biot-et-dpa complex was prepared as described in Chapter 3. The metallocofactors Co^{II} -biot-et-dpa, Ni^{II} -biot-et-dpa, and Zn^{II} -biot-et-dpa complexes were prepared by treating biot-et-dpa with 1.1 equivalents of the corresponding metal chloride salts in ethanol and stirring at room temperature for 4 hours. The solutions were concentrated by rotary evaporation followed by precipitation with diethyl ether. Solid was collected via filtration. Attempts to prepare and isolate Mn^{II} -biot-et-dpa were unsuccessful due to low incorporation of Mn. The ArMs were prepared by addition of a solution of excess metal cofactor to a solution of Sav in potassium phosphate buffer (KPi 50 mM, pH 6.9). Samples were separated from excess metal co-factor and concentrated using Amicon Ultra 10K centrifugal filter devices and washed with 5X volume of buffer.

Structural Characterization of ArMs by X-ray Diffraction

X-ray diffraction (XRD) crystal structures of native QDs have informed on key mechanistic steps, so I first sought to prepare and structurally characterize the ArMs for comparison to the structures of

QDs. Single crystals of Co^{II}-2xm-S112E-Sav, Ni^{II}-2xm-S112E-Sav, Cu^{II}-2xm-S112E-Sav, and Zn^{II}-2xm-S112E-Sav were prepared by the previously described soaking method^{59,60} and diffracted to 1.9 Å (Co) and 1.4 Å (Ni, Cu, Zn). In Cu-2xm-S112E, the Cu-center is in a distorted trigonal bipyramidal geometry, similar to the geometry of one conformation of the resting state of Cu-QD from *A. japonicus* (**Figure 4.2 A**, PDB: 1JUH).³⁵ In both Cu-2xm-S112E-Sav and Cu-QD, the Cu-center is coordinated by O atoms from a water molecule and a glutamate residue bound in a κ^1 fashion. In the resting state of native Cu-QD, the carboxylate coordination represents a minor species, indicating some flexibility in this residue that is diminished upon substrate binding.¹⁹ For the Cu ArM, the remaining coordination sites are occupied by three N atoms from the dpa ligand, which is coordinated in a meridional fashion. In the major species of Cu-QD, the remaining coordination sites are also occupied by three N atoms, but the three His residues coordinate in a facial manner (**Figure 4.2 B** and **Tables 4.S1** and **4.S2**).

Even more striking are the similarities between the active sites of Ni-QD from *Streptomyces* (**Figure 4.2 C**, PDB: 5FLH)⁴⁰ and Ni-2xm-S112E-Sav (**Figure 4.3 D** and **Tables 4.S1** and **4.S3**). In both structures, the Ni-center is in a pseudo-octahedral geometry, where Ni is facially coordinated by 3 N atoms, a glutamate residue bound in a κ^1 fashion, and two *cis*-O atoms from water molecules. The coordinated water molecules also participate in hydrogen bonding (H-bonding) with the glutamate residue or surrounding water molecules. Although the structures of native Co-QD or Zn-QD have not been reported, I prepared and determined the structures of Co-2xm-S112E-Sav and Zn-2xm-S112E-Sav, which exhibit similar metal ion coordination geometries to that found in Ni-2xm-S112E-Sav (**Figure 4.S1** and **Tables 4.S1** and **4.S3**).

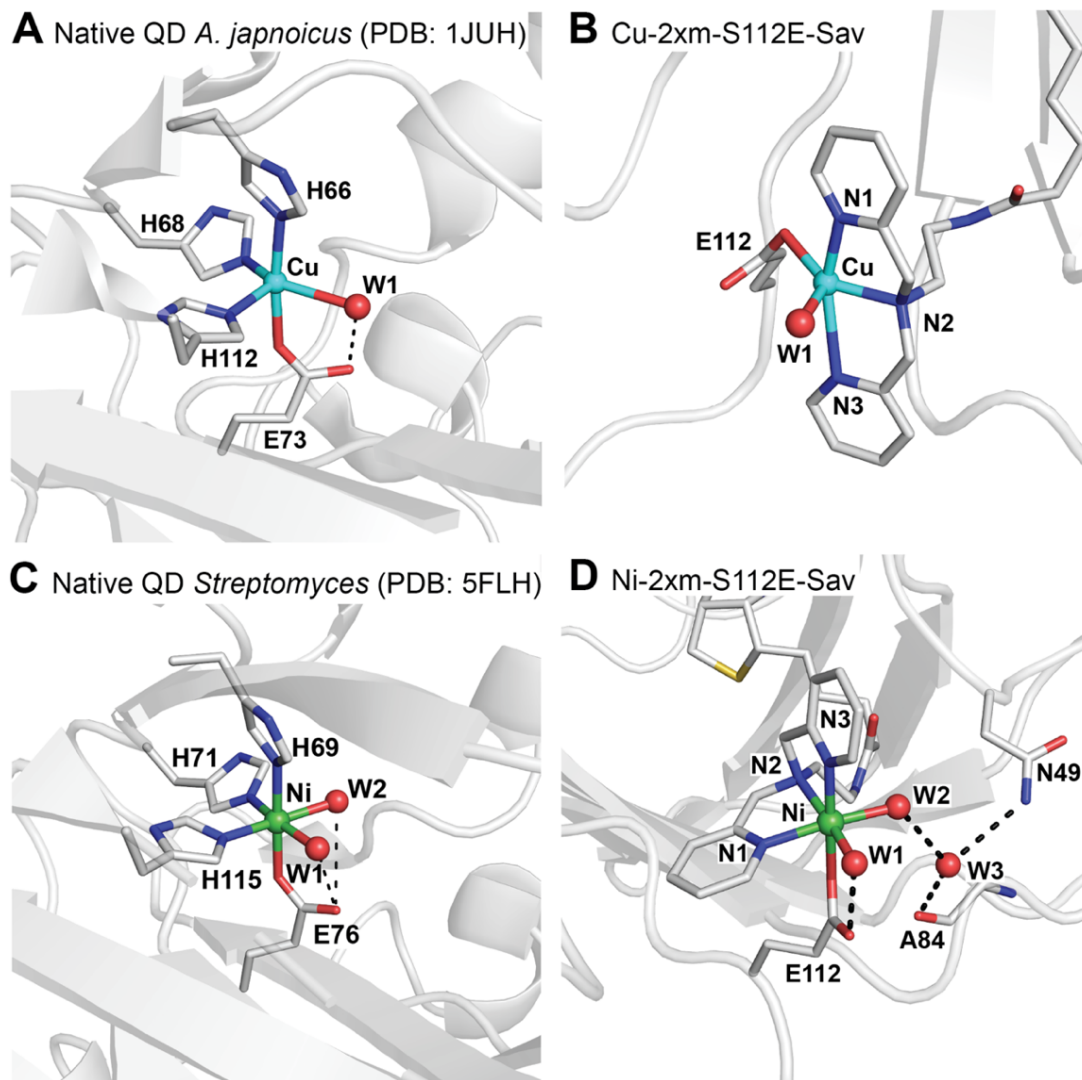


Figure 4.2. Comparison of ArMs and resting-state structures of native QDs. The protein is displayed as a cartoon and the cofactor, including ligated residues, as sticks. Cu is colored in cyan, Ni in green and water molecules are represented as red spheres. Hydrogen bonds are suggested by black dashed lines. (A) Resting-state active site of Cu-QD from *Aspergillus japonicus* (PDB: 1JUH) (B) Molecular structure of Cu-2xm-S112E-Sav. (C) Resting-state active site of Ni-QD from *Streptomyces* sp. FLA recombinantly expressed in *E. coli* BL21 (PDB: 5FLH). (D) Molecular structure of Ni-2xm-S112E-Sav.

In all ArMs employing the 2xm-S112E-Sav variant, the coordinated water molecules were positioned within a solvent accessible pocket and engaged in a hydrogen bonding (H-bonding) network of well-ordered solvent molecules within the active site, so I reasoned that these exposed sites could be accessed by small-molecule substrates. We also know from previous work about the importance of N49 in the stabilization of these second sphere H-bond networks on intermediate

stabilization.⁵⁹ I, therefore, reasoned that the effects of the secondary coordination sphere H-bonding could also impact substrate binding. So, an additional Sav variant, 2xm-N49A-S112E-Sav, was prepared with the idea that disruption of the H-bonding network surrounding the aqua ligands could increase the likelihood of substitution by substrate (**Figure 4.3** and **Table 4.S4**). Molecular structures of these variants support this suggestion because in addition to disrupting the H-bonding network, the B-factors, or atomic displacement parameters (ADPs), of the coordinated water molecules in the 2xm-N49A-S112E-Sav variant are increased relative to those in 2xm-S112E-Sav (**Table 4.1**). This increase in B-factors indicates that the aqua ligands exhibit a greater degree of thermal disorder when the H-bonding network is disrupted. The 2xm-N49A-S112E-Sav variant was therefore used for binding studies discussed in the following section.

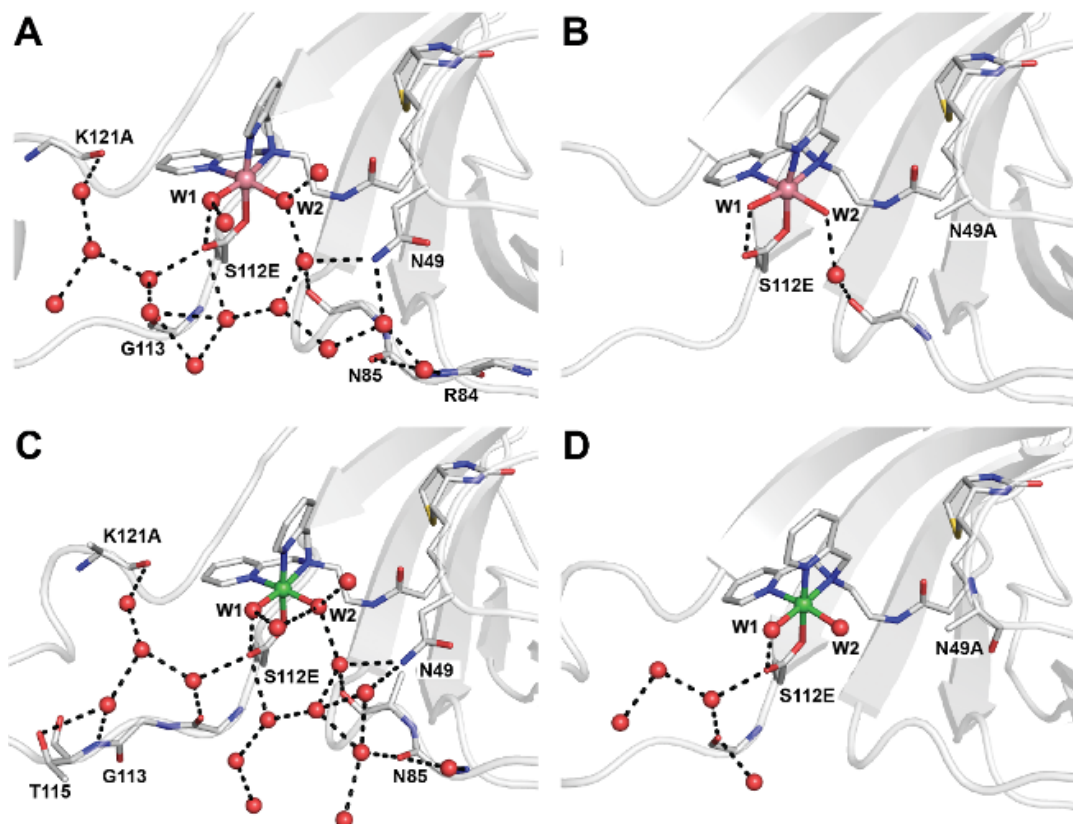


Figure 4.3. Molecular structures of (A) Co-2xm-S112E-Sav, (B) Co-2xm-N49A-S112E-Sav, (C) Ni-2xm-S112E-Sav, and (D) Ni-2xm-N49A-S112E-Sav. The protein is displayed as a cartoon and the cofactor, including ligated residues, as sticks. Residues involved in H-bonding are also displayed as sticks. Co is colored in pink, Ni in green and water molecules are represented as red spheres. Hydrogen bonds are suggested by black dashed lines.

Table 4.1. Comparison of resolution and B-factors of coordinated water molecules for XRD crystal structures.

Sav Variant	Resolution (Å)	B-Factor of W1 (Å ²)	B-Factor of W1 (Å ²)
Co-2xm-N49A-S112E-Sav	1.4	26.4	31.45
Co-2xm-S112E-Sav	1.9	26.6	27.5
Ni-2xm-N49A-S112E-Sav	1.4	27.2	23.0
Ni-2xm-S112E-Sav	1.4	23.2	21.7

Maltol Binding Measured by Isothermal Titration Calorimetry

In native QD enzymes, the regiospecific binding of quercetin to the metal center is facilitated by electrostatic interactions surrounding the active site metallofactor (**Figure 4.4**).^{35,40} Without these interactions to position quercetin to coordinate the metal center via the 3-hydroxy O atom, quercetin may also chelate metal ions between the 3',4'-hydroxy groups or between 5-hydroxy and 4-carbonyl group. To mitigate this possibility, the substrate maltol was chosen as a substrate mimic for quercetin, where alternative binding modes are eliminated. Maltol consists of the γ -pyrone ring found at the core of the native substrate quercetin (**Figure 4.5**) and has been reported to form stable chelates with transition metal ions such as iron and copper and is less prone to autooxidation than flavones, which can complicate substrate binding studies.^{61,62}

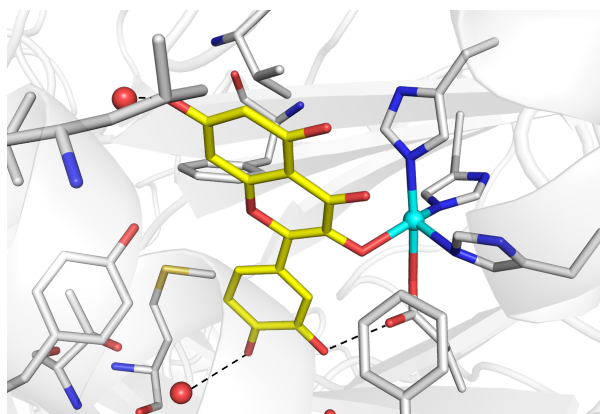


Figure 4.4. Anaerobic quercetin-bound active site of Cu-QD from *A. japonicus* (PDB: 1H1I). The protein is displayed as a cartoon and the Cu-cofactor as well as coordinated residues H66, H68, E73, and H112, as well as secondary sphere residues as sticks. Cu is colored in cyan and water molecules are colored as red spheres. The substrate quercetin is displayed in yellow sticks and is positioned in the active site by H-bonding interactions, shown in black dashed lines, and electrostatic interactions with the secondary sphere residues shown in sticks.

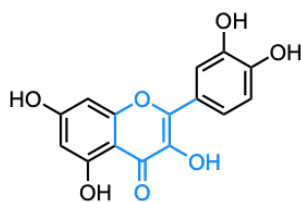


Figure 4.5. Chemical structure of quercetin, a naturally occurring flavonoid and the substrate for QD, showing the characteristic hydroxyl groups positioned at the 3, 3', 4', 5, and 7 positions on the flavonoid skeleton. The γ -pyrone ring that is maltol is highlighted in blue.

To study the binding of maltol to Co-, Ni-, and Cu- 2xm-N49A-S112E-Sav, isothermal titration calorimetry (ITC) was employed. Across the metal-substituted 2xm-N49A-S112E-Sav variants, the dissociation constants, K_d values, were determined to be 72.7 μM for Co, 3.57 μM for Ni, and 315 μM for Cu (**Table 4.2** and **Figure 4.S2**). To my surprise, the binding stoichiometry for Co- and Ni- 2xm-N49A-S112E-Sav was determined to be two molecules of maltol per metal site, while Cu-2xm-N49A-S112E-Sav was fit to one molecule of maltol per metal site (**Table 4.2** and **Figure 4.S2**). I reasoned that the binding stoichiometry could be altered by mutating amino acid residues surrounding the metal center through noncovalent interactions with the substrate. Comparison of ITC binding studies between Ni-Sav variants with other point mutations supported this notion and revealed that Ni-S112E-Sav also binds two molecules of maltol per Ni site, while Ni-2xm-S112E-Sav binds only one molecule of maltol per metal site, with a K_d of 1.09 μM (**Table 4.3** and **Figure 4.S3**). While consistent with the original proposal that residues in the second sphere, like N49, could impact substrate binding, the results of the ITC measurements drove me to return to the use of 2xm-S112E-Sav variant for further substrate binding studies because of the 1:1 binding stoichiometry.

Table 4.2. Comparison of dissociation constants and binding stoichiometries for maltol between metal-substituted Sav.

Sav Variant	K_d Trial 1 (μM)	K_d Trial 2 (μM)	K_d Trial 3 (μM)	K_d Average (μM)	Binding Sites
Co-2xm-N49A-S112E-Sav	42.7	98.0	72.8	72.7 ± 12.0	2
Ni-2xm-N49A-S112E-Sav	4.04	1.26	5.42	3.57 ± 1.22	2
Cu-2xm-N49A-S112E-Sav	315	--	--	--	1

Table 4.3. Comparison of dissociation constants and binding stoichiometries for maltol between Ni-Sav mutants.

Sav Variant	K_d Trial 1 (μM)	K_d Trial 2 (μM)	K_d Trial 3 (μM)	K_d Average (μM)	Binding Sites
Ni-et-dpa (no Sav)	15.4	7.75	12.2	11.8 ± 2.2	1
Ni-S112E-Sav	13.9	23.2	12.9	16.7 ± 3.3	2
Ni- 2xm -S112E-Sav	0.992	1.17	1.11	1.09 ± 0.05	1
Ni-2xm- N49A -S112E-Sav	4.04	1.26	5.42	3.57 ± 1.22	2

Quercetin Binding

With the success of the maltol binding experiments and identification of a Sav variant that exhibited 1:1 binding stoichiometry, I wanted to identify whether our ArMs could bind the native substrate, quercetin. However, the sensitivity of quercetin and related flavone compounds to autooxidation precluded the ability to study binding by ITC in our current configuration. Fortunately, flavone compounds exhibit two major absorption bands in the UV-vis region, band I (300–380 nm, attributed to ring B, cinnamoyl motif, **Figure 4.6**) and band II (240–295 nm, attributed to ring A, benzoyl motif, **Figure 4.6**).⁶³ Flavones which contain a 3-hydroxyl group can form metal chelates involving the carbonyl at position 4, resulting in a bathochromic shift in band I.^{64,65} Flavones which contain a 5-hydroxyl group may form metal chelates involving the carbonyl at position 4, resulting in a bathochromic shift in band II;^{64,65} however, band II is obscured by the strong absorption from the protein aromatic residues.

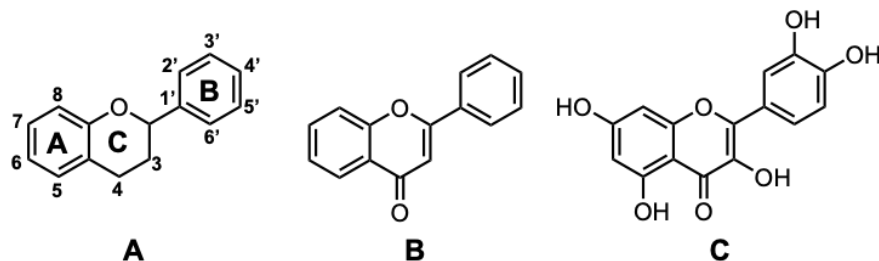


Figure 4.6. (A) Chemical structure and labeling scheme of flavonoid compounds. (B) Generic chemical structure of flavones. (C) Chemical structure of quercetin, a naturally occurring flavone and the substrate for QD.

