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Lessons from Nature: A Bio-Inspired Approach to Molecular Design

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Abstract

Metalloproteins contain active sites with intricate structures that perform specific functions with high selectivity and efficiency. The complexity of these systems complicates the study of their function and the understanding of the properties that give rise to their reactivity. One approach that has contributed to the current level of understanding of their biological function is the study of synthetic constructs that mimic one or more aspects of the native metalloproteins. These systems allow individual contributions to the structure and function to be analyzed and also permit spectroscopic characterization of the metal cofactors without complications from the protein environment. This Current Topics is a review of synthetic constructs as probes for understanding the biological activation of small molecules. These topics are developed from the perspective of seminal molecular design breakthroughs from the past that provide the foundation for the systems used today.

Introduction

Nearly one third of all proteins utilize metal ions as co-factors to promote function. The roles of the metal ions vary from simple structural units to those that are directly involved in catalytic processes.¹ Understanding how metal ions promote chemical function within the active sites of proteins has been one of the central avenues of research in metallobiochemistry for the last 50 years. Since the initial molecular structure of myoglobin (Mb) was obtained in 1958, a considerable amount of effort has been put forth to determine the structural factors that govern the reactivity of metal cofactors.^{2,3} The investigations have relied on key advances in structural biology, biophysics, computational chemistry, and spectroscopy to obtain the necessary structural information. Moreover, rapid measurements such as stop-flow kinetics and freeze-quench methods have provided essential functional information. What has emerged from these studies are the crucial roles that both the primary coordination environment and the protein-derived active site structures have in determining the properties of the metal cofactors. While the ligands that are directly coordinated to the metal ion determine the electronic properties of the metal center(s) and therefore strongly influence the reactivity, no catalyst operates in isolation without interacting with its local

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environment.⁴ The importance of this local environment is clear from the change in reactivity upon isolation of the metal cofactor from its active site, which often greatly reduces or completely eliminates function. However, knowing the arrangement of the ligands and surrounding residues in an active site is not enough to understand its function—the local environments are dynamic and must respond to the changes during turnover that are necessary to achieve high efficiencies and selectivities. For instance, nitrogenases and cytochrome c oxidase (Cco) are enzymes with active sites composed of catalytic metaloclusters that reduce inherently stable compounds: nitrogenase takes N₂ to two equivalents of NH₃ and Cco converts O₂ to H₂O.^{5,6} While the mechanisms are obviously different, both processes require a variety of non-covalent interactions with nearby functional groups to orchestrate the precise transfer of protons and electrons during turnover. Moreover, nitrogenases and Cco accomplish these difficult transformations at faster rates and lower thermodynamic overpotentials than other reported systems.

The complexities of the enzymes described above impede the investigation of individual contributions to function. One approach that has allowed us to deconvolute the complex nature of metalloprotein active sites is the use of synthetic constructs that emulate various structural features.⁷ Advances in preparative techniques now allow for remarkable synthetic control that permits placement of high impact functional groups in specific locations within a molecule. This ability to achieve structural fidelity within a molecule permits the establishment of detailed structure–function correlations that can be used to gain a detailed knowledge of how proteins accomplish difficult chemical processes. Synthetic systems are often more amenable to accurate determinations of their structural and physical features and therefore can be used as probes for single properties found in proteins. This point is especially relevant in metalloproteins, in which the metal center(s) within active sites can be spectroscopically analyzed without interference from the rest of the protein.

In this Current Topics article, we examine the role of synthetic chemistry in determining properties associated with the biological activation of small molecules. Two general topics will be analyzed: the binding and activation of dioxygen and the oxidative conversion of water to dioxygen. Both of these processes have enormous importance in biology, energy science, and chemical processes. These topics will be developed by first describing seminal discoveries in synthetic metallobiochemistry that occurred in the past. Our aim is to trace the lineages in molecular design from earlier synthetic constructs that had a major impact on the field to similar, more current influential systems. We highlight the observation that several current systems that are being used to probe metalloproteins own similar key design features to systems that were first introduced decades ago. Principles of molecular architecture that were uncovered in the past have now been refined and recast to produce synthetic constructs that emulate features of metalloprotein active sites.

Reversible Binding of Dioxygen

The era of molecular bioinorganic chemistry began with the seminal discoveries of the structures of myoglobin (Mb) and hemoglobin (Hb) by Kendrew and Perutz in the 1960s.^{8–10} These structures gave us the first picture of the active site of a metalloprotein from which key structural requirements for function could be inferred and applied to the

rational design of synthetic molecules. One key feature of the active site that was confirmed by these and later structural studies was the differentiation of the two faces of the heme unit by the protein environment (Figure 1). On one side, known as the proximal face, a histidine residue coordinates to the iron center, forming the only direct linkage between the heme and the protein scaffold. On the opposite face (distal face), a second histidine residue lies in close proximity to the iron center but is not close enough to coordinate. This creates an open coordination site on the iron center to which dioxygen and other small molecules can bind.

Prior to the seminal structural studies by Kendrew and Perutz, significant work had already been directed towards studying the chemistry of porphyrin complexes with dioxygen. However, these early systems primarily utilized simple porphyrin units and often suffered from irreversible autooxidation to oxo-bridged dimers.^{11–13} The elucidation of the structures of Mb and Hb showed how this detrimental reaction is prevented in the protein; the active site resides within a cleft of the protein that protects the iron center from interactions with other heme units that would result in auto-oxidation. Furthermore, the arrangement of amino acid residues within this cleft regulates access to the active site while also maintaining an open coordination site on the iron center—a property that is known to be essential for function.

The apparent involvement of the protein scaffold in establishing the function of Hb and Mb highlighted two major challenges associated with the preparation of a synthetic complex with properties that mimic Hb and Mb. First, the faces of the porphyrin must be differentiated so that an axial ligand can bind to only one face while the second face remains open. This was first accomplished in a synthetic system by Collman, who showed that steric interactions between a sterically encumbered axial ligand (2-methylimidazole) and the porphyrin ring prevented a second ligand from binding on the opposite face by pulling the iron center out of the plane of the porphyrin towards the axial ligand. The second major challenge in designing a synthetic system that is capable of reversibly binding O₂ is preventing irreversible oxidation to oxo-bridged dimers. In the absence of the protective protein scaffold, the strong thermodynamic driving force for the formation of [(Fe(porphyrin))₂O] dimers requires very low temperatures for the stabilization of dioxygen adducts with simple iron porphyrins, making their characterization difficult.^{14,15} Collman and coworkers addressed this challenge synthetically by constructing a protective fence around one face of the corresponding iron-porphyrin complex (Figure 2) using an atropisomer of an ortho-substituted meso-tetraphenylporphyrin.¹⁶ When combined with a sterically encumbered imidazole, this fence limited coordination of an axial ligand to the unhindered side of the iron center while still allowing small molecules such as dioxygen to bind within the cavity of the hindered face and also protected the coordinated O₂ unit from further reaction with a second iron center. These properties lead to the first crystallographic characterization of an iron-dioxygen adduct in either a protein or a synthetic complex and provided the first structural evidence of the proposed end-on coordination mode of dioxygen in Hb and Mb.^{17–20}

The development of Collman's picket fence porphyrin system provided a means to study the origin of one functional aspect of Hb—the cause(s) for a two-state model for O₂ binding in which an increase in the O₂ binding affinity occurs upon partial oxygenation.²¹ Perutz

suggested that changes in the position of the iron center within the porphyrin ring in the deoxy state (T state) compared to the ligated state (R state) was the source for the increased binding affinity.²² Collman's original picket fence porphyrin iron complex acted as a suitable model for the T state because the sterically hindered 2-methyl imidazole that was required to maintain an open coordination site on the iron center caused displacement of the metal ion from the porphyrin plane in a manner similar to what is observed in the T state of Hb. Furthermore, the measured binding affinity of this complex is comparable to that of the native protein. In order to construct a model for the R state in which the iron center is not displaced from the porphyrin plane, Collman prepared a 'tailed' picket fence porphyrin that appended a sterically unhindered imidazole to the open face of the porphyrin (Figure 3). This modification was required because the steric bulk of the 'fence' was not great enough to prevent coordination of the analogous free unhindered imidazole to both faces of the porphyrin. The metal center in the resulting five-coordinate complex was positioned within the porphyrin plane and reversibly bound dioxygen with an affinity that was greater than the T state model with the hindered imidazole. This difference in affinity between the two synthetic complexes provided support for the proposal that the position of the iron center within the plane of the porphyrin modulates the O₂ binding affinity.

Intramolecular Hydrogen Bonding Networks

A key structural parameter for the stabilization of the O₂ unit within the active sites of Hb and Mb are hydrogen bonding (H-bonding) interactions on the distal face that involve the Fe(III)–superoxo unit. Changes in these H-bonding networks are known to have a significant impact on dioxygen binding. For instance, in human Hb, a single bifurcated intramolecular H-bond between H65 of the distal histidine residue and the Fe(III)–superoxo unit has been directly linked to the reversible binding of dioxygen (Figure 4).¹⁰ In Hb isolated from the parasitic nematode *Ascaris suum*, two distinct H-bonds are formed to the O₂ unit from tyrosine residue B10 and glutamate residue E7.²³ This difference in H-bonding network is thought to be partly responsible for the 10⁴ increase in O₂ affinity of the nematode Hb over human Hb and demonstrates the influence of H-bonding in stabilizing reactive species.

The correlation between structure and function as it relates to H-bonding interactions within metallo-active sites is not limited to heme-based respiratory proteins; the necessity for intramolecular H-bonds in stabilizing reactive intermediates is clear from a myriad of examples of H-bonding interactions within protein scaffolds and particularly within active sites. These H-bonding interactions serve a variety of functions, including substrate binding, proton shuttling, and stabilization of exogenous ligands to a metal cofactor. Incorporation of these non-covalent interactions has therefore been a goal of synthetic chemists who desire to replicate the reactivity of metalloproteins. However, the design of synthetic systems in which intramolecular H-bonding interactions influence the properties or reactivity of the complex is not an easy task. Even if H-bond donors/acceptors are successfully incorporated, their positioning must allow them to interact with molecules coordinated to the metal center(s). This difficulty is apparent in the picket fence porphyrin system that was discussed above; although the amide linkages that form the fence could potentially act as H-bond donors to the Fe(III)–superoxo unit in a similar manner as the H65 residue in human Hb, these groups

are too far removed from the Fe(III)–superoxo unit (a distance of over 5 Å) to form intramolecular H-bonds (Figure 5).

