Published in final edited form as:

Methods Enzymol. 2016 ; 580: 501–537. doi:10.1016/bs.mie.2016.05.050.

Design of Heteronuclear Metalloenzymes

Ambika Bhagi-Damodaran^{1,†}, Parisa Hosseinzadeh^{1,‡}, Evan Mirts[#], Julian Reed[‡], Igor D. Petrik[†], and Yi Lu^{†,‡,*,#}

[†]Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801

[‡]Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801

[#]Center for Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

Abstract

Heteronuclear metalloenzymes catalyze some of the most fundamentally interesting and practically useful reactions in nature. However, the presence of two or more metal ions in close proximity in these enzymes makes them more difficult to prepare and study than homonuclear metalloenzymes. To meet these challenges, heteronuclear metal centers have been designed into small and stable proteins with rigid scaffolds to understand how these heteronuclear centers are constructed and the mechanism of their function. This chapter describes methods for designing heterobinuclear metal centers in a protein scaffold by giving specific examples of a few heme-nonheme bimetallic centers engineered in myoglobin and cytochrome *c* peroxidase. We provide step-by-step procedure on how to choose the protein scaffold, design a heterobinuclear metal center in the protein computationally, incorporate metal centers in the protein and characterize the resulting metalloprotein, both structurally and functionally. Finally, we discuss how an initial design can be further improved by rationally tuning its secondary coordination sphere, electron/proton transfer rates, and the substrate affinity.

Metal ions have long been known to play important roles in biology as essential structural elements to stabilize protein tertiary structure, as multi-valent redox-active elements to promote long-range electron transfer (ET), or as active sites to catalyze a variety of otherwise difficult reactions (Malmström, 1990, Gray & Winkler, 1996, Lu et al., 2009, Lu et al., 2009, Gray & Winkler, 2010, Liu et al., 2014). An important class of metalloenzymes contains active sites consisting of two or more distinct metal centers, called heteronuclear metalloenzymes (Table 1) (Holm, 1995, Collman & Wang, 1999, Kim et al., 2004, Collman et al., 2004).

Heteronuclear metalloenzymes are responsible for catalyzing some of the most fundamentally interesting and practically useful reactions found in Nature, including the oxygen reduction reaction by heme-copper oxidase (HCO) that is known to be important for bioenergetics and fuel cells research (Garcia-Horsman et al., 1994, Gao et al., 2012), NO reduction to N₂O by nitric oxide reductase (NOR) that contributes to the global nitrogen

*Corresponding author: yi-lu@illinois.edu.

[†]These authors contributed equally

cycle (Richardson & Watmough, 1999, Flock et al., 2005), and Mn^{2+} oxidation to Mn^{3+} by manganese peroxidases (MnP) that is critical to degrading lignin, the second most abundant biopolymer on earth (Hofrichter, 2002) (Fig. 1). The importance of the reactions catalyzed by these and similar enzymes (Table 1) have rendered them attractive targets for biochemical and biophysical investigations and for biomimetic studies to design artificial catalysts or enzymes with similar active site structures and activities (Fontecave & Pierre, 1998, Mahadevan et al., 2000).

Despite the importance of these heteronuclear metalloenzymes in catalysis, they are generally more difficult to study because many of them (e.g., HCO and NOR) are membrane enzymes that are relatively difficult to purify to homogeneity in high yields, and contain other metal-binding sites, making it difficult to focus on spectroscopic study of the heteronuclear metal-binding sites. In the case of MnP, even though it is a water-soluble protein containing only a heme-Mn center, it has been difficult to construct and express mutations. To overcome these limitations, much effort have been devoted toward making synthetic models using small organic molecules as ligands, whose study has provided deeper insight into the physical underpinnings of the chemical reactions facilitated by these enzymes by allowing researchers to isolate oxidation states, reactive intermediates, and find structural details in the primary coordination geometry of catalytic metal sites (Fontecave & Pierre, 1998, Sanders, 1999, Mahadevan et al., 2000, Punniyamurthy et al., 2005, Que & Tolman, 2008, Tard & Pickett, 2009, Koziol et al., 2012). Despite the progress made, the majority of these synthetic models tend to exhibit low activity. (Lilie, 2003, Petrik et al., 2014)

To complement the study of native enzymes and synthetic models, we and others have employed a biosynthetic approach of designing heteronuclear metalloenzymes using small, stable scaffold proteins that can already be expressed in high yields with exceptional purity through well-established purification schemes (DeGrado et al., 1989, DeGrado et al., 1999, Gibney et al., 1999, Lu et al., 1999, Reedy & Gibney, 2004, Thomas & Ward, 2005, Zanghellini et al., 2006, Lu et al., 2009, Lu et al., 2009, Richter et al., 2011, Zastrow & Pecoraro, 2013, Lu et al., 2013, Marshall et al., 2013, Chakraborty et al., 2014). Protein scaffolds are innately optimized to function under physiological conditions, offer a broad ligand set that can be expanded through the use of non-canonical amino acids, and comprise a genetically-programmable platform for bottom-up engineering of metal binding sites. A key advantage of protein-based biosynthetic models is the ability to construct and tune the non-covalent secondary sphere interactions around the primary coordination sphere using the much more rigid protein scaffold.

Study of native enzymes and their models has shown the importance of the secondary sphere interactions in tuning the enzymatic activities (Varadarajan et al., 1989, Butland et al., 2001, Miller, 2008, Marshall et al., 2009). The secondary interactions can confer their roles in many ways: engaging in a hydrogen bond or salt bridge to the primary ligands, changing the overall electrostatic environment of the metal binding site by changing charge or hydrophobicity, engaging in proton or electron transfer, or providing space for the metal cofactors (Hosseinzadeh & Lu, 2015). The stability and reactivity of heteronuclear metal-binding sites depends heavily on secondary sphere interactions that may contribute to

electronic coupling between metal cofactors, the redox potential (E°) of one or both metals, and the stabilization of substrates and reactive intermediates (Yikilmaz et al., 2002, Jackson & Brunold, 2004, Marshall et al., 2009, Berry et al., 2010, Shook & Borovik, 2010, New et al., 2012, Petrik et al., 2016). Engineering such sites into easily expressible protein scaffolds makes the process of studying an isolated heteronuclear site as well as its secondary coordination sphere less complicated by excluding the complex global effects present in native enzymes while working on a stable, three-dimensional platform that can be altered rationally.

In this chapter, we will describe methods of engineering heteronuclear, bimetallic active sites into the small, stable protein scaffolds sperm whale myoglobin (swMb) and yeast cytochrome *c* peroxidase (CcP) from the incipient stages that make use of computational methods to identify and design metal binding sites, through expression and quality-assessment of the biosynthetic models, and finally to specialized characterization techniques that take advantage of the physiologically relevant nature of biosynthetic models to probe their structural, electronic, and catalytic properties.

Step 1: Computational design of heteronuclear metal-binding sites in Mb or CcP

In this step we describe the computational design process of a heteronuclear center beginning with choosing a scaffold, continuing through designing secondary sphere interactions for tuning activity, and finally to *in silico* generation and assessment of the models. An overall flow diagram of the described process is shown in Fig. 2.

1.1. Structure-guided design of primary ligands

The design process begins with the choice of an appropriate protein scaffold and finding residues to mutate as primary ligands. As a general rule, the chosen scaffold should be relatively small, stable, easy to express recombinantly in large quantities, easy to crystallize, and devoid of any additional ligand or cofactor unless required as part of the design. It is also advisable to choose a scaffold that can achieve some of the chemistry of the reaction of interest. For example, for the design of a Cu_B-heme center for investigating oxygen reduction activity, the scaffold protein should contain a heme cofactor and bind oxygen but not natively catalyze its reduction. SwMb is a small, very well studied protein that can be easily crystallized and expressed in large quantities in *Escherichia coli* (Springer & Sligar, 1987). It possesses a histidine coordinated heme group (similar to HCOs) that is known to bind to oxygen, making it an excellent scaffold for studying the reactivity of the Cu_B site in HCOs (Fig. 3a,b) (Varadarajan et al., 1989). Similarly, MnP (Hofrichter, 2002, Annele et al., 2003) shares strong structural similarity and shares enzymatic intermediates with yeast cytochrome *c* peroxidase (CcP) (Finzel et al., 1984, Sundaramoorthy et al., 1994), making CcP an excellent starting point for mimicking MnP activity (Yeung et al., 1997, Wilcox et al., 1998).

- 1 Choosing scaffolds: There are several methods and tools to perform an advanced search to find appropriate scaffold(s). The protein data bank (PDB, www.rscb.org) (Berman et al., 2000) contains over one hundred thousand

biological macromolecular structures and has a very straightforward and versatile advanced search module that can narrow down proteins based on size, desired ligands, and even expression system. In addition to PDB, several other servers such as FATCAT (<http://fatcat.burnham.org>) (Ye & Godzik, 2004), MarkUs (http://wiki.c2b2.columbia.edu/honiglab_public/index.php/Software:Mark-Us) (Fischer et al., 2011), Dali (http://ekhidna.biocenter.helsinki.fi/dali_server) (Holm & Rosenstrom, 2010), and SwissProt (<http://www.expasy.ch/sprot/> and <http://www.ebi.ac.uk/swissprot/>) (Bairoch & Apweiler, 2000) also provide a variety of tools that can assist in searching the scaffolds.

The next step is to design the primary ligands of the second metal-binding site. This process can be carried out in one of several ways depending on the similarity between the target protein and the scaffold and the availability of initial structural data.