The bathochromic shift observed on mixing M^{2+} -Sav with quercetin allowed for determination of the binding constants. In 10% methanol, 50 mM KPi pH 6.9, quercetin band I is observed at 372 nm which shifts to 446 nm upon addition of Cu-2xm-S112E-Sav. While standard binding curves exhibiting 1:1 binding stoichiometry are fit to **Equation 4.1a**, titration of Cu-2xm-S112E-Sav into a solution of quercetin resulted in a saturation binding curve that was fit according to **Equation 4.1b** to determine the dissociation constant, K_d , of 94 μ M (**Figure 4.7** and **Table 4.S5**). **Equation 4.1b** models a one-site binding model that includes a nonspecific binding component. This nonspecific component (NS) accounts for the overlapping absorption that comes from the growth of the Cu d-d band as Cu-2xm-S112E-Sav is titrated into the solution. For native Cu-QD isolated from *A. flavus*, which contains two Cu sites, anaerobic spectral titration of enzyme into quercetin revealed that two moles of quercetin were bound per mole of enzyme, consistent with a 1:1 stoichiometry, and K_d was extrapolated to be ~ 30 μ M, on the same order of magnitude as Cu-2xm-S112E-Sav.⁶⁵ Titration of Co-, Ni-, or Zn- 2xm-S112E-Sav into a solution of quercetin resulted in no shift of band I, preventing the determination of the K_d for these ArMs (**Figure 4.S4**). We note that it is also possible that these ArMs bind quercetin at a different position, which would not result in the same shift in band I.

Equation 4.1a. $Y = B_{max} * X / (K_D + X)$

Equation for the one-site binding model, where B_{max} is the maximum specific binding, K_D is the equilibrium dissociation constant, Y is the fraction of sites occupied by the substrate, and X is the concentration of the substrate.

Equation 4.1b. $Y = [B_{max} * X / (K_D + X)] + NS * X$

Equation for the one-site binding model with nonspecific binding component, where B_{max} is the maximum specific binding, K_D is the equilibrium dissociation constant, NS is slope of nonspecific binding component, Y is the fraction of sites occupied by the substrate, and X is the concentration of the substrate.

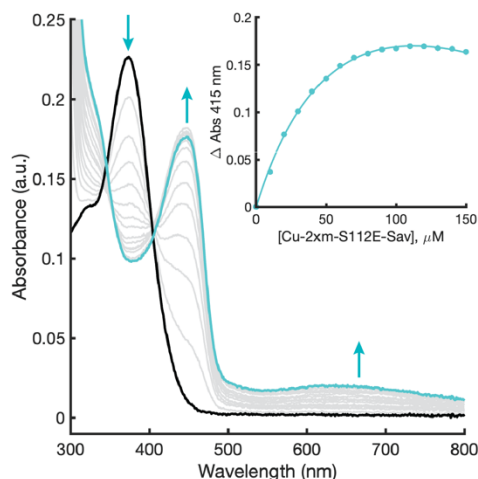


Figure 4.7. Representative UV-vis titration of Cu-2xm-S112-Sav into quercetin in KPi pH 7. Initial quercetin spectrum shown in black, titration of 10 μM aliquots of ArMs shown in grey and final spectrum after addition of 200 μM Cu-2xm-Sav shown in cyan.

Flavonol Binding: Comparison Between Metal-Substituted ArMs

As mentioned previously, in contrast to QD, the 2xm-S112E-Sav ArMs lack the residues surrounding the metal site that help position quercetin to coordinate via the 3-hydroxy O atom. To encourage binding at this position in the 2xm-S112E-Sav ArMs, the substrate 3-hydroxyflavonol (flav, **Figure 4.8**) was selected as a more faithful mimic of quercetin, but with potential limited binding modes. Both flav and quercetin are flavone compounds that have been used in studies that model QD activity.

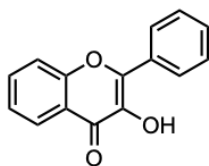


Figure 4.8. Chemical structure of 3-hydroxyflavone (flav)

In methanol, flav is reported to exhibit three absorption bands (given as λ_{max} , nm (ϵ_{M} , $\text{M}^{-1} \text{cm}^{-1}$): 239 (16200), 305 (10400), and 344 (14800). Flavonoid-metal complexes were first reported with aluminum ions, where addition of AlCl_3 to flav resulted in decrease of band I at 344 nm and increase

in a new band at 402 nm.⁶⁶ More recently, flav has also been reported to chelate Co^{2+} at pH 7.5 resulting in a decrease of band I at ~ 345 nm and an increase in a new band at ~ 420 nm.⁶⁷

I found that in 10% methanol at pH 6.9, flav exhibits three absorption bands 243 (13700), 312 (8680) and 343 (10700). Upon addition of M^{2+} -2xm-S112E-Sav to a solution of flav, a decrease in the absorption band at 342 nm, and an increase in a new band (denoted $\lambda_{\text{M-flav}}$) is observed. The absorption maximum is dependent on the metal complex (Co 415 nm, Ni 430 nm, Cu 411 nm, and Zn 400 nm) suggesting flav was binding to metallocofactors in each ArM at the 3-hydroxy position. Increasing amounts of M^{2+} -2xm-S112E-Sav were titrated into a 10 μM solution of flav, and the increase in $\lambda_{\text{M-flav}}$ was monitored to determine a dissociation constant, K_d , for flav binding (**Figure 4.9** and **Tables 4.S6–S10**). For Ni-2xm-S112E-Sav, the saturation binding curve was modelled a one-site binding model according to **Equation 4.1a**, and K_d was determined to be $36.59 \pm 6.98 \mu\text{M}$. For Cu^{2+} -2xm-S112E-Sav, the saturation binding curve was modelled according to **Equation 4.1b** as a one-site binding model with a non-specific binding component to account for the overlapping absorption due to the growth of the Cu d-d band upon titration of the Cu-2xm-S112E-Sav. The K_d for flav binding to Cu^{2+} -2xm-S112E-Sav was determined to be $31.90 \pm 5.60 \mu\text{M}$. The K_d for Zn^{2+} -2xm-S112E-Sav could not be determined under these conditions because the shift in band I was not observed but can be extrapolated to be much larger than for Ni- or Cu- 2xm-S112E-Sav (**Figure 4.9**). As the binding affinity of flavonoid compounds for native or recombinantly expressed Zn-QD has not been reported, this result offers a plausible explanation for the lack of Zn-QD activity—if the flavonoid substrate does not bind to the metal center, then the catalytic cycle will not be initiated.

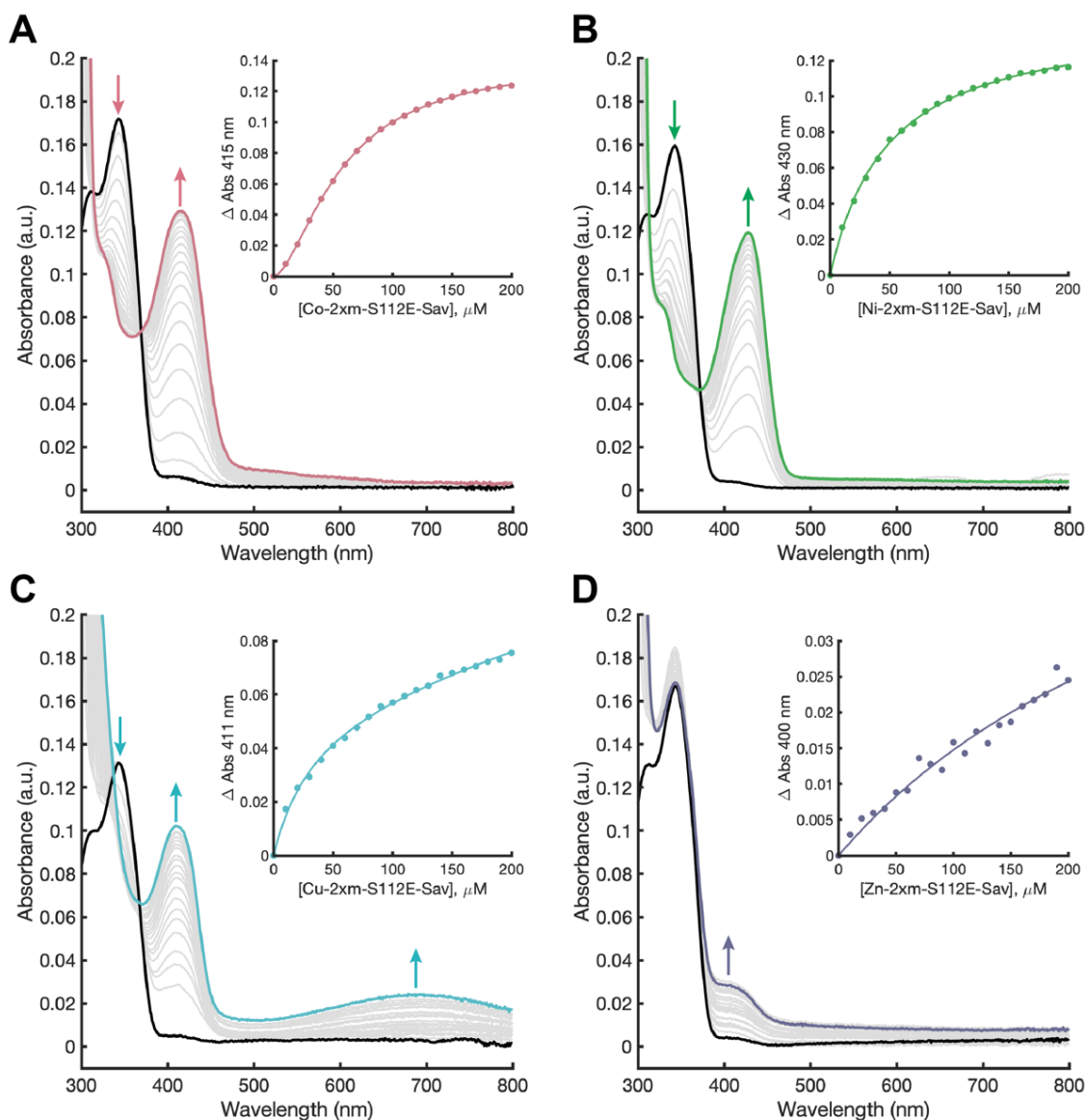


Figure 4.9. Representative UV-vis titrations of ArMs into flav in KP pH 7 and simulated binding curves (insets). Initial flav spectra shown in black, titration of 10 μM aliquots of ArMs shown in grey and final spectra after addition of 200 μM ArMs shown in colors. **(A)** Titration of Co-2xm-S112E-Sav into flav. **(B)** Titration of Ni-2xm-S112E-Sav into flav. **(C)** Titration of Cu-2xm-S112E-Sav into flav. **(D)** Titration of Zn-2xm-S112E-Sav into flav.

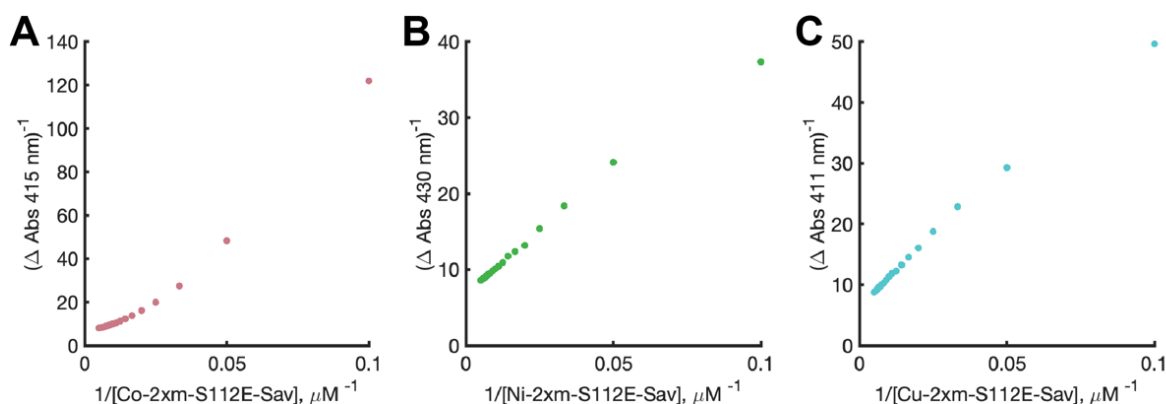


Figure 4.10. Double-reciprocal plots of UV-vis titrations of ArMs into flav in KPi pH 7. **(A)** Titration of Co-2xm-Sav into flav. **(B)** Titration of Ni-2xm-Sav into flav. **(C)** Titration of Cu-2xm-Sav into flav.

Of note, K_d for flav and Ni- or Cu-2xm-S112E-Sav were determined from spectral titrations performed open to atmosphere. Under anaerobic conditions, no significant differences in flav binding were observed for Ni- or Cu- 2xm-S112E-Sav. In contrast, anaerobic titration of Co-2xm-S112E-Sav into a solution of flav resulted in the formation of a precipitate, which redissolved in the cuvette upon exposure to air. While this result is not fully understood, it points to a potential Co/dioxygen interaction that may mediate flav binding. Further examination of the double reciprocal plots of the aerobic titrations exhibits significant deviations from linearity, potentially indicating some form of cooperativity in the binding of flav to Co-2xm-S112E-Sav (**Figure 4.10**). Thus, the binding curve for Co^{II} -2xm-S112E-Sav was best fit according to **Equation 4.1c** which represents a one-site binding model with a Hill coefficient. The Hill coefficient is applied phenomenologically—it does not inherently explain any underlying mechanism of the observed behavior. It instead allows us to account for complex interaction between Co^{II} -2xm-S112E-Sav and flav that may be attributable to some combination of changes in speciation, interactions with O_2 or true cooperative binding of substrate. In the absence of further evidence for the origin of this interaction **Equation 4.1c** allows us to benchmark an approximate binding constant for comparison to our other ArMs and native QD

systems. Using **Equation 4.1c**, the K_d was determined to be $82.59 \pm 8.67 \mu\text{M}$ with a Hill coefficient of 1.62, which would reflect positive cooperative binding in the original interpretation.

Equation 4-1c. $Y = B_{\text{max}} * X^h / (K_D^h + X^h)$

Equation for the one-site binding model, where B_{max} is the maximum specific binding, K_D is the equilibrium dissociation constant, h is the Hill coefficient, Y is the fraction of sites occupied by the substrate, and X is the concentration of the substrate.

To try to clarify the origins of this behavior, revisiting the structural models shows that adjacent Co centers are positioned 11.2 Å apart, a distance too far to suggest cooperativity between metal sites or multiple substrates. Moreover, the observation of precipitate upon anaerobic titration of Co-2xm-S112E-Sav into flav, led me to speculate that the cooperativity arises from Co interactions with dioxygen to form a transient putative Co^{III} species. In support of this hypothesis, preliminary electron paramagnetic resonance (EPR) spectroscopy experiments indicate a decrease in high-spin Co^{II} population upon addition of flav (**Figure 4.11**).

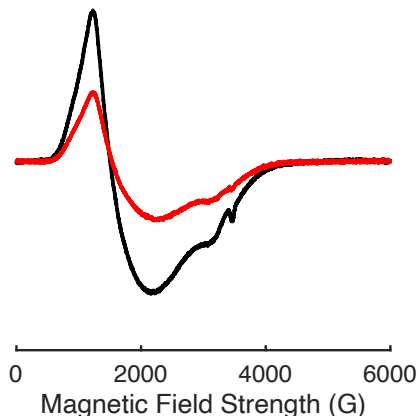


Figure 4.11. X-band EPR spectra of Co-2xm-S112E-Sav (black) with 1 eq flav (red).

To better understand the species formed upon binding of flav to ArMs, singular value decomposition (SVD) was applied to the spectral titrations to aid in identifying the potential number of species involved in each titration. In cases of substrate binding with a 1:1 stoichiometry and no intermediates, it is expected that the scree plots of the singular values of the titrations would exhibit two dominant singular values reflecting two independent states involved in the spectral series:

unbound and bound flav in solution. As expected, the scree plots of the singular values for the Ni-2xm-S112E-Sav titration exhibits two dominant singular values, and the scree plots of the singular values for the Cu-2xm-S112E-Sav titration exhibits three dominant singular values, where the third component arises from contributions of the increasing Cu d-d band throughout the titration (**Figure 4.12 A and B**).

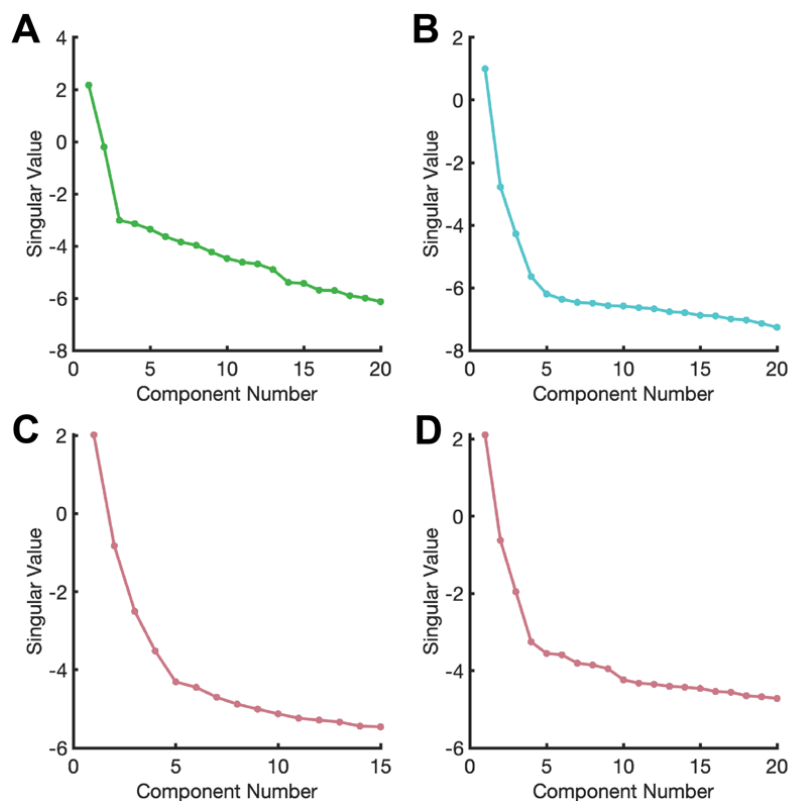


Figure 4.12. Scree plots of UV-vis titrations of ArMs into flav in KPi pH 7. **(A)** Titration of Ni-2xm-S112E-Sav into flav. **(B)** Titration of Cu-2xm-S112E-Sav into flav. **(C)** Titration of Co-2xm-S112E-Sav into flav. **(D)** Titration of Co-2xm-S112E-Sav after bubbling O₂ through solution into flav.

As expected, the Co-2xm-S112E-Sav titration exhibited a more complex scree plot with up to four dominant components (**Figure 4.12 C**). Bubbling O₂ through the solution of Co-2xm-S112E-Sav before titration into a solution of flav resulted in a saturation binding curve that could be fit to a one-site binding model with a Hill coefficient of 1.24, notably closer to 1 (where a Hill coefficient of 1 indicates no cooperative or speciation changes) than for the “unoxidized” Co-2xm-S112E-Sav. The

scree plot of the titration of “oxidized” Co-2xm-S112E-Sav into a solution of flav has changed, exhibiting three dominant singular values, rather than four for “unoxidized” Co-2xm-S112E-Sav, further implicating that an oxidized Co^{III} species binds flav, which may be important for the interpretation of native QD mechanisms (**Figure 4.12 D**).