Despite the inability of Collman's picket fence porphyrin to participate in H-bonding with the coordinated O₂ unit, this system showed how functional groups could be positioned within a molecule for H-bonding via incorporation into a rigid scaffold. Our group and others have utilized this concept in the design of rigid ligand frameworks that establish local C₃ symmetry around a metal center.^{24–30} Our ligand precursor (referred to as H₆buea) contains three ethyl-urea groups that, when singly deprotonated, simultaneously provide anionic donors to bind a metal ion and position three H-bond donating groups proximal to a metal center (Figure 6A).³¹ These anionic donors and the adjacent central nitrogen atom enforce a trigonal pyramidal coordination environment, which places the remaining NH components of the urea groups nearly perpendicular to the trigonal plane and pointing inward towards an open binding site on the metal center. This ligand design allowed us to prepare Fe(II) complexes that also bind O₂; however, the Fe–O₂ adducts are unstable and further reacted via cleavage of the O–O bond to produce the Fe(III)–oxo complex [Fe(III)H₃buea(O)]^{2–}.³² The importance of Fe–oxo complexes will be discussed in the next section, but these type of metal–oxo species are proposed to be the kinetically competent oxidants in numerous oxidative processes. In proteins, the Fe–oxo units are often too reactive to isolate or even detect, which has hindered our understanding of their properties and functions. The isolation of [Fe(III)H₃buea(O)]^{2–} gave the field the first look at the molecular structure of a complex with a discrete Fe–oxo core and showed that intramolecular H-bonds were essential for its stabilization; XRD studies showed that the NH groups are in the correct position and orientation to form intramolecular H-bonds to the oxo ligand coordinated to the metal center (Figure 6B). In fact, the oxo ligand was positioned nearly coplanar with the hydrogen-bonding urea N–H bonds with an average N–O_{oxo} distance of ~2.7 Å. We postulated that isolation of this mononuclear complex was made possible only by the design principles incorporated into the [H₃buea]^{3–} scaffold: the rigid, sterically protected hydrogen-bonding cavity. These design features have given us the ability to stabilize and characterize a number of biologically relevant metal complexes that we have used to study certain aspects of the native metalloproteins; the contributions of several of these complexes to the current understanding of enzymatic function will be described in later sections.

Expanding Characterization Methods of Non-Heme Fe(IV)–oxo Species

Iron–oxo intermediates have been implicated in a variety of C–H bond activation transformations by non-heme containing enzymes such as the α-ketoglutarate dependent dioxygenases and the halogenases.^{33–36} In these enzymes, the active oxidant is formulated as a monomeric Fe(IV)–oxo species with an S = 2 spin state whose primary coordination sphere is variable across classes of enzymes. Synthetic examples of Fe(IV)–oxo complex were pioneered by Que and Nam, but most of their systems had S = 1 spin states, leading to further questions about how electronic structure influences function. We have been able to access such a high-spin Fe(IV)–oxo species in our synthetic system via one-electron oxidation of [Fe(III)H₃buea(O)]^{2–} to give [Fe(IV)H₃buea(O)][–].³⁷ Crystallographic characterization of this complex revealed a shortening of the Fe–O bond length (1.68 Å vs

1.81 Å) relative to the Fe(III)–oxo complex that indicated a reduction in the extent of hydrogen-bonding to the oxo ligand. However, the steric bulk of the tert-butyl groups protected the Fe(IV)–oxo unit, creating a stable complex that could be fully characterized.

The isolation of our Fe(IV)–oxo complex in high purity provided a unique opportunity to fully characterize this usually-transient intermediate in search of new spectroscopic handles for detection. The primary method for characterizing iron species in metalloproteins, including Fe(IV)–oxo intermediates, has been Mössbauer spectroscopy due to its sensitivity to the oxidation state and electronic environment of the iron center. However, this technique is only sensitive to the ^{57}Fe nucleus (2.2% natural abundance), and as a result, protein samples usually have to be enriched with this isotope in order for measurable signals to be obtained. An alternative method for investigating the electronic properties of paramagnetic active sites in metalloproteins is EPR spectroscopy. Although the standard EPR spectrometer is limited to systems that contain an odd number of electrons, species with an even number of electrons, such as Fe(IV) systems, can be detected via a modified technique in which the microwave frequency is aligned parallel to the applied magnetic field instead of perpendicular. However, the first parallel mode EPR signal of a non-heme Fe(IV)–oxo species was not detected until 2010 when we obtained a spectrum for our $[\text{Fe(IV)H}_3\text{buea(O)}]^-$ complex that contained two low-field features at g-values of 8.19 and 4.06 that both originate from excited states within the $S = 2$ spin manifold (Figure 7).

The detection of the EPR signal for $[\text{Fe(IV)H}_3\text{buea(O)}]^-$ supports the utility of EPR spectroscopy as a tool for analyzing metalloproteins that are proposed to proceed through Fe(IV)–oxo units. EPR theory suggests that signals for these Fe(IV)–oxo species should become more intense as the arrangement of ligands becomes less symmetric, and since the ligand environment imposed by metalloproteins has much lower symmetry than our $[\text{H}_3\text{buea}]^-$ ligand system, Fe(IV)–oxo intermediates in metalloproteins should produce intense EPR signals. We therefore suggest that parallel-EPR spectroscopy be added to the repertoire of techniques that are typically used to characterize proposed Fe(IV) intermediates. The information gained from these EPR studies can not only be used to detect proposed Fe(IV) species but can also provide information about their molecular and electronic structures and the bonding between the iron and oxo centers. For example, a property of interest in metal-oxo species is the amount of spin-density localized on the oxo ligand, which provides information about the bonding in the Fe–oxo unit. This spin density can be measured for species that have detectable EPR signals using ^{17}O labeling. A spin density of 0.56 was determined for our $[\text{Fe(IV)H}_3\text{buea(O)}]^-$ complex, which indicated a high degree of covalency in the Fe–oxo bond.³⁸ This type of detailed experimental information about bonding is currently lacking for biological Fe(IV)–oxo units and is often described using computation methods instead. Hence, additional data such as those obtained from parallel-mode EPR spectroscopy are important for correlating experimental and computational findings as they relate to Fe(IV)–oxo systems in biology.

C–Bond Functionalization

An important class of heme metalloproteins that proceed through an Fe(IV) intermediate are the cytochrome P450 monooxygenases. These enzymes serve several important functions

including the breakdown of toxins, metabolism of pharmaceuticals, and oxidation of substrates for the synthesis of many biologically relevant molecules.³⁹ Unlike the histidine-ligated globins described above that reversibly bind O₂, the unique thiolate-ligated heme of P450 enzymes promotes not only the binding of O₂ but also the subsequent cleavage of the O–O bond to generate a high-valent Fe species that is kinetically competent to effect the hydroxylation of strong aliphatic C–H bonds approaching 100 kcal/mol.^{40,41} The impressive ability of the P450 enzymes to perform these oxidation reactions brought about a period of intense study to understand the mechanism of hydroxylation and the identity of the active oxidant.

Elucidation of the mechanism of hydroxylation was hindered by a lack of knowledge about the identity of the key intermediate, known as Compound I from peroxidase enzymes, which is now known to be an Fe(IV)–oxo species with a delocalized ligand radical (Figure 8).⁴² The characterization of Compound I proved challenging, as the species could not be directly observed under normal turnover conditions. In lieu of the capture of the active oxidant, model reactions using synthetic compounds were studied to gain a greater understanding of the identity of Compound I and the mechanism of substrate hydroxylation. Groves was one of the first to begin studying a model reaction to understand enzymatic C–H bond hydroxylation. He chose to study Fenton oxidation chemistry in which an aliphatic substrate is hydroxylated upon addition of hydrogen peroxide to a solution of ferrous perchlorate.⁴³ Groves theorized that the active oxidant in the Fenton oxidations was actually an iron-bound species and not a free hydroxyl radical. Through detailed mechanistic studies using deuterium-labeled substrates, Groves was able to determine that the oxidant is an iron-bound species that coordinates with the substrate prior to H–atom abstraction, then quickly recombines with the ensuing carbon-based radical. Through these experiments with the model reactions and similar mechanistic studies in enzymatic systems, Groves developed his theory of oxygen-rebound describing the recombination of the carbon radical generated from the initial C–H bond cleavage step with the iron-bound oxidant that must operate in P450 enzymes.^{44,45}

Groves and others proposed that the identity of Compound I generated in P450 enzymes must be the same as that characterized in the peroxidase family.^{46–48} This proposal was based on the intriguing property of peroxidase and P450 enzymes to use peroxides as both the source of electrons and oxygen atoms in the absence of O₂ and NADPH in a reaction pathway that became known as the peroxide shunt. The peroxide shunt allowed for generation of a relatively stable Compound I species in the peroxidases that was characterized as an Fe(IV)–oxo exchange-coupled to a porphyrin radical.⁴⁹ However, due to the lack of spectroscopic evidence for such an iron species in P450 enzymes, alternative mechanisms such as a protein-bound oxidant were proposed. In order to explore the potential for a high-valent iron species to be operative in P450 enzymes, Groves again studied the peroxide shunt pathway using synthetic ferric porphyrin complexes.^{46,50,51} Unlike the Mb model compounds discussed earlier that required more complicated substituted porphyrins, Groves found that a simple tetra-aryl-substituted porphyrin was sufficient for his experiments. From the reaction of a synthetic porphyrin complex with peroxide mimics at low temperatures, he successfully produced a species characterized as an Fe(IV)–oxo with porphyrin radical just like the Compound I intermediate of peroxidase

enzymes. He was also able show that this species oxidized relatively inert C–H bonds, providing evidence that the peroxide shunt pathway can produce a Compound I species outside of the peroxidase heme systems and therefore is likely to be relevant to P450 chemistry.