- 2 Finding primary ligand positions: (a) homologous target and scaffold: If the two proteins share high structural similarity, the first method is to perform structural alignment and identify the residues in the scaffold protein that occupy the same location as the metal ligands in the target protein. These residues can then be directly mutated to the corresponding residues of the target protein. This strategy was successfully applied to design a manganese-binding site near the heme in CcP based on structural similarity with manganese peroxidase (Fig. 4). Several programs and online services can perform structure-based alignment including VMD (Humphrey et al., 1996), Dali (http://ekhidna.biocenter.helsinki.fi/dali_server) (Holm & Rosenstrom, 2010), FATCAT (<http://fatcat.burnham.org>) (Ye & Godzik, 2004), SSAP (<http://v3-4.cathdb.info/cgi-bin/SsapServer.pl>) (Sillitoe et al., 2015), CLUSTAL omega (<http://www.clustal.org/omega/>) (Sievers et al., 2011), and many more.
- 3 Finding primary ligand positions: (b) non-homologous target and scaffold: If the structures of the target and scaffold are known and contain a common cofactor but are not closely related, the two proteins can be aligned relative to this cofactor followed by visual inspection of the scaffold protein for positions that can potentially accommodate ligands of the target metal site in the appropriate geometry. This strategy was successfully used to design a Cu_B-heme center in swMb by aligning the heme of swMb with the catalytic heme of HCO (Sigman et al., 1999, Sigman et al., 2000). This strategy can also be extended to model the active sites of proteins with unsolved structures. For example, the sequence similarity within HCOs and NORs was used to model the Fe_B-heme center in Mb at a time when the X-ray crystal structure of NOR had not been obtained (Figure 3c) (Yeung et al., 2009). If the ligands of the desired metal binding site are mostly within a loop, loop-directed mutagenesis is a good strategy for incorporation of the ligands (Hay et al., 1998). In this method, a loop in the scaffold protein is replaced with the metal binding loop of the target protein.

Tip. Often in the design of heteronuclear bimetallic sites, the chosen scaffold should already contain one of the desired metal centers, e.g. heme in the examples discussed in the review, to eliminate the additional difficult step of designing a heme-binding site.

Tip. For more complicated cases, the Rosetta software suite – specifically the Matcher program (Richter et al., 2011) – can be used to find sites within protein scaffolds that can satisfy constraints of the target site. While this method is powerful in searching for large cofactors, it still has limitations for small cofactors, such as metal ions, especially if there is no *a priori* information about the expected position of the metal center.

1.2. Design of secondary coordination interactions

While the design of the primary metal-binding ligands is the first necessary step in achieving functional biosynthetic models, it has been observed in many cases that these features are not sufficient to recapitulate the complete activity of the target system (Yeung et al., 1997, Wilcox et al., 1998, Sigman et al., 1999, Sigman et al., 2000, Yeung et al., 2009) in part due to the essential role of secondary coordination sphere interactions. There are several approaches to design these secondary interactions successfully. We briefly describe each approach here and refer the readers to more comprehensive reviews and articles.

- 1 Using evolutionary information and biochemical studies as a guide: Use of evolutionary relationships between members of a class of native metalloproteins combined with biochemical studies of the conserved residues is one of the most effective approaches to identify the secondary sphere interactions essential for the activity of interest. It is known that conserved residues found upon sequence or structural alignment of homologous proteins with similar functions play important roles in the observed activity (Worth et al., 2009, Dib & Carbone, 2012) and that mutation of these residues usually abolishes the activity of the enzyme under study (Sollewijn Gelpke et al., 1999). Thus, it is expected that adding these residues to the design will impart or improve the desired activity. A substantial amount of software is available for alignment of proteins with similar structure and function (homologous proteins) at the DNA sequence, amino acid sequence, or structural levels. The proteins in the alignment can be further separated and clustered based on stricter homology criteria to identify subfamilies. Each subfamily usually has a common feature that separates it from others. The residues that are conserved within members of each subfamily and those that differ from others are potential targets for design. For instance, a Glu in the secondary coordination sphere of NORs was suggested to be important by biochemical studies (Butland et al., 2001), and incorporation of an additional Glu into the NOR model Fe_BMb increased the NOR activity by two fold (Zhao et al., 2006). Similarly, presence of a Tyr in HCOs has long been known to be essential for their activity based on mutagenesis studies (Das et al., 1998). Addition of Tyr in the right orientation in Cu_BMb imparted oxygen reduction activity within a factor of 300 of a native HCO and with 1000 turnovers (Miner et al., 2012). Similarly, addition of an imidazole-Tyr residue resulted in substantial improvements in activity (Liu et al., 2012).

Tip. Sometimes general features rather than residues are conserved – e.g. positive charge in a certain position – and in those cases the residue that satisfies this feature and fits the design scaffold best should be chosen. This method has been successfully used to enhance the manganese peroxidase activity in a designed mimic called MnCcP (Hosseinizadeh et al., 2016). Similarly; it has been used to increase the potential of Fe/Mn superoxide dismutase (Miller, 2008).

- 2 Structural requirements: After the primary ligands are found and put in place, one should carefully inspect the structure to find out whether the ligands fit well into the protein structure and whether there are any clashes or unsatisfied buried charges to be removed. For example, by incorporating a Gly in the loop that contains primary Mn-binding ligands into MnCcP and giving the ligands more flexibility, this designed mimic of MnP could achieve binding affinities for Mn comparable to the native system (Hosseinizadeh et al., 2016). Removing a disfavored hydrogen bond to another Mn ligand in the same mimic increased the activity and binding affinity (Hosseinizadeh et al., 2016).
- 3 Activity requirements: Information about the activity can be of prime importance in figuring out what secondary interactions should be designed. Biological reactions in heteronuclear metalloproteins are usually highly coupled to proton and/or electron transfer (Malmström, 1990, Chang et al., 2004, Blomberg & Siegbahn, 2006, Reece & Nocera, 2009, Gray & Winkler, 2010, Weinberg et al., 2012, Liu et al., 2014, Fukuda et al., 2016). The metal centers need to shuttle electrons and protons from the environment through redox active amino acids or hydrogen bonding networks involving water and/or charged/polar amino acids. In order to improve the activity, similar residues should be incorporated as well (Weinberg et al., 2012).

1.3. Computational analysis of the design

Design hypotheses and methods often suggest several if not many possible solutions. Before experimental analyses of all of these designs, they should first be analyzed computationally. In this section, we briefly explain the steps for generating *in silico* models and analyzing them.

- 1 Generation of *in silico* designs: After a decision has been made of which residues to mutate, a molecular model containing those mutations should be generated. There are several ways to achieve this starting from an experimentally determined structure—and the reader may use tools they are most familiar with—but the easiest methods are offered by programs like chimera (Pettersen et al., 2004), pymol, and coot (Emsley et al., 2010) which enable easy replacement of a residue with another residue and even a choice of rotamers in coot or pymol.

Tip. For loop directed mutagenesis, manual alignment and splicing of the loop structure into the scaffold structure can be done, but an easy way to carry out this design is to feed the amino acid sequence into a protein structure prediction servers like I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>) (Zhang, 2008, Roy et al., 2010, Yang et al.,

2015) or use the remodel mover in the Rosetta suite (explained in detail in Rosetta documentation) (Hu et al., 2007).

Assuming that the initial mutagenesis reveals no major problems, such as geometric clashes after incorporation of the desired feature, the designs should be further evaluated for feasibility. One easy way to evaluate the designs and eliminate those that are unlikely to work is to subject them to 1–5 ns of molecular dynamics simulation. These simulations can be carried out using one of several software packages, such as CHARMM (Brooks et al., 2009), NAMD (Phillips et al., 2005), Amber (Case et al., 2005), and GROMACS (Pronk et al., 2013), which implement various macromolecular force fields, such as CHARMM, AMBER, GROMOS, and OPLS. The authors are most familiar with NAMD employing the CHARMM force field and VMD for simulation preparation and will give general procedural advice for this packages.

- 2 Preparing models for molecular dynamics simulation: Initialization of molecular dynamics simulation requires the definition of two related properties: (1) the exact connectivity of the atoms in the simulation, known as the topology; and (2) the physical constants of the atoms in the molecule, such as mass, charge, and interatomic force constants for bonds, three-atom angles, and four-atom torsional values. In CHARMM, the topologies of individual residues of a polymer and other monomers, such as water, lipids, and many exogenous cofactors, have been determined and are available with the default for force field parameter package. In VMD/NAMD, the overall topology of macromolecules is generated by a script called “psfgen”. Instructions for general use of this script to produce topologies for macromolecules are readily available on the NAMD/VMD website. However, the authors would like to point out that special actions must usually be taken when additional cofactors are present. For instance, while parameters for heme are included in the standard CHARMM force field, bonds between His or Cys residues and the heme iron must be explicitly defined using patches. Even when the heme is unligated, a patch must be applied to ensure proper planarity of the Fe in the ring.

Tip. For some cofactors, such as FeS clusters, topology and force field parameters are not included. In such cases, parameters can sometimes be found in the literature or from the websites of various biophysical research labs (Chang & Kim, 2009). More often, these properties are not readily available for the desired force field. There are several avenues to pursue in these cases: (1) if parameters are available from another force field, they can sometimes be translated into parameters for the desired force field; (2) if parameters for similar bonds exist, and quantitative precision is not critical, the parameters can be approximated from existing parameters; (3) otherwise, the ligand and its interactions with the macromolecule must be parameterized by quantum mechanical calculations. Guidance for such parameterization is provided by the developers of the relevant force fields. Depending on the model, it may be necessary to constrain the metal atom to prevent it from moving out of the protein pocket.

