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Using Biosynthetic Models of Heme-Copper Oxidase and Nitric Oxide Reductase in Myoglobin to Elucidate Structural Features Responsible for Enzymatic Activities

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Abstract

In biology, a heme-Cu center in heme-copper oxidases (HCOs) is used to catalyze the four-electron reduction of oxygen to water, while a heme-nonheme diiron center in nitric oxide reductases (NORs) is employed to catalyze the two-electron reduction of nitric oxide to nitrous oxide. Although much progress has been made in biochemical and biophysical studies of HCOs and NORs, structural features responsible for similarities and differences within the two enzymatic systems remain to be understood. Here, we discuss the progress made in the design and characterization of myoglobin-based enzyme models of HCOs and NORs. In particular, we focus on use of these models to understand the structure-function relations between HCOs and NORs, including the role of nonheme metals, conserved amino acids in the active site, heme types and hydrogen-bonding network in tuning enzymatic activities and total turnovers. Insights gained from these studies are summarized and future directions are proposed.

1. Introduction

It is estimated that approximately 90% of oxygen reduction in atmosphere is catalyzed by heme-copper oxidases (HCOs).^[1] HCOs are large membrane proteins found both in bacteria and in the mitochondria of eukaryotes. They are terminal enzymes in the respiratory electron transport chain, and use a heme-copper center to catalyze the four-electron reduction of oxygen to water efficiently (Figure 1a–b).^[2] Interestingly, the HCOs exhibit high sequence and structure homology with nitric oxide reductases (NORs), which utilize a heme-nonheme diiron center to perform two-electron reduction of NO to N₂O (Figure 1c–d).^[3] NORs constitute an integral part of denitrification processes – conversion of nitrates/nitrites to nitrogen.^[4] The similarities between the two classes of enzyme are not only in sequence and structure, but in their function as well. Cross-reactivity has been observed, where some NORs are able to perform oxygen reduction to water – albeit at significantly lower rates as compared to HCOs (less than 10%) – and vice-versa.^[5] At the same time, the two enzyme classes possess notable differences, such as (1) the identity of nonheme metals: HCOs use copper while NORs use iron as the nonheme metal ion;^[6] (2) coordination of the nonheme metals: copper in HCOs is coordinated by three histidines, while the nonheme iron in NORs

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This review is dedicated to Prof. Harry B. Gray in celebration of his 80th birthday

is coordinated by a glutamate in addition to three histidines;^[7, 3a] (3) conserved secondary coordination sphere (SCS) residues: HCOs contain a conserved tyrosine crosslinked to a copper coordinating histidine, while NORs contain several conserved glutamates;^[8] (4) the heme types: NORs use exclusively heme *b* at their catalytic center while HCOs utilize a variety of heme types, such as heme *a*, *o* and *b*.^[9] These subtle differences in the structure and function of these otherwise homologous proteins raise a fundamental question of how these and other structural features are responsible for fine-tuning their specificities and cross-reactivities (Fig. 1).

Elucidating the structural features responsible for HCO and NOR functions will not only provide deeper insights into their roles in biological respiration and nitrogen metabolic processes, but also molecular bases for designing efficient catalysts for the oxygen reduction reaction (ORR) in fuel cells and nitrogen cycles. For example, a recent DFT study compared the best platinum-based oxygen reduction catalysts with HCO, and concluded that HCO is a better catalyst for fuel-cell based energy applications, as it exhibits much lower overpotential than current platinum-based catalysts.^[10] Additionally, HCOs use earth-abundant metal ions (iron and copper) for catalysis. Thus, understanding the principles employed by HCOs to catalyze fast ORR ($k \sim 1000/\text{s}$) at low overpotentials under physiological conditions, will help us design low-cost and energy-efficient fuel cell catalysts. At the same time, NOR is one of the key enzymes in the nitrogen cycle, responsible for the return of fixed nitrogen back to the atmosphere.^[4a] NORs are suggested to be involved in pathogenesis of organisms such as *P. aeruginosa*,^[11] especially in cystic fibrosis patients, and the NOR reaction can be used as a model for mammalian enzymes utilizing NO as an essential signaling molecule.^[12] Probing HCO and NOR functions will shed light upon these and various other human health, energy, and environmental processes.

An elegant way to evaluate and understand the structure-function relations *between* HCOs and NORs would be to reproduce the structural features of one enzyme within the other. For example, by replacing copper in HCOs with nonheme iron and probing how it impacts the activity, one may be able to determine the role of nonheme metal ion in the two enzymatic functions. However, it is difficult, if not impossible, to perform such metal ion substitution without significantly perturbing the structure and function of the enzyme, because HCOs and NORs are large (MW ~ 100 – 200 KDa) membrane-bound proteins, and incorporation of copper into HCO and nonheme iron into NOR often requires specific chaperone proteins. Furthermore, HCOs and NORs contain multiple metal centers, such as Cu_A and heme *a* centers in bovine CcO, that deliver electrons into the heme-copper center, making it difficult to focus on and specifically study the catalytic metal center. To overcome these limitations with studying the native HCOs and NORs, a number of small-molecule based model systems have been synthesized and studied, and the results obtained have contributed immensely to the understanding of the mechanism of oxygen and NO activation in the two protein systems.^[13] As an alternative approach, we have designed biosynthetic models of HCOs and NORs in a small (MW ~ 17.4 KDa), easy-to-purify, soluble protein, myoglobin (Mb), called Cu_BMb^[14] and Fe_BMb,^[15] respectively (Fig. 1e–f). Table 1 shows some limitations in studying native HCOs/NORs, and how their small-molecular models and biosynthetic models can help overcome these limitations. As can be discerned from the table, Mb-based biosynthetic models have key advantages over both native proteins and

synthetic models, and are excellent systems to elucidate the structure-function relations of HCOs and NORs.

In this review, we discuss the design and comprehensive characterization of Mb-based biosynthetic models of HCO and NORs that mimic the native enzymes, not only structurally but also functionally, some with activity similar to that of native enzymes. In the process, we have shown that these models not only help elucidate structural features responsible for different reactivities of these two classes of enzymes, but also offer deeper insight into the fundamental mechanisms of the two reactions, including key intermediate states. The major contribution from the studies of biosynthetic models is a clear demonstration of the importance of the non-covalent secondary sphere interactions, such as H-bonding interactions involving water, in fine-tuning the electron transfer (ET) rates, enzyme reactivity, product selectivity, and turnover numbers.