Despite the advances that Groves made in understanding the mechanism of P450s and the identity of Compound I, the direct detection and complete characterization of P450 Compound I continued to elude scientists for another 30 years. This drought ended in 2010 when Green reported the generation of Compound I in a remarkable 70% yield using the thermophilic bacterial enzyme, Cyp119.^{52–54} The key advancement that led to such a high yield was the development of a new purification method by Green that strictly excluded any potential substrates during isolation of the protein. Reaction of the purified ferric enzyme with mCPBA, a peroxide mimic, produced Compound I via the peroxide shunt pathway, which was previously shown by Groves to generate a Compound I species, as discussed above (Scheme 1). The high yield of Compound I allowed for detailed Mossbauer and EPR spectroscopic studies that confirmed the proposed identity of the intermediate as an Fe(IV)-oxo center coupled to a ligand-based radical. Furthermore, the observed reactivity and kinetic isotope effects of the substrate oxidation by P450 Compound I support the mechanism of H-atom abstraction from the substrate to produce an Fe(IV)-hydroxo species, Compound II, that recombines with the resulting carbon radical, solidifying the place of Compounds I and II in the mechanism of P450 enzymes. Green's experiments support the 30-year-old predictions made by Groves based on his work with synthetic model reactions about both the electronic structure of Compound I and the oxygen rebound mechanism of P450 enzymes.

Contribution of Oxo Basicity to C–H Reactivity

Both heme and non-heme enzymes generate high-valent Fe–oxo species in the functionalization of C–H bonds. Although the level of oxidation and the spin states differ for these two classes of enzymes, they share similar mechanistic features that include oxidation to the active species by O₂, C–H bond cleavage of the substrate, and rebound into the resulting carbon radical by the exogenous ligand on the iron center.

The high reactivity of the iron-oxo species in both heme and non-heme enzymes raises an obvious question—how can such a potent oxidant be generated within a relatively fragile protein scaffold? It is often assumed that the active oxidants must possess a high reduction potential in order to react with inert substrates, but this would effect deleterious electron transfer reactions and destroy the delicate protein framework. Another factor must therefore be at play that accounts for the high turnover of these metalloenzymes. Indeed, an often-overlooked property that contributes to the reactivity of metal–oxo species is the basicity of the oxo ligand. Mayer has extensively described the relationship between the pK_a and the one-electron reduction potential of the high-valent metal-oxo in determining the reactivity for transfer of H atoms.⁵⁵ He asserts that the reactivity of the active oxidant with C–H bonds can be predicted by comparing the bond dissociation energy of the O–H bond in the resulting M–OH species to that of the C–H bond to be cleaved, and this O–H bond energy is determined by both the reduction potential of the metal-oxo species and its basicity. This

relationship is clearly illustrated in the schematic representation of Hess's law shown in (Figure 9).

The implications of Mayer's description of metal-oxo reactivity for heme oxygenases were examined by Green using Compounds I and II in P450 enzymes.⁵⁶ Green determined the pKa of the hydroxide ligand in Compound II to be ~ 12, which he attributed to the strong electronic donation from the thiolate ligand. This high basicity compensates for the relatively low reduction potential of Compound I and allows for abstraction of H-atoms from strong C-H bonds. The lower reduction potential explains how such a highly reactive species like Compound I can be generated without destroying the protein residues surrounding it.

Our [H₃buea]³⁻ ligand provided a series of complexes that are ideally suited to studying the interplay between oxo basicity and reactivity in non-heme systems; in addition to the Fe(III)-oxo and Fe(IV)-oxo complexes described above, we have also isolated the corresponding Fe(III)-OH and Fe(II)-OH complexes that 10).^{31,57,58} This gave us the rare opportunity to evaluate the effect of pKa and reduction potential on the reactivity of a set of non-heme Mⁿ-O(H) compounds that differ by only one electron or one proton. Note that our approach differs from that of Nam, who examined the reactivity of a series of S = 1 Fe(IV)-oxo complexes with differing reduction potentials.^{59,60} In our studies, we found that both the [Fe(III)H₃buea(O)]²⁻ and [Fe(IV)H₃buea(O)]⁻ complexes were competent in performing hydrogen-atom abstraction from sufficiently weak organic substrates despite the extremely negative reduction potentials of <-2 V vs Fc^{0/+} for the Fe(III)-oxo complex and -0.90 V for the Fe(IV)-oxo complex!^{32,37} These low reduction potentials are typically associated with reductants and not with species that are capable of oxidizing C-H bonds. This surprising reactivity suggested that the basicity of the metal-oxo moiety dominates the H-atom affinity in the same manner as was observed by Green for Compound I of P450.

To help explain the reactivity of our synthetic Fe(III)- and Fe(IV)-oxo complexes, Shaik performed a computational study of the observed H-atom transfer reactivity and showed that this reactivity can be attributed almost entirely to the extreme basicity of the metal-oxo unit in [Fe(III)H₃buea(O)]²⁻.⁶¹ The reactivity of the two complexes manifests from the diverse mechanistic pathways traversed by the two metal-oxos; while [Fe(IV)H₃buea(O)]⁻ abstracts a hydrogen-atom in a more traditional mechanism of near-concerted proton and electron transfer, the [Fe(III)H₃buea(O)]²⁻ mechanisms are dominated by a more step-wise proton transfer followed by electron transfer. It should also be noted that the analogous Mn complexes, [Mn(III)H₃buea(O)]²⁻ and [Mn(IV)H₃buea(O)]⁻, exhibit the same trends in reactivity, extending the relationship of pKa and reduction potential beyond Fe alone.^{62,63} These experimental and theoretical observations demonstrate that the basicity of the oxo ligand can greatly attenuate reactivity to promote H-atom abstraction and therefore maintain reduction potentials that are low enough for a highly reactive Fe(IV)-oxo intermediate to be safely generated within a protein matrix.

Subsite Differentiated Metalloclusters: Fe–S Clusters

In addition to single-metal active sites discussed above for heme and non-heme iron systems, nature also utilizes multiple metal ions in a cluster within an active site. These metalloclusters are found in a variety of different protein active sites and perform a diverse set of functions. One of the most common types of clusters contains one or multiple Fe–S units (Figure 11) and was first discovered in electron transfer proteins.^{64,65} These Fe–S clusters are now known to also play key roles in several enzymatic processes, including dinitrogen reduction and radical SAM chemistry, although their most common role is still in electron transfer processes.³ One class of electron transfer proteins that have been studied extensively are ferredoxins, which contain the basic Fe₄S₄ cubane-type cluster. Beginning in 1972 and continuing until today, there have been several synthetic examples of these clusters that have paved the way toward our understanding of their function.^{66,67} The landmark discovery that initiated this field was the preparation by Holm of self-assembled, low-molecular weight synthetic analogs in highly pure and crystalline form whose properties could be examined independent of the protein matrix.⁶⁸ The contributions of this and later self-assembled clusters to the field of bioinorganic chemistry have been reviewed extensively and will not be discussed here.^{69–71}

Although the early synthetic self-assembled Fe₄S₄ clusters provided important information about their functions in proteins, they failed to replicate one important property of the natural clusters known as subsite differentiation. Many proteins are known to contain specific iron subsites that have different structural and reactivity properties than the other iron centers. For example, within the Fe₄S₄ ferredoxins, one iron center of the cluster can have a different ancillary ligand or coordinate to another type of metallocluster, thus distinguishing it from the other iron centers. In the enzyme aconitase, an inactive cuboidal Fe₃S₄ cluster is converted to a fully active Fe₄S₄ cluster by the addition of iron ions; the newly incorporated iron center is different from the other three iron centers in the cubane cluster and becomes a specific subsite that binds substrates and inhibitors.^{72,73} This differentiation of one or more Fe sites within a Fe₄S₄ cluster is difficult to achieve in a synthetic system. Attempts to duplicate this type of chemistry and extend it to make heterometallocluster (that is, MFe₃S₄ where M is another metal ion) were made difficult by the lack of synthetic control in determining the position of individual metal ions and the inherent instability of the cuboidal Fe₃S₄ cluster precursors outside of the protein. It thus became a challenge to develop molecular systems that could subsite differentiate one or more of the iron centers within the Fe₄S₄ cluster.

To apply the idea of subsite differentiation to synthetic clusters, Holm and Stack designed a molecular system that employed a tridentate thiolate ligand to capture an Fe₄S₄ cluster (Figure 12).⁷⁴ The design of this ligand utilized the cavitand concept put forth by Cram, which states that molecular recognition events can occur more favorably if the host molecule is pre-organized toward binding of the guest molecule. In order to incorporate this concept into a tridentate ligand, Holm and Stack adapted the work of MacNicol on hexamethylbenzene, which showed that the methyl groups adopt an alternating up-down (also referred to as *ababab*) conformation that reproducibly positions three of the methyl groups on each face of the aryl ring.^{75–78} By incorporating alternating arylthiolate “legs”

along the central ring of the hexa-substituted benzene, they were able to prepare a semi-rigid trithiol compound that had the correct orientation to bind a cubane cluster in which one of the iron centers contained a different ligand than the other three centers (Figure 13).

Exploration of the reactivity of this cluster yielded insight into ligand exchange reactions and electron transfer properties related to differentiation of the fourth iron center. Further elaborations on this chemistry lead to the isolation of the first purely synthetic cuboidal Fe_3S_4 cluster via removal of one Fe center from the subsite-differentiated cubane Fe_4S_4 cluster.⁷⁹ The generation of this unique cluster allowed for comparison of its electronic and structural properties to the native cluster. These experiments corroborated the observation that the inactive Fe_3S_4 cluster found in natural systems such as aconitase could be reversibly converted into the active Fe_4S_4 form by addition of an iron ion. Ultimately, a series of heterometallic clusters were generated from the basic cuboidal Fe_3S_4 cluster by the addition of metal ions to give clusters of the general formula MFe_3S_4 that are supported by the LS_3 ligand scaffold.^{80–82} The discovery of these metal ion substitution reactions would prove to be an invaluable tool in cluster chemistry

Subsite Differentiated Metalloclusters: Oxygen Evolving Center in Photosystem II

The concepts outlined for subsite differentiation in synthetic Fe–S clusters can be applied to other metalloclusters, especially those having different metal ions. One of the most investigated metalloclusters of this type is the oxygen-evolving cluster (OEC) within the enzyme photosystem II (PSII). This cluster orchestrates the transfer of four electrons and four protons in the oxidation of two water molecules to dioxygen—one of the most important reactions in biology. A unique feature of the OEC is the incorporation of a calcium ion within a CaMn_3O_4 cuboidal cluster, the structure of which was recently outlined in high-resolution structures obtained from XRD measurements (Figure 14).⁸³ This calcium ion is known to be essential for the function of the enzyme, but its contribution to that function is not well understood.⁸⁴ Furthermore, its substitution for any other metal ion except Sr^{2+} completely shuts down the function of the enzyme.^{85–87} Although a possible role for the $\text{Ca}^{2+}/\text{Sr}^{2+}$ ion can be rationalized based on comparison of the properties of these ions to those of other ions, their true contributions to the function have been difficult to elucidate due to the complexity of the cluster and of the mechanism leading to dioxygen evolution.^{88–90} The design of synthetic analogues is therefore an obvious approach to examining the contributions of the Ca^{2+} ion.