- 3 Simulation parameters: After defining the macromolecular topologies and corresponding parameters, the structure should be solvated in explicitly modeled water, such as TIP3 parameterized water, and the charge balanced with ions of choice. Further general considerations about simulation parameters, including size of the simulation box and ensemble are discussed in literature and software documentation. As a start, the authors suggest 1,000–2,000 steps of minimization and a 2-ns simulation (2 fs/step) in an NPT ensemble with a box that is at least twice as long in each direction as the longest protein dimension,. More complicated methods for simulation are also available that are not within the scope of this chapter.
- 4 Analysis of the simulation: After the simulation is over, the “.dcd” file (or any file that contains the simulation trajectory) should be loaded and the overall movement of the protein should be evaluated, especially in the designed area to make sure no undesired movements of the ligand, changes in the structure, or solvation of the metal site is observed. General structural RMSDs should be calculated with respect to the starting scaffold to ensure that there are no major structural changes. The VMD package has several useful extensions for these analyses such as a RMSD calculator, hydrogen bond and electrostatic interaction predictor, and a pKa calculator that the user can apply when required.

Tip. Make sure to align all the scaffolds in the whole trajectory before running RMSD measurements.

Tip. It is important to note that simulations cannot guarantee that the designed variants will work in an experimental setting, but they are extremely useful to filter those variants that are likely to fail. Overall, if a variant causes drastic changes in the protein structure after 2 ns of simulation, it probably is very destabilizing and should not be further pursued.

Tip. It is also important to note that all force fields, including CHARMM, are currently limited in handling electrostatic interactions to evaluate metal binding and thus we don't suggest using the simulation as a way to assess metal affinity. However, the results from simulation can be a guide to get some ideas about factors that can enhance metal binding affinity.

Step 2: Purification and Structural Characterization of Rationally Designed Proteins

In this step, we provide a detailed description of rationally designed heteronuclear metalloprotein characterization aimed at identifying binding events and interactions of designed metal binding sites. Single mutation site-directed mutagenesis is typically performed using the QuikChange Site Directed Mutagenesis Kit (Sigman et al., 1999). Where applicable, Gibson assembly (Gibson, 2011) and Mega-Primer PCR (Tyagi et al., 2004) can be also used. Detailed descriptions of these methods are beyond the scope of this chapter, and have been covered previously (Sraphin&Kandels-Lewis, 1996, Weissensteiner et al., 2003, Nakayama & Shimamoto, 2014, Casini et al., 2015). The presence of desired

mutations in the DNA sequence is confirmed by DNA sequencing. General purification of our designed heteronuclear metalloproteins will not be discussed in great detail here, but generally falls into two categories: inclusion body and cytosolic purification. The inclusion body protocol is used extensively in models such as Cu_BMb and Fe_BMb and has been covered previously in those works (Springer & Sligar, 1987, Sigman et al., 2000, Sigman et al., 2000, Sigman et al., 2003, Pfister et al., 2005, Zhao et al., 2005, Zhao et al., 2006, Yeung et al., 2009, Lin et al., 2010, Lin et al., 2010, Miner et al., 2012, Chakraborty et al., 2014, Bhagi-Damodaran et al., 2014, Petrik et al., 2016). Additionally, cytosolic purification of CcP and its rationally designed mutants has also been described in great detail by our group (Yeung et al., 1997, Sigman et al., 1999, Feng et al., 2003, Pfister et al., 2007, Miner et al., 2014, Hosseinzadeh et al., 2016). After purification, the protein mass is determined by SDS-PAGE (Brunelle & Green, 2014) and ESI-MS (Strupat, 2005). A pyridine hemochromogen assay is used to determine the molar extinction coefficient of the Soret band in the designed mutants (Berry & Trumpower, 1987). Additionally, circular dichroism (CD) is a very useful technique to assess the overall folding state of the designed proteins (Greenfield, 2006). Using these techniques, it is possible to immediately assess whether the protein is correctly folded.

A crucial component of designing heteronuclear metalloenzymes is being able to perform a quick check of metal ion binding at the designed metal center, a procedure for which a number of techniques are helpful:

2.1. UV-Vis Spectroscopy

Metalloenzymes containing transition metal ions typically exhibit characteristic UV-Vis signals that are of great advantage in detecting the binding of metal ions. For example, the titration of Cu(II) in apo-WT azurin (a copper containing ET protein) results in an obvious increase in broad absorption bands centered at ~450 and ~850 nm due to ligand to metal charge transfer (LMCT) and *d-d* transitions, respectively (Hadt et al., 2012). However, detecting metal binding using UV-Vis is not as straightforward for heme-based metalloenzymes; heme exhibits strong UV-Vis signals from 400 to 650 nm. As a result, we monitor the changes in the heme UV-Vis signals as metal ions are titrated into the protein (Fig. 5a). These are small but significant changes resulting from the alteration in the heme electronic environment by addition of a new metal ion in its vicinity (Yeung & Lu 2008). It should be noted that if no changes are observed, it does not preclude the possibility of metal still being bound to the site. In addition, it should be verified that the changes in the UV-Vis signals are not due to (1) unfolding of the protein, which will be apparent by an increase in the absorbance at 280 nm, or (2) removal of heme from the protein, which will be apparent by an increase in 360/280 nm signals due to free heme. We discuss the protocol for titration of nonheme Fe in Fe_BMb below (Yeung et al., 2009).

2.1.1. UV-Vis spectroscopic titration of nonheme Fe(II) in E-Fe_BMb

1. Degas 1000 µL of 7 µM protein solution in 50 mM Bis-Tris (bis (2-hydroxyethyl) iminotris (hydroxymethyl) methane) pH 7 buffer on a Schlenk line using standard freeze-pump-thaw techniques.

2. Bring the degassed empty Fe_BMb (that is Fe_BMb with no nonheme metal added called E-Fe_BMb) solution into an anaerobic chamber.
3. Reduce the protein using a small amount of solid dithionite, leaving an excess to keep the protein reduced during titration.
4. Record an initial spectrum of the protein solution.
5. Prepare a solution of 50 mM FeCl₂ in the anaerobic chamber by dissolving 6.33 mg solid FeCl₂ in 1 mL degassed water.
6. Dilute 28 µL of the 50 mM FeCl₂ solution to 1.4 mM by adding 972 µL degassed water, total volume 1 mL.
7. Add 5 µL of FeCl₂ solution to the protein solution with rapid stirring and incubate for 5 minutes.
8. Collect a UV-Vis spectrum of the metal-added protein.
9. Repeat steps 7 and 8 for a total of 2 molar equivalents nonheme metal added.
10. Dilute 56 µL of the 50 mM FeCl₂ solution to 2.8 mM by adding 944 µL degassed water, total volume 1 mL.
11. Repeat step 9 for a total of 5 molar equivalents nonheme metal added.
12. Changes in the absorbance profile of the Soret (433 nm → 434 nm redshift) and visible (formation of split peak at 550 nm and 572 nm) regions are indicative of nonheme metal binding (Yeung et al., 2009).
13. Dissociation constants based on difference spectra (Abs vs. λ) can be calculated using previously described protocols (Bidwai et al., 2003).

Tip. Vary the concentration of your protein so that the Soret absorption is ~1 or within an acceptable range for the particular spectrometer.

Tip. If the titration is incomplete after addition of 5 molar equivalents of nonheme metal, adjust the concentration of FeCl₂ such that a 5 µL addition is a larger molar equivalent (eg. 1, 2, 4 etc...).

Tip. Run a control experiment with WT protein (WT swMb in this case) to make sure that the change in the UV-Vis spectrum is due to binding of metal in the designed binding site and not an undesired endogenous site close to the heme iron.

Tip. If no binding was observed, the protein should be treated with chelating agents (e.g. EDTA) to make sure there is no exogenous metal binding that prevents the binding of the desired metal ion.

Tip. While it is difficult in the glovebag, the titration of metal ions should ideally be performed with the protein at ~4°C. This practice helps stabilize some mutant proteins that are not very stable at room temperature.

2.2. Mass spectrometry (MS)

The addition of metal ions to the protein, given that the binding is strong, will lead to an increase in mass which can be detected using MS. Care should be taken that the protocol used for MS does not lead to protein disruption or loss of metal ions. In order to do so, a milder electrospray ionization (ESI) method is used which is called “syringe pump ESI”. In this technique the protein is exchanged into water or buffer with very low ionic strength. The lines are washed with water thoroughly and then the protein is loaded into the ESI instrument using a syringe at very low flow rates (~5 µl/min) without addition of formic acid. There are also reports of using matrix assisted laser desorption/ionization-time of flight MS (MALDI-TOF MS) with specific resins to find the mass of metal-bound species (Zeng et al., 2007). In general, MS has been useful in detecting metal binding for metalloproteins with high binding affinities to the metal ion such as azurin, but for proteins that exhibit low binding affinities for nonheme metal ions (~ 10 µM) such as Mb and CcP, it is more difficult to obtain accurate MS results by this method.

While UV-Vis and MS are excellent methods to perform initial characterization of the designed metalloenzymes, more specific methods are required for their complete characterization of properties including coordination, geometry, oxidation state, and spin-state. Next, we describe methods that can be used to determine them:

2.3. EPR

Monitoring the changes in the spin-state of metal ions is another approach through which metal binding can be readily detected. For example, addition of nonheme Fe(II) close to high spin ($S=5/2$) heme iron(III) leads to spin-coupling between the two metals that causes attenuation of the $g=6$ signal (Figure 5b). The spin coupling between nonheme Fe and heme Fe in I107E Fe_BMb will be described as an example. The nonheme Fe(II) addition was performed in the reduced form of Fe_BMb, which is EPR-silent. After metal addition, the resulting Fe-Fe_BMb was oxidized using azurin.