Substrate Binding: Investigating the Role of Carboxylate Coordination to Metal Center

Beyond the identity of the metal ion at the active site, the primary coordination sphere has been implicated in QD activity. In fungal Cu-QD, mutation of E73 inhibits enzymatic function, implying that the carboxylate residue is essential for function, though its direct role has not been determined. It has been suggested that initial deprotonation of the 3-hydroxy group may result from the lowering of $\text{p}K_{\text{a}}$ upon binding Cu, in addition to the proper positioning of residue E73.²³ Moreover, E73 can participate in H-bonding with the 4'-hydroxy group on quercetin to further stabilize the substrate complex. To probe the role of carboxylate coordination in substrate binding, I prepared a Sav variant lacking the S112E mutation (2xm-Sav), where the metal center is not coordinated by an O atom donor from a carboxylate group. As discussed in Chapter 3, the XRD crystal structure Cu-2xm-Sav reveals a square planar Cu-center coordinated by 3 N atoms of the dpa ligand in a meridional fashion and one O atom from an aqua ligand. The caveat with studies of 2xm is the lack of an endogenous ligand to the metal center. Upon titration of Cu-2xm-Sav into a solution of quercetin, the expected shift in band I to indicate binding at the 3-hydroxy group was not observed, so K_{d} could not be determined (**Figure 4.S4**). Given that, in contrast to Cu-2xm-Sav, Cu-2xm-S112E-Sav binds quercetin with a K_{d} of $94.42 \pm 8.608 \mu\text{M}$, these results suggest an important role of H-bonding between the carboxylate residue and quercetin that promotes quercetin binding at the 3-hydroxy position to the Cu center.

To further probe the role of carboxylate coordination in substrate binding, I again turned to flav, which does not have a 4'-hydroxy group to participate in H-bonding with the carboxylate residue.

Titration of Zn^{2+} -2xm-Sav into a solution of flav did not result in the shift in flav band I so K_d could not be determined by UV-vis spectral titrations (**Figure 4.13**). Titration of Co^{2+} -2xm-Sav or Cu^{2+} -2xm-Sav into a solution of flav resulted in binding curves that indicate much weaker binding compared to the respective 2xm-S112E-Sav variants. These results suggest a role for carboxylate coordination in promoting substrate binding, consistent with reported findings in synthetic biomimetic systems and computational studies.^{22,39,50,68} Surprisingly for Ni-2xm-Sav, K_d was determined to be $22.69 \pm 0.77 \mu\text{M}$, indicating tighter binding than in Ni-2xm-S112E-Sav (**Table 4.S11**). Further reactivity studies will determine if carboxylate coordination plays additional roles in tuning reactivity beyond initial substrate binding in the Ni-2xm-Sav and Ni-2xm-S112E-Sav variants.

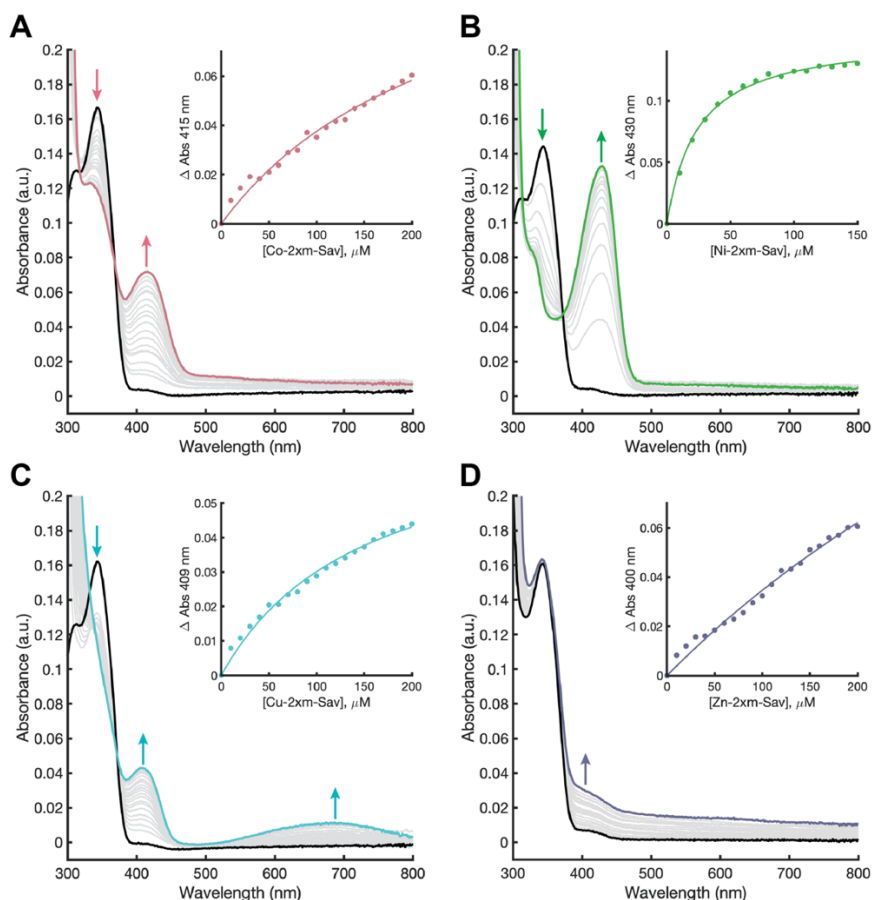


Figure 4.13. Representative UV-vis titrations of metal substituted 2xm-Sav into flav in KPi pH 7 and simulated binding curves (insets). Initial flav spectra shown in black, titration of 10 μM aliquots of ArMs shown in grey and final spectra after addition of 200 μM ArMs shown in colors. (A) Titration of Co-2xm-Sav into flav. (B) Titration of Ni-2xm-Sav into flav. (C) Titration of Cu-2xm-Sav into flav. (D) Titration of Zn-2xm-Sav into flav.

At present, reactivity studies reported in this section are preliminary. Reactivity of Ni-2xm-S112E-Sav with flav and O₂ was monitored by the decrease in absorbance of band I of flav upon injection of 2 mL O₂ into a sealed low-volume cuvette containing a solution of flav and ArM in a 10:1 molar ratio (notably, at concentrations of flav exceeding 20 μ M, the rate of flav autooxidation complicates the analysis of degradation of flav by Ni-2xm-S112E-Sav, so the concentration of flav must be kept at 10 μ M or less). This decrease in band I occurs over approximately 35–40 minutes, followed by an abrupt plateau (**Figure 4.14**). For the decay of band I to be observed, the cuvette must be sealed and somewhat over pressurized with injected O₂—reactivity does not occur when open to atmosphere over several hours nor if O₂ saturated buffer is used instead.

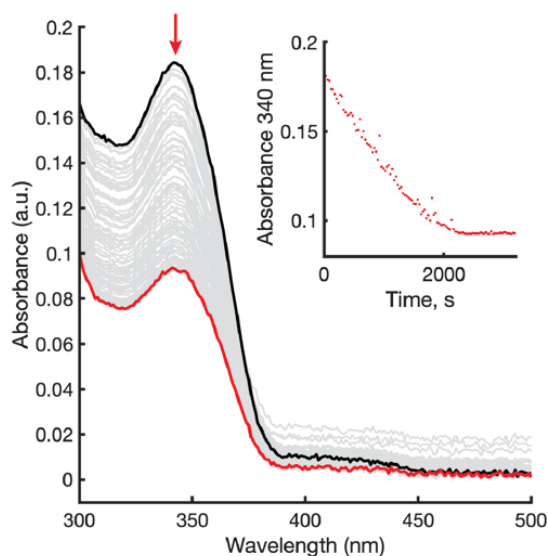


Figure 4.14. UV-vis spectra following reaction of flav with Ni-2xm-S112E-Sav and O₂. Starting spectrum of 10 μ M flav with 1 μ M Ni-2xm-S112E-Sav shown in black, after O₂ injection spectra taken every 30 s shown in grey, and final spectrum after 53 min shown in red. The absorbance of band I of flav at $\lambda_{\text{max}} = 340$ nm was followed over time (inset).

After the decrease in band I absorbance reaching a plateau, it was assumed that the reaction was completed (or perhaps degradation of the ArM was leading to loss of function), and the sample was either i) acidified with HCl, extracted into ethyl acetate, dried under a stream of N₂, and redissolved

into 50/50 methanol/water; or ii) taken directly from the cuvette for analysis by liquid chromatography high resolution electrospray ionization quadrupole time-of-flight tandem mass spectrometry (LC-HR-ESI-Q-ToF MS/MS). Regardless of sample preparation, a small peak in the chromatogram corresponding to the proposed depside product was observed (**Figure 4-15**). However, the amount of depside product observed did not correlate the amount of flav degraded.

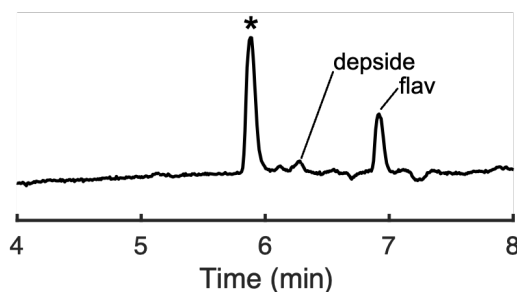
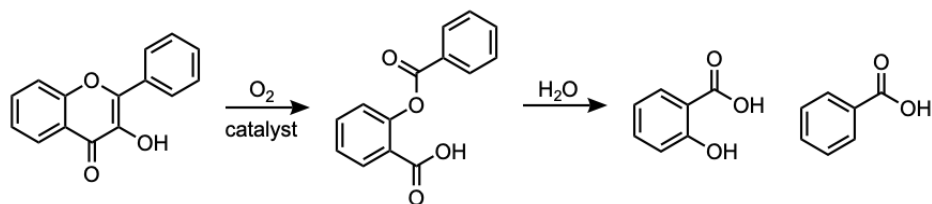


Figure 4.15. Chromatogram resulting from negative-ion mode LCMS of reaction of O₂ with a solution of 1:10 flav:Ni-2xm-S112E-Sav. Flav (substrate) elutes at 6.97 min, depside (hypothesized product) elutes at 6.24 min, and an unknown compound elutes at 5.87 min indicated by the asterisk. This chromatographic peak was integrated at full-width, half-maximum for analysis of resulting MS.

Scheme 4.3. Catalyst activates dioxygen for oxidation of flav to produce the depside 2-(benzoyloxy)benzoic acid, which is hydrolyzed in water to form salicylic acid and benzoic acid.



The inconsistency in expected product formation could be due to the hydrolysis of the depside product in methanol/water to form salicylic acid (SA) and benzoic acid (BA), as previously observed (**Scheme 4.3**).^{68,69} Instead, a peak eluting at 5.87 min, which did not match the retention times of any of the standards of depside, SA or BA was observed (**Figure 4.14**), and analysis of the MS and MS/MS led us to propose a dichlorinated salicylic acid product with chemical formula [C₇H₅O₃Cl₂]⁻ (**Figure**

4.15). When 10 μ M depside or SA were reacted with Ni-2xm-S112E-Sav under the same conditions, this product is also observed suggesting that the chlorination event occurs after oxidation of flav.

Attempts to eliminate any sources of chloride in the reaction mixture are underway. The starting metal complexes have been prepared from triflate salts rather than chloride salts, buffer has been pH adjusted with potassium phosphate mono/di basic rather than HCl, and Sav has been dialyzed into nanopure water. Analysis of the products resulting from reaction of flav with ArMs prepared in these conditions and O₂ are underway.

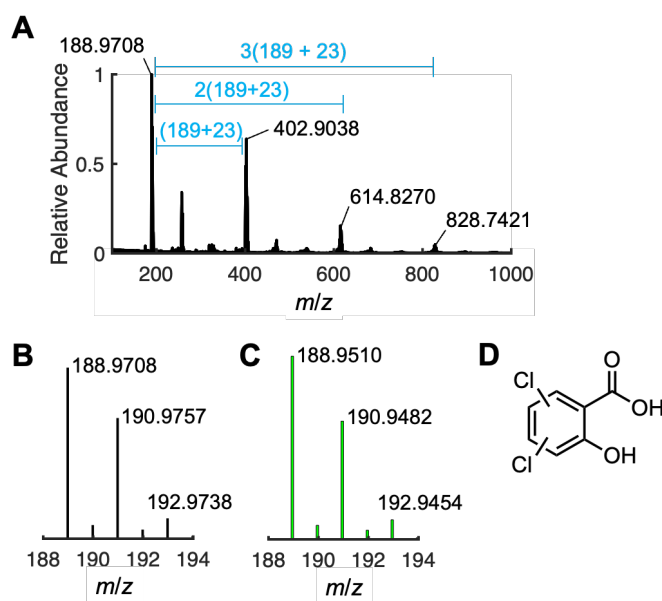


Figure 4.16. (A) Mass spectrum resulting from integration of the chromatographic peak eluting at 5.87 minutes. Sodiated aggregates of the most abundant ion ($m/z = 188.9708$) are observed. (B) The most abundant ion at $m/z = 188.9708$ exhibits an isotope pattern that suggests the inclusion of two chlorine atoms. (C) The simulated MS of an ion with the chemical formula $[C_7H_5O_3Cl_2]^-$ which matches the proposed product showed in (D).

Conclusions

This chapter described the design, preparation, and characterization of a series of metal-substituted ArMs to examine their activities with flavone compounds. The structural characterization of Co-, Ni-, Cu- and Zn- 2xm-S112E-Sav reveals that their coordination geometries closely resemble

those of native QDs. Co-, Ni- and Zn- 2xm-S112E-Sav all exhibit an octahedral geometry, where S112E is coordinated in a monodentate fashion, the dpa ligand is coordinated facially and the remaining coordination sites are occupied by two cis water molecules. Cu-2xm-S112E exhibits a distorted trigonal bipyramidal geometry, where S112E is also coordinated in a monodentate fashion, but the dpa ligand is coordinated meridionally and only one water molecule is coordinated to the Cu center. Spectral titrations of these ArMs into quercetin, the native substrate for QD, allowed for the determination of K_d for only the Cu-substituted ArM, perhaps due to the unique coordination geometry and special location of the Cu-cofactor compared to the other metal-substituted ArMs, reinforcing the hypothesis that metal coordination geometries play a crucial role in enzymatic function.

To eliminate off-target binding modes and make further comparisons of substrate binding behaviors between the metal cofactors, substrate binding was further interrogated with flav as a substrate mimic for quercetin. Comparison of K_d of flav with the metal-substituted 2xm-S112E-Sav ArMs reveal that Co binds weaker than Ni and Cu, which have the same affinity for flav within experimental error. Additionally, the observed cooperative flav binding behavior in Co-2xm-S112E-Sav suggests a potential interaction with dioxygen, warranting further investigation into the formation of putative cobalt-oxygen intermediates. Flav binding to Zn-2xm-S112E-Sav was not observed, which could offer a plausible explanation for the lack of native Zn-QD activity—if the flavonoid substrate does not bind to the metal center, then the catalytic cycle will not be initiated.

In addition to examining how different metal ions can modulate substrate binding, the impact of the primary coordination sphere was explored. ArMs lacking the S112E mutation (2xm-Sav) were prepared to evaluate how lack of carboxylate coordination impacts substrate binding. Without the carboxylate coordination, spectral titrations could only determine K_d for flav in the Ni-substituted variant, suggesting carboxylate coordination aids in substrate binding for Co- and Cu- Sav. Reactivity

studies and kinetic analysis will reveal if carboxylate coordination plays additional roles in the activity of Ni-2xm-Sav compared to Ni-2xm-S112E-Sav.

Beyond the primary coordination sphere, XRD structures demonstrated how mutations in secondary coordination sphere to surrounding amino acid residues, specifically N49A, eliminates secondary coordination sphere H-bonding interactions that impact substrate binding. ITC methods revealed differences in the binding stoichiometry of maltol between Ni-2xm-N49A-S112E and Ni-2xm-S112E, suggesting that H-bonding networks influence the likelihood of displacing aqua ligands for substrate binding.

Although about one-third of enzymes are metalloenzymes, the use of Ni or Co as a cofactor is much less common compared to Fe, Cu, or Zn. Understanding the reaction mechanisms, coordination chemistry, and redox properties underlying Ni/Co metalloenzymes may uncover why these enzymes select Ni or Co as a catalytic center. The development of artificial metalloproteins (ArMs) offers an innovative approach to understanding and replicating the function of native dioxygenase enzymes. By incorporating different metal ions into a conserved protein scaffold, these systems provide a unique platform for studying cambialistic enzyme behavior, oxygen activation, and selective substrate oxidation. The ability to fine-tune primary and secondary coordination sphere interactions enables modulation of metal ion reactivity and stabilization of reactive intermediates, and influences reaction selectivity.

Materials and Methods

Preparative Methods

All commercially available reagents were purchased of the highest purity and used as received. Acetonitrile, ethanol, and diethyl ether were degassed with argon and dried by vacuum filtration through activated alumina according to the procedure outlined by Grubbs.⁷⁰ Nanopure water was

obtained from Millipore Super-Q Plus water purification system, 120 V, 4-stage purification system with pump. Thin-layer chromatography (TLC) was performed on Whatman 250 μm layer 6 Å aluminum-backed silica plates and visualized using ultraviolet (UV) light. Silica gel chromatography was performed using Fisher reagent silica gel 60 (230-400 mesh) with indicated solvents. Biotin pentafluorophenol ester (biot-PFP)⁷¹ and di-(2-picolyl)amine (dpa)⁷² were prepared according to literature procedures.

¹H and ¹⁹F nuclear magnetic resonance (NMR) spectra were recorded at 500 and 400 MHz respectively. ¹H NMR spectra are reported in ppm on the δ scale and referenced to tetramethylsilane or solvent residual. The data are presented as follows: chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, quin=quintet, m=multiplet, br=broad), and integration.

Elemental analyses (carbon, hydrogen and nitrogen) were carried out using a Thermo Scientific FlashSart CHNS/O Elemental Analyzer (UC Irvine Materials Research Institute).

Preparation of cofactors. The ligand (biot-et-dpa) was prepared as published previously.⁶⁰ ¹H (500 mHz, DMSO-d₆) δ 8.48 (d, 2H), 7.76 (td, 3H), 7.53 (d, 2H) 7.25 (t, 2H), 6.40 (s, 1H), 6.33 (s, 1H), 4.29 (t, 1H), 4.12 (t, 1H), 3.78 (s, 4H), 3.20 (q, 2H), 3.08 (q, 1 H), 2.81 (dd, 1 H), 2.59 (s, 1H), 2.56 (t, 2H), 2.04 (t, 2H), 1.47 (m, 6H).

The Cu-complex [Cu^{II}(biot-et-dpa)(NO₃)₂] was prepared by addition of a solution of Cu(NO₃)₂•3H₂O (85.2 mg, 0.353 mmol) in 10 mL ethanol to a solution of biot-et-dpa (150.2 mg, 0.324 mmol) in 50 mL ethanol. The pale blue solution was allowed to stir for 4 h before concentrating the solution to ~1 mL followed by precipitation with diethyl ether. Blue solid was collected via filtration, washed with diethyl ether and dried under vacuum.

HR-MS (ES, MeOH) m/z calcd for C₂₆H₃₂CuN₈O₈S [M – (NO₃)[–]]⁺ 621.2, found 621.4.

The Ni-complex [Ni^{II}(biot-et-dpa)(OH₂)₃]Cl₂ was prepared by addition of a solution of NiCl₂•6H₂O (12.9 mg, 0.0543 mmol) in 10 mL ethanol to a solution of biot-et-dpa (23.1 mg, 0.0493

mmol) in 50 mL ethanol. The pale green solution was allowed to stir for 4 h before concentrating the solution to ~1 mL followed by precipitation with diethyl ether. Pale green solid was collected via filtration, washed with diethyl ether and dried under vacuum.