The earliest preparations of synthetic analogues of the OEC primarily relied on self-assembly driven by the thermodynamic stability of oxide and hydroxide bridging ligands.^{91,92} As discussed above for the Fe_4S_4 clusters, this synthetic method makes the preparation of multimetallic clusters difficult, but in the case of Mn, not impossible. However, the reported self-assembled clusters contain many more metal centers than the OEC, and their synthetic routes do not allow for the systematic exchange of single metal ions so that a series of complexes with similar structures can be prepared and their properties compared.^{93–97} In an adaptation of the approach developed by Holm and Stack for iron

sulfur clusters, Agapie and coworkers described the rational synthesis of heterometallic clusters supported by a templating ligand. This ligand positioned three sets of dipyridylhydroxymethyl chelating groups on one face of an aryl ring that coordinated three Mn^{II} ions in the presence of base (Figure 15A).^{98,99} This trinuclear cluster provided a precursor to which a fourth, redox inactive metal ion could be systematically added to form a variety of higher valent, tetranuclear clusters. Two types of structures were obtained depending on the extent of oxidation of the trinuclear precursor; when four new bridging oxo ligands were introduced via KO_2 , the ligand rearranged to support a cubane structure in which the redox inactive metal ion occupied the fourth binding site (Figure 15B).^{100,101} When only two bridging oxo ligands were added via the reagent iodosylbenzene, the trinuclear core remained intact, and the redox inactive metal ion coordinated via one μ_4 -oxo and one μ_2 -oxo ligand to the trimanganese cluster (Figure 15C).¹⁰²

Agapie's synthetic route to tetranuclear clusters made it possible to develop a series of complexes in which the core structure remained unchanged but the identity of the fourth metal ion was varied. Consequently, the effects of the fourth metal ion on the properties of the cluster could be examined. Agapie compared the electrochemical properties of clusters containing five different redox-inactive metal ions and observed a linear relationship between the one-electron reduction potential of the cluster and the Lewis acidity of the redox-inactive metal ion, which was quantified by the $\text{p}K_{\text{a}}$ of the metal aqua ion ($\text{M}(\text{OH}_2)_m^{n+}$).^{101,102} This indicates that the redox-inactive metal ion plays an important role in tuning the electrochemical properties of the Mn ions even though it cannot directly participate in electron transfer. Furthermore, the clusters containing a Ca^{2+} or Sr^{2+} ion exhibited nearly identical reduction potentials, which is in agreement with the ability of both these ions to establish function in the OEC. These results provide one possible explanation for why the identity of the redox-inactive metal ion is important for the function of the OEC—the Ca^{2+} ion tunes the redox potential of the cluster into a range in which oxidation becomes feasible, and all other ions besides Sr^{2+} push the potential out of this range.

Merging Synthetic and Biological Chemistries

The previous cases studies illustrated an approach that utilized synthetic systems to assist in the investigation of complicated biochemical processes that involve metal ions. For all of the many successes that this approach has had, there are still limitations, the most notable being the inability of synthetic metal complexes to function at rates and with the selectivity found in metalloproteins. The most direct reason for the lack of functional mimicry is that synthetic complexes cannot fully recreate the local environments found within the active sites of metalloproteins. As discussed above for mimics of myoglobin, it has been particularly difficult to synthesize constructs that incorporate non-covalent interactions near a metal center in a similar manner as found in metalloproteins. These weaker interactions are now known to have a substantial effect on the function of most proteins, especially those involving metal ions, and their absence has deleterious effects on function. Our group has developed relatively small compounds that use intramolecular H-bonding networks around transition metal ions to regulate O_2 activation; this type of control allowed us to prepare a series of M -oxo complexes that are purported to be intermediates in catalytic cycles of metalloproteins (see above). However, these synthetic systems only provide H-bonds in the

volume of space closest the metal centers, often referred to as the secondary coordination sphere of the metal ion, while the control of function often extends outward to encompass a much larger volume of space that is referred to as the outer sphere. For instance, Brudvig recently commented on the importance of the outer sphere of the OEC in water oxidation, which consists of an extensive volume of water molecules surrounding the active site.¹⁰³ One important feature within this outer sphere is a H-bonded water channel (Figure 16) that is thought to provide a pathway for proton movement out of the active site toward the lumen of the plant.

So we are left with questions of how the secondary and outer spheres dictate function at an active site metal cofactor: it is at this interface of structure and function that many of the newer challenges exist. To tackle these issues, new approaches have emerged that combine synthetic constructs and proteins/peptides to help simulate many of the important architectural features found in metalloproteins. Some recently reported systems offer modifications of existing small molecules to manage both the primary and secondary coordination spheres of synthetic complexes. An excellent example of this approach is the family of Ni complexes that function as electrocatalysts for H₂ production/oxidation–reactions that are similar to those performed by hydrogenase enzymes. DuBois first introduced mononuclear Ni complexes that function as electrocatalysts for H₂ production/oxidation–reactions that are similar to those performed by hydrogenase enzymes. DuBois first introduced mononuclear Ni complexes that have two diphosphine ligands (P^R₂N^{R'}₂) and pendant amine moieties within the secondary coordination sphere that are essential for function (Figure 17).^{104,105} These basic amine sites act as proton relays that assist in producing high catalytic rates for the interconversion of H⁺/H₂ at reasonable overpotentials. Shaw has recently elaborated on this system through the placement of amino acids or small peptides on the periphery of the complex to modulate the outer coordination sphere. Impressive rates of catalysis were observed in many of these systems; for example, the Ni complex [Ni(PCy₂N^{Arg}₂)₂]²⁺ had turnover frequency of 210 s⁻¹ in water with a low overpotential of 180 mV for the oxidation of H₂, which is the most active catalysts in this series.¹⁰⁶ Note that that modified ligand PCy₂N^{Arg}₂ contains arginine groups appended to the amino nitrogen atom in the ligand, which the authors suggest helps regulate the outer coordination sphere around the catalytic Ni center.

Combining metal ions with longer peptides has produced a wide variety of larger molecular assemblies that contain more elaborate architectures. The most studied are *de novo* assemblies of peptides in which the most common motifs are single chained peptides that fold into either 3- or 4-helix bundles to produce a metal binding site within the interior of the artificial protein.¹⁰⁷ DeGrado recently demonstrated the power of this approach by designing a *de novo* protein for the catalytic *N*-hydroxylation of arylamines.¹⁰⁸ The starting point was a single-chain four-helix bundle whose interior was initially designed to simulate the active sites of di-iron proteins that activate O₂ and have also shown catalytic activity for the two-electron oxidation of hydroquinones. Two major structural aspects had to be altered before this protein could function as a hydroxylase. First, a more accessible channel for substrates to reach the active site had to be installed, which was accomplished through mutation of four alanine residues within the interior of the protein to glycines. The second alteration was inspired by the enzyme *p*-aminobenzoate *N*-oxygenase AurF, which also

catalyzes the hydroxylation of amines and contains a di-iron center within its active site. The site in this AurF enzyme has an additional histidine residue coordinated to the one of the iron centers relative to the original di-iron protein, which DeGrado correctly predicted would elicit function in his artificial protein. However, incorporation of another histidine residue within the interior of the 4-helix bundle caused steric mismatches with neighboring residues. To circumvent this problem, three additional mutations were required that installed a network of H-bonds to stabilize this new imidazole residue. The structure of the Zn analog was solved by NMR methods and furnished a picture of a protein whose active site is significantly different from that of the original construct (Figure 18). The resulting functional effect of these structural changes was a 10^6 -fold increase in relative rate between *N*-arylamine hydroxylation and hydroquinone oxidation.

The structures of the helix bundles described above were created by folds within the peptide that produced interfaces between the helices. The formation of the interfaces was driven by networks of non-covalent interactions and the binding of metal ions. This assembly process has been further exploited to direct the formation of more complex protein oligomers having well-defined structures. For instance, Tezcan has introduced a design approach called *metal templated interface redesign* that has produced a number of new artificial proteins.^{109,110} The basic concept is to introduce a small number of surface mutations into a natural-occurring protein to create a metal ion binding site(s) at the interface between two proteins. Using cytochrome *cb₅₆₂* (cyt *cb₅₆₂*) as a building block, Tezcan designed and built a variety of artificial proteins with new structural and functional properties by changing the conditions that influenced metal ion binding. These modifications led to the development of various 1-D, 2-D, and 3-D architectures that resemble those found in biology, such as microtubules and helical viruses. Moreover, the environment surrounding the metal ions could be modulated through changes to the residues at the interfaces of the assemblies. Through these multiple levels of structural control, Tezcan was able to create a tetrameric protein oligomer that contained a metal binding site that was selective for Zn(II) ions over other divalent metal ions, including Cu(II), which should have had stronger intrinsic binding properties. He further showed that one of his Zn(II)-containing protein-protein assemblies displayed enzymatic activity in *Escherichia coli* cells—a rare demonstration of an artificial protein functioning *in vivo*.¹¹¹ The target was the hydrolysis of ampicillin, a lactam antibiotic that was shown to prevent the growth of *E. coli* cells. Cells cultured in the presences of the artificial protein were able to grow and survive despite the introduction of this antibiotic, which provides compelling evidence that the artificial protein was able to function *in vivo*.