1. Prepare a solution of degassed protein as described in “Metal Titration, steps 1–3.”
2. Remove the excess dithionite by passing the protein solution down a size exclusion PD-10 column (GE Healthcare) pre-equilibrated with 50 mM Bis-Tris pH 7.
3. Add varying molar equivalents of FeCl₂ (eg. 0.5, 1.0, 1.5, 2.0) in one addition to the protein solution(s) with rapid stirring for 20 minutes.
4. Add 3 molar equivalents of blue copper Cu(II)-azurin to the nonheme metal-added protein solution(s) with rapid stirring for 5 minutes.
5. Add glycerol to 10% final concentration as a glassing agent.
6. Transfer the protein solution(s) into separate EPR tubes and flash freeze using liquid N₂.

Tip. Final protein concentrations before flash freezing should be 0.5 mM Fe_BMb and 1.5 mM azurin.

Tip. Other metal ions, like Cu(II) and Zn(II), can bind Fe_BMb in the oxidized state and neither need to be reduced nor kept in an anaerobic environment (Lin et al., 2010, Lin et al., 2010).

2.4. X-ray crystallography

Crystallization is a standard technique for metalloprotein characterization to obtain overall structure, metal coordination, and geometry. As mentioned, one criterion for choosing the scaffold proteins for designing heteronuclear metalloenzymes is ease of crystallization; thus, extensive screening is often not required to obtain crystals of their mutants. For example, to model HCOs and NORs, we have used swMb, which was the first protein whose X-ray crystal structure was obtained. Thus, crystallization conditions for myoglobin are well-known and standardized. Here, we discuss the protocol to obtain the structure of nonheme metal-added Fe_BMb variants.

1. Start with the crystallization of E-Fe_BMb and screen for crystallization conditions. Details and protocols used for screening are described in the following reviews (Luft et al., 2014, Skarina et al., 2014).
2. For Mb variants, the following crystallization conditions obtain diffraction-quality crystals with the variation in the pH of Tris/MES buffer and PEG molecular weights: Well buffer: 0.1 M TRIS/MES, 0.2 M Sodium Acetate, 30% PEG 10,000 kDa MW; Protein: ~1 mM in 20 mM potassium phosphate buffer
3. Once the crystallization conditions for E-Fe_BMb are finalized, we prepare the Fe-Fe_BMb protein as described previously and set up the crystal trays in an anaerobic chamber at 4°C using conditions determined for E-Fe_BMb. The resulting crystals have obtained the structure of Fe-Fe_BMb at 1.7 Å resolution (Yeung et al., 2009).
4. Alternatively, for certain variants of Fe_BMb, crystals obtained for empty protein are transferred to the anaerobic chamber, reduced using 20 mM dithionite in crystallization buffer, and soaked with crystallization buffer containing ~10 mM FeCl₂ for ~1 hr to obtain the nonheme Fe containing crystal structure.
5. For metal ions like Cu(II) and Zn(II) that are stable in the presence of oxygen (unlike Fe(II) which tends to oxidize to Fe(III) and precipitate out), crystal trays can be set in aerobic conditions. The protein is mixed with 1, 2, and 3 molar equivalents of ZnCl₂ or CuSO₄, and conditions similar to E-Fe_BMb are used for crystallization (Lin et al., 2010, Miner et al., 2012).
6. At the time of X-ray data collection, diffraction should be obtained at the metal-edge (for example at Cu-edge if the nonheme metal is copper) to be sure that the electron density at the designed metal center is indeed due to the presence of a metal ion and not, for example, a water molecule.

Tip. Avoid any crystallization buffers that may chelate the metal ion out of the protein e.g. citrate or cacodylate buffer.

Step 3: Functional characterization of designed heterobinuclear metalloenzymes

In this section, we describe methods for functional characterization of designed metalloenzymes that include activity assays, mechanistic studies, redox potential (E° '), and characterization of reaction intermediates.

3.1. Activity assays

There are three measures for assessing the functional efficiency of a designed metalloenzyme, namely the rate of reaction, the product selectivity, and the turnover number. As an example, we will detail how the designed Mb-based HCO mimics ($\text{Cu}_\text{B}\text{Mb}$) were tested for all of these measures in the presence or absence of nonheme metal ions.

3.1.1. Catalytic efficiency of the reaction—The activity assays used for testing designed metalloenzymes should ideally be the same as that of native enzymes. For example, an oxygen electrode is the technique commonly utilized for measuring oxygen reduction rates in native HCOs and was also employed to characterize $\text{Cu}_\text{B}\text{Mb}$ variants. The reduction of oxygen requires a constant supply of electrons, thus a mixture of TMPD/ascorbate was used for electron donation, again the same system used for native HCOs. The rate of oxygen reduction is measured as a slope of concentration of oxygen with respect to time. To further optimize the reaction conditions, similar experiments should be performed at different pH values and different concentrations of reductant. Finally, the reaction should be repeated in presence of the nonheme metal ion of interest (copper in this case). Additionally, redox inactive metal ions such as Ag(I) or Zn(II) should also be used as controls (Fig. 6a).

3.1.2. Product selectivity—One important feature of an enzyme-catalyzed reaction is its high selectivity and specificity with minimal byproduct formation. Hence, the products should be analyzed to evaluate the quality of the designed protein as a model compared with its native counterpart and whether it can recapitulate the native activity. The assessment of products can be performed with methods similar to those applied to the native enzyme. For example, a hallmark of an oxidase is that it performs complete four-electron reduction of oxygen to water. An incomplete reduction of oxygen can produce a multitude of reactive oxygen species (ROS) namely O_2^- , O_2^{2-} , and $\text{OH}^\bullet$. Thus, it is important to obtain the ratio of water with respect to ROS produced during oxygen reduction by Mb-based HCO mimics. To measure this ratio, the reaction is repeated in the presence of superoxide dismutase (that reacts with superoxide converting it to oxygen and peroxide) and catalase (that reacts with peroxide converting it to oxygen). Thus, if superoxide or peroxide is produced during the reaction, the rate of oxygen reduction is decreased. These assays when performed for swMb converted oxygen almost exclusively to ROS, while $\text{Cu}_\text{B}\text{Mb}$ variants performed selective reduction of oxygen to water (Fig. 6b). (Miner et al., 2012, Yu et al., 2014, Bhagi-Damodaran et al., 2014) Other methods such as nuclear magnetic resonance (NMR), high

performance liquid chromatography (HPLC), and gas chromatography-MS (GC-MS) can also be used to identify and quantify the products of a reaction (Yeung et al., 2009, Hosseinzadeh et al., 2016).

3.1.3. Turnover Numbers—Native enzymes often exhibit turnover numbers in the order of thousands while most synthetic small-molecule models exhibit turnover numbers less than 10. Thus, turnover number is an important parameter to evaluate the efficiency of designed proteins. There are several ways to assess the turnover number of a reaction, but all follow a similar procedure of continuously providing substrate under reaction conditions until the reaction ceases. For example, to measure turnover number of oxygen reduction by G65Y-Cu_BMb, after the oxygen in the reaction chamber was exhausted, the chamber was vented with pure oxygen to boost the oxygen concentration to ~0.6 mM. Over the course of the reaction, the sacrificial reductant (ascorbate) is also consumed, so its concentration must also be maintained. These experiments showed that G65Y-Cu_BMb could perform up to 1000 turnovers without any degradation. (Miner et al., 2012)

Tip. It is a good idea to perform a secondary assay to confirm findings, especially when measuring the product selectivity where a number of variables are at play. For example, the assay for oxidase activity involved two enzymes—SOD and catalase—that could be inhibited upon addition of metal ions. Thus, we used ¹⁷O₂-NMR spectroscopy to analyze the product of ¹⁷O₂ reduction in parallel to the quantitative oxygen electrode experiments described above (Fig. 6c).

3.2. Redox potential measurement

The redox potentials ($E^{\circ'}$) of metalloenzymes provide significant insights into the electronic nature of metal ions, their ligands, and environment. For example, E-Fe_BMb possesses a heme $E^{\circ'}$ of -159 mV (vs SHE), which is ~200 mV lower than that of Cu_BMb, suggesting that the presence of negatively charged Glu68 in the distal pocket of Fe_BMb increases the electron density on the heme iron to favor its Fe(III) state over Fe(II) state (Yeung et al., 2009). Upon addition of nonheme Fe(II), the $E^{\circ'}$ of Fe_BMb increases to -60 mV due to increased distance between negatively charged Glu68 and the heme iron by coordinating to nonheme Fe and the presence of the positively charged nonheme Fe close to the heme center. Thus, changes (generally increase) in $E^{\circ'}$ upon addition of a second metal ion can be an indication of the proximity of two metal centers in the protein. Heme proteins in particular exhibit strong UV-Vis spectroscopic signals characteristic to their oxidized and reduced states that are useful to deduce their heme $E^{\circ'}$. Protocols for measurement of heme $E^{\circ'}$ for Fe^{3+/2+} couple through spectroelectrochemistry are fairly standardized and discussed extensively in other reviews (Taboy et al., 1999, Hosseinzadeh & Lu, 2015). Cyclic voltammetry (CV) can also be used to measure $E^{\circ'}$ of a heterobinuclear center. Along with determining $E^{\circ'}$, a CV set-up can also be utilized to perform electrocatalysis that yields mechanistic information, specifically the number of electrons involved in the reaction as well as the rate of reaction (Armstrong, 2002, Evans & Armstrong, 2014). Recently, *in situ* electrocatalysis has been coupled with vibrational spectroscopy to detect reaction intermediates (Mukherjee et al., 2015). As the Fe^{3+/2+} transition of heme in swMb is accompanied by the loss of a water ligand, CV usually does not produce reversible signals in

Mb variants and hence most studies on these models used spectroelectrochemistry (Van Dyke et al., 1996). CcP models, however, produce reversible CV signals (Becker et al., 2009).