2. Developing a structural and functional HCO mimic in myoglobin

2.1. Design of a Cu_B site in Mb and its spectroscopic and structural characterizations

Mb serves as an excellent scaffold to design the catalytic heme-copper center, as in HCOs, because Mb already contains a high-spin heme iron coordinated by a histidine and binds oxygen with high affinity.^[16] The heme-copper center in HCOs utilizes three histidines to coordinate a copper, called Cu_B, in the distal heme pocket (Fig. 1b). The wild type myoglobin (WTMb) contains a single H64 in the distal heme pocket. Based on visual inspection and comparison with the heme-copper center in bovine CcO, L29 and F43 residues were identified to be in corresponding positions to accommodate two additional His residues in the distal pocket. The resulting mutant, F43HL29HMB, possessing three histidine residues capable of coordinating copper in the distal heme pocket, similar to the Cu_B center in HCOs, was named Cu_BMb (Fig. 2a).^[14] It is important to note that Cu_BMb is purified without any metal in the Cu_B site, but with heme *b* as in native Mb. The binding of copper to the Cu_B center in Mb was observed through UV-Vis titrations that revealed that Cu_BMb binds Cu(II) with changes in heme Soret and visible band, and a K_D of 9 μM (Fig. 2c). Finally, a crystal structure of copper-bound Cu_BMb mutant showed that the copper binds to three histidines and a water molecule ~ 5 Å away from heme iron (Fig. 2b).^[17] Thus, the Cu_BMb is a good structural model of HCO that binds copper at the Cu_B center, similar to HCOs.

2.2. The effects of cyanide and chloride on HCO structure and function

Further investigation of the heme-copper center was performed using CN⁻, a high affinity ligand to several heme proteins including Mb and HCOs.^[13d] The presence of Cu(II) at the Cu_B center increased affinity for negatively charged CN⁻ by ~ 20-fold. Furthermore, addition of ~ 1.4 eq. of Cu(II) to cyanide bound Cu_BMb resulted in attenuation of low-spin heme signals at *g* = 2.06, suggesting spin-coupling of heme-copper center similar to HCOs (Fig. 2d). Control EPR experiments performed with Cu(II)-added WTMb or Zn(II)-Cu_BMb showed no decrease in heme signal.

In addition to CN^- , spectroscopic studies on HCOs suggest that chloride binds to the heme-copper center in cases where the HCO purification processes are not rendered rigorously chloride-free.^[18] This binding of chloride to the heme-copper center induces spectroscopic and chemical changes such as lowering its redox potential^[19] and preventing fast reactions with CN^- and NO .^[20] Based on these studies, the possible role of chloride as a mimic of hydroxide ligand to the Cu_B center has been suggested.^[21] To test this hypothesis, the spectroelectrochemical titration of $\text{Cu(II)-Cu}_\text{B}\text{Mb}$ was performed in the presence of 500 molar equivalents of chloride.^[22] Upon the first electron transfer to the Cu_B center, one of the His ligands of Cu_B center dissociated and coordinated to the heme iron, forming a six-coordinate low-spin ferric heme center and reduced Cu_B center (Fig. 2f). The second electron transfer reduced the ferric heme and caused the release of the coordinated His ligand. Therefore, the fully reduced state of the heme-copper center contained a five-coordinate ferrous heme and a reduced Cu_B center, ready for O_2 binding. In the absence of chloride, formation of the low-spin heme species was not observed (Fig. 2e). These results showed that binding of chloride to the Cu_B center can induce redox-dependent structural changes. Additionally, chloride and hydroxide in the heme-copper center may play different roles in the redox-linked enzymatic reactions, probably because of their different binding affinity to the Cu_B center and relatively high concentration of chloride under physiological conditions.

2.3. The roles of Cu_B center in O_2 binding and reduction

The design of a Cu_B site in the Mb scaffold helped us probe its roles in O_2 binding and reduction, with and without nonheme metal ions such as Cu(II) and Cu(I) , and their redox-inactive controls, Zn(II) and Ag(I) , respectively.^[23] First, the O_2 -binding affinity of heme-containing $\text{Cu}_\text{B}\text{Mb}$ in which the Cu_B site is empty (more simply called E- $\text{Cu}_\text{B}\text{Mb}$) was compared with $\text{Ag(I)-Cu}_\text{B}\text{Mb}$, i.e., with Ag(I) occupying the Cu_B site. While the deoxy E- $\text{Cu}_\text{B}\text{Mb}$ formed only partial heme-oxy form (with ~50% protein in O_2 -free deoxy-form) upon exposure to O_2 , the $\text{Ag(I)-Cu}_\text{B}\text{Mb}$ formed heme-oxy completely (Fig. 3a), suggesting that the presence of a metal ion, such as Ag(I) , in the Cu_B center increased the O_2 -binding affinity. Next, the reaction of $\text{Cu(II)-Cu}_\text{B}\text{Mb}$ with O_2 was investigated in the presence of a reductant, ascorbate, and a redox mediator, N,N,N',N' -tetramethyl-p-phenylenediamine (TMPD), and kinetics monitored by electronic absorption spectroscopy in the ultraviolet and visible region (UV-vis). These measurements showed a gradual decrease in the Soret band of $\text{Cu}_\text{B}\text{Mb}$ (at ~408 nm) and concomitant increase in absorption at 680 nm. A spectral analysis of the product along with mass spectrometry confirmed a gradual ($k = 0.028 \text{ s}^{-1}$) degradation of heme to form verdoheme, similar to what occurs naturally in heme oxygenase (HO) (Fig. 3b–c). Similar experiments performed with $\text{Ag(I)-Cu}_\text{B}\text{Mb}$ and $\text{Zn(II)-Cu}_\text{B}\text{Mb}$ showed no evidence of verdoheme formation, suggesting that the redox active copper ion was required for the heme degradation reaction. These results suggest a pathway wherein the heme-copper center activates oxygen to form a peroxy form (2e^- reduced oxygen). While the heme-peroxy species can be protonated and undergo heterolytic O-O bond cleavage to form a ferryl species, as observed in HCOs and cytochrome P450s, the heme-peroxy in $\text{Cu}_\text{B}\text{Mb}$ evidently attacked the heme and followed the HO pathway to produce verdoheme (Fig. 3d). The reason for this difference was proposed to be that the first-generation HCO model ($\text{Cu}_\text{B}\text{Mb}$) did not possess the elaborate H-bonding network found in HCOs or P450s to

deliver the extra proton to activated forms of oxygen. To support this proposal, a reaction of H_2O_2 with met- $\text{Cu}_\text{B}\text{Mb}$ was carried out, because 2e^- reduction of O_2 produces a species equivalent in oxidation state to H_2O_2 . This so-called “peroxide-shunt” reaction is known in HO, P450, and HCO. When H_2O_2 was reacted with ferric $\text{Cu}_\text{B}\text{Mb}$, a ferryl-heme was observed instead of verdoheme. These results suggest that the extra protons brought into the $\text{Cu}_\text{B}\text{Mb}$ active site by the H_2O_2 may allow the $\text{Cu}_\text{B}\text{Mb}$ to switch from the HO reaction pathway into the P450 and HCO pathway. These studies with $\text{Cu}_\text{B}\text{Mb}$ demonstrate that the presence of a metal ion in the Cu_B site increases oxygen binding affinity. While O_2 reduction has been observed, the lack of proton delivery through a hydrogen-bonding network in the distal heme pocket of the HCO model makes it go through the HO pathway to produce verdoheme, instead of ferryl species important for HCO reaction to produce water.