Another method for creating artificial proteins is to combine naturally-occurring proteins with unnatural inorganic complexes. Artificial metalloproteins of this type have their roots in the work of Whitesides, who treated streptavidin (Sav) with a biotinylated Rh complex to produce an asymmetric hydrogenation catalyst.¹¹² The advantage of this design resides in the strong binding affinity that Sav has for biotin ($K_a \sim 10^{13}$), which anchors the biotinylated metal complex to a specific location within the protein. Ward has furthered developed this approach to encompass a wide range of artificial metalloenzymes with impressive catalytic functions.^{113,114} In particular, site-directed mutagenesis methods have been used in conjunction with the binding of exogenous metal complexes to rationally tune the secondary coordination spheres within newly created active sites. This *chemogenetic* concept was

illustrated by Ward and Rovis in the development of a bifunctional catalyst that consisted of a biotinylated Rh(III)Cp* complex (Cp*, pentamethylcyclopentadiene) within SAV.¹¹⁵ Their aim was to catalyze the asymmetric C–H bond functionalization reactions between pivaloyl-activated benzhydroxamic acid and acrylates. Prior work on synthetic Rh catalysts found that the rates of this reaction were greatly accelerated in the presence of base (normally an acetate ion), but the reaction had not been made enantioselective due to difficulties in designing a Rh(III) complex supported by an asymmetric ligand. With this prior knowledge in hand, Ward and Rovis redesigned Sav to include two mutations that produced a more active *and* selective catalyst; mutation of lysine to glutamate provided a local base in the form of a carboxylate ion near the Rh(III) center that accelerated the reaction, and mutation of serine to lysine established enantioselectivity. These improvements produced a highly active, asymmetric catalyst with enantiometric ratios of 90:10 and overall yields of greater than 90%. Importantly, this high level of function was clearly correlated with changes within the secondary coordination sphere of the metal complexes that were provided by SAV. These types of second sphere modifications are difficult to achieve in a purely synthetic constructs, which highlights the power of combining synthetic complexes and proteins to produce functional systems.

Artificial metalloproteins have also been prepared by treating an apo-form of a metalloprotein with a synthetic metal complex. For example, Ménage has shown that incorporation of the metal complex $[\text{Fe}(\text{L})\text{OH}]^-$ ($[\text{L}]^{4-}$, *N*-benzyl-*N'*-(2-hydroxybenzyl)-*N,N'*-ethyldiaminediacetate) within the nickel-binding protein NikA produced an artificial enzyme with hydroxylase activity.¹¹⁶ The docking of $[\text{Fe}(\text{L})\text{OH}]^-$ within the NikA protein was assisted by a series of intramolecular H-bonding interactions to residues within the binding pocket. The mechanism of the hydroxylation was probed by XRD methods, and several intermediates formed during the hydroxylation process were identified, including a rare example of an Fe–O₂ adduct. The basics of this approach have also been applied to probe the properties of [Fe,Fe]-hydrogenases, in which catalysis occurs at a unique di-iron cofactor (Figure 19A). The formation of these enzymes is accomplished through a multienzymatic biosynthetic route in which the cofactor is first assembled on the maturation enzyme (HydF) and then transferred into the apohydrogenase to produce the functional hydrogenase (HydA1). The unusual structure of the di-iron cofactor has inspired the preparation of many synthetic systems whose structures closely resemble that of the natural di-iron complex (Figure 19B).^{117–121} However, none of these synthetic constructs have the activity of HydA1, presumably because their secondary and outer spheres differ significantly from those of the protein. In a recent report, Fontecave and Lubitz have shown that synthetic analogs of the di-iron cofactor can be inserted into HydA1 and exhibit activity similar to the natural hydrogenase.¹²² To produce this functional protein, HydF was loaded with synthetic di-iron complex, which transferred this unnatural cofactor into apo-HydA. Amazingly, this artificial hydrogenase had specific activity approaching 800 $\mu\text{mol H}_2$ per min per mg of HydA1, which is comparable to that found in the WT enzyme.

Summary

In this Current Topics article, we have described several important contributions that synthetic chemistry has made to the understanding of key topics in biology. The comparative

simplicity of these synthetic systems has permitted examination of specific aspects of active sites whose studies were hindered by the complexity of the native metalloenzyme. This approach has been particularly useful in providing evidence for proposals put forth about the functions of metalloenzymes, such as the binding mode of dioxygen in myoglobin and mechanistic aspects of C–H bond functionalization by P450s. Moreover, the ability to manipulate structure in specific ways has produced synthetic systems whose properties can be independently evaluated and compared. These types of systems have further lead to the detection of relatively unstable species that often are invoked as key intermediates in biochemical processes but are too fleeting to fully characterize in biomolecules. The new spectroscopic handles provided by these synthetic constructs can then be used to probe intermediates, and thus mechanisms, related to catalytic turnover in metalloenzymes.

From our perspective, one major challenge for the continued advancement of synthetic bioinorganic chemistry is the development of systems that can be applied to the study of how the local environments around metal ions affect function. As the understanding of how metalloenzymes function increases, the essential role of the environment surrounding the active site becomes more apparent. Within this context, we discussed the secondary and outer coordination spheres of metal ions and their critical roles in substrate binding and orientation, proton shuttling, and electron transfer. We also provided examples of artificial proteins that demonstrated how function could be regulated through changes in the secondary and outer coordination spheres; these illustrations highlight the potential of approaches that combine inorganic and protein chemistry to probe problems in metallobiochemistry. Continued development of new systems of this type will allow us to deconvolute the complex mechanisms of enzymatic catalysis so that we can begin to understand how proteins are able to orchestrate the precise movement of protons and electrons that is important in all types of chemical transformations.

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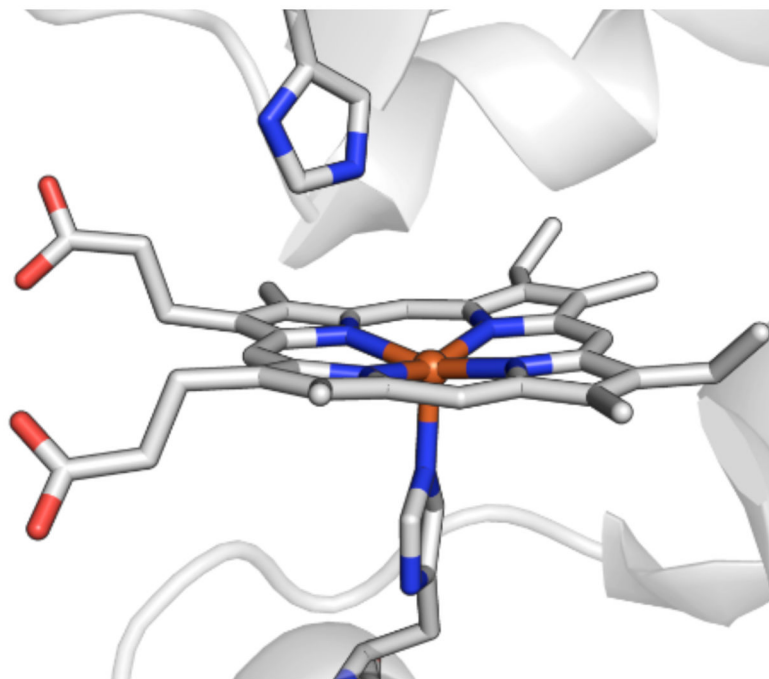


Figure 1.
Molecular structure of deoxy-hemoglobin determined from XRD (PDB: 3HHB).

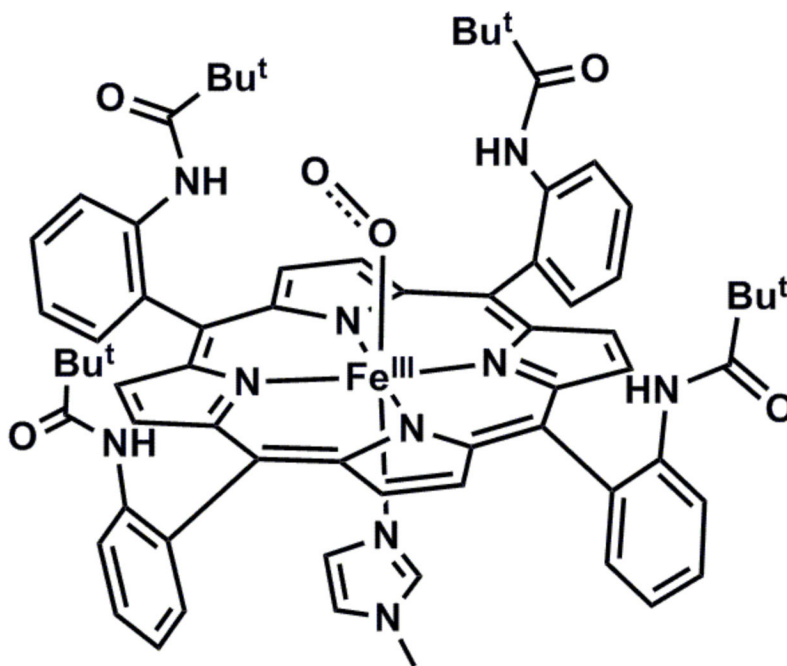


Figure 2.
An Fe(III)-superoxo adduct of a picket-fence porphyrin complex.

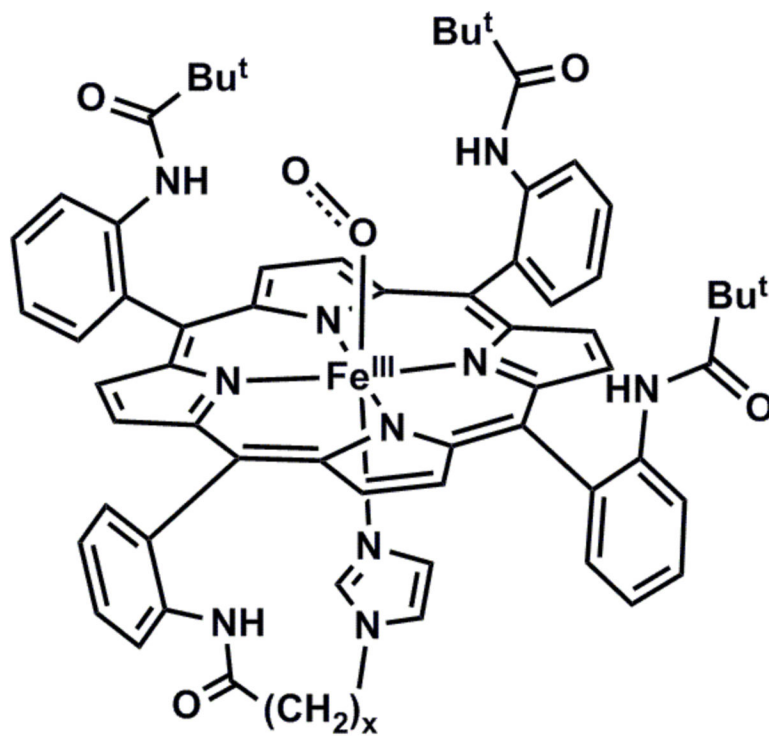


Figure 3.
The Fe(III)–superoxo adduct of a tailed picket-fence porphyrin complex.