Tip. Typically an electrolyte is added to make the protein solution conducting for spectroelectrochemical titrations. It should be made sure that the electrolyte does not react with the nonheme metal of interest. For example, when measuring the heme E°' of Ag(I)-Cu_BMb, NaNO₃ should be used as an electrolyte and not NaCl which will precipitate any Ag(I) bound to the protein.

Tip. In order to get a high quality CV signal, vary the scan rates and the initial potential. Pyrolytic edge graphite (PEG) electrodes are usually well-suited for protein samples. However, other electrodes such as glassy carbon or gold may yield preferable behavior at the electrode surface, which is variable depending on the protein.

3.3. Kinetic and Mechanistic studies

Respiratory enzymes like HCOs and NORs contain a number of metal cofactors, thus it is difficult to selectively study their heterobinuclear catalytic center. Designed heteronuclear metalloenzymes offer an easy alternative route to study reaction mechanism under physiological conditions.

Initial mechanistic studies are performed using a stopped-flow apparatus wherein the protein is mixed with the reactant and the reaction is monitored in a time-resolved fashion using either UV-Vis spectroscopy or vibrational spectroscopy. The strong and well-characterized UV-Vis signals of heme proteins, in particular, make UV-Vis stopped-flow spectroscopy a direct and easy method to probe their mechanism. Here, we describe how the mechanism of NO reduction by I107E-Fe_BMb (an NOR mimic) was investigated using UV-Vis stopped-flow spectroscopy (Matsumura et al., 2014). The reduction of NO to N₂O involves the cleavage of an N-O bond and formation of an N-N bond. This reaction can happen through three possible pathways: the *cis*-heme *b*₃, *cis*-Fe_B, or the *trans* mechanisms (Figure 7a).

1. To investigate the reaction mechanism, stopped-flow experiments were performed with an SX20 apparatus (Applied Photophysics) with a 1-cm path length cell equilibrated at 4 °C inside an anaerobic chamber.
2. Briefly, 10 μM of Fe(II)-I107E-Fe_BMb in 50 mM Bis-Tris pH 7.3 buffer was mixed with 0.8 mM NO in the same buffer in 1:1 ratio and the reaction was investigated with exponentially increasing time range for up to 50s.
3. The results obtained (shown in Fig. 7b) showed two distinct intermediates depicted in red (4 ms) and blue (128 ms) followed by a final species in green (11s).
4. A comparison of the UV-Vis spectra obtained at 128ms and 11s with literature values revealed them to be hemeFe(II)-NO and hemeFe(III)-NO species, respectively. These results strongly suggest the *trans*-mechanism of NO reduction for Fe_BMb variants (Fig. 7a).

Tip. To get a better understanding of the role of nonheme metal in the reaction mechanism, experiments should be repeated with empty protein or redox-inactive metal controls like Zn(II). For example, when E-I107E-Fe_BMb was mixed with NO under conditions described above, the reaction stopped at the formation of hemeFe(II)-NO, suggesting the importance of nonheme Fe for N-N bond formation and subsequent reactions.

3.4. Capture and characterization of reaction intermediates

For a deeper insight into the reaction mechanism of the designed metalloenzymes, it is necessary to capture and identify the intermediates involved in the reaction. The use of transition metal ions with rich spectroscopic features provides researchers with a wealth of techniques with which they can characterize these intermediates. Techniques such as X-ray absorption Spectroscopy (XAS), electron paramagnetic resonance (EPR), Mössbauer, nuclear magnetic resonance (NMR), magnetic circular dichroism (MCD), resonance Raman (rR), nuclear resonance vibrational spectroscopy (NRVS), and other techniques have been extensively used to characterize heteronuclear metal centers. Table 2 shows a list of these techniques, the information gained from each, and some studies in which these techniques have been used on designed heteronuclear metalloenzymes.

Usually, the presence of an intermediate can be observed through stopped-flow UV-Vis experiments. These intermediates can be captured at millisecond time points using rapid freeze-quench/chemical-quench (Matsumura&Moenne-Loccoz, 2014) techniques and further characterized using various spectroscopic techniques described in Table 1. To give an overview of this process, below we describe the capture and vibrational characterization of intermediates obtained during reaction of Fe-Fe_BMb with NO.

1. Rapid freeze-quench (RFQ) was used to capture the reaction mixture at early time points. Protocols for preparation of RFQ samples and their resonance Raman analysis have been discussed in detail in this chapter (Matsumura&Moenne-Loccoz, 2014).
2. Briefly, glass syringes (1 or 2 mL) were loaded with protein solutions (0.6 mM reduced Fe_BMbs in 50 mM Bis-Tris, pH 7.0) and NO solutions (2 mM ¹⁴NO or ¹⁵NO in 50 mM Bis-Tris, pH 7.0) inside an anaerobic chamber before mounting them to the System 1000 Chemical/Freeze Quench Apparatus (Update Instruments).
3. Reaction times were controlled by varying the syringe displacement rate or by varying the length of the reactor hose after the mixer.
4. Mixed volumes of 250 µL were ejected into a glass funnel attached to NMR tubes filled with liquid ethane at or below 120 °C. The frozen samples were packed into the tube as the assembly sat within a Teflon block cooled to 120 °C. Liquid ethane was subsequently removed by incubation of samples at 80 °C for 2 h.

The rR spectra recorded on all of the RFQ samples shown in Figure 6c display a prominent high frequency vibration at 1755 cm⁻¹ corresponding to ν(NO) of nonheme-Fe(II)-NO with a continuous increase in vibration at 1660 cm⁻¹ with time corresponding to ν(NO) of heme-

Fe(II)-NO (Fig. 7c). These results suggest that the 6 ms species obtained in UV-Vis stopped-flow spectroscopy corresponds to a nonheme-Fe(II)-NO species. Thus, NO binds first to nonheme iron following which it binds to the heme iron. Overall, stopped-flow experiments in tandem with RFQ-rR experiments strongly support the *trans*-mechanism for NO reduction for Mb-based NOR mimic (Matsumura et al., 2014).

Tip. Generally, the strong spectroscopic signature of heme iron along with its high affinity makes characterization of the nonheme metal ion challenging. To overcome this challenge, the heme can be replaced with Zn protoporphyrin IX. This replacement has been successfully done in Fe_B-Mb protein for the complete characterization of nonhemeFe(II)-NO species via UV-Vis, EPR, and Mössbauer spectroscopy (Chakraborty et al., 2014).

Step 4: Further improvement of designed heteronuclear metalloenzymes: Case studies

Here, we discuss a few examples of how the information obtained from structural, functional, and mechanistic studies of heteronuclear metalloenzymes was used to further improve their activity and robustness.

4.1. Improving electron transfer to the heme center

Increasing the concentration of an electron donor (TMPD/ascorbate) led to a direct increase in the oxygen reduction rates of G65Y-Cu_BMb, suggesting that electron transfer is the rate-limiting step in oxygen reduction of Mb-based HCO mimics (Miner et al., 2012). Thus, an efficient method to increase the overall oxidase activity of HCO mimics is to enhance electron transfer rates to the catalytic heme center. As a result, a triple lysine mutant of G65Y-Cu_BMb called G65Y-Cu_BMb(+6) was created that displayed very strong interactions with the native redox partner of Mb, cytochrome *b*₅ (Livingston et al., 1985). The G65Y-Cu_BMb(+6) mutant in the presence of cyt. *b*₅ (Figure 8a) reduced oxygen at a rate (52 s⁻¹) comparable to that of a native HCO (cyt. *cbb*₃ oxidase, 50 s⁻¹) (Yu et al., 2015).

4.2. Tuning heme reduction potential

The heme E°' of F33Y-Cu_BMb is rather low (95 mV) relative to native HCO, and hence has a higher over-potential for biofuel cell applications, which require oxygen reduction catalysts with higher E° (close to 0.8 V at pH 7). To increase the heme E°' of the Mb-based HCO mimic, we used several strategies including tuning of the H-bonding interactions of the proximal histidine and introducing non-native heme cofactors which contain electron-withdrawing carbonyl group conjugated to the porphyrin macrocycle. Four F33Y-Cu_BMb variants were obtained with systematically increasing heme E°' (Figure 8b). Interestingly, the HCO mimics with higher heme E°' showed a corresponding increase in oxygen reduction rates (Bhagi-Damodaran et al., 2014).

4.3. Increasing the binding affinity of Mn²⁺ in MnCcP

The initial activity assays on MnCcP mimics showed much lower rates of manganese oxidation than native MnPs. One contributor to this effect is the lower affinity for Mn²⁺ in the designed variants compared with their native counterparts. A series of mutations in the

secondary coordination sphere of the Mn binding site resulted in variants with higher activities. In particular, the Ile40Gly variant showed K_M values comparable with native MnPs (Figure 8c) (Hosseinzadeh et al., 2016).

Conclusions

The process of designing biosynthetic models of heteronuclear metalloenzymes is intricate and requires simultaneous consideration of many variables. Scaffold selection should favor the desired properties of the target metal binding site while maintaining a useful level of plasticity for investigative purposes; this initial choice carries forward for subsequent design considerations, and sometimes a promising first-choice may not ultimately be the best. The presence of and ability to rationally tune secondary sphere interactions (and beyond) is a powerful tool for accessing new regimes of catalysis or for generating states found in nature that cannot be stabilized by primary interactions alone. The design space for these outer sphere interactions increases correspondingly, and predicting optimal changes to achieve the desired outcome is not trivial. Fortunately, a large assortment of tools is available to assist in the design process, and the versatility and accuracy of computational modeling suites is already quite high and constantly improving: the task need not be daunting. A rich knowledge base exists for physical and spectroscopic characterization of metalloprotein active sites, much of which has been honed so that meaningful results can be obtained while working with dilute samples at varying levels of purity. A major advantage of the biosynthetic approach is the ability to make use of these techniques with large concentrations of highly pure samples. Experiments can target individual parameters such as redox potential, pK_a , or catalytic kinetic parameters such as K_M to both probe and improve upon nature, and all of these changes benefit from direct comparison through the same scaffold. Biosynthetic models have already contributed greatly to our understanding of heteronuclear metalloprotein catalysis, and as the techniques in design and genetic manipulation continue to improve, they are likely to continue to grow in importance for increasingly complex designed biological and biomimetic systems.