HR-MS (ESI, MeOH) m/z calcd for $C_{24}H_{32}Cl_2NiN_6O_2S [M - (Cl)]^+$ 561.65. Found: 561.4.

Elemental Analysis for $C_{24}H_{38}Cl_2NiN_6O_5S$: C 44.19, H 5.87, N 12.88. Found: C 43.428, H 5.768, N 12.455.

The Co-complex $[Co^{II}(\text{biot-et-dpa})(OH_2)_3]Cl_2$ was prepared by addition of a solution of $CoCl_2$ (9.72 mg, 0.0749 mmol) in 10 mL ethanol to a solution of biot-et-dpa (31.9 mg, 0.0656 mmol) in 50 mL ethanol. The purple solution was allowed to stir for 6 h before concentrating the solution to ~1 mL followed by precipitation with diethyl ether. Purple solid was collected via filtration, washed with diethyl ether and dried under vacuum.

HR-MS (ESI, MeOH) m/z calcd for $C_{24}H_{32}Cl_2CoN_6O_2S [M - (Cl)]^+$ 562.65. Found: 562.1.

Elemental Analysis for $C_{24}H_{38}Cl_2CoN_6O_5S$: C 44.18, H 5.87, N 12.88. Found: C 43.397, H 5.288, N 12.572.

The Zn-complex $[Zn^{II}(\text{biot-et-dpa})Cl_2]$ was prepared by addition of a solution of $ZnCl_2$ (19.3 mg, 0.142 mmol) in 10 mL ethanol to a solution of biot-et-dpa (60.3 mg, 0.129 mmol) in 50 mL ethanol. The solution was allowed to stir for 6 h before concentrating the solution to ~5 mL followed by precipitation with diethyl ether. Pale yellow solid was collected via filtration, washed with diethyl ether, and dried under vacuum.

1H (600 MHz, DMSO- d_6) δ 8.86 (d, 2H), 8.12 (t, 2H), 7.76 (t, 1H), 7.66 (t, 2H), 7.62 (d, 2H), 6.39 (s, 1H), 6.34 (s, 1H), 4.34 (t, 1H), 4.29 (t, 2H), 4.10 (t, 1H), 3.44 (q, 2H), 3.38 (q, 1H), 3.16 (q, 2H), 3.05 (q, 1H), 2.81 (dd 1H), 2.61 (t, 2H), 2.56 (d, 1H), 1.98 (t, 2H), 1.42 (m, 6H). A downfield shift in protons on the pyridine ring is observed, consistent with coordination to Zn.

HR-MS (ESI, MeOH) m/z calcd for $C_{24}H_{32}Cl_2ZnN_6O_2S [M - (Cl)]^+$ 567.13. Found 567.13.

Preparation of streptavidin variants. The construction of E101Q-K121A-S112E-Sav (denoted 2xm-S112E-Sav) was achieved by site-directed mutagenesis (SDM) using the codon optimized E101Q-K121A-pET24a-Sav plasmid, the following primers, and Phusion polymerase.

S112E_fwd: 5'- CTG ACC GAA GGC ACC ACC GAA GCA AAT C

S112E_rev: 5'- GGT GCC TTC GGT CAG CAG CCA CTG GGT C

SDM reaction mixtures were transformed into *Escherichia coli* bl21 cells followed by rescue with lysogeny broth (LB) media. The solution was spread aseptically onto LB/kanamycin agar plates and incubated overnight at 38 °C with shaking at 200 rpm. Of the overnight cultures, 600 µL was saved to prepare glycerol stocks to be stored in 25% glycerol at -80 °C. From the remaining liquid culture, plasmids were isolated using Qiagen Miniprep kit according to manufacturer's instructions. DNA sequencing was performed by Genewiz. Glycerol stocks described above were used to inoculate overnight liquid cultures for expression of Sav variants. Expression and purification of Sav variants was carried out as reported previously.⁷³

Protein Crystallization, X-ray Diffraction Data Collection and Processing

Crystallization of M-Sav. Apo-Sav protein was crystallized by the sitting-drop vapor diffusion method. Diffraction-quality crystals were grown at ambient temperature and pressure by mixing 2.5 µL protein solution (26 mg/mL lyophilized 2xm-S112E-Sav in nanopure water) and 2.5 µL crystallization buffer (2.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4). The droplet was equilibrated against a reservoir of 100 µL crystallization buffer solution. Single crystals of Co-Sav were prepared by soaking apo-Sav crystals in a solution of 9 µL soaking buffer (3.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4) mixed with 1 µL of Co-complex (10 mM Co-complex, 100 mM CoCl₂ in nanopure water) overnight. Single crystals of Ni-Sav were prepared by soaking apo-Sav crystals in a solution of 9 µL soaking buffer (3.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4) mixed with 1

μL of Ni-complex (10 mM Ni-complex, 100 mM NiCl₂ in nanopure water) overnight. Upon soaking Sav crystals with Ni-complex, crystals turned from colorless to pale green. Single crystals of Cu-Sav were prepared by soaking apo-Sav crystals in a solution of 9 μL soaking buffer (3.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4) mixed with 1 μL of Cu-complex (10 mM Cu-complex, 100 mM CuCl₂ in nanopure water) overnight. Upon soaking Sav crystals with Cu-complex, crystals turned from colorless to blue. Single crystals of Zn-Sav were prepared by soaking apo-Sav crystals in a solution of 9 μL soaking buffer (3.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4) mixed with 1 μL of Zn-complex (10 mM Zn-complex, 100 mM ZnCl₂ in nanopure water) overnight. After soaking in metal-complex, crystals were transferred to a cryoprotectant solution of 30% glycerol in soaking buffer and shock frozen in liquid nitrogen.

X-ray diffraction data collection and processing. X-ray diffraction data were collected at the Advanced Light Source (ALS, BL 5.0.2, BL 8.2.2, BL 8.2.1) at a wavelength of 1 Å. Diffraction images were integrated with software XDS⁷⁴ or iMOSFLM⁷⁵ and scaled with AIMLESS (CCP4 Suite).⁷⁶ For molecular replacement, Phaser-MR (PHENIX Suite)⁷⁷ was used with PDB: 2QCB with water molecules removed as the input model. Structural refinement was carried out in PHENIX.REFINE^{77,78} and COOT.⁷⁹ Restraints for biot-et-dpa (ligand ID: QG7) were created using eLBOW (PHENIX Suite). For water and solvent picking, electron density and structure visualization, the software COOT was used. Figures were drawn with PyMOL (Schrodinger, LLC Version 2.5.5). Crystallographic details, processing and refinement statistics are given in **Tables 4.S1** and **4.S2**.

Overall structures. Sav crystals soaked in metal complex solution constituted space group I4₁22 with unit cell parameters reported in **Tables 4.S1** and **4.S2**. One Sav monomer was obtained per asymmetric unit after molecular replacement. Protein residues 2–11 and 135–159 of the N- and C-termini, respectively, were not resolved in electron density, presumably due to disorder.

General M-complex modelling. For all structures of Sav crystals soaked with M-complex, the following general observations were made: i) residual electron density in the F_o-F_c map in the biotin-binding pocket, and ii) one peak in the anomalous density map in the biotin vestibule superimposed with the electron density peak. The residual electron density was fit with the M-complex which projected Co/Ni/Cu/Zn to the position of the strong anomalous density peak. The occupancy of Co, Ni, Cu or Zn was refined to 1.00, 1.00, 0.75, or 0.70 respectively. We also observed negative electron density surrounding the side chain of S112E, suggesting a rotamer

Substrate Binding Studies

General preparation of M-Sav in solution. A solution of lyophilized Sav was prepared in nanopure water. This solution was diluted to 150 μ M in potassium phosphate buffer (KPi, 50 mM, pH 6.9), and five equivalents of Co-, Ni-, Cu-, or Zn- complex were added from a 10 mM stock solution in nanopure water. Samples were separated from excess M-complex and concentrated using Amicon Ultra 10K centrifugal filter devices and washed with 5X volume of nanopure water or buffer. The concentration of the M-Sav stock solution was determined based on $\epsilon_M = 167,760$ at $\lambda_{\max} = 280$ nm and diluted to the final working concentration as specifically indicated for each experiment described below.

General preparation of maltol, quercetin or flav in solution. Maltol (Spectrum Chemical), quercetin (Sigma Aldrich) or flav (Indofine Chemical Company) solution was prepared fresh for each experiment. A 10X stock solution of maltol was prepared in DMSO and diluted to appropriate working concentration in aqueous buffer. A 10X stock solution of quercetin or flav was prepared in MeOH, with sonication to aid in dissolving, and diluted to appropriate working concentration in aqueous buffer.

Maltol Binding Studied by Isothermal Titration Calorimetry. All experiments were performed with a MicroCal PEAQ-ITC instrument (Malvern). All solutions were made in MES buffer (100 mM, pH6)

and 10% DMSO. All experiments were performed at 25 °C. The reaction cell contained 10 mM maltol solution and the syringe contained 50 μ M ArM solution. Each titration experiment was performed using 37 injections of 2 μ L with 1 s duration and 120 s intervals between injections. Reference power of 5 μ cal/s, high-feedback mode, and stirring speed of 750 rpm were used for all experiments. All data were analyzed using the MicroCal PEAQ-ITC analysis software. To obtain the binding enthalpies, observed enthalpy values were corrected for the enthalpy of dilution obtained under identical conditions with the sample cell containing buffer alone (**Figures 4.S3 and 4.S3**)

Quercetin or Flavonol Binding Measured by Spectral Titrations. Electronic absorbance spectra were recorded with an Agilent Cary 50 UV-vis spectrophotometer in a 1 cm quartz cuvette. To a solution of quercetin or flav (10 μ M in 10% methanol, 50 mM KPi pH 6.9), aliquots of M-Sav (625 μ M with respect to Sav tetramer, or 2.5 mM with respect to metal sites, in mQ water) were titrated and resulting UV-vis spectra were recorded following mixing and equilibration. If a shift in band I was observed, the resulting λ_{max} was monitored until saturation. Saturation binding curves were modelled according to **Equations 4.1 a–c**.

Electron Paramagnetic Resonance Spectroscopy. Co-2xm-S112E-Sav was diluted to a final concentration of 250 μ M (1 mM Co) in 50 mM MES buffer pH 6. The solution was aliquoted into two EPR tubes and either DMSO or 10X stock solution of flav in DMSO was added. Solution was mixed and allowed to equilibrate for 5 minutes then flash-frozen in liquid nitrogen. Continuous wave CW) X-band EPR spectra were recorded by Dr. Alec Follmer with assistance from Dr. Paul Oyala (CalTech EPR Facility) on a Bruker EMX spectrometer equipped with a 4119HS-W1 cavity and X-Band CW microwave Bridge. Spectra were collected at 5 K, 2.1 mW, 9.8 GHz and averaged over 5 scans.

Reactivity with O₂

To a solution of flav (10 μ M in 10% methanol, 50 mM KPi pH 6.9), Ni-2xm-S112E-Sav was added to a final concentration of 1 μ M (with respect to Ni, or 250 nM with respect to Sav tetramer). The cuvette was sealed with a silicone septa cap and solution was allowed to equilibrate for 5 minutes. The reaction was held at 23 °C with stirring, as 2 mL gaseous O₂ were injected via gas-tight syringe. The reaction was monitored over 1 hour, following the decay of flav band I at λ_{max} =340 nm.

After 1 hour, the reaction mixture was either i) taken directly from the cuvette for LCMS analysis, or ii) acidified with 1 M HCl, extracted into 3x 500 μ L ethyl acetate, dried under a stream of N₂, then redissolved in 50/50 methanol/water for LCMS analysis. Mass spectra were measured with an ACQUITY ultra-performance liquid chromatography (UPLC) H-class system, Xevo G2-XS quadrupole time-of-flight (QTOF) (Waters Corporation). Separation of analytes was performed by reverse-phase chromatography at 45 °C using a reverse-phase C18 column (ACQUITY UPLC Oligonucleotide BEH C18 column, 130 Å, 1.7 μ m, 2.1 mm X 50 mm, Waters Corporation). The 15-minute LC method was used with a flow rate of 0.3 mL/min of a gradient of 0.1% formic acid in water (Buffer A), and acetonitrile (Buffer B) according to the gradient in **Table 4.S12**. The first minute was diverted to direct buffer components to waste.

The Xevo Z-spray ionization source was operated with a capillary voltage of 3000 V in positive ion mode or 2000 V in negative ion mode. Data was acquired across the 50–1000 Da range in fast data dependent acquisition (FDDA) mode with alternating 0.2 s scans at alternating collision energy: low energy, 0 V, and a high collision energy ramp (10–45 V). The high energy data corresponds to the MS/MS secondary fragmentation of the low energy scans of a similar time.

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Supplementary Tables and Figures

Structural Characterization of ArMs by XRD

Table 4.S1. X-ray crystallography data processing and refinement statistics

Sav Variant	2xm-S112E	2xm-S112E	2xm-S112E	2xm-S112E
Cofactor	Co-et-dpa	Ni-et-dpa	Cu-et-dpa	Zn-et-dpa
PDB Code	Pending Deposition	Pending Deposition	Pending Deposition	Pending Deposition
Data Processing				
Unit Cell (a, b, c, α, β, γ)	57.69 Å, 57.69 Å, 183.402 Å, 90°, 90°, 90°	57.67 Å, 57.67 Å, 183.56 Å, 90°, 90°, 90°	57.614 Å, 57.614 Å, 183.509 Å, 90°, 90°, 90°	57.778 Å, 57.778 Å, 184.277 Å, 90°, 90°, 90°
Space Group	I4 ₁ 22	I4 ₁ 22	I4 ₁ 22	I4 ₁ 22
Resolution (Å)	41.96 – 1.9	45.89 – 1.4	45.88 – 1.4	46.07 – 1.4
Highest resolution shell (Å)	1.968 – 1.9	1.45 – 1.4	1.45 – 1.4	1.45 – 1.4
R-merge (%)	0.07 (0.60)	0.89 (0.99)	0.78 (0.99)	0.2447 (4.176)
No. unique reflections	12673 (1191)	7747 (3038)	8900 (3063)	313337 (3065)
Multiplicity	11.5 (7.4)	9.7 (8.9)	12.5 (11.8)	24.5 (24.9)
Mean I/sig(I)	23.68 (4.08)	8.33 (6.53)	11.49 (6.05)	11.95 (1.15)
Completeness	99.58 (96.11)	99.08 (99.31)	99.74 (99.97)	96.93 (100.0)
CC(1/2)	0.999 (0.894)	0.253 (0.543)	0.719 (0.484)	0.999 (0.422)
Structure Refinement				
R-work	0.1691 (0.3174)	0.1731 (0.4143)	0.1736 (0.4079)	0.2217 (0.3297)
R-free	0.1924 (0.3030)	0.2074 (0.4451)	0.2060 (0.4127)	0.2562 (0.3554)
RMSD bond length (Å)	0.017	0.010	0.004	0.004
RMSD bond angle (°)	1.34	1.03	0.78	0.81
Avg B-factor (Å²)	27.99	21.90	22.54	19.45
Macromolecules	26.77	19.56	21.45	17.93
Ligands	29.95	22.53	24.99	23.00
Solvent	36.75	36.82	32.18	31.64
Occupancy of Metal Ion	1.00	1.00	0.75	0.70

Table 4.S2. Select bond lengths (Å) and angles (°) for Cu-QD and Cu-2xm-S112E-Sav according to atom labeling shown in Figure 4.2

Cu-QD PDB: 1JUH		Cu-2xm-S112E-Sav	
Cu–N _{H112}	2.1	Cu–N1	2.1
Cu–N _{H68}	2.1	Cu–N2	2.1
Cu–N _{H66}	2.2	Cu–N3	2.6
Cu–O1 _{E73}	2.2	Cu–O1 _{E112}	2.0
Cu–O _{W1}	2.6	Cu–O _{W1}	1.9
O2 _{E73} –O _{W1}	3.0	O2 _{E112} –O _{W1}	3.9
N _{H68} –Cu–N _{H112}	114.4	O1 _{E112} –Cu–O _{W1}	125.0
O1 _{E73} –Cu–O _{W1}	84.1	O _{W1} –Cu–N2	90.9
O _{W1} –Cu–N _{H68}	141.8	N2–Cu–O _{E112}	142.5
N _{H112} –Cu–O _{E73}	86.3	N1–Cu–O _{E112}	83.0
N _{H112} –Cu–N _{H66}	96.6	N1–Cu–N2	84.5
N _{H112} –Cu–O _{W1}	101.0	N1–Cu–O _{W1}	94.4
O _{W1} –Cu–N _{H68}	144.8	O _{E112} –Cu–N3	120.3
N _{H66} –Cu–N _{H68}	99.4	N2–Cu–N3	74.8
O _{W1} –Cu–N _{H66}	89.8	O _{W1} –Cu–N3	75.1

Table 4.S3. Select bond lengths (Å) and angles (°) for Ni-QD, Ni-2xm-S112E-Sav, Co-2xm-S112E-Sav, and Zn-2xm-S112E-Sav according to atom labeling shown in Figure 4.S1

Ni-QD PDB: 5FLH		Ni-2xm-S112E-Sav		Co-2xm-S112E-Sav		Zn-2xm-S112E-Sav	
Ni–N _{H115}	2.0	Ni–N1	2.0	Co–N1	2.2	Zn–N1	2.2
Ni–N _{H71}	2.0	Ni–N2	2.1	Co–N2	2.2	Zn–N2	2.3
Ni–N _{H69}	2.1	Ni–N3	2.1	Co–N3	2.1	Zn–N3	2.1
Ni–O1 _{E76}	2.0	Ni–O1 _{E112}	2.1	Co–O1 _{E112}	2.2	Zn–O1 _{E112}	2.2
Ni–O _{W1}	2.1	Ni–O _{W1}	2.0	Co–O _{W1}	2.1	Zn–O _{W1}	1.9
Ni–O _{W2}	2.1	Ni–O _{W2}	2.1	Co–O _{W2}	2.1	Zn–O _{W2}	2.4
O2 _{E76} –O _{W1}	2.7	O2 _{E112} –O _{W1}	2.6	O2 _{E112} –O _{W1}	2.5	O2 _{E112} –O _{W1}	3.1
O2 _{E76} –O _{W2}	3.4	O2 _{E112} –O _{W2}	4.0	O2 _{E112} –O _{W2}	4.0	O2 _{E112} –O _{W2}	4.4
O1 _{E76} –Ni–O _{W1}	88.4	O1 _{E112} –Ni–O _{W1}	91.3	O1 _{E112} –Co–O _{W1}	92.2	O1 _{E112} –Zn–O _{W1}	101.5
O1 _{E76} –Ni–O _{W2}	95.2	O1 _{E112} –Ni–O _{W2}	90.9	O1 _{E112} –Co–O _{W2}	85.5	O1 _{E112} –Zn–O _{W2}	94.2
O1 _{E76} –Ni–N _{H71}	86.0	O1 _{E112} –Ni–N2	93.6	O1 _{E112} –Co–N2	90.5	O1 _{E112} –Zn–N2	89.6
O1 _{E76} –Ni–N _{H115}	91.8	O1 _{E112} –Ni–N1	79.4	O1 _{E112} –Co–N1	81.8	O1 _{E112} –Zn–N1	77.9
N _{H115} –Ni–N _{H71}	98.9	N1–Ni–N2	79.8	N1–Co–N2	73.3	N1–Zn–N2	94.2
N _{H71} –Ni–O _{W2}	91.3	N2–Ni–O _{W2}	96.6	N2–Co–O _{W2}	98.5	N2–Zn–O _{W2}	98.5
O _{W2} –Ni–O _{W1}	78.1	O _{W2} –Ni–O _{W1}	89.6	O _{W1} –Co–O _{W2}	94.3	O _{W1} –Zn–O _{W2}	88.8
O _{W1} –Ni–N _{H115}	92.5	O _{W1} –Ni–N1	94.9	O _{W1} –Co–N1	94.6	O _{W1} –Zn–N1	99.0
N _{H69} –Ni–N _{H115}	89.0	N3–Ni–N1	101.4	N3–Co–N1	101.7	N3–Zn–N1	103.3
N _{H69} –Ni–N _{H71}	94.8	N3–Ni–N2	82.7	N3–Co–N2	81.5	N3–Zn–N2	79.2
N _{H69} –Ni–O _{W1}	83.9	N3–Ni–O _{W1}	92.6	N3–Co–O _{W1}	97.0	N3–Zn–O _{W1}	90.3
N _{H69} –Ni–O _{W2}	90.7	N3–Ni–O _{W2}	88.7	N3–Co–O _{W2}	89.5	N3–Zn–O _{W2}	83.1

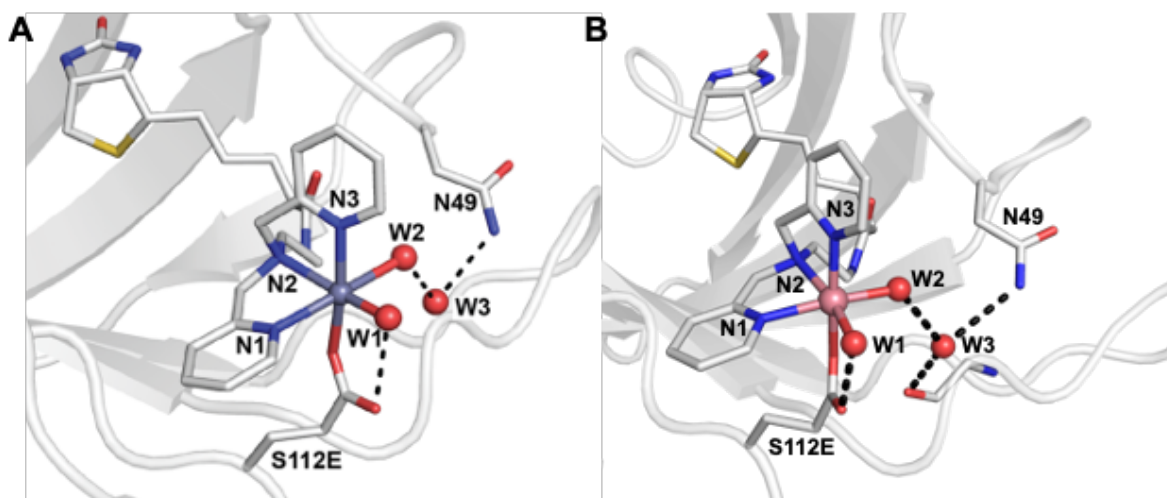


Figure 4.S1. Molecular structures of (A) Zn-2xmS112E-Sav and (B) Co-2xm-S112E-Sav.