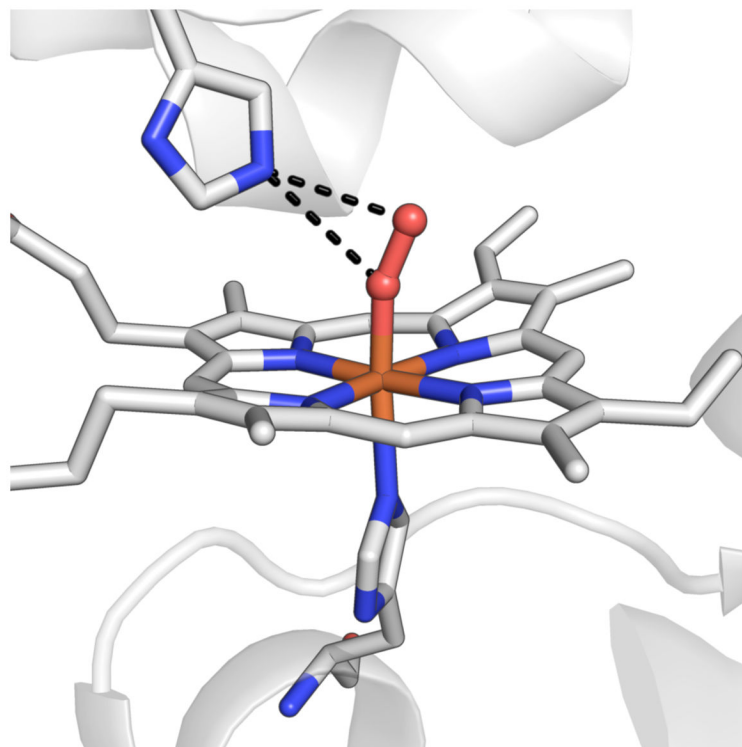


Figure 4. Structure of oxy-hemoglobin depicting the hydrogen bonding interactions to the bound dioxygen (PDB: 1GZX).

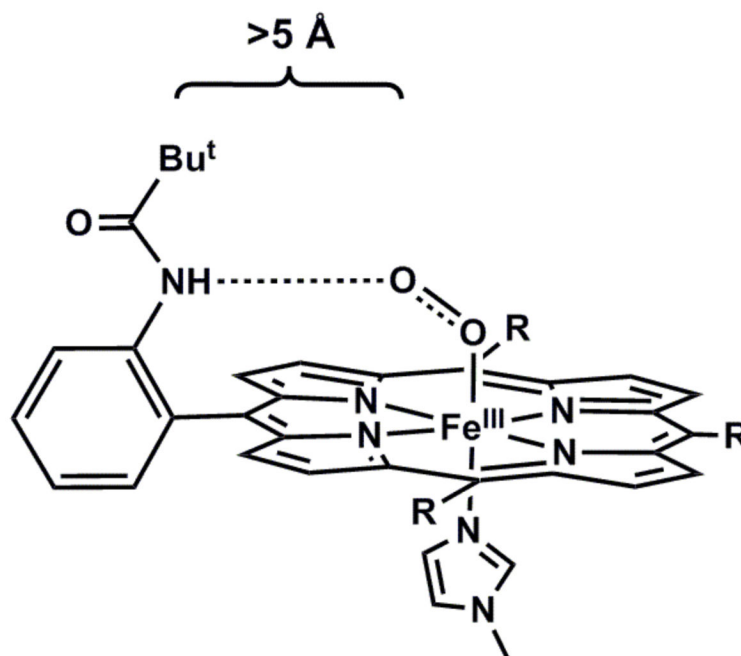


Figure 5. Relative distance between H-bond donor and acceptor in the Fe(III)–superoxo adduct within the picket fence porphyrin complex. Only one of the meso-substituents is shown for clarity.

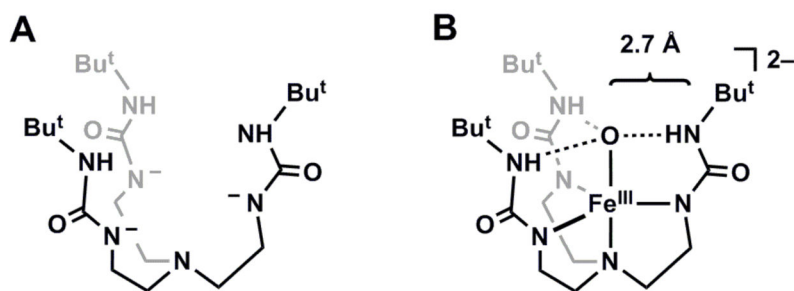


Figure 6. Depiction of the ligand [H₃buea]³⁻ (A) and the intramolecular hydrogen bonds formed with the Fe–O unit in the [Fe(III)H₃buea(O)]²⁻ complex (B).

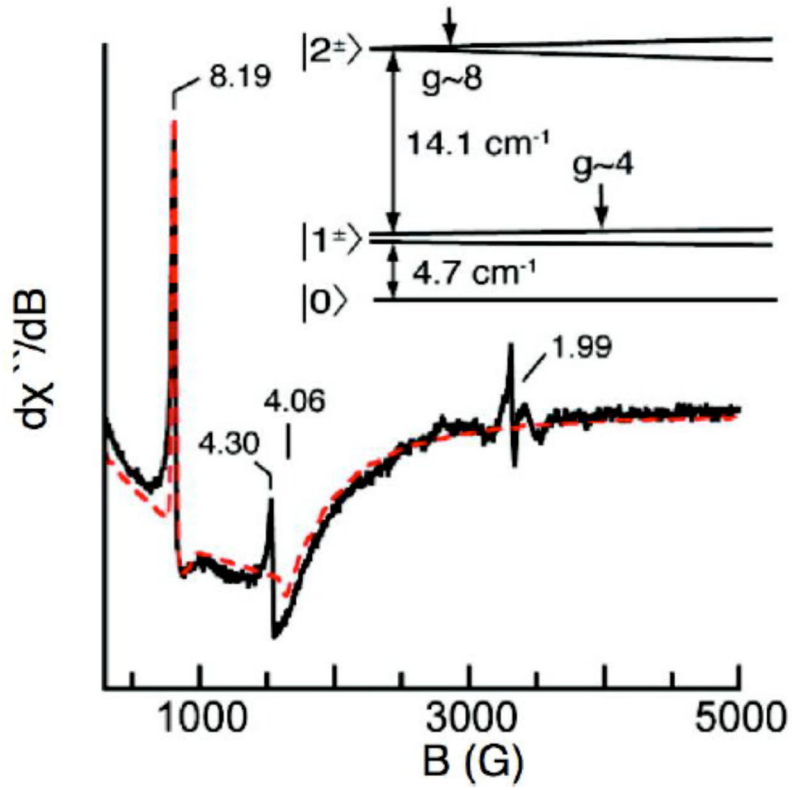


Figure 7. Parallel mode X-band EPR spectrum of the high spin $[\text{Fe(IV)H}_3\text{buea(O)}]^-$. Inset is the spin manifold that give rise to the spectral features.

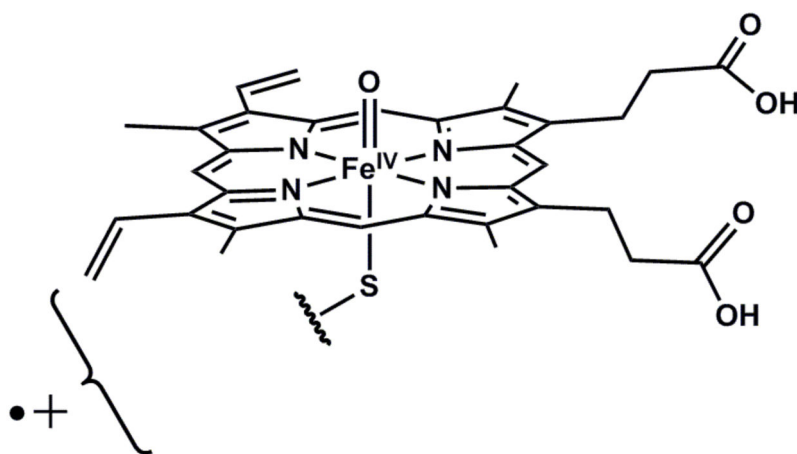


Figure 8.
Consensus structure of Compound I in cytochrome P450.

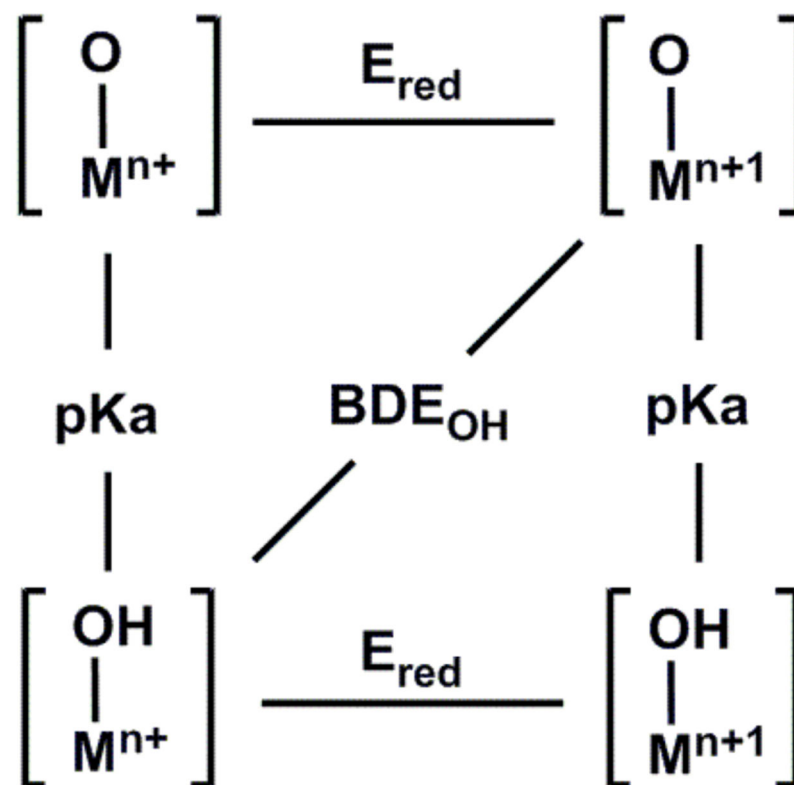


Figure 9. A thermodynamic square scheme showing the relationship between pKa , redox potential, and bond dissociation energy for M-OH complexes.