Acknowledgments

We wish to thank all the former and current Lu group members who have contributed to the development of the protocols and obtaining results published in papers cited in this chapter. The Lu group research described in papers cited in this chapter has been supported by the US National Institute of Health (R01GM06211) and National Science Foundation (CHE 14-13328).

References

- Annele, H.; Taina, L.; Martin, H.; Pekka, M. Applications of Enzymes to Lignocellulosics. Washington, DC: American Chemical Society; 2003. Manganese Peroxidase and Its Role in the Degradation of Wood 685 Lignin.
- Armstrong FA. Protein film voltammetry: revealing the mechanisms of biological oxidation and reduction. Russ J Electrochem. 2002; 38:49–62.
- Bairoch A, Apweiler R. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res. 2000; 28:45–8. [PubMed: 10592178]
- Becker CF, Watmough NJ, Elliott SJ. Electrochemical Evidence for Multiple Peroxidatic Heme States of the Diheme Cytochrome c Peroxidase of *Pseudomonas aeruginosa*. Biochemistry. 2009; 48:87–95. [PubMed: 19072039]

- Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank. *Nucleic Acid Research*. 2000; 28:235–42.
- Berry EA, Trumpower BL. Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra. *Anal Biochem*. 1987; 161:1–15. [PubMed: 3578775]
- Berry SM, Baker MH, Reardon NJ. Reduction potential variations in azurin through secondary coordination sphere phenylalanine incorporations. *J Inorg Biochem*. 2010; 104:1071–8. [PubMed: 20615551]
- Bhagi-Damodaran A, Petrik ID, Marshall NM, Robinson H, Lu Y. Systematic Tuning of Heme Redox Potentials and Its Effects on O₂ Reduction Rates in a Designed Oxidase in Myoglobin. *J Am Chem Soc*. 2014; 136:11882–5. [PubMed: 25076049]
- Bidwai A, Witt M, Foshay M, Vitello LB, Satterlee JD, Erman JE. Cyanide binding to cytochrome c peroxidase (H52L). *Biochemistry*. 2003; 42:10764–71. [PubMed: 12962501]
- Blomberg MR, Siegbahn PE. Different types of biological proton transfer reactions studied by quantum chemical methods. *Biochim Biophys Acta*. 2006; 1757:969–80. [PubMed: 16483535]
- Brooks BR, Brooks CL 3rd, Mackerell AD Jr, Nilsson L, Petrella RJ, Roux B, et al. CHARMM: the biomolecular simulation program. *J Comput Chem*. 2009; 30:1545–614. [PubMed: 19444816]
- Brunelle JL, Green R. One-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE). *Methods Enzymol*. 2014; 541:151–9. [PubMed: 24674069]
- Butland G, Spiro S, Watmough NJ, Richardson DJ. Two conserved glutamates in the bacterial nitric oxide reductase are essential for activity but not assembly of the enzyme. *J Bacteriol*. 2001; 183:189–99. [PubMed: 11114916]
- Case DA, Cheatham TE 3rd, Darden T, Gohlke H, Luo R, Merz KM Jr, et al. The Amber biomolecular simulation programs. *J Comput Chem*. 2005; 26:1668–88. [PubMed: 16200636]
- Casini A, Storch M, Baldwin GS, Ellis T. Bricks and blueprints: methods and standards for DNA assembly. *Nat Rev Mol Cell Biol*. 2015; 16:568–76. [PubMed: 26081612]
- Chakraborty, S.; Hosseinzadeh, P.; Lu, Y. Metalloprotein design and engineering. In: SCOTT, RA., editor. *Encyclopedia of Inorganic and Bioinorganic Chemistry*. Chichester: John Wiley and Sons, Ltd; 2014.
- Chakraborty S, Reed J, Ross M, Nilges MJ, Petrik ID, Ghosh S, et al. Spectroscopic and computational study of a nonheme iron nitrosyl center in a biosynthetic model of nitric oxide reductase. *Angewandte Chemie International Edition*. 2014; 126:2449–53.
- Chakraborty S, Reed J, Sage JT, Branagan NC, Petrik ID, Miner KD, et al. Recent Advances in Biosynthetic Modeling of Nitric Oxide Reductases and Insights Gained from Nuclear Resonance Vibrational and Other Spectroscopic Studies. *Inorg Chem*. 2015; 54:9317–29. [PubMed: 26274098]
- Chang CH, Kim K. Density Functional Theory Calculation of Bonding and Charge Parameters for Molecular Dynamics Studies on [FeFe] Hydrogenases. *J Chem Theory Comput*. 2009; 5:1137–45. [PubMed: 26609623]
- Chang CJ, Chang MC, Damrauer NH, Nocera DG. Proton-coupled electron transfer: a unifying mechanism for biological charge transport, amino acid radical initiation and propagation, and bond making/breaking reactions of water and oxygen. *Biochim Biophys Acta*. 2004; 1655:13–28. [PubMed: 15100012]
- Collman JP, Boulatov R, Sunderland CJ, Fu L. Functional analogues of cytochrome c oxidase, myoglobin, and hemoglobin. *Chemical reviews*. 2004; 104:561–88. [PubMed: 14871135]
- Collman JP, Wang Z. Synthetic heme chemistry: the cradle of functional molecules and catalysts. *Chemtracts*. 1999; 12:229–63.
- Das TK, Pecoraro C, Tomson FL, Gennis RB, Rousseau DL. The post-translational modification in cytochrome c oxidase is required to establish a functional environment of the catalytic site. *Biochemistry*. 1998; 37:14471–6. [PubMed: 9772174]
- Degrado WF, Summa CM, Pavone V, Nistri F, Lombardi A. *De novo* design and structural characterization of proteins and metalloproteins. *Annual review of biochemistry*. 1999; 68:779–819.
- Degrado WF, Wasserman ZR, Lear JD. Protein design, a minimalist approach. *Science*. 1989; 243:622–8. [PubMed: 2464850]

- Dib L, Carbone A. Protein fragments: functional and structural roles of their coevolution networks. *PLoS One*. 2012; 7:e48124. [PubMed: 23139761]
- Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr*. 2010; 66:486–501. [PubMed: 20383002]
- Evans RM, Armstrong FA. Electrochemistry of Metalloproteins: Protein Film Electrochemistry for the Study of *E. coli* [NiFe]-Hydrogenase-1. *Methods Mol Biol (N Y, NY, U S)*. 2014; 1122:73–94.
- Feng M, Tachikawa H, Wang X, Pfister TD, Gengenbach AJ, Lu Y. Resonance Raman spectroscopy of cytochrome c peroxidase variants that mimic manganese peroxidase. *J Biol Inorg Chem*. 2003; 8:699–706. [PubMed: 14505074]
- Finzel BC, Poulos TL, Kraut J. Crystal structure of yeast cytochrome c peroxidase refined at 1.7-Å resolution. *J Biol Chem*. 1984; 259:13027–36. [PubMed: 6092361]
- Fischer M, Zhang QC, Dey F, Chen BY, Honig B, Petrey D. MarkUs: a server to navigate sequence-structure-function space. *Nucleic Acids Res*. 2011; 39:W357–61. [PubMed: 21672961]
- Flock U, Watmough NJ, Ädelroth P. Electron/proton coupling in bacterial nitric oxide reductase during reduction of oxygen. *Biochemistry*. 2005; 44:10711–9. [PubMed: 16060680]
- Fontecave M, Pierre JL. Oxidations by copper metalloenzymes and some biomimetic approaches. *Coordination Chemistry Reviews*. 1998; 170:125–40.
- Fukuda Y, Tse KM, Nakane T, Nakatsu T, Suzuki M, Sugahara M, et al. Redox-coupled proton transfer mechanism in nitrite reductase revealed by femtosecond crystallography. *Proc Natl Acad Sci U S A*. 2016
- Gao Y, Meyer B, Sokolova L, Zwicker K, Karas M, Brutschy B, et al. Heme-copper terminal oxidase using both cytochrome c and ubiquinol as electron donors. *Proc Natl Acad Sci U S A*. 2012; 109:3275–80. [PubMed: 22334648]
- Garcia-Horsman JA, Barquera B, Rumbley J, Ma J, Gennis RB. The superfamily of heme-copper respiratory oxidases. *Journal of Bacteriology*. 1994; 176:5587–600. [PubMed: 8083153]
- Gibney BR, Rabanal F, Skalicky JJ, Wand AJ, Dutton PL. Iterative Protein Redesign. *Journal of the American Chemical Society*. 1999; 121:4952–60.
- Gibson DG. Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol*. 2011; 498:349–61. [PubMed: 21601685]
- Gray HB, Winkler JR. Electron transfer in proteins. *Annual Review of Biochemistry*. 1996; 65:537–61.
- Gray HB, Winkler JR. Electron flow through metalloproteins. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 2010; 1797:1563–72. [PubMed: 20460102]
- Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc*. 2006; 1:2876–90. [PubMed: 17406547]
- Hadt RG, Sun N, Marshall NM, Hodgson KO, Hedman B, Lu Y, et al. Spectroscopic and DFT studies of second-sphere variants of the type 1 copper site in azurin: Covalent and nonlocal electrostatic contributions to reduction potentials. *Journal of the American Chemical Society*. 2012; 134:16701–16. [PubMed: 22985400]
- Hay MT, Ang MC, Gamelin DR, Solomon EI, Antholine WE, Ralle M, et al. Spectroscopic characterization of an engineered purple Cu_A center in azurin. *Inorganic Chemistry*. 1998; 37:191.
- Hayashi T, Lin JJ, Chen Y, Fee JA, Moënné-Loccoz P. Fourier transform infrared characterization of a Cu_B-nitrosyl complex in cytochrome *ba*₃ from *Thermus thermophilus*: Relevance to NO reductase activity in heme-copper terminal oxidases. *Journal of the American Chemical Society*. 2007; 129:14952–8. [PubMed: 17997553]
- Hofrichter M. Review: lignin conversion by manganese 682 peroxidase (MnP). *Enzyme Microb Technol*. 2002; 30:454–66.
- Holm L, Rosenstrom P. Dali server: conservation mapping in 3D. *Nucleic Acids Res*. 2010; 38:W545–9. [PubMed: 20457744]
- Holm RH. Chemical approaches to bridged biological metal assemblies. *Pure Appl Chem*. 1995; 67:217–24.
- Hosseinzadeh P, Lu Y. Design and fine-tuning redox potentials of metalloproteins involved in electron transfer in bioenergetics. *Biochim Biophys Acta, Bioenerg*. 2015 Ahead of Print.