Table 4.S4. X-ray crystallography data processing and refinement statistics

Sav Variant	2xm-N49A-S112E	2xm-N49A-S112E
Cofactor	Co-et-dpa	Ni-et-dpa
PDB Code	220912_D07	220912_E08
Data Processing		
Unit Cell (a, b, c, α , β , γ)	57.77 Å, 57.77 Å, 183.52 Å, 90°, 90°, 90°	57.51 Å, 57.51 Å, 175.67 Å, 90°, 90°, 90°
Space Group	I4 ₁ 22	I4 ₁ 22
Resolution (Å)	37.32 – 1.4	41.03 – 1.4
Highest resolution shell (Å)	1.45 – 1.4	1.45 – 1.4
R-merge (%)	0.05 (1.31)	0.09 (1.01)
No. unique reflections	31195 (3055)	29593 (2901)
Multiplicity	13.9 (13.2)	13.7 (13.0)
Mean I/sig(I)	28.89 (2.04)	16.60 (2.57)
Completeness	99.90 (99.93)	99.60 (99.97)
CC(1/2)	1 (0.782)	0.999 (0.849)
Structure Refinement		
R-work	0.1565 (0.2360)	0.1762 (0.2642)
R-free	0.1733 (0.2819)	0.1999 (0.2778)
RMSD bond length (Å)	0.068	0.012
RMSD bond angle (°)	1.02	1.20
Avg B-factor (Å ²)	25.42	23.72
Macromolecules	24.43	22.55
Ligands	27.50	29.31
Solvent	36.21	34.78
Occupancy of Metal Ion	1.00	0.82

Maltol Binding Measured by Isothermal Titration Calorimetry

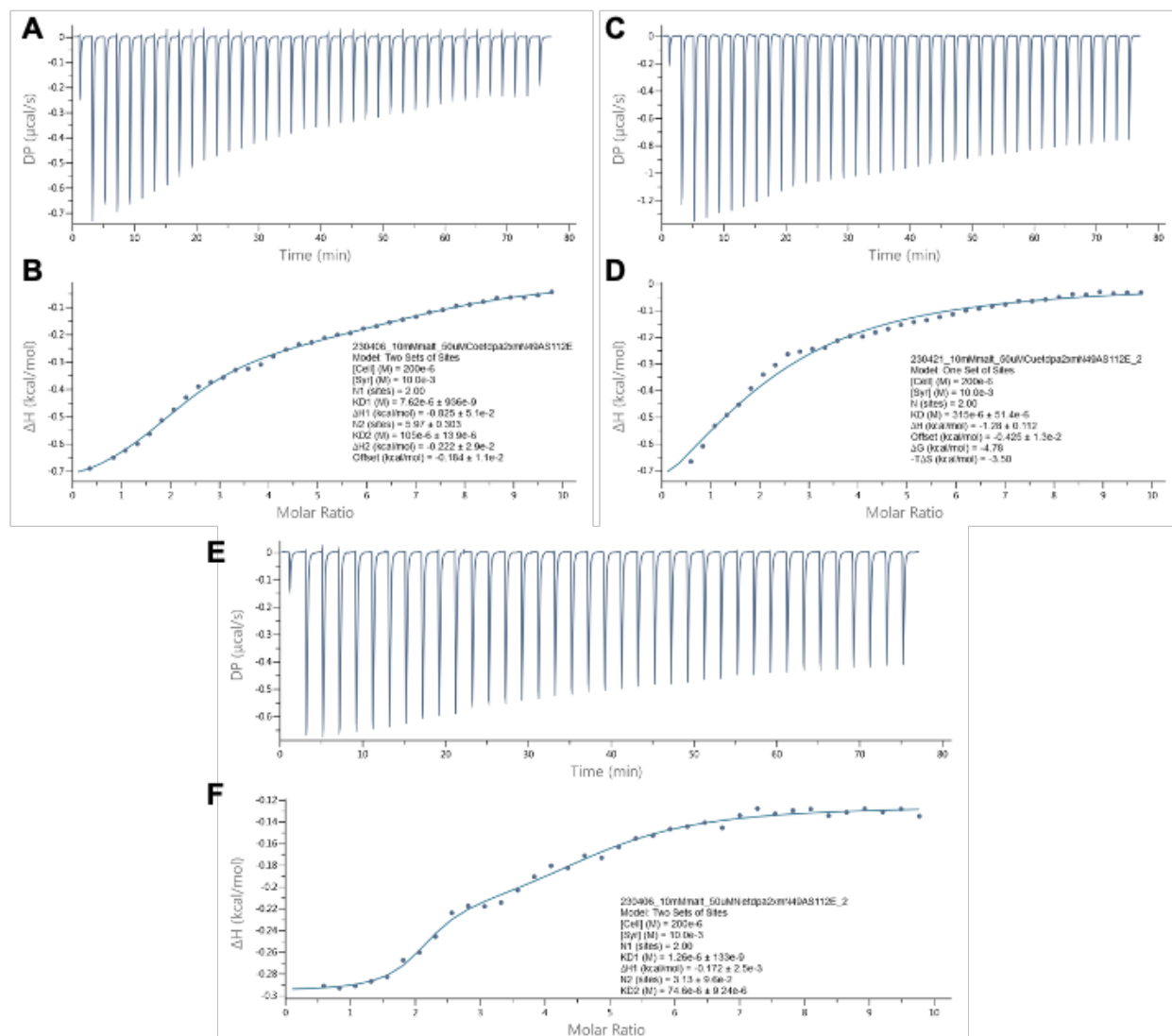


Figure 4.S2. Isothermal titration calorimetry data. (A) Raw heat of injections for Co-2xm-N49A-S112E-Sav into maltol, and (B) fit of data to two-site binding model. (C) Raw heat of injections for Cu-2xm-N49A-S112E-Sav into maltol, and (D) fit of data to one-site binding model. (E) Raw heat of injections for Ni-2xm-N49A-S112E-Sav into maltol, and (F) fit of data to two-site binding model.

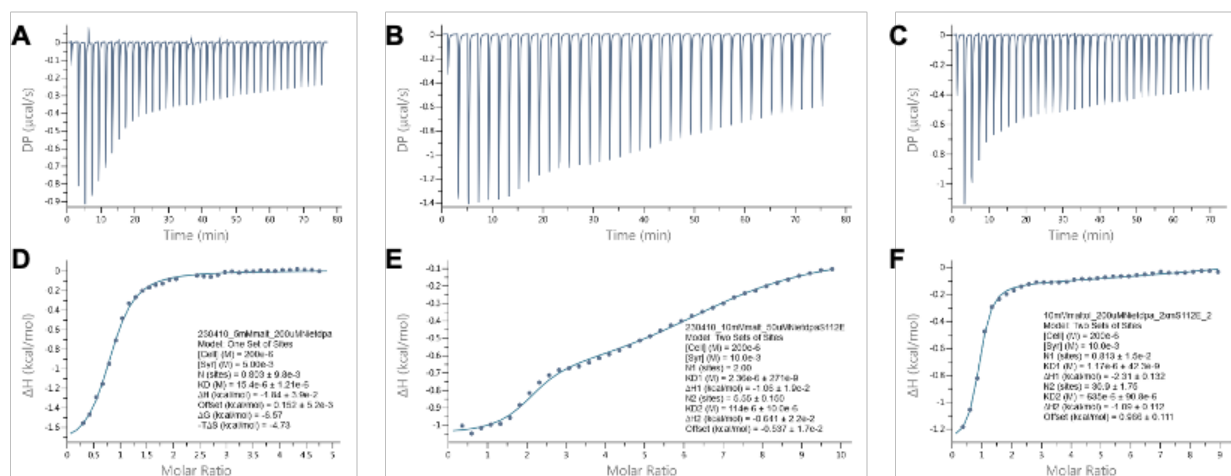


Figure 4.S3. Isothermal titration calorimetry data. Raw heat of injections for (A) Ni-et-dpa, (B) Ni-S112E-Sav, or (C) Ni-2xm-S112E-Sav into maltol. The data was fit with Malvern PEAQ ITC Analysis software to a one- or two-site binding model shown in D–F.

Quercetin Binding

Table 4.S5. Binding curve fits for titration of Cu-2xm-S112E-Sav into quercetin (with 95% confidence intervals) according to Equation 4.1b. Average of values for each fit reported $\pm$ standard error of mean (SEM)

	Trial 1	Trial 2	Trial 3	Average of Fits
$B_{\max}$ (ΔAbs_{430})	0.576 (0.4638, 0.6882)	0.7255 (0.4026, 1.048)	0.48 (0.3335, 0.6266)	0.5938 ± 0.0714
K_d (μM)	94.47 (77.14, 111.8)	109.3 (65.98, 152.5)	79.48 (54.96, 104)	94.42 ± 8.6
NS	0.001269	0.001631	0.001067	0.001322 ± 0.000165
R-square	0.999	0.9957	0.9964	

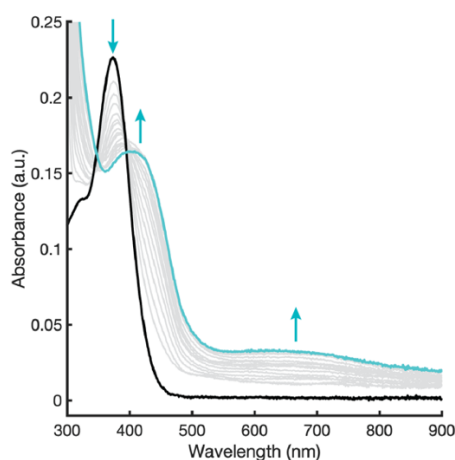


Figure 4.S4. Representative UV-vis titration of Cu-2xm-Sav into quercetin in KPi pH 7. Initial quercetin spectrum shown in black, titration of 10 μM aliquots of ArMs shown in grey and final spectrum after addition of 200 μM Cu-2xm-Sav shown in cyan.

Table 4.S6. Binding curve fits of titration of Co-2xm-S112E-Sav into flav (with 95% confidence intervals) according to Equation 4.1c. Average of values for each fit reported $\pm$ standard error of mean (SEM).

	Trial 1	Trial 2	Trial 3	Trial 4	Average of Fits
B_{max} (ΔAbs₄₃₀)	0.123 (0.1176, 0.1285)	0.1073 (0.09425, 0.1203)	0.1187 (0.1104, 0.1269)	0.1255 (0.1243, 0.1268)	0.1186 $\pm$ 0.0040
K_d (μM)	46.88 (43.33, 50.44)	51.67 (42.53, 60.81)	49.98 (44.56, 55.39)	57.42 (56.51, 58.33)	51.49 $\pm$ 2.21
Hill Coefficient	1.45 (1.341, 1.559)	1.761 (1.374, 2.147)	1.619 (1.423, 1.815)	1.651 (1.618, 1.685)	1.620 $\pm$ 0.06438
R-square	0.9991	0.9920	0.9976	0.9999	

Table 4.S7. Binding curve fits of titration of Ni-2xm-S112E-Sav into flav (with 95% confidence intervals) according to Equation 4.1a. Average of values for each fit reported $\pm$ standard error of mean (SEM).

	Trial 1	Trial 2	Trial 3	Average of Fits
B_{max} (ΔAbs₄₃₀)	0.1475 (0.1443, 0.1507)	0.1909 (0.1823, 0.1995)	0.1751 (0.1713, 0.1792)	0.1712 $\pm$ 0.0127
K_d (μM)	49.6 (46.72, 52.49)	34.48 (29.44, 39.52)	25.69 (25.53, 27.85)	36.59 $\pm$ 6.98
Hill Coefficient	1.015 (0.9514, 1.079)	0.9812 (0.7548, 1.208)	1.022 (1.019, 1.026)	1.006 $\pm$ 0.013
R-square	0.9991	0.9937	0.9976	

Table 4.S8. Binding curve fits of titration of Cu-2xm-S112E-Sav into flav (with 95% confidence intervals) according to Equation X-4. Average of values for each fit reported $\pm$ standard error of mean (SEM).

	Trial 1	Trial 2	Trial 3	Average of Fits
B_{max} (ΔAbs₄₃₀)	0.04934 (0.041, 0.05769)	0.05978 (0.04925, 0.0703)	0.0648 (0.05108, 0.07852)	0.0580 $\pm$ 0.0046
K_d (μM)	20.95 (12.94, 28.97)	35.3 (24.7, 45.91)	39.44 (26, 52.88)	31.90 $\pm$ 5.60
NS	0.0001541	0.0001254	0.0001069	0.0001288 $\pm$ 1.37e-05
R-square	0.9945	0.997	0.9961	

Table 4.S9. Binding curve fits of titration of Co-2xm-S112E-Sav (O₂ bubbled through solution) into flav (with 95% confidence intervals) according to Equation X-2. Average of values for each fit reported $\pm$ standard error of mean (SEM).

	Trial 1	Trial 2	Trial 3	Average of Fits
B_{max} (ΔAb_{s430})	0.1689 (0.151, 0.1868)	0.1367 (0.1199, 0.1534)	0.1499 (0.1421, 0.1576)	0.1518 $\pm$ 0.0093
K_d (μM)	94.91 (76.64, 113.2)	87 (66.36, 107.6)	65.85 (59.77, 71.93)	82.59 $\pm$ 8.67
Hill Coefficient	1.222 (1.093, 1.352)	1.153 (1.004, 1.302)	1.333 (1.231, 1.434)	1.236 $\pm$ 0.052
R-square	0.9977	0.9966	0.9986	

Table 4.S10. Binding curve fits of titration of Ni-2xm-Sav into flav (with 95% confidence intervals). Average of values for each fit reported $\pm$ standard error of mean (SEM)

	Trial 1	Trial 2	Trial 3	Average of Fits
B_{max} (ΔAb_{s430})	0.1532 (0.1492, 0.1572)	0.1545 (0.1513, 0.1577)	0.1499 (0.1443, 0.1555)	0.1525 $\pm$ 0.0014
K_D (μM)	23.86 (21.41, 26.31)	22.96 (21.03, 24.89)	21.25 (17.94, 24.56)	22.69 $\pm$ 0.77
R-square	0.9967	0.9976	0.9923	

Table 4.S11. Small molecule LC-MS gradient method

Time (mins)	Flow (mL/min)	% A	%B	Curve
1.00	0.3	97.0	3.0	6
10.0	0.3	97.0	3.0	6
11.0	0.3	3.0	97.0	6
12.0	0.3	3.0	97.0	6
13.0	0.3	97.0	3.0	6
14.0	0.3	97.0	3.0	6
15.0	0.05	97.0	3.0	6

CHAPTER 5

Ironing Out the Gut Microbiome's Role in Parkinson's Disease

Preface

To me, one of the most exciting aspects of my undergraduate work was investigating intricate connections between human health/disease and metal micronutrients including zinc, copper and iron ions—I wanted to continue investigating these connections in my graduate studies. The old saying “you are what you eat” took on a whole new meaning when I began researching the chemistry of the gut microbiome. The gut microbiome consists of trillions of microscopic organisms that reside within a person's gastrointestinal tract. Each person has a unique microbiome composition, which is shaped by many factors including the environment we live in and the foods we eat. In turn, the microorganisms in the gut are responsible for many processes critical to host function. For example, they play important roles in digesting, absorbing and synthesizing an array of essential nutrients. On the other hand, factors such as illness, stress, or poor-quality diet can cause the gut microbiome to become imbalanced and contribute to disease. Accordingly, many human diseases have been linked to the gut microbiome, though our understanding of these relationships is limited, and direct causative roles are often highly debated. The early part of my graduate work focused on understanding the gut microbiome's role in Parkinson's disease (PD). While there is a clear connection between PD symptoms and brain damage, increasing evidence suggest that PD may originate in the gut. This work required development of chemistry, molecular biology, and microbiology techniques to probe the molecular-level mechanisms and metabolic pathways that drive protein misfolding in the context of the gut microbiome.