2.4. Introducing a hydrogen-bonding network with a different heme type and its effect in HCO activity

Given the changes in O_2 reactivity imparted by the above design of the primary Cu_B site into myoglobin, and the observation that the reaction may be limited by protonation of intermediates, we sought to model the active site of native HCOs more closely by modeling the secondary coordination sphere (SCS) residues of HCOs. One interesting difference is that HCOs use a variety of heme types at their catalytic center. In particular, A- and B-type HCOs contain heme *o* and heme *a*, which incorporate a hydroxyethylfarnesyl moiety onto the heme *b* structure.^[24] The HCOs remained mostly active when heme substitution occurred between *a*- and *o*-type hemes, however, when either *a*- or *o*-type heme was replaced by heme *b* at the catalytic center, the enzyme lost its activity.^[25] The hydroxyl group of the farnesyl side chain is suggested to be an essential part of the active site hydrogen-bonding network, with internal water molecules bridging the gap between the hydroxyl group of cross-linked tyrosine and the bound oxygen at the heme-copper site.^[26] To probe the importance of the heme *o* hydroxyl group in tuning the oxygen chemistry, the heme *b* in $\text{Cu}_\text{B}\text{Mb}$ was replaced with the heme *o* mimic Fe-hydroxyethylvinyldeuteroporphyrin (Fe-HVD) – heme *b* possessing a hydroxyethyl group similar to heme *o* (Fig. 4a). This substitution resulted in a ~4 nm blue shift in the heme Soret band and ~20 mV decrease in the heme E° , suggesting that the addition of a hydroxyethyl group only mildly impacts the electronic properties of heme iron. In contrast, such replacement caused a significant difference in oxygen reactivity: the heme degradation reaction of $\text{Cu}(\text{II})\text{-Cu}_\text{B}\text{Mb}$ was slowed by ~19-fold in the heme *o* mimic-substituted $\text{Cu}_\text{B}\text{Mb}$ (from 0.028/s to 0.0015/s for heme *b*, Fig. 4b). These results strongly suggest the critical role of the hydroxyl group of heme *o* in modulating HCO activity through participation in a hydrogen-bonding network.

2.5 Incorporating the conserved tyrosine into $\text{Cu}_\text{B}\text{Mb}$ and its effect on enzyme activity and robustness

Another key and novel structural feature of HCOs is a conserved tyrosine residue near the Cu_B site, which forms a covalent bond with one of the Cu_B coordinating His ligands (Fig. 1b).^[28, 2b] To incorporate a tyrosine into the $\text{Cu}_\text{B}\text{Mb}$ models, structural overlays with different types of HCOs were performed. In type A- and B-type HCOs, the tyrosine is

located on a helix, four residues downstream from one of the His ligands to which it is covalently attached.^[28a, 2b] In C-type HCOs, the conserved tyrosine is located on an adjacent helix.^[28c] Therefore, two different models were designed to incorporate tyrosine into the active site of Cu_BMb: (1) F33Y-Cu_BMb, in which the tyrosine is four residues downstream from His 29, on the same helix, and (2) G65Y-Cu_BMb, in which the tyrosine is on a helix adjacent to the one containing H29 and the position of the Y65 hydroxyl group is predicted to be in approximately the same position with respect to the heme as seen in C-type HCOs.^[17] The structure of F33Y-Cu_BMb and structural model of G65Y-Cu_BMb shown in Fig. 5a–b closely resemble the active sites of native HCOs.^[17] The functional properties of F33Y-Cu_BMb and G65Y-Cu_BMb were evaluated by kinetic assays of oxygen reduction using an oxygen electrode (Figure 5c).^[17] These assays were carried out using previously reported ascorbate/TMPD as the reductant system. Formation of water was additionally confirmed by detecting isotopically labeled H₂¹⁷O in the presence of ¹⁷O₂ (Figure 5d). The results of these assays showed that presence and positioning of tyrosine in the active site of Cu_BMb plays a significant role in improving oxygen reduction to water.^[17] Moreover, F33Y-Cu_BMb and G65Y-Cu_BMb were able to achieve over 500 and 1000 turnovers of oxygen reduction, respectively.^[17]

The observed importance of Tyr in promoting ORR in the Cu_BMb models makes it an ideal choice for investigating what structural features are responsible for the activity. It is proposed that in native HCOs, the conserved tyrosine participates by donating an H radical during turnover.^[29a, 1c, 29b–d] Additionally, the conserved tyrosine in the Cu_B site constitutes the end of the K-proton channel, which is responsible for delivering two of the catalytic protons for complete oxygen reduction.^[28a, 2b] EPR of F33Y-Cu_BMb under reductive conditions in the presence of O₂ was performed, and revealed the formation of tyrosyl radical (Fig. 6d).^[30] This observation provides strong support for the direct involvement of Tyr in the redox process of O₂ reduction in F33Y-Cu_BMb. Further investigations into the role of tyrosine were carried out by incorporating unnatural tyrosine analogues with a range of pK_a's and reduction potentials,^[31] and observing the effects on activity (Figure 6a–c). The investigation revealed (1) that the overall rate of reductive oxygen consumption increases with increasing reduction potential, and (2) that the ratio of water produced increases with decreasing pK_a. Together, these results suggest that tyrosine plays an important role in both electron and proton transfer to oxygen.

Finally, the structural interactions that promote oxygen activation in F33Y-Cu_BMb were investigated using EPR of cryoreduced oxy-F33Y-Cu_BMb (Fig. 7a).^[33] This technique gives information about the electronic structure of the heme-bound oxygen and its interactions with the active site.^[34] The results showed that oxygen bound to F33Y-Cu_BMb can be more polarized than in WTMB, withdrawing electron density from the Fe onto the O₂, likely due to strong hydrogen bond donation, as confirmed by ENDOR. A significant hydrogen-bonding network consisting of waters and connecting the tyrosine and histidine residues with the oxygen was confirmed in oxy-F33Y-Cu_BMb by X-ray crystallography (Fig. 7b). This study further supports that a key role of tyrosine in Cu_BMb activity is the activation and protonation of oxygen by hydrogen-bonding.

2.6. The role of His-Tyr crosslink in HCO activity and turnovers

The His-Tyr crosslink is a unique post-translational structural feature found naturally in all HCOs. This feature was first identified using X-ray crystallography^[35] and was confirmed *via* mass spectrometry and Edman degradation.^[36] The proposed function of the crosslink ranges from merely structural to donation of a proton^[24a] and/or an electron for complete catalytic oxygen reduction.^[37] An elegant method of understanding the role of His-Tyr crosslink in modulating O₂ reduction activity will be by comparing function of His and Tyr residues at the same positions in the same protein with and without cross-linking. We attempted to do this comparison by incorporating an unnatural amino acid, imidazolyl-tyrosine (imiTyr), that mimics a crosslink between H43 and Y33 residues in the Cu_BMb model (Fig. 6e).^[32] The resulting mutant, imiTyrCu_BMb, could bind Cu(II) at the Cu_B center with ~5-fold higher binding affinity as compared to F33Y-Cu_BMb (with no His-Tyr crosslink). Additionally, the F33imiTyr-Cu_BMb could perform much faster and cleaner oxidase activity, and exhibited almost twice as number of turnovers as F33Y-Cu_BMb. These results suggest that the His-Tyr crosslink plays an important role in tuning the oxygen reduction activity and turnovers of HCOs.