- Hosseinzadeh P, Mirts EN, Pfister TD, Gao YG, Mayne C, Robinson H, et al. Enhancing Mn(II)-Binding and Manganese Peroxidase Activity in a Designed Cytochrome c Peroxidase through Fine-Tuning Secondary-Sphere Interactions. *Biochemistry*. 2016; 55:1494–502. [PubMed: 26885726]
- Hosseinzadeh P, Mirts EN, Pfister TD, Gao YG, Mayne C, Robinson H, et al. Enhancing Mn(II)-Binding and Manganese Peroxidase Activity in a Designed Cytochrome c Peroxidase through Fine-Tuning Secondary-Sphere Interactions. *Biochemistry*. 2016
- Hu X, Wang H, Ke H, Kuhlman B. High-resolution design of a protein loop. *Proc Natl Acad Sci U S A*. 2007; 104:17668–73. [PubMed: 17971437]
- Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996; 14:33–8. 27–8. [PubMed: 8744570]
- Jackson TA, Brunold TC. Combined spectroscopic/computational studies on Fe- and Mn-dependent superoxide dismutases: insights into second-sphere tuning of active site properties. *Acc Chem Res*. 2004; 37:461–70. [PubMed: 15260508]
- Kim E, Chufan EE, Kamaraj K, Karlin KD. Synthetic models for heme-copper oxidases. *Chemical Reviews*. 2004; 104:1077–133. [PubMed: 14871150]
- Koziol L, Valdez CA, Baker SE, Lau EY, Floyd WC 3rd, Wong SE, et al. Toward a small molecule, biomimetic carbonic anhydrase model: theoretical and experimental investigations of a panel of zinc(II) aza-macrocyclic catalysts. *Inorg Chem*. 2012; 51:6803–12. [PubMed: 22671132]
- Lillie H. Designer proteins in biotechnology. International Titisee Conference on protein design at the crossroads of biotechnology, chemistry and evolution. *EMBO Rep*. 2003; 4:346–51. [PubMed: 12671677]
- Lin YW, Yeung N, Gao YG, Miner KD, Lei L, Robinson H, et al. Introducing a 2-His-1-Glu nonheme Iron center into myoglobin confers nitric oxide reductase activity. *Journal of the American Chemical Society*. 2010; 132:9970–2. [PubMed: 20586490]
- Lin YW, Yeung N, Gao YG, Miner KD, Tian S, Robinson H, et al. Roles of glutamates and metal ions in a rationally designed nitric oxide reductase based on myoglobin. *Proc Natl Acad Sci U S A*. 2010; 107:8581–6. [PubMed: 20421510]
- Liu J, Chakraborty S, Hosseinzadeh P, Yu Y, Tian S, Petrik ID, et al. Metalloproteins containing cytochrome, iron-sulfur or copper redox centers. *Chemical reviews*. 2014; 114:4366–469. [PubMed: 24758379]
- Liu X, Yu Y, Hu C, Zhang W, Lu Y, Wang J. Significant increase of oxidase activity through the genetic incorporation of a tyrosine-histidine cross-link in a myoglobin model of heme-copper oxidase. *Angewandte Chemie, International edition*. 2012; 51:4312–6. [PubMed: 22411709]
- Livingston DJ, McLachlan SJ, La Mar GN, Brown WD. Myoglobin:cytochrome b5 interactions and the kinetic mechanism of metmyoglobin reductase. *J Biol Chem*. 1985; 260:15699–707. [PubMed: 4066692]
- Lu C, Zhao X, Lu Y, Rousseau DL, Yeh SR. Role of copper ion in regulating ligand binding in a myoglobin-based cytochrome c oxidase model. *Journal of the American Chemical Society*. 2010; 132:1598–605. [PubMed: 20070118]
- Lu, Y.; Chakraborty, S.; Miner, KD.; Wilson, TD.; Mukherjee, A.; Yu, Y., et al. Metalloprotein design. In: REEDIJK, J.; POEPELMEIER, K., editors. *Comprehensive inorganic chemistry II*. Elsevier Science; 2013.
- Lu, Y.; Garner, DK.; Zhang, J-L. Artificial metalloproteins: design and engineering. In: BEGLEY, TP., editor. *Wiley encyclopedia of chemical biology*. John Wiley & Sons, Inc; 2009.
- Lu, Y.; Wang, X.; Sigman, JA.; Gengenbach, A.; Berry, SM.; Brueschoff, PJ. Redesigning metalloproteins. *Book of Abstracts, 218th ACS National Meeting*; New Orleans. Aug. 22–26; 1999. p. INOR-403
- Lu Y, Yeung N, Sieracki N, Marshall NM. Design of functional metalloproteins. *Nature*. 2009; 460:855–62. [PubMed: 19675646]
- Lu Y, Yeung N, Sieracki N, Marshall NM. Design of functional metalloproteins. *Nature*. 2009:855.
- Luft JR, Newman J, Snell EH. Crystallization screening: the influence of history on current practice. *Acta Crystallogr, Sect F: Struct Biol Commun*. 2014; 70:835–53. [PubMed: 25005076]

- Mahadevan V, Gebbink RK, Stack TD. Biomimetic modeling of copper oxidase reactivity. *Curr Opin Chem Biol.* 2000; 4:228–34. [PubMed: 10742191]
- Malmström BG. Structural control of electron-transfer properties in metalloproteins. *Biology of Metals.* 1990; 3:64–6.
- Marshall NM, Garner DK, Wilson TD, Gao YG, Robinson H, Nilges MJ, et al. Rationally tuning the reduction potential of a single cupredoxin beyond the natural range. *Nature.* 2009; 462:113–U27. [PubMed: 19890331]
- Marshall, NM.; Miner, KD.; Wilson, TD.; Lu, Y. Rational design of protein cages for alternative enzymatic functions. In: UENO, T.; WATANABE, Y., editors. *Coordination chemistry in protein cages.* John Wiley & Sons, Inc; 2013.
- Matsumura H, Hayashi T, Chakraborty S, Lu Y, Moënne-Loccoz P. The production of nitrous oxide (N₂O) by the heme/non-heme diiron center of engineered myoglobins (Fe₂Mbs) proceeds through a trans iron-nitrosyl dimer. *Journal of the American Chemical Society.* 2014; 136:2420–31. [PubMed: 24432820]
- Matsumura H, Moenne-Loccoz P. Characterizing Millisecond Intermediates in Hemoproteins Using Rapid-Freeze-Quench Resonance Raman Spectroscopy. *Methods Mol Biol (N Y, NY, U S).* 2014; 1122:107–23.
- Miller AF. Redox tuning over almost 1 V in a structurally conserved active site: lessons from Fe-containing superoxide dismutase. *Acc Chem Res.* 2008; 41:501–10. [PubMed: 18376853]
- Miner KD, Mukherjee A, Gao YG, Null EL, Petrik ID, Zhao X, et al. A designed functional metalloenzyme that reduces O₂ to H₂O with over one thousand turnovers. *Angewandte Chemie International Edition.* 2012; 51:5589–92. [PubMed: 22539151]
- Miner KD, Pfister TD, Hosseinzadeh P, Karaduman N, Donald LJ, Loewen PC, et al. Identifying the elusive sites of tyrosyl radicals in cytochrome *c* peroxidase: implications for oxidation of substrates bound at a site remote from the heme. *Biochemistry.* 2014; 53:3781–9. [PubMed: 24901481]
- Mukherjee S, Mukherjee M, Dey A, Mukherjee A, Bhagi-Damodaran A, Lu Y. A biosynthetic model of cytochrome *c* oxidase as an electrocatalyst for oxygen reduction. *Nat Commun.* 2015; 6:8467. [PubMed: 26455726]
- Nakayama H, Shimamoto N. Modern and simple construction of plasmid: saving time and cost. *J Microbiol.* 2014; 52:891–7. [PubMed: 25359266]
- New SY, Marshall NM, Hor TSA, Xue F, Lu Y. Redox tuning of two biological copper centers through non-covalent interactions: same trend but different magnitude. *Chemical Communications.* 2012; 48:4217–9. [PubMed: 22441412]
- Petrik ID, Davydov R, Ross M, Zhao X, Hoffman B, Lu Y. Spectroscopic and Crystallographic Evidence for the Role of a Water-Containing H-Bond Network in Oxidase Activity of an Engineered Myoglobin. *J Am Chem Soc.* 2016; 138:1134–7. [PubMed: 26716352]
- Petrik ID, Davydov R, Ross M, Zhao X, Hoffman B, Lu Y. Spectroscopic and Crystallographic Evidence for the Role of a Water-Containing H-Bond Network in Oxidase Activity of an Engineered Myoglobin. *J Am Chem Soc.* 2016; 138:1134–7. [PubMed: 26716352]
- Petrik ID, Liu J, Lu Y. Recent advances in functional metalloprotein design. *Current Opinion in Structural Biology.* 2014; 19:67–75.
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem.* 2004; 25:1605–12. [PubMed: 15264254]
- Pfister TD, Mirarefi AY, Gengenbach AJ, Zhao X, Danstrom C, Conatser N, et al. Kinetic and crystallographic studies of a redesigned manganese-binding site in cytochrome *c* peroxidase. *Journal of Biological Inorganic Chemistry.* 2007; 12:126–37. [PubMed: 17021923]
- Pfister TD, Ohki T, Ueno T, Hara I, Adachi S, Makino Y, et al. Monooxygenation of an aromatic ring by F43W/H64D/V68I myoglobin mutant and hydrogen peroxide. Myoglobin mutants as a model for P450 hydroxylation chemistry. *J Biol Chem.* 2005; 280:12858–66. [PubMed: 15664991]
- Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, et al. Scalable molecular dynamics with NAMD. *Journal of Computational Chemistry.* 2005; 26:1781–802. [PubMed: 16222654]