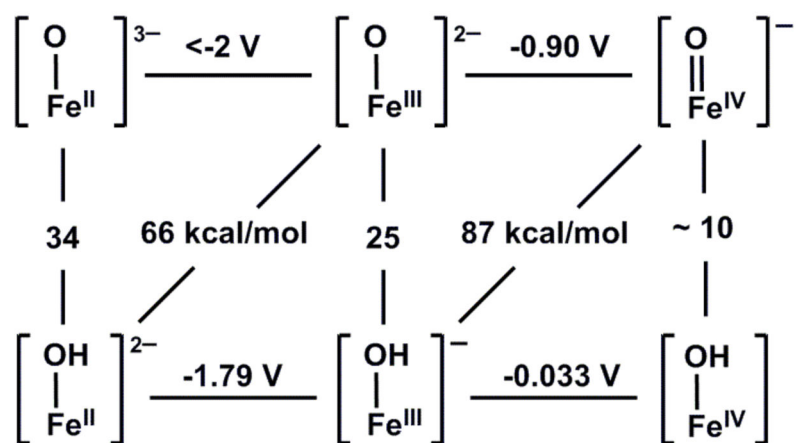


Figure 10. Thermodynamic square schemes for the complexes supported by the $[\text{H}_3\text{buea}]^{3-}$ ligand. Reported values are measured in DMSO at 25 °C and referenced to the $\text{Fc}^{0/+}$ couple.

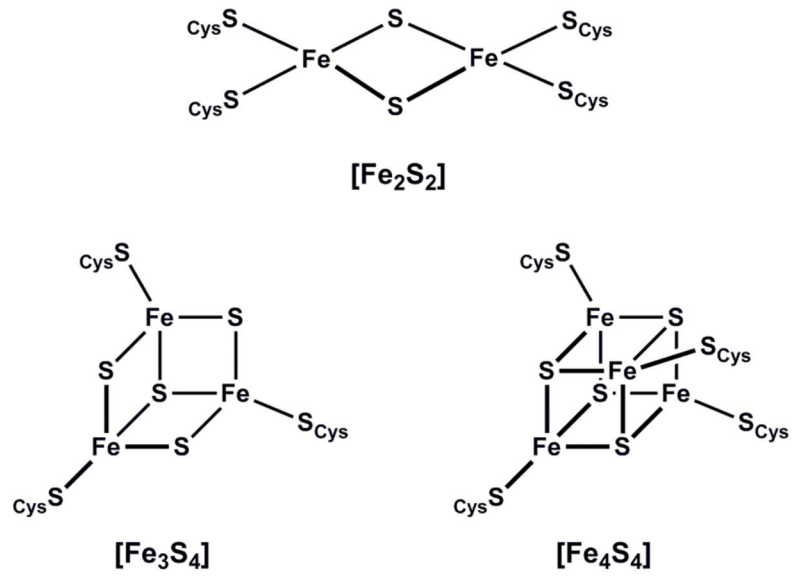


Figure 11.
Various [Fe-S] clusters found in nature.

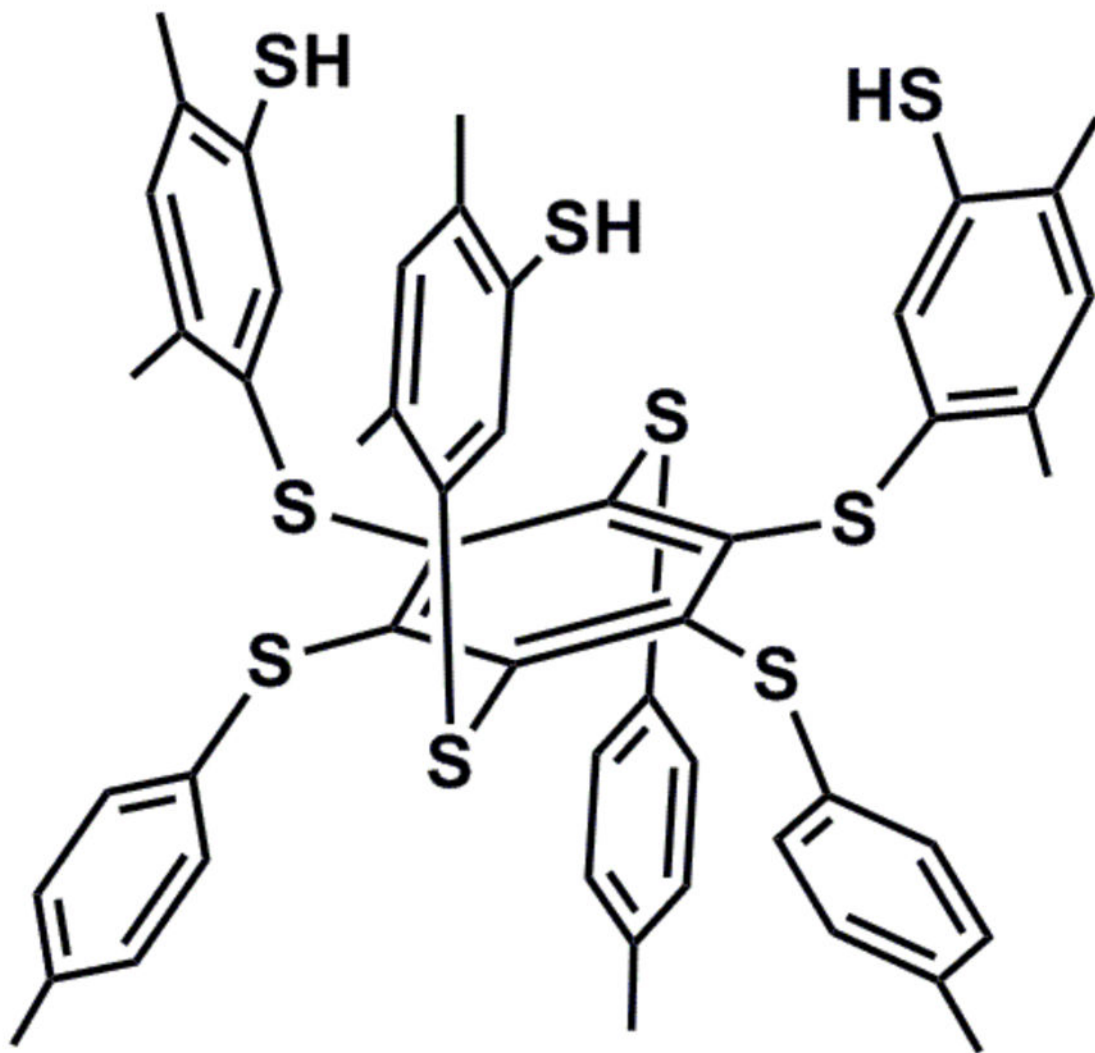


Figure 12.
The site-differentiating ligand LS₃.

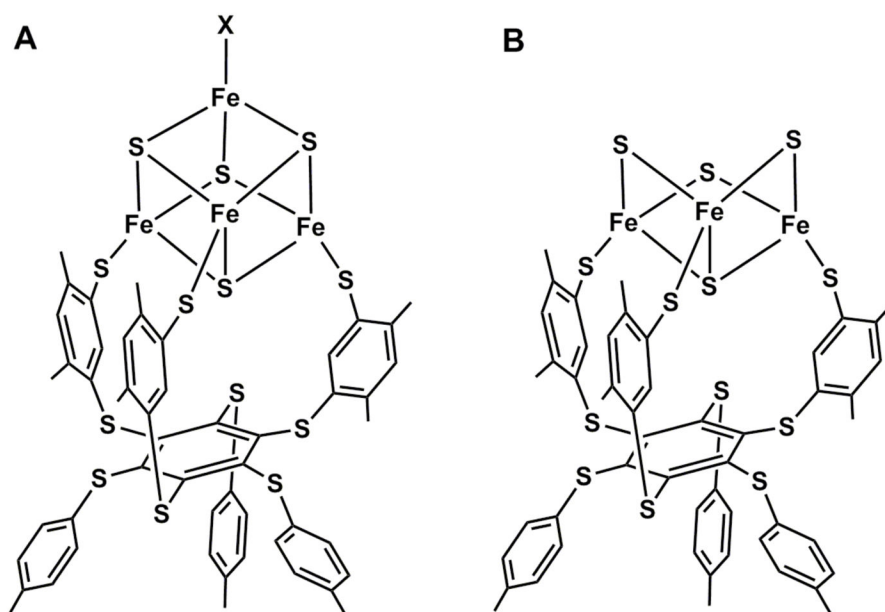


Figure 13. Structures of [Fe₄S₄] (A) and [Fe₃S₄] (B) clusters supported the site differentiating ligand LS₃.