2.7. Importance of heme redox potential and electron transfer rates in tuning HCO reactivity

The catalytic heme centers in various HCOs exhibit a wide (~400 mV) range of heme E° values, with the highest reported heme E° of +365 mV for bovine CcO^[39] and lowest heme E° of -59 mV for *cbh₃* oxidase.^[40] The reasons for such variation of heme E° in HCOs and its impact on oxidase activity (if any) is not yet understood. This is because of the difficulty of systematically tuning the E° of catalytic heme iron in HCOs without impacting the E° of other metal ions (e.g. the Cu_B center). To investigate the role of heme E° in controlling HCO activity and turnovers, the heme E° in F33Y-Cu_BMb were tuned using two approaches:

1. Tuning the H-bonding environment of heme iron in the proximal side: Previous reports on Mb and other metalloproteins have shown that modulating the H-bonding environment of the metal ion is an efficient method to tune its E°.^[41] For the Mb-based HCO model specifically, the Cu_B site and Tyr residues were in the distal side of the heme, and in order to avoid perturbing the O₂ and Cu_B binding properties, we concentrated on the proximal side of heme. Based on crystal structure and NMR studies on Mb, the proton in H93 forms a H-bond with the lone pair on the OH group of S92 (Fig. 8a). Mutation of this S92 to A in principle should increase the positive charge of this proton, in turn decreasing the electron donating capabilities of H93. Indeed, the resulting mutant S92A-F33Y-Cu_BMb displayed ~27 mV (E° = 123 mV) higher heme E° than F33Y-Cu_BMb (E° = 95 mV)^[42]
2. Using different heme types with higher heme E°: Certain A- and B-type HCOs exhibiting high heme E° possess a heme *a* at their active site. The heme *a* contains formyl group, which due to its electron-withdrawing nature, should increase the heme E°. Thus, heme *a* mimics such as

diacetyl heme (DA-heme), monoformyl-heme (MF-heme) and diformyl-heme (DF-heme) were incorporated in the HCO mimics (Fig. 8b). The resulting F33Y-Cu_BMb variants exhibited heme E°' values of 175 mV, 210 mV and 320 mV respectively.

The above set of HCO models with systematically tuned heme E°' provided an ideal system to probe the importance of E°' on oxidase activity. Interestingly, the increase in heme E° resulted in ~6-fold increase in oxygen reduction rates with a direct correlation between heme E° and oxygen reduction rates (Fig. 8c). The HCO mimic with highest heme E°, F33Y-Cu_BMb (DF-heme) exhibited more than 1200 turnovers.

The concurrent increase in oxygen reduction rates with increase in heme E°' suggest that electron transfer (ET) to the heme center is a rate-limiting step in the designed HCO mimics. To increase the ET – and concomitantly the oxygen reduction rate – further, we followed a report by Hoffman and coworkers, which showed that replacing three negatively charged amino acids (D44, D60, and E85) on the surface of Mb with positively charged lysines improved electrostatic interaction between Mb and its negatively charged redox partner, cyt *b*₅.^[43] and resulted in 200-fold increase in ET rates between Mb and cyt *b*₅. Based on these studies, a triple-lysine variant of the HCO model G65Y-Cu_BMb, called G65Y-Cu_BMb(+6), was produced (Fig. 8d), which exhibited a 400-fold enhancement in ET rate and an oxygen reduction rate of 52 s⁻¹.^[43] Remarkably, the engineered HCO mimic exhibits oxygen reduction rates comparable to that of native cytochrome *cbb*₃ oxidase (50 s⁻¹) under the same conditions (Fig. 8e).

2.8. Using Mb-based HCO mimics to perform electrocatalytic oxygen reduction reaction

The performance of G65Y-Cu_BMb as an electrocatalyst was tested by immobilizing a single layer of protein on a gold electrode surface using modified porphyrin groups.^[45] The rate of oxygen reduction and product selectivity of G65Y-Cu_BMb immobilized on the electrode ($k = 1.98 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, 6% ROS) was far superior to a HCO synthetic model tested under same conditions ($k = 1.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, 10% ROS). Moreover, the first-order oxygen reduction rate of G65Y-Cu_BMb (~5000 s⁻¹) is at least 10-fold higher than that of native HCO on electrode. *In situ* resonance Raman analysis of these reactions suggest that electron transfer shunt from the electrode circumvents the slow dissociation of a ferric hydroxide species, which slows down native HCOs in comparison to its Mb-based mimic. The efficient electrocatalytic reduction of oxygen to water by Mb-based HCO models suggests that they can potentially be employed as fuel cell catalysts. However, low protein stability remains to be an issue with enzyme-based fuel cell electrodes which can be addressed *via* protein design and engineering of more robust and thermophilic proteins. While these protein-based catalysts may never replace fuel cell catalysts in car batteries, they are ideal for biomedical applications, such as powering diagnostic and imaging devices *in vivo*, where biocompatibility is essential and power requirement is less.

2.9. NO reduction cross-reactivity of Mb-based HCO mimic

It has been observed that certain HCOs, such as *ba*₃/*caa*₃ oxidases from *Thermus thermophilus* and cytochrome *cbb*₃ oxidase from *Pseudomonas stutzeri*, display NOR reactivity.^[46] Likewise, many NORs display HCO activity. On the other hand, CcO from

bovine heart shows no NOR activity and instead is inhibited by nanomolar concentrations of NO.^[47] To understand such differences in NO reduction cross-reactivity of HCOs, the NO reduction activity of Cu_BMb was investigated by GC-MS. In the presence of Cu(I), Cu_BMb catalyzed the selective reduction of NO to N₂O, as shown by the appearance of a second peak at a longer retention time in the GC, which corresponds to a 44 MW peak (N₂O) in the MS (Figure 8f).^[44a] The relative GC peak intensity of N₂O:NO increased as a function of time, indicating further reduction of NO to N₂O as the reaction proceeds. The turnover number for NO reduction was calculated to be ~2 mol NO/mol Cu_BMb/min, close to the 3 mol NO/mol enzyme/min reported for the *ba*₃ oxidases from *T. thermophilus*.^[9c] Control experiments performed with Cu_BMb alone or with WTswMb in the presence of Cu(I) showed no reduction of NO. These results demonstrated that Cu(I) plays a critical role in the reduction of NO to N₂O in Cu_BMb.

3. Developing a structural and functional NOR mimic in myoglobin

3.1. Design of a nonheme iron binding Fe_B center in Mb

The NORs contain a heme-nonheme diiron catalytic center that catalyzes the two-electron reduction of NO to N₂O, an important step in the biological denitrification process. At the time the Mb model of NOR was designed, the crystal structure of NOR was not available. Nevertheless, the structural and sequence homology of NORs with HCOs predicted that all three His ligands in HCOs (coordinating to Cu_B) were conserved in the same position in NORs.^[48] Additionally, the NORs contained several conserved glutamates (not found in HCOs) that were required for the nonheme iron binding and/or catalysis.^[8a] Based on this information, and knowledge that nonheme iron typically prefers an octahedral geometry where one of the ligands is a carboxylate (amino acids D/E), we embarked to design the putative nonheme iron binding site (Fe_B) in Mb, consisting of three histidines and one glutamate. The histidine residues were introduced first, as crystal structures of HCOs containing three conserved histidines were readily available (e.g., bovine CcO). Based on overlays of the minimized structure of swMb and CcO, the distal His64 in swMb was chosen as one of the histidines, while L29 and F43 were mutated to histidines, to produce a nonheme metal binding site as in Cu_BMb. Next, V68E mutation to swMb was chosen based on its proximity and angle to the heme and the three His. The minimized computer model of the resultant triple mutant was named Fe_BMb, is shown in Fig. 9a–b. The Fe_B metal (modeled as Zn(II)) was found within bonding distance to Nε of all three His residues and both O atoms of E68, indicating that the proposed mutations would support iron binding in myoglobin, forming an Fe_B site.