Background

The accumulation of α -synuclein (α -syn) protein aggregates in the *substantia nigra*, the brain site of motor control, leads to neuronal degradation and motor dysfunction symptoms of Parkinson's disease (PD).¹ It has been demonstrated in dopaminergic neurons in brain tissues that the accumulation of iron, often associated with aging, can cause oxidative stress that results in increased oxidation of labile cytosolic ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}).² Dopamine can also induce the dissolution of precipitated iron and release of Fe^{3+} from the storage protein ferritin.³ Fe^{3+} ions can then oxidize dopamine, a catecholamine hormone and neurotransmitter that is highly abundant in dopaminergic neurons, to dopamine-quinone.^{2,4} These highly reactive *ortho*-quinones can interact with the Y₁₂₅EMPS₁₂₉ peptide of α -syn to cause misfolding and aggregation that leads to the formation of pathogenic α -syn oligomers (**Figure 5.1 A**).⁵⁻⁹ The toxic pair of Fe^{3+} ions and dopamine in brain tissues has been suggested as a possible root cause of PD especially in non-genetic cases. There is accumulating evidence supporting an alternative proposal that the starting point of α -syn aggregation occurs in the gastrointestinal (GI) tract at least eight years prior to symptoms of motor dysfunction.¹⁰⁻¹² However, how pathogenic protein aggregation proceeds within the gut is not understood. α -Syn aggregates formed in the GI tract can spread to the brain in a putatively prion-like fashion via the Vagus nerve, a nerve connecting the brain stem to the GI tract.¹³⁻¹⁶ Although there are no reports that explain the mechanisms initiating α -syn aggregation within the GI tract, several factors suggest that the GI tract environment may be poised to seed α -syn aggregation. First, α -syn expression is not limited to the brain, as enteroendocrine cells and enteric neurons also express α -syn.^{17,18} Additionally, an astounding 46% of the body's dopamine is harbored within the GI tract, which also exhibits concentrations of iron ions reaching as high as 25 mM.^{19,20} In contrast to the oxidizing environment of the aging brain, the anoxic, reducing environment of a healthy GI tract is unlikely to promote

dopamine oxidation, thus α -syn aggregation is unlikely to occur. However, there are several processes that can disrupt the healthy GI tract environment, creating conditions that promote α -syn aggregation.

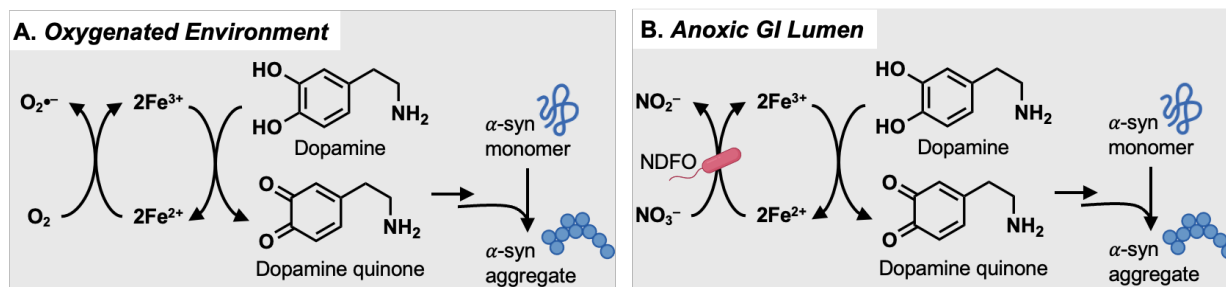


Figure 5.1. (A) Upon oxidation of Fe^{2+} to Fe^{3+} in brain dopaminergic neurons, dopamine can be oxidized to *ortho*-quinones that cause α -syn to misfold and aggregate. (B) Findings presented in this chapter lead us to speculate that nitrate-dependent iron oxidation by *E. coli* can occur in the anoxic GI tract, elevating levels of Fe^{3+} and triggering a similar cascade of reactions leading to α -syn aggregation. This pathway provides a target for early detection and/or prevention of PD.

Support for this proposal is derived from the observation that the gut microbiota of people with PD is distinct compared to non-diseased controls, though a causative role remains controversial.^{21–25} Murine models implicate the gut microbiome in PD etiology, where gnotobiotic mice overexpressing α -syn that are colonized with fecal samples from PD patients exhibit motor impairment and brain pathologies compared to mice colonized with fecal samples of healthy controls.²⁶ In another study, induction of intestinal inflammation resulted in accumulation of α -syn in the GI tract, followed by accumulation of pathogenic α -syn aggregates in the brains of α -syn overexpressing mice.^{27,28} Intestinal inflammation is commonly associated with blooms in *Enterobacteriaceae*, a bacterial family that is enriched in the fecal microbiomes of people with PD, and the abundance of which is correlated to the severity of motor symptoms.^{23,24,29,30} Facultative anaerobic *Enterobacteriaceae* can oxidize Fe^{2+} to Fe^{3+} as a consequence of bacterial nitrate-dependent iron oxidation (NDFO), suggesting a bacterial metabolic pathway that may be involved in anaerobic dopamine-mediated α -syn aggregation (**Figure 5.1 B**).^{31,32}

In addition to bacterial NDFO, other processes in the gut may cause dysbiosis and alter iron speciation and bioavailability, and there is a long history in studying iron dysregulation and

accumulation in PD patients, especially within brain tissues.^{33–35} As such, clinical studies have shown promise for the use of iron chelators to slow PD progression.^{36–39} Additionally, orally dosed antibiotics have been correlated with elevated risk for development of PD and are known to alter the redox potential of the gut microbiome environment, which may in turn alter iron ion speciation.^{40,41} Other studies demonstrate that diet alters the gut microbiome composition, and consumption of dietary (poly)phenolic compounds is correlated with lower risk of development of PD and may even ameliorate PD motor systems, though the mechanisms behind these observations within the context of the GI tract remain undefined.^{42–45}

Given the links between gut microbiome compositions, iron ion regulation, α -syn aggregation, and PD, there is a critical need to elucidate the mechanisms for how the gut microbiome contributes to PD. This chapter details a new approach to understanding the gut microbiome's role in PD, corroborating the proposal that dopamine and Fe^{3+} can induce α -syn aggregation in an anoxic environment like the GI tract. Evidence is presented identifying gut bacterial nitrate respiration as a potential trigger for initiating α -syn aggregation in the GI tract.⁴⁶ Then, three strategies are investigated to manipulate gut microbiome redox potential, which could alter iron availability to inhibit dopamine oxidation and α -syn aggregation in the gut.

Results and Discussion

Dopamine and ferric iron induce α -syn aggregation in vitro:

Previously published works demonstrated that in oxygenated environments, dopamine can be oxidized to cause α -syn misfolding and aggregation.^{2,4} In anoxic conditions, dopamine oxidation can be catalyzed by Fe^{3+} ions; however, subsequent α -syn aggregation had not been confirmed under these conditions. First, I investigated whether addition of dopamine, Fe^{3+} , and α -syn, in varying orders of addition could lead to α -syn aggregation under anaerobic, reducing atmosphere at 37 °C similar to the

conditions of the GI tract. All samples containing dopamine, FeCl_3 , and α -syn, regardless of the order in which they were added to the solution, showed formation of higher molecular weight α -syn oligomers after as soon as 4 h incubation, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 5.2**). This contrasts with samples containing FeCl_2 , dopamine and α -syn, which did not induce α -syn aggregation (**Figure 5.2**). This supported the idea that the presence of Fe^{3+} ions and dopamine in an anoxic environment, like the human gut, can trigger α -syn aggregation. Investigation of how the gut microbiome may create these conditions for α -syn aggregation will be discussed next.

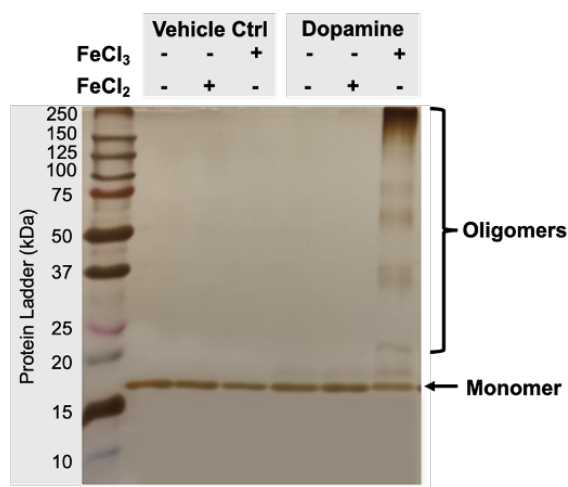


Figure 5.2. SDS-PAGE analysis of solutions of monomeric α -syn incubated under anaerobic conditions for 4 h with Fe^{2+} or Fe^{3+} and in the presence or absence of dopamine. The presence of higher molecular weight α -syn oligomers is only observed in samples containing the combination of α -syn, dopamine and Fe^{3+} .

Escherichia coli nitrate respiration initiates a cascade of reactions that lead to α -syn aggregation:

After demonstrating that dopamine-mediated α -syn aggregation is plausible in an anoxic environment, we sought to identify how the gut microbiome may contribute to this process. Previously published work reports that nitrate generated as a by-product of the host inflammatory response fuels a bloom of nitrate-respiring *Enterobacteriaceae* by working as a final electron acceptor in the electron transport chain.^{28,47} *Escherichia coli*, a member of the *Enterobacteriaceae* family which are enriched in the fecal microbiotas of people with PD compared to healthy controls, are facultative

anaerobes capable of both fermentative and anaerobic reparative metabolism. In comparison to fermentation conditions (in media supplemented with FeCl_2 500 μM , and glucose 20 mM as the sole carbon source), anaerobic respiration conditions (in the same media but supplemented with 50 mM nitrate as the final electron acceptor, which is endogenously produced by the human host and elevated during inflammation) afforded a 1.6-fold increase in *E. coli* K-12 bacterial growth after 12 h of incubation as measured by optical density of cultures at 600 nm (OD_{600}) (**Figure 5.3 A**). This result is consistent with nitrate respiration being more energetically lucrative than fermentative metabolism.

Bacteria cultured in respiration conditions reduced nitrate to nitrite, as measured by Griess assay. (**Figure 5.3 C**). Because nitrite is a redox-active molecule, we reasoned that redox potential of the media would shift to oxidizing conditions as a function of the production and accumulation of this metabolite. To test this hypothesis, fluctuations in redox potential relative to sterile controls were measured. In bacterial cultures grown under fermentation conditions, a reducing redox potential was measured, while bacterial cultures grown under respiration conditions demonstrated a steadily increasing oxidizing potential (**Figure 5.3 B**). Increases in oxidative redox potential coincided with the accumulation of nitrite in culture media, supporting the idea that bacterial nitrate respiration can shift the redox potential of the environment. Next, the impact of oxidizing conditions generated during nitrate respiration on iron ion speciation was assessed. Using the ferrozine assay, Fe^{2+} ions were found to be the predominant species under fermentative conditions (**Figure 5.3 D**), while Fe^{3+} ions were the predominant species under respiration conditions (**Figure 5.3 E**). These findings demonstrate that the presence of nitrate enhances *E. coli* K-12 growth and creates an oxidizing environment that correlates with nitrite production and shifts the predominant speciation of iron ions in culture media. Moreover, these results point to a scenario where intestinal inflammation may create optimal conditions for triggering the cascade of reactions implicated in dopamine-mediated α -syn aggregation.

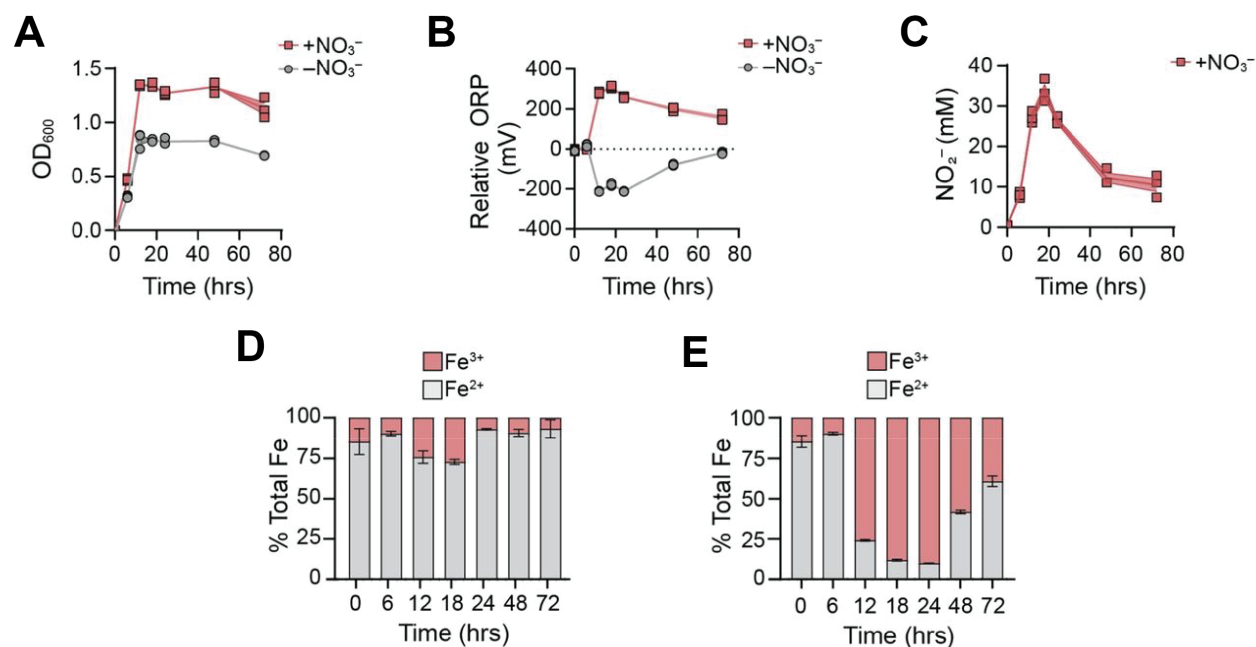


Figure 5.3. *E. coli* K-12 was incubated under anaerobic conditions in mineral media with or without nitrate. (A) Growth was measured by optical density at 600 nm (OD₆₀₀). (B) Oxidation-reduction potential (ORP) was measured using an electrode, in culture media relative to sterile media. (C) Nitrite was quantified in cultures supplemented with nitrate using Griess assay. (D) Iron speciation measured using ferrozine assay in bacterial cultures without nitrate or (E) supplemented with nitrate. n=3 biological replicates; bars denote mean $\pm$ S.E.M.

We next sought to determine whether nitrate respiration by *E. coli* K-12 could lead to dopamine oxidation and α -syn aggregation. Again, *E. coli* K-12 was anaerobically cultured until stationary phase under fermentation or respiration conditions, now with the addition of α -syn monomer (20 μ M) and dopamine (500 μ M) or its vehicle control. As in the previous experiment, bacteria cultured under nitrate-respiring conditions reduced nitrate to nitrite (**Figure 5.4 A**), coinciding with an increase in the relative abundance of Fe³⁺ ions (**Figure 5.4 B**) compared to cultures without nitrate supplementation. Nitrate-respiring cultures supplemented with dopamine resulted in lower relative abundance of Fe³⁺ ions compared to conditions without dopamine, putatively suggesting dopamine oxidation being coupled to reduction of Fe³⁺ ions (**Figure 5.4 B**). In nitrate-respiring cultures containing dopamine, formation of a dark pigment characteristic of polymerization of quinones was observed (**Figure 5.4 D**) and presence of quinones was detected by redox-cycling stain nitroblue tetrazolium (NBT) (**Figure**

5.4 E). In contrast, no dark pigment was observed in dopamine-supplemented fermentative culture conditions, and the presence of quinones was not detected. In the absence of dopamine supplementation to culture media, quinones were not detected in nitrate-respiring nor fermenting bacterial cultures. These findings indicate that dopamine-dependent quinone formation occurs in the oxidizing conditions resulting from bacterial nitrate respiration. Finally, in nitrate-respiring cultures supplemented with dopamine, we observe α -syn aggregation by dot blot immunostaining on membranes spotted with culture media (Figure 5.4 F). Significantly greater amounts of α -syn aggregates formed in nitrate-respiring conditions supplemented with dopamine compared to fermentation conditions with or without dopamine (Figure 5.4 C). These results highlight the critical role that bacteria, oxidative stress and dopamine play in α -syn aggregation under anaerobic conditions.

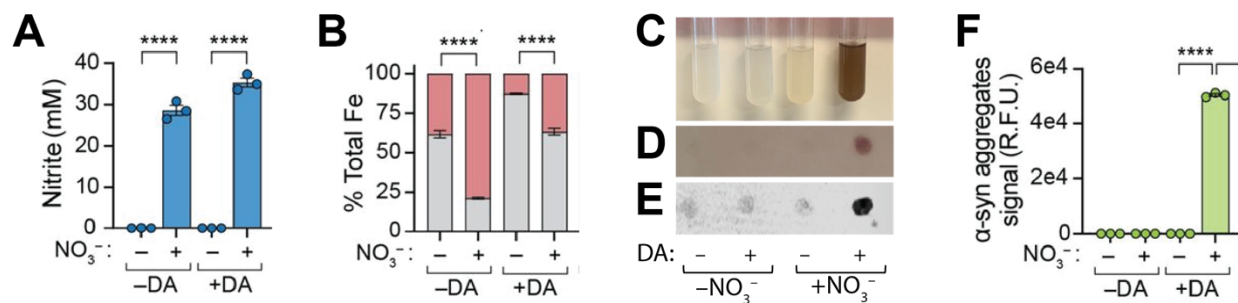


Figure 5.4. *E. coli* nitrate respiration instigates dopamine-dependent quinone formation and α -syn aggregation. *E. coli* K-12 was cultured for 14 h in mM9 media with α -syn monomer in the presence of nitrate (+NO₃⁻) or its absence (-NO₃⁻) and with dopamine (+DA) or without (-DA). Quantification of (A) nitrite (using Griess assay), (B) labile Fe³⁺ and Fe²⁺ (using ferrozine assay), and (C) α -syn aggregates in dot blots (R.F.U.: relative fluorescence units). Representative images of (D) bacterial cultures as well as (E) membrane-stained quinone formation (using NBT stain) or (F) dot blots stained for α -syn aggregate formation (using immunostaining with MJFR-14 anti-fibril α -syn as the primary antibody). *n* = 3 biological replicates; bars denote means $\pm$ S.E.M.; significance was determined using ordinary one-way ANOVA with Sidak's multiple comparisons test; and ****: *P* < 0.0001, ns: not significant.

Disruption of Escherichia coli nitrate respiration with sodium tungstate supplementation inhibits dopamine oxidation and α -syn aggregation:

After confirming that *E. coli* metabolism could induce dopamine oxidation and α -syn aggregation in anoxic conditions, we investigated ways to perturb bacterial nitrate respiration as a means of

inhibiting this cascade of reactions. In *E. coli* K-12, the nitrate reductases involved in nitrate respiration contain a molybdenum cofactor, and it has previously been demonstrated that enzymatic respiration may be inhibited by incorporation of tungstate as an alternative to molybdate.^{27,48} We evaluated the effects of supplementing sodium tungstate (Na_2WO_4 , 0.5–100 mM) on *E. coli* K-12 cultured until stationary phase under previously mentioned respiration conditions in the presence of dopamine and α -syn monomer. Dose-dependent relationships between increasing concentrations of Na_2WO_4 and decreasing production of nitrite, decreasing oxidizing redox potentials of cultures, and decreasing relative abundance of Fe^{3+} were observed, indicating inhibition of NDFO by Na_2WO_4 (**Figure 5.5 A-D**). Increasing concentrations of Na_2WO_4 further correlated with decreased visible pigmentation of cultures and NBT-stained quinone, as well as decreased formation of α -syn aggregates (**Figure 5.5 E-H**). These results further corroborate the role of bacterial anaerobic respiration in dopamine oxidation and α -syn aggregation. Moreover, the reveal Na_2WO_4 may be an effective strategy to ameliorate the effects of bacterial *E. coli* NDFO on dopamine oxidation and α -syn aggregation.