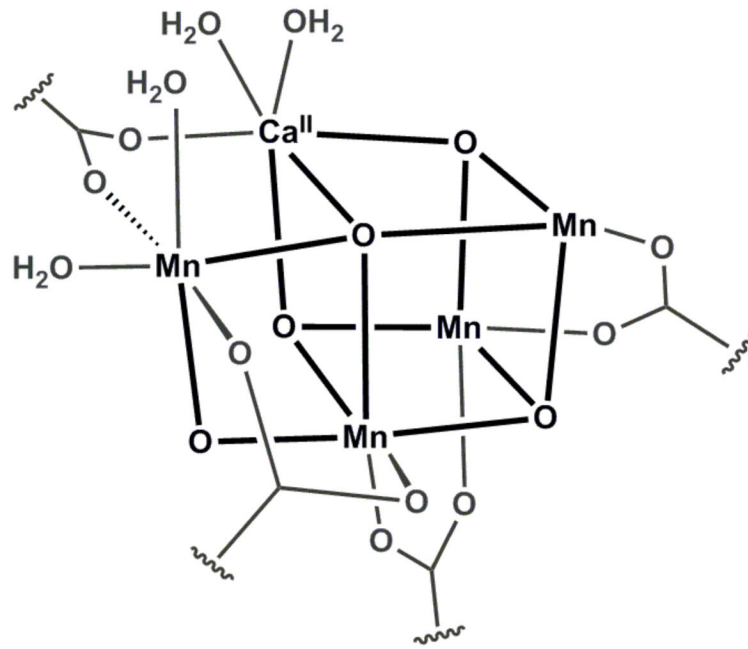


Figure 14.
Depiction of the oxygen-evolving complex composed of a Mn_4CaO_5 cluster that is found within Photosystem II.

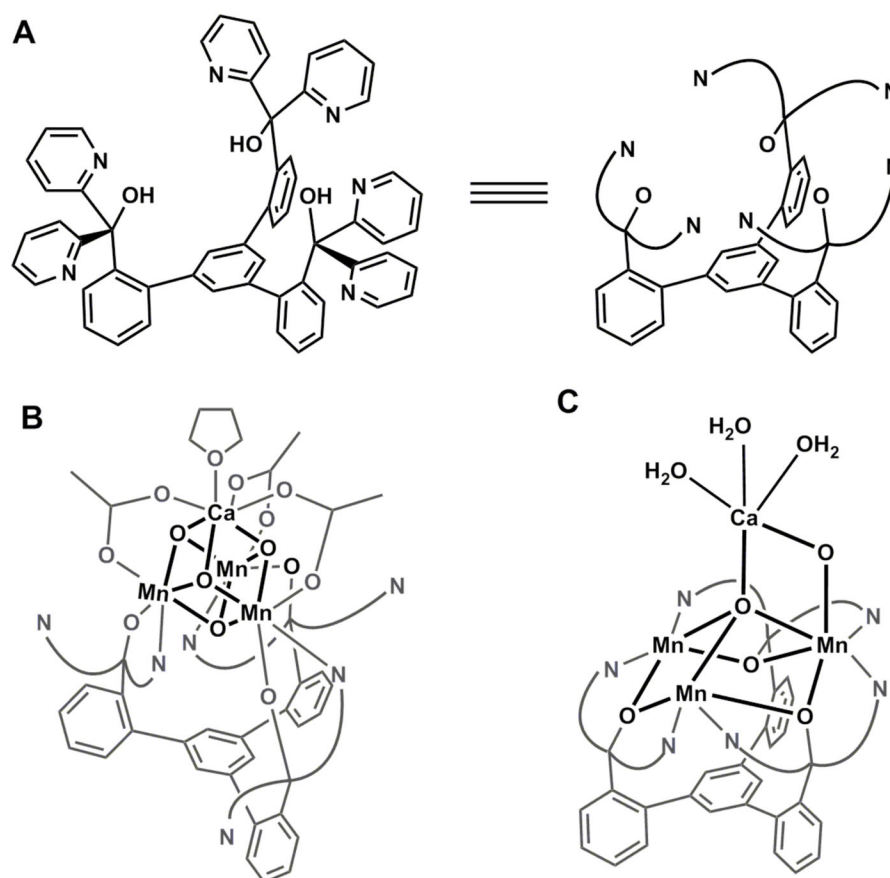


Figure 15. Ligand 1-H₃ and the icon used to represent it (A) and the Mn₃CaO₄ clusters that resemble the OEC (B and C).

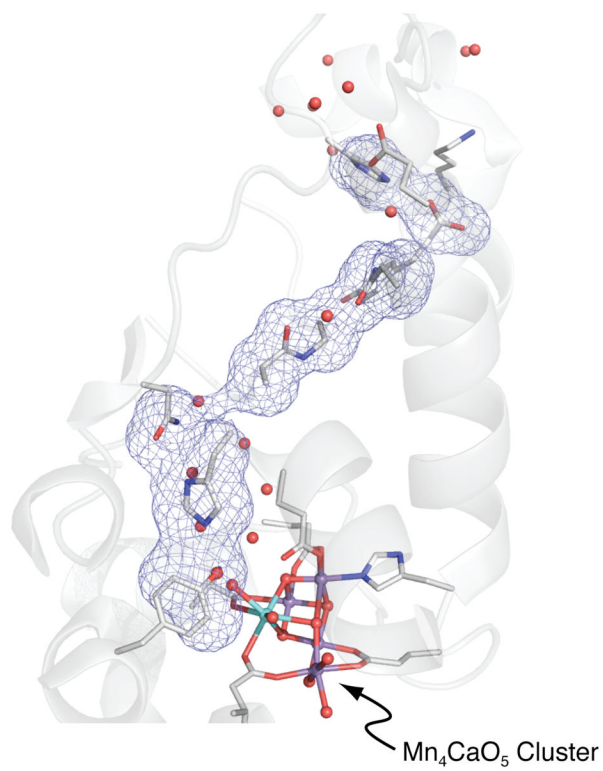


Figure 16.
The Mn₄CaO₅ cluster of the OEC with water channel as indicated by blue mesh (PDB: 3ARC).

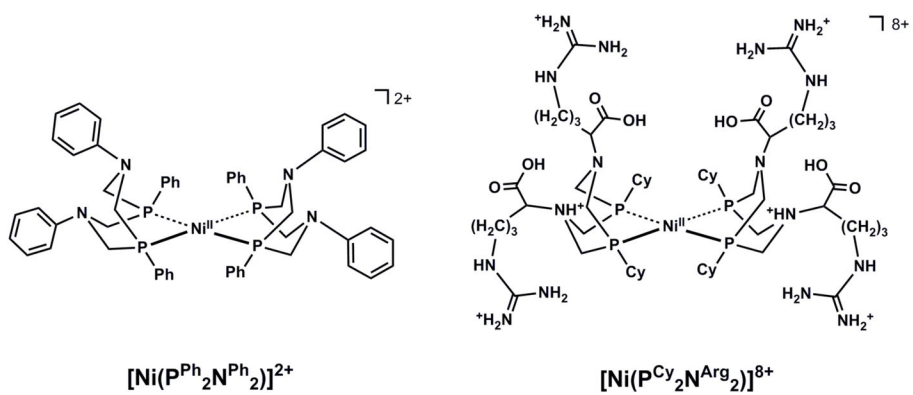


Figure 17. Schematic of Ni(II) complexes with pendant amines within the secondary coordination sphere.

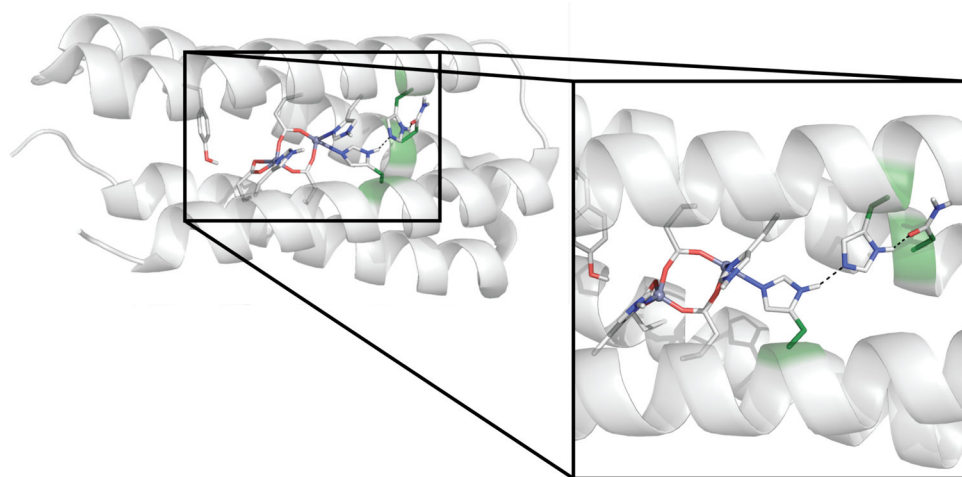


Figure 18. Structure of the Zn₂ adduct of the artificial 4-helix bundle protein that hydroxylates arylamines. The insert illustrates the H-bonding network within the active site and the green highlights indicates mutation sites.

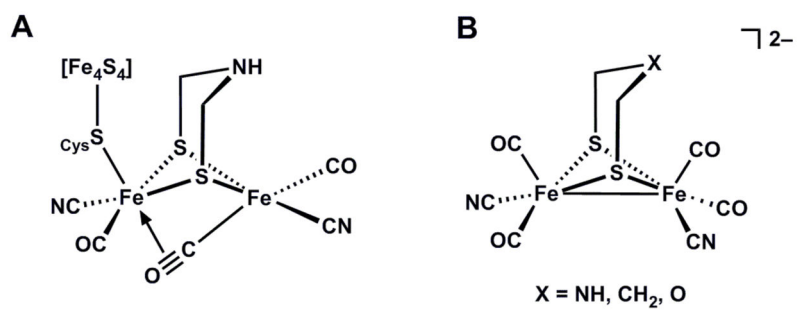
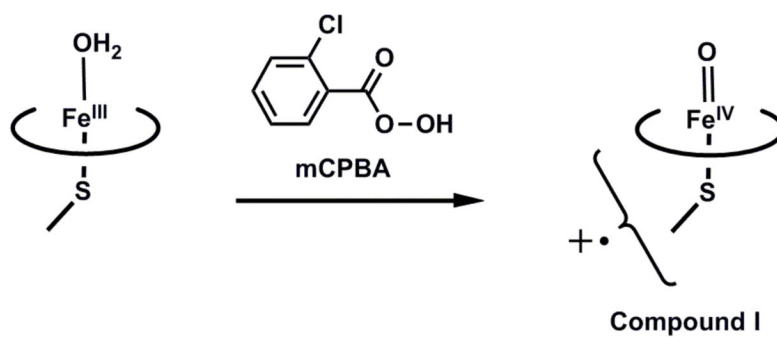


Figure 19. The natural [Fe,Fe]-hydrogenase cofactor (A) and Fe₂-synthetic mimics (B).



Scheme 1.
Generation of Compound I using the peroxide shunt pathway.