Following the computational design, Fe_BMb was tested for nonheme iron binding *via* UV-Vis and EPR spectroscopy. The modulation of heme Soret and visible bands in the UV-Vis spectra (Fig. 9c–d) of Fe_BMb, along with the attenuation of g=6 EPR signals, suggested the binding of nonheme iron in the designed Fe_B center. Further evidence of Fe²⁺ binding to the designed Fe_B site comes from a high resolution crystal structure (1.72 Å) of Fe(II)-Fe_BMb (Fig. 9b). The nonheme iron in the Fe_BMb was five coordinate, with bonds to all three histidines and E68. The crystal structure was found to be consistent with the proposal that a glutamate in the active site of NOR helps stabilize iron binding to the Fe_B site. To test the

NO reactivity of Fe_BMb, GC-MS assays were conducted. The Fe_BMb performed selective two-electron reduction of NO to N₂O in the presence of nonheme iron (Fig. 9e), while similar experiments performed with only nonheme iron or E-Fe_BMb produced negligible N₂O. Overall, Fe_BMb was the first designed protein that modeled both the structure and function of NOR, offering the insight that the active site glutamate is required for both iron binding and activity. These results also showed that structural and functional metalloproteins can be rationally designed *in silico* based on sequence and structural homologies, even before the crystal structure of native enzymes are available.

To understand the implications of nonheme metal in modulating NO reactivity, the reaction of one molar equivalent NO with E-, Zn-, Cu- and Fe-Fe_BMb was investigated by UV-Vis, EPR, resonance Raman and FTIR spectroscopies. These experiments revealed the binding of first NO molecule to the ferrous heme in all cases, but while the E-, Cu- or Zn-loaded proteins showed characteristic EPR signatures of S = 1/2 six-coordinate heme {FeNO}⁷ species, the nonheme Fe-loaded proteins were EPR silent. Vibrational modes from the heme [Fe-N-O] unit were identified in resonance Raman (rR) and FTIR spectra using ¹⁵NO and ¹⁵N¹⁸O. The E- and Cu(I)-bound proteins exhibit ν(FeNO) and ν(NO) that were only marginally distinct from those reported for native myoglobin. However, binding of nonheme Fe at the Fe_B site shifts the heme ν(FeNO) by 17 cm⁻¹ and the ν(NO) by ~50 cm⁻¹ to 1549 cm⁻¹. This low ν(NO) was without precedent for a six-coordinate heme {FeNO}⁷ species and suggested that the NO group adopts a strong nitroxyl character stabilized by electrostatic interaction with the nearby nonheme Fe (Fig. 9f). A similarly low ν(NO) was detected in the Zn(II)-loaded protein that supported this interpretation.^[49]

3.2. Role of secondary coordination sphere (glutamates) towards NOR activities

Further structural investigations and mutagenesis studies of native NORs revealed that the presence of additional glutamates near the Fe_B site are important for NOR activity.^[8a, 50, 13j] Based on these observations, a second generation model of Fe_BMb was engineered, in which Glu was introduced near the heme at position 107. The resulting mutant called I107E-Fe_BMb retained binding of metal ions, including Fe²⁺ to the Fe_B site, as was confirmed crystallographically (Fig. 10a).^[51] Moreover, the new mutant retains the position of Fe(II) and its coordination, as compared to Fe_BMb. EPR of fully oxidized Fe(II)-I107E-Fe_BMb revealed a decrease in the heme signals relative to E-I107E-Fe_BMb, suggestive of spin coupling between the metals, as observed in native NORs. Titration of Cu²⁺ resulted in similar apparent coupling, while titration of Zn²⁺ resulted in an increase in the heme signals, suggestive of weakening of the heme-Fe coordination. Binding of these metals and their interactions were again confirmed crystallographically. Cu retained similar geometry, while Zn showed absence of metal-coordinating water molecule and weakening of the Fe-glutamate coordination, consistent with EPR results. The heme E° of I107E-Fe_BMb was measured as -134 mV, ~20 mV higher than the -158 mV measured for Fe_BMb itself. The heme E° with Fe(II), Cu(II), and Zn(II) bound were measured to be -64 mV, -137 mV, and -105 mV respectively.

I107E-Fe_BMb showed a ~100% increase in NOR activity compared to Fe_BMb (Fig. 10b). This increase is attributable to the presence of a 2.32 Å hydrogen bond between the mutated

I107E and a Fe_B water ligand, which was observed crystallographically.^[51] Such a hydrogen-bonding network might be expected to activate NO by hydrogen bonding to the oxygen atom and facilitating protonation. NOR activity was also observed in the presence of Cu(I), but not Zn(II). EPR of these proteins upon reaction with NO showed formation of five-coordinate Fe-NO heme, due to His-Fe bond cleavage, unlike the weakening observed in Fe_BMb, suggesting it may be important for NOR activity.

Subsequent studies using stopped-flow UV-Vis and freeze-quench rR spectroscopies showed that the presence of Glu107 stabilizes the six-coordinate heme-nitrosyl state and prevents dissociation of the proximal histidine, which leads to a dead-end product.^[52] In Fe(II)-Fe_BMb two sets of rR signal were observed after reaction with NO – $\nu\{\text{FeNO}\}_{\text{heme}}$ and $\nu\{\text{NO}\}_{\text{heme}}$ at 522 and 1660 cm⁻¹, from five-coordinate heme-NO, and $\nu(\text{NO})_{\text{FeB}}$ at 1755 cm⁻¹, for nonheme Fe_B-NO. Transient kinetics studies showed that this 5-coordinate species is a result of His dissociation from the transient 6-coordinate NO-bound state. This dissociation is substantially slowed in I107E-Fe_BMb, allowing the heme-nitrosyl to combine with the non-heme nitrosyl and decompose to the desired product, N₂O, with a rate of 0.7 s⁻¹ at 4 °C (Fig. 10c). These observations support the trans-mechanism of NOR function and at the same time show that the H-bonding network introduced through incorporation of I107E residue is critical for NOR activity. Further insights into the role of H-bonding was obtained by probing the reaction of I107F-Fe_BMb with excess NO.^[53] The I107F-Fe_BMb variant with its large, hydrophobic distal I107F residue formed the heme-nitrosyl dead-end product at a much faster rate ($k = 5.3 \text{ mM}^{-1} \text{ s}^{-1}$) as compared to both Fe_BMb ($k = 2.1 \text{ mM}^{-1} \text{ s}^{-1}$) and I107E-Fe_BMb ($k < 1 \text{ mM}^{-1} \text{ s}^{-1}$). Overall, these results suggest that production of N₂O from the $[\text{6cLS heme } \{\text{FeNO}\}^7 / \{\text{Fe}_\text{B}\text{NO}\}^7]$ trans iron nitrosyl dimer intermediate requires a proton transfer event facilitated by an outer-sphere residue such as E107 in Fe_BMb and E280 in *P. aeruginosa* cNOR.