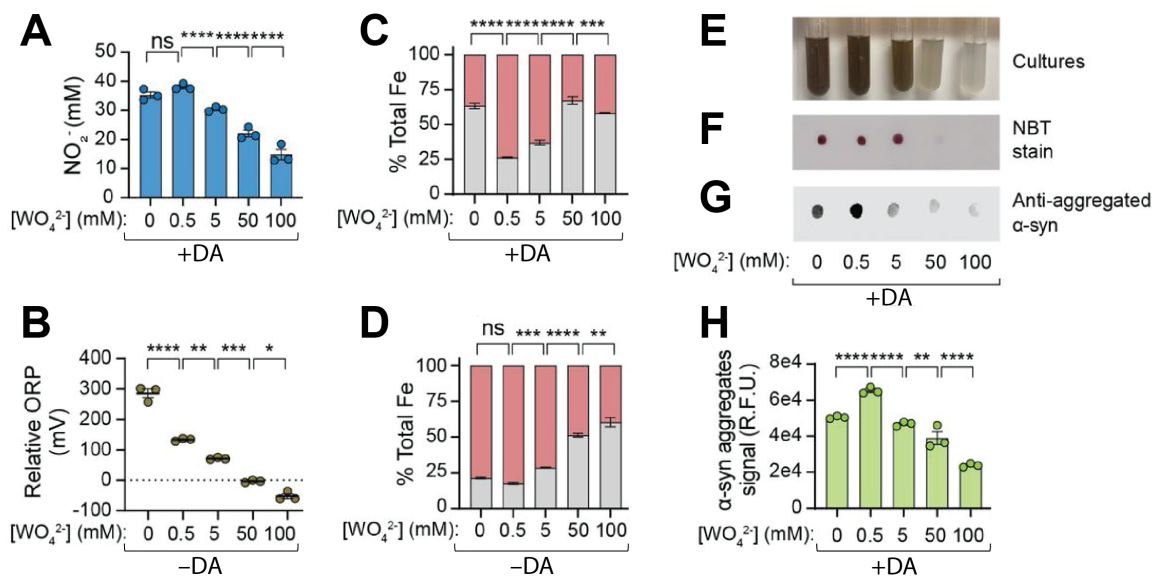


Figure 5.5. *E. coli* K-12 was cultured for 14 h in mineral media with α -syn monomer in the presence of nitrate (+ NO_3), with dopamine (+DA) or without (-DA), and with sodium tungstate (WO_4^{2-}) at varying concentrations (0–100 mM). Quantification of (A) nitrite (using Griess assay), (B) ORP relative to sterile media (using redox electrode), and labile Fe^{3+} and Fe^{2+} (using ferrozine assay) in cultures (C) with dopamine or (D) without dopamine. (E) bacterial cultures of *E. coli* K-12 incubated in mineral media in the presence of nitrate

and dopamine, supplemented with tungstate (0–100 mM), (**F**) membranes stained for quinone formation (using NBT stain), and (**G**) dot blots stained for α -syn aggregate formation (using immunostaining with MJFR-14 anti-fibril α -syn as the primary antibody). (**H**) Quantification of α -syn aggregates in dot blots. R.F.U.: relative fluorescence units. $n = 3$ biological replicates; bars denote means $\pm$ S.E.M.; significance was determined using ordinary one-way ANOVA with Sidak's multiple comparisons test; and ****: $P < 0.0001$, ***: $P < 0.0007$, **: $P < 0.0026$, *: $P = 0.0168$, ns: not significant.

Inhibiting dopamine-dependent α -syn aggregation by supplementing iron chelators:

Recent clinical trials investigating iron chelation strategies in patients with early PD have provided contradictory insights. It has been hypothesized that iron chelation may ameliorate PD symptoms by mitigating oxidative stress and mitochondrial dysfunction involved in cell death, particularly in dopamine-producing neurons in the brain. Given our observations that the combination of dopamine and Fe^{3+} ions triggers α -syn aggregation, precluding dopamine-iron interactions could provide a tangible target for preventing α -syn aggregation before accumulation of aggregates causes irreversible damage to neurons in the brain. In addition to dopamine, many (poly)phenolic compounds derived from diet, pharmaceuticals, gut bacterial metabolism or host metabolism are found in the GI tract. Previous reports suggest that diets rich in polyphenols may ameliorate symptoms of neurodegenerative diseases including PD, though the mechanisms behind these observations in the GI tract remain unclear, leading us to hypothesize that these observations may be due, at least in part, to altering iron homeostasis in the GI tract, which may ultimately influence α -syn aggregation. Preliminary work in the Bess lab demonstrated that under anaerobic *in vitro* conditions described above, incubation of monomeric α -syn with Fe^{3+} ions and tyramine (a non-catecholic precursor to dopamine), 5,6-dihydroxyindole (a catecholic oxidation product of dopamine) or dihydrocaffeic acid (HA, a common dietary catechol implicated in ameliorating PD symptoms, **Figure 5.6 A**) did not result in formation of α -syn oligomers as determined by SDS-PAGE and dot blot immunostaining. I thus began to screen a library of compounds for their abilities to induce α -syn aggregation in cultures of nitrate-respiring *E. coli* K-12.

Ethylenediaminetetraacetic acid (EDTA) is an aminopolycarboxylic acid that is widely used to bind metal ions, with a particularly high affinity for Fe^{3+} . Compared to cultures of *E. coli* K-12 in the presence of nitrate, dopamine and α -syn, supplementation with EDTA (500 μM) resulted in significantly less α -syn aggregation (**Figure 5.6**). This result led us to further investigate potential iron-chelating compounds for their abilities to prevent dopamine oxidation and α -syn aggregation.

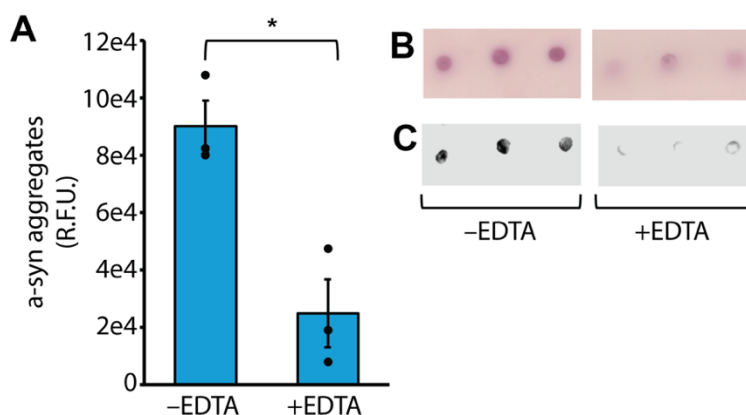


Figure 5.6. *E. coli* K-12 was cultured for 14 h in mineral media with dopamine and α -syn monomer in the presence of nitrate (+ NO_3), with or without EDTA. **(A)** Quantification of α -syn aggregates in dot blots. R.F.U.: relative fluorescence units. $n = 3$ biological replicates; bars denote means $\pm$ S.E.M, $t(4)=4.43$, *: $p<0.01$. **(B)** Membranes stained for quinone formation (using NBT stain), and **(C)** dot blots stained for α -syn aggregate formation (using immunostaining with MJFR-14 anti-fibril α -syn as the primary antibody).

Under fermentation conditions mentioned previously, supplementation of levodopa (LDOPA, a catecholic precursor to endogenous dopamine production as well as an anti-Parkinsonian drug, **Figure 5.7 A**), 3,4-dihydroxyphenylacetic acid (DOPAC, a catecholic metabolite of dopamine, **Figure 5.7 A**), or carbidopa (a catecholic drug given to PD patients, **Figure 5.7 A**), catechol (**Figure 5.7 A**), or HA did not result in formation of quinones detected by NBT stain, nor α -syn aggregation detected by dot blot immunostaining (**Figure 5.7 C–E**).

Under respiration conditions, supplementation of dihydroxyphenylacetic acid (DOPAC, a catecholic metabolite of dopamine) or carbidopa (a catecholic drug dosed to PD patients) resulted in quinone formation and α -syn aggregation, similar to dopamine (**Figure 5.7 C–E**). Of note,

supplementation of carbidopa at the time of inoculation hindered *E. coli* K-12 growth and resulted in significantly less nitrite production as indicated by Griess assay (**Figure 5.7 B**), which suggests an effect on *E. coli* metabolism as an alternative explanation for α -syn aggregation. Supplementation of LDOPA resulted in quinone formation detected by NBT staining, but not α -syn aggregation (**Figure 5.7 C–E**). On the other hand, supplementation of HA did not result in quinone formation detected by NBT, but did lead to α -syn aggregation (**Figure 5.7 C–E**). These results suggest that certain catechols can alter α -syn aggregation compared to dopamine, though there are other mechanisms besides catechol oxidation to quinone that determine whether α -syn will aggregate. In the complex milieu of the gut microbiome, it is important to understand the interplay between these molecules and future experiments are needed to corroborate a competitive effect of these catechols when dopamine is also present.

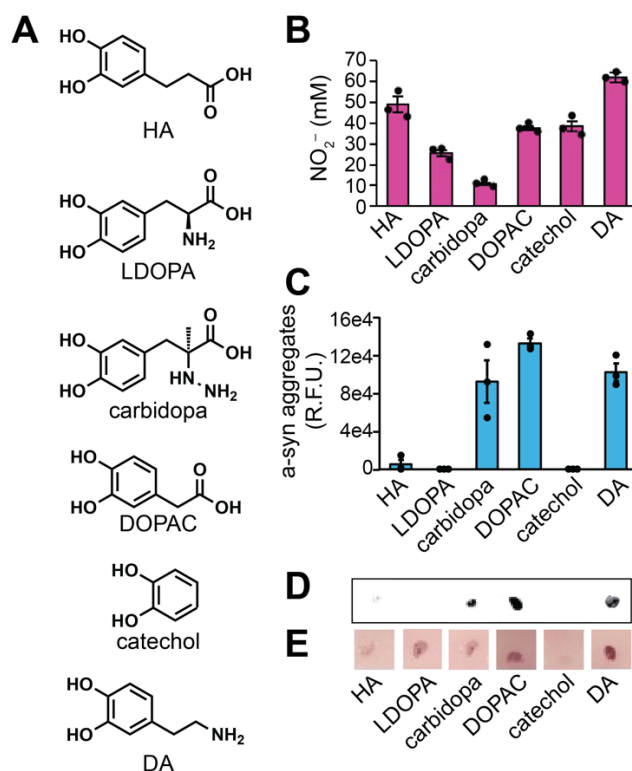


Figure 5.7. *E. coli* K-12 was cultured for 14 h in mineral media with α -syn monomer in the presence of nitrate (+NO₃), with catecholic compounds shown in (A). Quantification of (B) nitrite (using Griess assay) and (C) α -syn aggregates in dot blots. R.F.U.: relative fluorescence units. $n = 3$ biological replicates; bars denote means $\pm$ S.E.M. (D) Membranes stained for quinone formation (using NBT stain), and (E) dot blots stained for α -syn aggregate formation (using immunostaining with MJFR-14 anti-fibril α -syn as the primary antibody).

Ga(NO₃)₃ does not oxidize dopamine and cause α -syn aggregation

Another way to inhibit Fe³⁺-mediated dopamine oxidation and α -syn aggregation could be to preclude Fe³⁺-dopamine interactions by binding to other non-iron metals. Gallium (Ga³⁺) has been employed as an Fe³⁺ mimic in structural studies of catechol siderophore complexes because of their similarities in atomic size, charge, and coordination chemistry; however, Ga³⁺ is not redox active, so it cannot participate in the oxidation of dopamine. Instead, catechols such as dopamine that would otherwise be oxidized by to quinones, can chelate Ga³⁺. Given the success of employing Ga³⁺ as a replacement for Fe³⁺ in studies of siderophore interactions, studies have explored its use as an antimicrobial agent to disrupt microbial iron homeostasis. In *in vivo* studies, Ga³⁺ has been used as a nonfunctional iron analog to perturb iron-dependent functions.

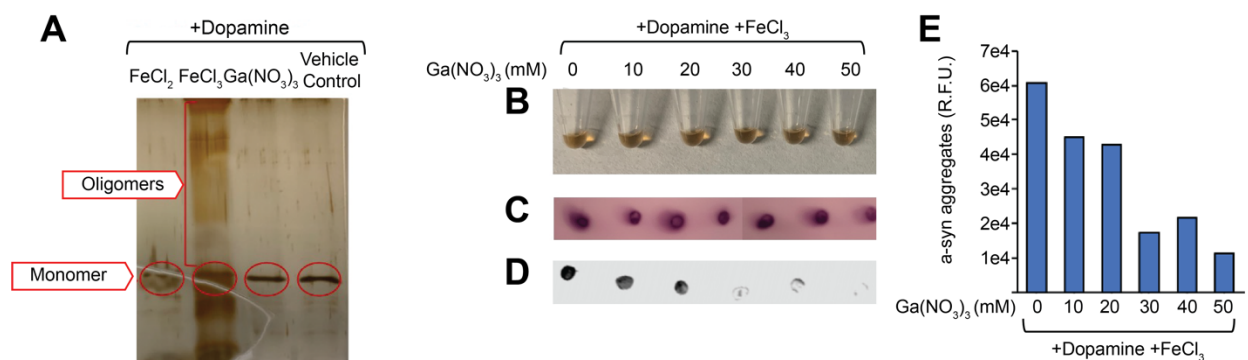


Figure 5.8. (A) SDS-PAGE analysis of solutions of monomeric α -syn incubated under anaerobic conditions for 4 h with dopamine and Fe²⁺ or Fe³⁺ or Ga³⁺. The presence of higher molecular weight α -syn oligomers is only observed in samples containing the combination of α -syn, dopamine and Fe³⁺. (B) solutions of mineral media in the presence of Fe³⁺ (50 mM) and dopamine, supplemented with gallium nitrate (0–50 mM), (C) membranes stained for quinone formation (using NBT stain), and (D) dot blots stained for α -syn aggregate formation (using immunostaining with MJFR-14 anti-fibril α -syn as the primary antibody). (E) Quantification of α -syn aggregates in dot blots. R.F.U.: relative fluorescence units.

In contrast to samples containing Fe³⁺ in the presence of dopamine and α -syn, samples containing gallium nitrate did not show formation of higher molecular weight α -syn oligomers after 4 h incubation under anaerobic, reducing atmosphere at 37 °C, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 5.8 A). When samples containing Fe³⁺ in the presence of dopamine and α -syn were supplemented with increasing amounts of gallium nitrate,

we observe quinone formation by NBT stain (**Figure 5.8 B and C**), as well as a dose-dependent decrease in α -syn aggregation as determined by dot blot immunostaining (**Figure 5.8 D and E**).

We further evaluated the effects of supplementing gallium chloride on *E. coli* K-12 cultured until stationary phase under previously mentioned respiration conditions in the presence of dopamine and α -syn monomer. Supplementation with gallium chloride (500 μ M) did not significantly affect *E. coli* K-12 growth. Compared to cultures of *E. coli* K-12 in the presence of nitrate, dopamine and α -syn, supplementation with gallium chloride (500 μ M) resulted in significantly less α -syn aggregation (**Figure 5.9**).

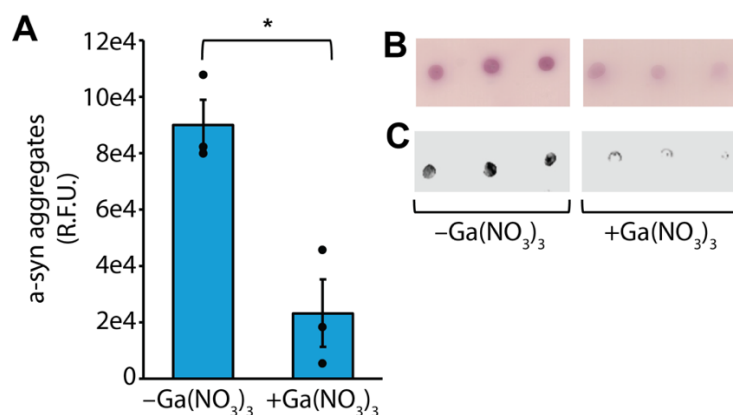


Figure 5.9. *E. coli* K-12 was cultured for 14 h in mineral media with dopamine and α -syn monomer in the presence of nitrate (+NO₃), with or without GaCl₃. **(A)** Quantification of α -syn aggregates in dot blots. R.F.U.: relative fluorescence units. $n = 3$ biological replicates; bars denote means $\pm$ S.E.M., $t(4)=4.50$, *: $p<0.01$. **(B)** Membranes stained for quinone formation (using NBT stain), and **(C)** dot blots stained for α -syn aggregate formation (using immunostaining with MJFR-14 anti-fibril α -syn as the primary antibody).

Conclusions and Future Directions

The work presented in this chapter outlines a possible mechanism by which gut bacterial metabolism influences the redox potential of the surrounding environment, disrupts iron homeostasis and may cause abnormal protein aggregation. In the presence of nitrate, anaerobic cultures of *E. coli* K-12 reduce nitrate to nitrite as part of nitrate dissimilatory metabolism, resulting in an oxidizing redox environment compared to cultures without nitrate. In contrast, it has been demonstrated in our

published work that nitrate supplied to *E. coli* K-12 $\Delta moaA$, bacteria with a nitrate respiration defect, resulted in neither nitrite production nor a more oxidizing redox potential.⁴⁶ This shift in redox potential coincides with a shift in iron ion speciation, where Fe^{3+} predominates over Fe^{2+} despite the anaerobic and reducing culture conditions simulating those of the GI tract. When nitrate-respiring cultures are supplemented with dopamine and α -syn, dopamine quinone and α -syn aggregates form, strongly implicating that gut bacterial nitrate metabolism triggers a cascade of reactions that ultimately causes α -syn to aggregate *in vitro*.

We further investigated three strategies to target this biochemical pathway to prevent α -syn aggregation. First, due to previously reported inhibition of *Enterobacteriaceae* nitrate respiration by tungstate, we sought to determine whether supplementing tungstate could inhibit α -syn aggregation in cultures of nitrate-respiring *E. coli*. Supplementation of tungstate lessened the shifts in redox potential and iron ion speciation compared to cultures without tungstate, ultimately limiting dopamine oxidation and α -syn aggregation in cultures of *E. coli* K-12 supplemented with nitrate. Based on previous reports of oral tungstate treatment effectively ameliorating murine colitis exacerbated by gut bacterial nitrate-respiration, our results may have further therapeutic implications for the use of tungstate in preventing α -syn aggregation in the GI tract.

Second, given our observations that the presence of Fe^{3+} ions is necessary for α -syn aggregation, we sought to determine whether supplementing compounds capable of chelating iron could inhibit α -syn aggregation in cultures of nitrate-respiring *E. coli*. Supplementation of EDTA to nitrate-respiring cultures of *E. coli* K-12 in the presence of dopamine and α -syn resulted in significantly less α -syn aggregation compared to without EDTA. Preliminary work supplementing nitrate-respiring cultures of *E. coli* K-12 with various catecholic compounds demonstrate that not all compounds behave like dopamine in triggering α -syn aggregation, which further supports the idea that it may be possible to limit dopamine-iron interactions when these compounds are present. Further work is needed to

understand how each compound interacts with iron and α -syn, as well as competition between various compounds within the GI tract. Further work by J. M. B. developed a laser-induced graphene-based electrochemical sensor chip to track α -syn aggregation and dopamine oxidation over time, which may be a valuable tool to assay a library of compounds for their abilities to inhibit dopamine oxidation and resulting α -syn aggregation.⁴⁹

Lastly, we investigated the use of gallium ions in disrupting dopamine oxidation by Fe^{3+} ions and subsequent α -syn aggregation due to the similarities between Ga^{3+} and Fe^{3+} ions in atomic size, charge, and coordination chemistry, though importantly, gallium is not redox active like iron. Supplementing gallium chloride to cultures of *E. coli* K-12 supplemented with nitrate did not influence bacterial growth and ultimately limited α -syn aggregation. Based on previous reports of intraperitoneally administered gallium nitrate as an effective antibiotic without causing adverse effects in murine models, these results may have further implications for the use of gallium in preventing α -syn aggregation in the GI tract.