3.3. Complete characterization of a nonheme iron nitrosyl center in Fe_BMb

A major barrier to understanding the binding and interaction of NO with the Fe_B center is the strong spectroscopic signals of heme iron that make it difficult to investigate the Fe_B center. To overcome this challenge, iron containing protoporphyrin IX (heme) in Fe_BMb was replaced by Zn protoporphyrin IX (ZnPPIX) to yield ZnPPIXFe_BMb (Fig. 11a).^[54] The low affinity of ZnPPIX for NO, along with lack of spectroscopic signals of Zn²⁺, was expected to help focus on the nonheme iron specifically. The binding of nonheme Fe²⁺ at the Fe_B center of ZnPPIXFe_BMb was confirmed using UV-Vis spectroscopy along with X-ray crystallography (Fig. 11b–c). The NO binding properties of nonheme iron were investigated using EPR and Mössbauer spectroscopy. More specifically, the EPR spectrum of Fe-ZnPPIXFe_BMb with 20 molar equivalents of NO added shows two well-resolved doublets at $g = 4.36, 3.58, \text{ and } 4.13, 3.73$, respectively (Fig. 11d). These two different components were ascribed to different orientations of NO bound to the Fe_B site, or slight changes in the orientation of Fe_B ligands upon NO binding. The electronic and spin state of the Fe_B nitrosyl species was further characterized by field-dependent Mössbauer measurements which revealed 35% unreacted Fe(II) species along with 65% iron-nitrosyl component. The Mossbauer parameters of the iron-nitrosyl component, $E_Q = (1.70 \pm 0.01) \text{ mm/s}$ and $\delta\text{Fe} = (0.69 \pm 0.01) \text{ mm/s}$, were indicative of an $S = 3/2$ six-coordinate $\{\text{FeNO}\}^7$ species

found in other proteins as well as small molecule models. To obtain further insight into the electronic structure of the $\{\text{FeNO}\}^7$ moiety, the Mulliken spin populations were calculated at the Fe and NO centers of the structures obtained from quantum mechanical/molecular mechanical (QM/MM) calculations as well as partially optimized active-site structures. The computational calculations supported the assignment of nonheme iron-nitrosyl species in $\text{Fe}_\text{B}\text{Mb}$ as a HS ferrous center ($S=2$) antiferromagnetically coupled to an NO radical ($S=1/2$) $[\text{Fe}^{2+}\text{-NO}^*]$. Overall, these findings are consistent with a trans-mechanism of NO reduction wherein the radical nature of the NO in Fe_B -nitrosyl would facilitate the radical coupling of the second heme-bound NO to promote N-N bond formation.

3.4. Influence of Fe_B coordination on NO reactivity: Design of a 2His, 1Glu Fe_B center in Mb

A sequence alignment of a unique quinone-oxidizing NOR, $g\text{NOR}$ predicted that it may exhibit a novel Fe_B site, with one of the 3-His ligands replaced by an Asp residue.^[55] This finding is quite interesting, as an inspection of the active site of nonheme iron containing enzymes in Nature indicates that the majority use a similar conserved 2-His-1-carboxylate (Asp/Glu) facial triad for iron binding and substrate oxidation. To test whether the unique 2-His-1-Asp/Glu nonheme metal center could be utilized to mimic NOR for NO reduction, a 2-His-1-Glu metal center was designed in swMb by replacing H29 in $\text{Fe}_\text{B}\text{Mb}$ with an E residue while keeping V68 intact ($\text{Fe}_\text{B}\text{Mb}$ H29E, E68V, or swMb L29E, F43H, H64, called $\text{Fe}_\text{B}\text{Mb}(-\text{His})$). UV-Vis and EPR spectroscopic measurements along with crystallography showed that $\text{Fe}_\text{B}\text{Mb}(-\text{His})$ could bind either iron or copper, with 2-His and 1-Glu coordinating to the nonheme metal (Cu) in a tetrahedral geometry (Fig. 11a–b). The NO reduction function of $\text{Fe}_\text{B}\text{Mb}(-\text{His})$ when probed using GC/MS under single turnover conditions showed that $\text{Cu}(\text{I})\text{-Fe}_\text{B}\text{Mb}(-\text{His})$ when exposed to excess NO produced N_2O with increased yield over time as estimated from the ratio of N_2O : NO peaks (~32% at 20 h). N_2O production was also observed for $\text{Fe}(\text{II})\text{-Fe}_\text{B}\text{Mb}(-\text{His})$ (~6% at 20 h). In contrast, no N_2O formation was observed for $\text{Zn}(\text{II})\text{-Fe}_\text{B}\text{Mb}(-\text{His})$ or WT Mb with Cu(I) or with Fe(II). Overall, the study presented a novel structural and functional protein model of NOR and provided insights into the newly discovered member of the NOR family, $g\text{NOR}$.

4. Conclusions and future directions

While studies of native HCOs and NORs have and will continue to provide valuable insight into their function, many questions on the structural features responsible for their functions and reaction mechanisms remain to be answered, despite many years of research. We have shown that these questions can be answered using small protein models of each of their active sites. For instance, our studies reveal that the presence and positioning of tyrosine is critically important for HCO activity and turnovers.^[17] Moreover, we have shown the first evidence for the formation of a Tyr radical upon reaction of a functional HCO mimic with oxygen.^[30] We have also directly demonstrated the key role of heme E^{O} on HCO activity in a system that decouples this from other effects of the protein.^[42] In understanding NOR reactivity, $\text{Fe}_\text{B}\text{Mb}$ provided valuable structural insights into a functional NOR before the structure of a native NOR was obtained.^[57] This model has proven invaluable for unraveling the mechanism of NO binding and N-N bond formation at heme-nonheme diiron sites.^[52, 54]

In both designed proteins, the importance of secondary coordination H-bonding effects was elegantly demonstrated using spectroscopic studies and rational engineering.^[51, 42]

The success of these system also enables the next generation of studies to address some important questions regarding the structure and activity of these enzymes that remain difficult to answer by studying native HCOs and NORs, including:

1. Preference of copper and nonheme iron in the active site of HCOs and NORs respectively: The HCOs and NORs are said to have evolved from a common ancestral protein,^[4b, 58] yet copper was chosen as the nonheme metal for HCOs and iron for NORs and each metal ion catalyzes its respective reactions much more efficiently than the other. The functional and chemical imperatives for these choices remain to be understood. HCO/NOR models where the nonheme metal can be successfully replaced is an efficient system to address this question and understand how the choice of a particular metal ion impacts the activity, mechanism, and turnovers of these enzymes.
2. Reasons for high affinity of nonheme metals: The HCOs and NORs bind nonheme metals in their catalytic center with very high affinities, such that even after thousands of turnovers their structure and activity remains unperturbed. In contrast, the Mb-based HCO and NOR models bind copper and nonheme iron with similar geometry and coordination, but exhibit low micromolar affinities.^[14–15] This comparison suggests that the SCS residues and the membranous nature of native HCO/NORs are critical for their strong metal ion affinities to the protein. For example, the structure of bovine CcO shows that the Ne of two of the coordinating His is strongly H-bonded to water molecules and backbone carbonyl. Even the coordinating glutamate in cNOR is strongly H-bonded to other conserved glutamates. This H-bonding interaction may modulate the electronic nature of His/Glu making them a strong sigma donor to nonheme metal. An effective method to test this theory would be to systematically tune H-bonding of coordinating His and Glu residues and test how that impacts the metal binding affinity, catalytic activity and turnovers.
3. Mechanism of His-Tyr crosslinking in HCOs: The His-Tyr crosslink is a unique post-translational modification found naturally in all HCOs. How the His-Tyr crosslink is formed is unknown, but it is believed to be formed during the early turnovers of the enzyme. An efficient method to test this hypothesis will be by reacting a crosslink-free HCO with oxygen and/or activated forms of oxygen to see if any crosslink is formed. However, HCOs lacking His-Tyr crosslink have not been isolated yet unless the crosslinked residues (His/Tyr) were mutated. Protein based HCO models are an ideal system to probe the mechanism of His-Tyr crosslink formation as well as investigate the conditions required for its formation e.g. the pH, oxygen concentration, presence of nonheme metal ions etc.

4. Reasons for high oxygen affinity of C-type HCOs: The C-type HCOs, specifically *cbb₃* oxidase, comprise ~20% of known HCOs and are essential respiratory enzymes for many different pathogenic bacterial species, e.g., *Helicobacter pylori*, *Vibrio cholerae*, and *Pseudomonas aeruginosa*. The *cbb₃* oxidases are characterized by ~6–8 fold higher oxygen affinity ($K_D \sim 7$ nM) as compared to A-type HCOs. A recent crystal structure of *cbb₃* oxidase^[59] has revealed two potential reasons for such high oxygen affinity of C-types HCOs: (a) Distortion of catalytic heme from planarity: The catalytic heme *b₃* in *cbb₃* oxidase is highly domed and distorted. By fast binding of oxygen to the heme iron, the protein may be able to relieve itself from this high-energy distortion and approach the more conventional planar form of heme similar to NO signaling H-NOX proteins;^[60] and (b) H-bonding pattern in proximal pocket: All *cbb₃* oxidases contain a conserved His-Glu-Trp triad similar to peroxidases,^[61] which may tune the electronics of heme iron and modulate its oxygen binding affinity. Protein models of HCOs are good systems to probe whether this unique H-bonding pattern or distortion of heme from planarity are a cause for high oxygen affinity of C-type oxidases.
5. Why do HCOs exhibit much higher heme $E^{\circ'}$ as compared to NORs?: Most HCOs exhibit high heme $E^{\circ'}$ values between +140 to +365 mV while NORs exhibit low heme $E^{\circ'}$ values between +60 to –170 mV. ^[62] The specific reasons for such variation in heme $E^{\circ'}$ between two homologous enzymes and its impact on their activity remains unknown. The systematic variation of heme $E^{\circ'}$ in HCOs/NOR models may be used to address this question and explain how $E^{\circ'}$ values are tuned in metalloproteins to control enzyme activities.

Overall, these experiments will provide broader insights into chemical properties driving evolution of bioenergetics reactions, as well as into structural features that will allow design of more efficient fuel cell catalysts.

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Biographies

Ambika Bhagi-Damodaran was born in Moradabad, India. She received her BSc (Hons.) from St. Stephen's College, Delhi in 2009, followed by an MSc in Chemistry from the Indian Institute of Technology, Delhi in 2011. Ambika completed her PhD in Chemical Biology at the University of Illinois, Urbana-Champaign in 2016 under guidance of Prof. Yi Lu focusing on the use of myoglobin-based enzyme models to probe structure-function relationships in heme-copper oxidases. Her research interests include enzyme design, spectroscopic and kinetic studies of metalloenzymes, protein-protein interactions and drug-design.

Igor Petrik was born in L'viv, Ukraine and grew up in Philadelphia, PA. From 2006 to 2009, he attended the University of the Sciences in Philadelphia, where he investigated the physicochemical properties of ionic liquids by NMR and MD techniques, under the mentorship of Prof. Guillermo Moyna. After graduating with a B.S. in Chemistry and a minor in Forensics, he continued his PhD studies in Chemical Biology in the lab of Prof. Yi Lu at the University of Illinois at Urbana-Champaign. He performed rational design of metalloenzymes, with a focus on understanding and improving activity of biosynthetic models of terminal oxidases, with the aid of sophisticated spectroscopic and crystallographic techniques.

Yi Lu received his BS degree from Peking University in 1986, and a PhD degree from University of California at Los Angeles in 1992 under Professor Joan S. Valentine. After 2 years of postdoctoral research in Professor Harry B. Gray's group at the California Institute of Technology, Lu started his own independent career in the Department of Chemistry at the University of Illinois at Urbana Champaign in 1994. He is now a Jay and Ann Schenck Professor of Chemistry and a HHMI Professor in the Departments of Chemistry, Biochemistry, Bioengineering and Materials Science and Engineering. He is also a member of the Center for Biophysics and Computational Biology and Beckman Institute for Advanced Science and Technology. His research interests lie at the interface between chemistry and biology. His group is developing new chemical approaches to provide deeper insight into biological systems. At the same time, they take advantage of recently developed biological tools to advance many areas in chemistry. Lu has received numerous research and teaching awards, including the Fellow of the American Association for the Advancement of Science (2007), Early Career Award, Society of Biological Inorganic Chemistry (2007), Howard Hughes Medical Institute Professor Award (2002), Camille Dreyfus Teacher-Scholar Award (1999), Alfred P. Sloan Research Fellowship (1998), Research Corporation Cottrell Scholars Award (1997), and the Beckman Young Investigators Award (1996).

Figure 1.

The complete structure of HCO *P. stutzeri* *cbb3* oxidase (a) and its catalytic heme-copper center (b) PDB: 5DJQ. The complete structure of *cNOR* (c) and its catalytic heme-nonheme diiron center (d) PDB: 3O0R. The structure of heme-copper center in $Cu_B Mb$ (e) and heme nonheme diiron center in $Fe_B Mb$ (f).

Figure 2.