Further work has been focused on expanding these findings to enteroendocrine cells (EECs),⁴⁶ which have been implicated in PD as a source of intestinal α -syn as well as the starting point of α -syn aggregates that may spread from EECs to the brain via the vagus nerve; however, α -syn aggregation in EECs is not well understood. In EECs, nitrite exposure (0.5–50 mM) resulted in a dose-dependent increase in α -syn aggregation, supporting the notion that bacterial nitrate respiration can induce α -syn aggregation in EECs which line the GI lumen.

These *in vitro* findings suggest a causative role of bacterial nitrate respiration on α -syn aggregation, prompting further investigations into the role of this biochemical pathway on neurodegeneration *in vivo* using a *Caenorhabditis elegans* model of PD.⁵⁰ Acute exposure of *C. elegans* to *E. coli* K-12 that reduced nitrate to nitrite caused significant acceleration of dopaminergic neurodegeneration as compared to treatment of cultures of *E. coli* K-12 without nitrate. Similarly, exposure to nitrite induced

neurodegeneration. Furthermore, mutation of α -syn residues Y₁₂₅EMPS₁₂₉, residues that have been identified to interact with dopamine quinone to cause misfolding and aggregation, prevented neurodegeneration. These findings suggest that acute exposure of *C. elegans* to the metabolic product of bacterial nitrate metabolism putatively induces formation of dopamine-derived quinones that interact with the C-terminus of α -syn to cause neurotoxic α -syn aggregation.

Materials and Methods

Chemicals:

Cell culture: M9 minimal salts were purchased from Sigma-Aldrich, and magnesium sulfate, calcium chloride, glucose, casamino acids and iron (II) chloride tetrahydrate were purchased from Fisher Scientific. Lysogeny broth (LB) was purchased from Neta Scientific. Ampicillin and isopropyl β -d-1-thiogalactopyranoside (IPTG) were purchased from Fisher Scientific. Bacteriological agar and glycerol were purchased from Sigma-Aldrich.

Osmotic shock buffer: Tris-HCl (1 M, pH 7.4) and magnesium chloride were purchased from Fisher Scientific. Ethylenediaminetriacetic acid sodium (EDTA) was purchased from Millipore Sigma. Sodium chloride was purchased from Sigma-Aldrich.

Protein quantification assays: Coomasi Brilliant Blue G-250 and 200 proof ethanol were purchased from VWR. Phosphoric acid was purchased from Fisher Scientific. Lyophilized bovine plasma gamma globulin was purchased from Sigma-Aldrich. Modified Lowry Protein Assay Kit was purchased from Bio Basic.

Denaturing gels: Acrylamide (30%), sodium dodecyl sulfate (SDS, 10%), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), and 4x Laemmli sample buffer were purchased from Bio-rad. Pierce Silver Stain kit was purchased from Thermo Fisher.

Griess assay: Naphthylethylenediamine dihydrochloride (NEDD), sulfanilamide (SULF), and hydrochloric acid (HCl, 36%) were purchased from Sigma-Aldrich. Sodium nitrite was purchased from Alfa Aesar. ACS-grade methanol was purchased from Sigma-Aldrich.

Dot blot: Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore Sigma. *p*-Nitroblue tetrazolium chloride (NBT) was purchased from Santa Cruz Biotechnologies. Glycine was purchased from Neta Scientific. Sodium tetraborate decahydrate was purchased from Sigma-Aldrich. Non-fat dry milk (blocking buffer) was purchased from Bio-Rad. Anti- α -synuclein antibody and anti- α -syn filament antibody were purchased from Abcam. Dopamine HCl was purchased from Alfa Aesar. Lyophilized polyphenol oxidase (tyrosinase) isolated from *Agaricus bisporus*, was purchased from Worthington Biochemical.

Effect of iron and dopamine on α -syn aggregation: Dopamine HCl was purchased from Alfa Aesar. Ferric chloride hexahydrate was purchased from Sigma-Aldrich. Ferrous chloride tetrahydrate was purchased from Fisher Scientific and stored in the anaerobic chamber to prevent oxidation.

Effect of tungstate on E. coli growth: Sodium tungstate was purchased from Sigma-Aldrich.

Effect of catechols on α -syn aggregation: 3,4-dihydroxyphenylacetic acid (DOPAC), levodopa (LDOPA), and catechol were obtained from Oakwood Chemicals. Dihydrocaffeic acid (HA) was purchased from Alfa Aesar. Carbidopa was obtained from the Balskus Lab at Harvard University. Gallium nitrate was purchased from Santa Cruz biotechnologies

Bacterial strains:

Escherichia coli K-12 was purchased from VWR. *E. coli* Rosetta 2 was obtained from the Turnbaugh lab at University of California, San Francisco. Cultures were stored as glycerol stocks at -80 °C.

General bacterial culture conditions:

All media for anaerobic experiments were equilibrated in the anaerobic chamber (COY Lab Products chamber filled with 5% hydrogen, 20% carbon dioxide, 75% nitrogen) for 12 h prior to use.

- Modified M9 mineral media, herein referred to as mM9 (3.37 mM Na₂HPO₄, 2.2 mM KH₂PO₄, 0.855 mM NaCl, 0.935 mM NH₄Cl, 1 mM MgSO₄, 0.3 mM CaCl₂, 20 mM glucose, 0.2% Casamino acids, 1% ATCC vitamins, 500 µM FeCl₂).
- Lysogeny broth supplemented with glucose, herein referred to as LBg (2 g LB/100 mL water, 20 mM glucose).
- Lysogeny broth supplemented with ampicillin, herein referred to as LBamp (2 g LB/100 mL water, 100 µg/mL ampicillin).

Denaturing gels:

SDS-12% polyacrylamide resolving gel mixture (Table X) was poured into cassettes, a thin layer of water was added to the top, and gels were allowed to polymerize for 45 minutes. Water was removed before pouring stacking gel mixture (Table X) and adding a comb, then allowed to polymerize for 45 minutes.

Into PCR tubes, sample to be analyzed by SDS-PAGE were aliquoted to 30 µL. To each sample, 10 µL of 4x Laemmli buffer was added, then samples were heated to 90 °C for five minutes in a thermocycler (Bio-Rad C1000 Touch Thermal Cycler). An aliquot of 10 µL was pipetted into each well of the gel. Gel electrophoresis was carried out in running buffer (10x dilution of solution detailed in Table X) at 120 mV. Gels were stained by Pierce silver stain according to manufacturer's instructions.

Overexpression and purification of α -syn:

Plasmid pET3a- α -synuclein was acquired from Addgene and transformed into *E. coli* Rosetta 2 by Dr. Ortiz de Ora. Purification of α -syn was performed according to Huang et al., 2005. An overnight culture of transformed *E. coli* Rosetta 2 with pET-3a was diluted 100-fold and induced at an A_{600} of 0.3–0.4 for 5 h with 100 μ M isopropyl β -D-1-thiogalactopyranoside (IPTG). All cultures were grown in LBamp at 37 °C. The cell pellet from a 1 L culture was resuspended in 100 mL osmotic shock buffer (30 mM Tris-HCl, 40% sucrose, 2 mM EDTA, pH 7.2) and incubated for 10 minutes at room temperature. After centrifugation at 12,000 rpm for 20 minutes, the pellet was resuspended quickly with 90 mL of cold water followed by addition of 37.5 μ L saturated $MgCl_2$ and kept on ice for 3 minutes. The supernatant containing periplasmic proteins was collected after centrifugation and dialyzed against 20 mM Tris-HCl and 2 mM EDTA, pH 8.0 overnight. After centrifugation at 12,000 rpm for 20 minutes, the supernatant was loaded onto a Q-Sepharose Fast Flow column (Amersham-Pharmacia Biotech) and eluted with 0–0.5 M NaCl gradient in 20 mM Tris-HCl pH 8.0. Elution fractions were analyzed by SDS-12% PAGE (**Fig. S10a**), and the fractions containing only 18 kDa band were combined for size exclusion chromatography in 0.00095% $MgCl_2$, 0.316% Tris-HCl, 0.58% NaCl, pH 7.5 (Abcam buffer). Fractions were analyzed by SDS-12% PAGE, and the fractions containing pure α -syn were aliquoted and stored at -20 °C (**Fig. S10b**). Protein was quantified using three methods: Lowry protein assay, Bradford protein assay, and nanodrop UV-visible spectroscopy (Thermo Scientific NanoDrop One/One Microvolume UV-Vis Spectrophotometer; $\epsilon=5,960\text{ M}^{-1}\text{ cm}^{-1}$), as detailed below. α -Syn aggregation was evaluated by dot blot immunostaining using the anti-fibril antibody (**Fig S10c**). The purified and concentrated sample was analyzed by SDS-PAGE, then visualized and quantified in a Chemidoc XRS+ imaging system. Monomeric α -syn was successfully purified, and it is >90% pure.

Lowry assay for protein quantification: Modified Lowry Protein Assay Kit was purchased from Bio Basic. Assay was conducted according to manufacturer's instructions using a plate reader (Spectrostar Nano, BMG Lab Technologies).

Bradford assay for protein quantification: Lyophilized bovine plasma gamma globulin was resuspended to 1, 5, 7.5, and 10 μ /mL in 0.15 M sodium chloride. 100 μ L of each standard or unknown protein sample were added to separate microcentrifuge tubes and 1.0 mL Bradford reagent (100 mg Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol, add 100 mL 85% phosphoric acid and dilute to 1 L) was added to each tube. After two minutes, absorbance of each sample was read at 595 nm. A calibration curve of standard concentration versus absorbance was created.

Griess assay for nitrite quantification:

Two solutions were prepared as follows: 0.2% NEDD was dissolved in water, and 4% SULF was dissolved in 5% HCl. The two solutions were mixed in a 1:1 ratio to make 0.1% NEDD and 2% SULF, herein referred to as Griess reagent. A standard curve for nitrite was prepared as follows: from a 50 mM stock solution, 1 mM nitrite solution in methanol was prepared in a 96-well plate. A two-fold serial dilution was carried out to create solutions from 0.97 to 1000 μ M to final volumes of 100 μ L. 50 μ L of sample was transferred to clean wells on the same 96-well plate. 50 μ L Griess reagent was added, with mixing by aspiration. Absorbance was read at 540 nM in a plate reader (Spectrostar Nano, BMG Lab Technologies). See **Fig. S11** for the calibration curve.

Bacterial cultures were analyzed by adding 1 μ L supernatant to 199 μ L methanol to precipitate proteins. Samples were then mixed and centrifuged at 2000 rpm for 10 minutes. 50 μ L of sample was transferred to a 96-well plate. 50 μ L Griess's reagent was added, with mixing by aspiration. Absorbance was read at 540 nM in a plate reader (Spectrostar Nano, BMG Lab Technologies). Concentration of nitrite present in samples was determined by comparison to calibration curve.

Dot blot analysis: Polyvinylidene difluoride (PVDF) membrane was activated in methanol for one minute, rinsed with nanopure water, then equilibrated in Tris buffered saline (TBS) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, filter sterilized). To detect the presence of quinone, monomeric α -syn and/or aggregated α -syn in samples, 2 μ L of sample was spotted onto the equilibrated membrane and allowed to dry completely. On the blots that were used to visualize presence of quinones, a negative control of 2 μ L of 50 μ M dopamine and a positive control of 2 μ L of oxidized dopamine (see protocol below) were also spotted onto the membrane. On the blots that were used to visualize presence of α -syn, 2 μ L of 20 μ M monomeric α -syn (received from Abcam) and 2 μ L of 20 μ M aggregated α -syn (received from Abcam) were also spotted onto the membrane. Blots were visualized by nitroblue tetrazolium (NBT) staining or immunostaining as detailed below.

Quinone-specific NBT staining:

To visualize presence of quinone, dried membranes were reactivated in methanol then TBS. Membranes were immersed in a solution of 0.6 mg/mL nitroblue tetrazolium (NBT) in potassium glycinate, pH 10 and incubated with gentle shaking for 45 minutes protected from light. Membranes were washed twice with 0.16 M sodium borate and submerged in 0.16 M sodium borate for 12 h. Quinone concentration was quantified by measuring stain intensity by pixels in Image Studio.

Oxidizing dopamine for NBT positive control:

To obtain dopamine-quinone for use as a control for NBT staining, dopamine was oxidized by tyrosinase. Lyophilized tyrosinase, isolated from mushroom *Agaricus bisporus*, was resuspended in 1000 μ L 50 mM phosphate buffer, 15% glycerol, pH 6.5, to create a 24 kU/mL solution (50 mg/mL, 390 μ M). 100 μ L single-use aliquots were prepared and stored at -20 °C. Aliquots were thawed on ice

shortly before use. 500 μ M dopamine was oxidized by adding 250 U (3.9 μ M) of tyrosinase. The solution was incubated for one hour at room temperature.

Immunostaining for α -syn and α -syn aggregate:

To visualize presence of α -syn, dried membranes were reactivated in methanol then TBS. Membranes were submerged in blocking buffer (5% non-fat dry milk in TBS) for one hour at room temperature. Membranes were then incubated in primary antibody solution (0.1% anti-monomeric α -syn or anti-aggregated α -syn, 0.2% Tween 20 in blocking buffer) and incubated for 2 h at room temperature with gentle shaking, then incubated at 4 °C for 12 h. Membranes were washed with TBS-T (0.1% Tween 20 in TBS) then incubated in secondary antibody solution (0.05% secondary antibody, 0.2% Tween 20, 0.02% SDS in blocking buffer) at room temperature and protected from light for 45 minutes. Membranes were again washed with TBS-T then TBS and imaged on an Odyssey Imaging System. Aggregate intensity was measured in Image Studio.

Effect of Fe^{3+} and dopamine on α -syn aggregation:

All solutions and consumables were equilibrated in the anaerobic chamber for 24 h, except for Fe^{3+} solutions to prevent reduction to Fe^{2+} . In PCR tubes, 20 μ M α -syn was incubated with 50 μ M $FeCl_3 \cdot 6H_2O$ or $FeCl_2 \cdot 4H_2O$ in the presence or absence of 50 μ M dopamine. Samples were made with differing order of addition of α -syn, iron, and dopamine. All samples were incubated at 37 °C under anaerobic conditions for 4 h. Then, 7 μ L of 4x Laemmli sample buffer was added to each tube, heated at 90 °C for five minutes and stored at 20 °C until further analysis. To assess the formation of SDS-resistant oligomers a 12% denaturing acrylamide gel was prepared. The gel was then stained using Pierce silver stain kit, according to the manufacturer's instructions.

Effect of tungstate on *E. coli* growth, NDFO, and dopamine oxidation:

All solutions and consumables were equilibrated in the anaerobic chamber for 24 h except for Fe^{3+} solutions to prevent reduction to Fe^{2+} . Into four hungate tubes, 5 mL of mM9 media was aliquoted into each tube. Tubes were inoculated in triplicate with *E. coli* K12 that was cultured on LBg agar plates. The additional tube served as a sterile control. Tubes were incubated with agitation at 37 °C under anaerobic conditions for 12 h. The cultures were diluted 100-fold into mM9 media in a 96-well flat-bottom plate in the presence or absence of 50 mM NaNO_3 , 25 mM sodium tungstate, 500 μM dopamine, or a combination of the three reagents. These cultures were incubated in a plate reader with agitation at 37 °C under anaerobic conditions for 18 h to evaluate optical density at 600 nm (OD₆₀₀). Oxidation of dopamine to quinone was detected by NBT quinone stain. Samples were also analyzed for nitrate respiration by Griess assay for nitrite quantification.

Effect of catechols on α -syn aggregation in the presence of *E. coli* K-12:

All solutions and consumables were equilibrated in the anaerobic chamber for 24 h except for Fe^{3+} solutions to prevent reduction to Fe^{2+} . Into four hungate tubes, 5 mL of mM9 media was aliquoted into each tube. Tubes were inoculated in triplicate with *E. coli* K12 that was cultured on LBg agar plates. The additional tube served as a sterile control. Tubes were incubated with agitation at 37 °C under anaerobic conditions for 12 h. The cultures were diluted 100-fold into mM9 media in a 96-well flat-bottom plate in the presence or absence of 50 mM NaNO_3 , and/or 500 μM catechol (dopamine, LDOPA, DOPAC, HA, carbidopa, or catechol). These cultures were incubated in a plate reader with agitation at 37 °C under anaerobic conditions for 18 h to evaluate optical density at 600 nm (OD₆₀₀). Oxidation of dopamine to quinone was detected by dot blot NBT stain. α -Syn aggregation was detected by dot blot immunostaining. Samples were also analyzed for nitrate respiration by Griess assay.

Effect of Ga(NO₃)₃ on E. coli growth:

All solutions and consumables were equilibrated in the anaerobic chamber for 24 h except for Fe³⁺ solutions to prevent reduction to Fe²⁺. Into four hungate tubes, 5 mL of mM9 media was aliquoted into each tube. Tubes were inoculated in triplicate with *E. coli* K12 that was cultured on LBg agar plates. The additional tube served as a sterile control. Tubes were incubated with agitation at 37 °C under anaerobic conditions for 12 h. The cultures were diluted 100-fold into mM9 media in a 96-well flat-bottom plate in the presence or absence of 50 mM NaNO₃, 500 μM Ga(NO₃)₃, and/or 500 μM dopamine. These cultures were incubated in a plate reader with agitation at 37 °C under anaerobic conditions for 18 h to evaluate optical density at 600 nm (OD₆₀₀).

Effect of Ga³⁺ and dopamine on α-syn aggregation:

All solutions and consumables were equilibrated in the anaerobic chamber for 24 h, except for Fe³⁺ solutions to prevent reduction to Fe²⁺. In PCR tubes, 20 μM α-syn was incubated with 20 μM GaCl₃, or 20 μM Ga(NO₃)₃, in the presence and absence of 50 μM dopamine to a final volume of 21 μL. As a control, 20 μM α-syn was also incubated with 50 μM FeCl₃•6H₂O or FeCl₂•4H₂O in the presence of 50 μM dopamine. Samples were incubated at 37 °C under anaerobic conditions for four h. Then, 7 μL of 4x Laemmli sample buffer was added to each tube, heated at 90 °C for five minutes and stored at -20 °C until further analysis. To assess the formation of SDS-resistant oligomers a 12% denaturing acrylamide gel was prepared. The gel was then stained using Pierce silver stain kit, according to the manufacturer's instructions.

Competition between Fe³⁺ and Ga³⁺ to induce dopamine oxidation and α-syn aggregation: All solutions and consumables were equilibrated in the anaerobic chamber for 24 h except for Fe³⁺ solutions to prevent reduction to Fe²⁺. In PCR tubes, 80 μM α-syn and 50 mM dopamine was incubated with 50 mM FeCl₃, 50 mM FeCl₃ and 10 mM Ga(NO₃)₃ (5:1 Fe:Ga), 50 mM FeCl₃ and 12.5 mM Ga(NO₃)₃ (4:1 Fe:Ga),

50 mM FeCl₃ and 16.6 mM Ga(NO₃)₃ (3:1 Fe:Ga), 50 mM FeCl₃ and 25 mM Ga(NO₃)₃ (2:1 Fe:Ga), 50 mM FeCl₃ and 50 mM Ga(NO₃)₃ (1:1 Fe:Ga), 25 mM FeCl₃ and 50 mM Ga(NO₃)₃ (1:2 Fe:Ga), 16.6 mM FeCl₃ and 50 mM Ga(NO₃)₃ (1:3 Fe:Ga), 12.5 mM FeCl₃ and 50 mM Ga(NO₃)₃ (1:4 Fe:Ga), 10 mM FeCl₃ and 50 mM Ga(NO₃)₃ (1:5 Fe:Ga), or 50 mM Ga(NO₃)₃. Solutions were incubated at 37 °C under anaerobic conditions for four h. Oxidation of dopamine to quinone was detected by dot blot NBT stain, and α -syn aggregation was detected by dot blot immunostaining.

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