X-ray crystal structure of Cu_BMb (a) and Cu(II) -added F33Y- Cu_BMb variant (b). c) UV-Vis difference spectra for Cu(II) titration in Cu_BMb and double reciprocal, hill plots used to determine the binding affinity for copper. d) X-band EPR spectra of $\text{Cu}_B\text{Mb-CN}$ before (dotted line) and after the addition of Cu(II) (solid line) and Zn(II) (dashed line). Redox-dependent structural changes of the Cu_B center in the absence of chloride (e) and in the presence of excess chloride (f). Figures have been adapted from ref [14] and [22].

a) UV-vis spectra of reduced Cu_BMb in air (solid line), reduced Cu_BMb in air after the addition of AgNO₃ (dashed line), and reduced WTMB in air (dotted line). b) UV-vis spectra of reaction of Cu_BMb (6 μM) with oxygen in the presence of 1,000 equivalents of ascorbate and 100 equivalents of TMPD in 20 mM Tris buffer (pH 8) at 25°C with copper. c) Time-dependent absorbance changes at 418 and 678 nm with copper (solid line), without copper (dotted line), Ag(I) (short dashed line), and Zn(II) (dotted and dashed line). d) Proposed reaction mechanism of Cu_BMb with oxygen. Figures adapted from ref. [23]

Figure 4.

a) Heme *o* and *b* found in HCOs. Heme *o* mimics used in the study. b) Rates of heme degradation for different heme types. The changes of Soret band absorbance plotted against the reaction time at 418 nm for Cu_BMb(heme *b*) (solid line), at 410 nm for Cu_BMb(heme *o*) (dashed line), and at 404 nm for Cu_BMb(meso-heme) (dashed-dotted line). Figures adapted from ref. [27]

Figure 5.

Structural overlays of F33Y-Cu_BMb (crystal structure, 4FWX) and G65Y-Cu_BMb (molecular model), with their inspirational native HCO structures. c) Oxygen consumption rates of indicated proteins leading to formation of water or reactive oxygen species (ROS), as indicated. d) NMR detection of H₂¹⁷O produced by reaction of proteins with ¹⁷O₂ under reductive conditions. Figures adapted from ref. [17]

Figure 6.

a) pK_a and redox potential of Tyr and its analogs affects HCO activity. Correlation of activity of Phe33Tyr-Cu_BMb, Phe33ClY-Cu_BMb, Phe33F₂Y-Cu_BMb, and Phe33F₃Y-Cu_BMb vs pK_a of phenols on the Tyr and its analogs. b) Correlation of water produced in oxygen reduction reaction performed by these proteins vs the pK_a of phenols on the Tyr and its analogs. c) Correlation of oxidase activity of Phe33Tyr-Cu_BMb, Phe33ClY-Cu_BMb, Phe33F₂Y-Cu_BMb, and Phe33F₃Y-Cu_BMb vs peak potential at pH 13 (E_p) of the corresponding Tyr and Tyr analogs. d) EPR spectra of ferric F33Y-Cu_BMb reacted with 1 equiv. of H₂O₂ (red) and ferrous F33Y-Cu_BMb with oxygen (black). e) Structure model overlay of imiTyrCuBMb (cyan) and F33YCuBMb (yellow). Figures adapted from ref. [32, 30–31]

Figure 7.

(A) EPR spectra of oxy-wtMb and oxy-F33Y-Cu_BMb after radiolytic reduction and subsequent stepwise annealing for one minute at indicated temperatures (sharp signal marked by asterisk is due to radiolytically generated hydrogen atoms at 77K in quartz EPR tube). (B) Crystal structure of oxy-F33Y-Cu_BMb determined at 1.27 Å resolution (PDB: 5HAV), compared with that of oxy-WTMB (PDB: 1A6M). Figures adapted from ref. [38]

Figure 8.

a) Overlay of the X-ray crystal structures of F33Y-Cu_BMb (4FWX, cyan) and S92A-F33Y-Cu_BMb (4TYX, orange). b) (A) Protein scaffold of F33Y-Cu_BMb, heme *b* cofactor present in F33Y-Cu_BMb, heme *a* present in the catalytic site of bovine CcO, diacetyl heme, monofomyl heme and diformyl heme incorporated in F33Y-Cu_BMb apo-protein. c) Variation of oxygen reduction activity with heme E^{o'} for F33YCu_BMb variants. d) Structures of G65Y-Cu_BMb(+6), showing the engineered lysines in blue, and cyt *b*₅ (PDB IDs 1CYO for cyt *b*₅ and 4FWY for F33Y-Cu_BMb). e) Oxidase activity of G65Y-Cu_BMb(+6) in comparison with those of native *cbb*₃ oxidase and G65Y-Cu_BMb at the same concentration. f) GC/MS chromatogram of NO reduction by Cu_BMb and Cu(I). The GC peaks have been normalized. Figures adapted from ref. [44a, 42, 44b]

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Figure 10.

a) Overlay of Fe(II)-I107E Fe_BMb (cyan) (PDB ID code 3M39) with Fe(II)-Fe_BMb (orange) (PDB ID code 3K9Z). b) Time-dependent N₂O production by Fe(II)-I107E Fe_BMb (▲) and Fe(II)-Fe_BMb (●) with ~50 eq. NO under single turnover conditions. The yield was determined by a comparison of the ratio of NO:N₂O peaks from the GC/MS chromatograms. c) Reaction steps leading to the production of N₂O in I107E-Fe_BMb. Figures adapted from ref. [51–52]

Figure 11.

a) Schematic representation of the replacement of Fe-protoporphyrin IX from Fe_BMb (green) with Zn-protoporphyrin IX, yielding ZnPPIXFe_BMb (magenta). b) UV-vis spectra of Fe_BMb (green), ZnPPIXFe_BMb (magenta), and ZnPPIXFe_BMb in the presence of 1.0 equivalent Fe(II) (blue) c) 1.52 Å X-ray structure of Fe(II)-ZnPPIXFe_BMb. d) X-band EPR spectrum of ZnPPIXFe_BMb in the presence of 1.0 eq. FeCl₂ and 20 equivalents of NO (blue trace) and the simulated spectrum (red trace). Figures adapted from ref. [54]

Figure 12.

a) UV-vis titration of ferric E-Fe₈Mb(-His) with Cu(II). b) Crystal structure of Cu(II)-CN--Fe₈Mb(-His) at 1.65 Å resolution (3MN0). c) (B) X-band EPR spectra of ferric E-Fe₈Mb(-His) in the presence of 1.0 or 3.0 equiv of Cu(II). Figures adapted from ref. [56]

Table 1

Limitations in studying native HCOs/NORs and how synthetic and biosynthetic models can help overcome the limitations

Native HCOs/NORs	Synthetic models	Biosynthetic models
Difficult to obtain homogeneous sample in large quantities	Multiple steps for synthesis of heteronuclear centers	Easy to obtain large quantities in short times
SCS interactions differ in same enzymes from different sources	Very difficult to incorporate SCS interactions into model	Role of SCS interactions can be studied through mutagenesis
Residing in different protein environments hampers the comparison between similar enzymes	Different active centers can be designed within the same overall scaffold	Different active centers can be designed within the same overall scaffold
Difficult to crystallize	Much easier to crystalize than native enzymes	Highly amenable to crystallization
Overlapping features of other metal cofactors	Focus only on the active center	Focus only on the active center
Difficult to remove cofactors such as heme or nonheme metal ions without loss of function and/or structure	Can use different cofactors	Easy to replace heme, nonheme metal ion, or other cofactors
Difficult to trap intermediates using site-specific labeling	Intermediates can be trapped	Site-specific labeling is easily achievable <i>via</i> techniques such as heme (^{57}Fe) incorporation
Difficult to perform systematic studies of specific features such as heme E° , pKa of residues, and ET rates	Amenable to systematic studies using different ligands or reaction conditions	Amenable to systematic studies using cofactor replacement or unnatural amino acid incorporation