- Pronk S, Pall S, Schulz R, Larsson P, Bjelkmar P, Apostolov R, et al. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics*. 2013; 29:845–54. [PubMed: 23407358]
- Punniyamurthy T, Velusamy S, Iqbal J. Recent advances in transition metal catalyzed oxidation of organic substrates with molecular oxygen. *Chem Rev*. 2005; 105:2329–63. [PubMed: 15941216]
- Que L Jr, Tolman WB. Biologically inspired oxidation catalysis. *Nature*. 2008; 455:333–40. [PubMed: 18800132]
- Reece SY, Nocera DG. Proton-coupled electron transfer in biology: results from synergistic studies in natural and model systems. *Annu Rev Biochem*. 2009; 78:673–99. [PubMed: 19344235]
- Reedy CJ, Gibney BR. Heme protein assemblies. *Chemical Reviews*. 2004; 104:617–49. [PubMed: 14871137]
- Richardson DJ, Watmough NJ. Inorganic nitrogen metabolism in bacteria. *Curr Opin Chem Biol*. 1999; 3:207–19. [PubMed: 10348621]
- Richter F, Leaver-Fay A, Khare SD, Bjelic S, Baker D. *De novo* enzyme design using Rosetta3. *PloS One*. 2011; 6:e19230. [PubMed: 21603656]
- Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc*. 2010; 5:725–38. [PubMed: 20360767]
- Sanders, JKM. Supramolecular Catalysis in Transition. In: UNGARO, R.; DALCANALE, E., editors. *Supramolecular Science: Where It Is and Where It Is Going*. Dordrecht: Springer Netherlands; 1999.
- Sarangi R, Gorelsky SI, Basumallick L, Hwang HJ, Pratt RC, Stack TDP, et al. Spectroscopic and density functional theory studies of the blue-copper site in M121SeM and C112SeC azurin: Cu-Se versus Cu-S bonding. *Journal of the American Chemical Society*. 2008; 130:3866. [PubMed: 18314977]
- Sraphin B, Kandels-Lewis S. An efficient PCR mutagenesis strategy without gel purification [correction of “purification”] step that is amenable to automation. *Nucleic Acids Res*. 1996; 24:3276–7. [PubMed: 8774913]
- Shanmugam M, Xue G, Que L Jr, Hoffman BM. 1H-ENDOR evidence for a hydrogen-bonding interaction that modulates the reactivity of a nonheme Fe(IV)=O unit. *Inorganic Chemistry*. 2012; 51:10080–2. [PubMed: 22984805]
- Shook RL, Borovik AS. Role of the secondary coordination sphere in metal-mediated dioxygen activation. *Inorg Chem*. 2010; 49:3646–60. [PubMed: 20380466]
- Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*. 2011; 7:539. [PubMed: 21988835]
- Sigman JA, Kim HK, Zhao X, Carey JR, Lu Y. The role of copper and protons in heme-copper oxidases: Kinetic study of an engineered heme-copper center in myoglobin. *Proc Natl Acad Sci U S A*. 2003; 100:3629–34. [PubMed: 12655052]
- Sigman JA, Kwok BC, Gengenbach A, Lu Y. Design and creation of a Cu(II)-binding site in cytochrome *c* peroxidase that mimics the Cu_B-heme center in terminal oxidases. *J Am Chem Soc*. 1999; 121:8949–50.
- Sigman JA, Kwok BC, Lu Y. From myoglobin to heme-copper oxidase: Design and engineering of a Cu_B center into sperm whale myoglobin. *J Am Chem Soc*. 2000; 122:8192.
- Sigman JA, Wang X, Lu Y. Coupled oxidation of heme by myoglobin is mediated by exogenous peroxide. *Journal of the American Chemical Society*. 2000; 123:6945–6.
- Sillitoe I, Lewis TE, Cuff A, Das S, Ashford P, Dawson NL, et al. CATH: comprehensive structural and functional annotations for genome sequences. *Nucleic Acids Res*. 2015; 43:D376–81. [PubMed: 25348408]
- Skarina T, Xu X, Evdokimova E, Savchenko A. High-Throughput Crystallization Screening. *Methods Mol Biol (N Y, NY, U S)*. 2014; 1140:159–68.
- Sollewijn Gelpke MD, Moenne-Loccoz P, Gold MH. Arginine 177 is involved in Mn(II) binding by manganese peroxidase. *Biochemistry*. 1999; 38:11482–9. [PubMed: 10471300]
- Springer BA, Sligar SG. High-level expression of sperm whale myoglobin in *Escherichia coli*. *Proc Natl Acad Sci U S A*. 1987; 84:8961–5. [PubMed: 3321062]

- Strupat K. Molecular weight determination of peptides and proteins by ESI and MALDI. *Methods Enzymol.* 2005; 405:1–36. [PubMed: 16413308]
- Sundaramoorthy M, Kishi K, Gold MH, Poulos TL. The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-Å resolution. *J Biol Chem.* 1994; 269:32759–67. [PubMed: 7806497]
- Taboy CH, Bonaventura C, Crumbliss AL. Spectroelectrochemistry of heme proteins: effects of active-site heterogeneity on Nernst plots. *Bioelectrochem Bioenerg.* 1999; 48:79–86. [PubMed: 10228573]
- Tard C, Pickett CJ. Structural and functional analogues of the active sites of the [Fe]-, [NiFe]-, and [FeFe]-hydrogenases. *Chem Rev.* 2009; 109:2245–74. [PubMed: 19438209]
- Thomas CM, Ward TR. Design of artificial metalloenzymes. *Applied Organometallic Chemistry.* 2005; 19:35–9.
- Tyagi R, Lai R, Duggleby RG. A new approach to ‘megaprimer’ polymerase chain reaction mutagenesis without an intermediate gel purification step. *BMC Biotechnol.* 2004; 4:2. [PubMed: 15070414]
- Van Dyke BR, Saltman P, Armstrong FA. Control of myoglobin electron-transfer rates by the distal (nonbound) histidine residue. *J Am Chem Soc.* 1996; 118:3490–2.
- Varadarajan R, Zewert TE, Gray HB, Boxer SG. Effects of buried ionizable amino acids on the reduction potential of recombinant myoglobin. *Science.* 1989; 243:69–72. [PubMed: 2563171]
- Wang X, Lu Y. Proton NMR investigation of the heme active site structure of an engineered cytochrome *c* peroxidase that mimics manganese peroxidase. *Biochemistry.* 1999; 38:9146–57. [PubMed: 10413489]
- Weinberg DR, Gagliardi CJ, Hull JF, Murphy CF, Kent CA, Westlake BC, et al. Proton-Coupled Electron Transfer. *Chemical Reviews.* 2012; 112:4016–93. [PubMed: 22702235]
- Weissensteiner, T.; Nolan, T.; Bustin, S.; Griffin, H.; Griffin, A. *PCR Technology: Current Innovations.* 3. CRC press; 2003.
- Wilcox SK, Putnam CD, Sastry M, Blankenship J, Chazin WJ, Mcree DE, et al. Rational design of a functional metalloenzyme: introduction of a site for manganese binding and oxidation into a heme peroxidase. *Biochemistry.* 1998; 37:16853–62. [PubMed: 9836578]
- Worth CL, Gong S, Blundell TL. Structural and functional constraints in the evolution of protein families. *Nature Reviews Molecular Cell Biology.* 2009; 10:709–20. [PubMed: 19756040]
- Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER Suite: protein structure and function prediction. *Nat Methods.* 2015; 12:7–8. [PubMed: 25549265]
- Ye Y, Godzik A. FATCAT: a web server for flexible structure comparison and structure similarity searching. *Nucleic Acids Res.* 2004; 32:W582–5. [PubMed: 15215455]
- Yeung BK, Wang X, Sigman JA, Petillo PA, Lu Y. Construction and characterization of a manganese-binding site in cytochrome *c* peroxidase: towards a novel manganese peroxidase. *Chemistry and Biology.* 1997; 4:215–21. [PubMed: 9115415]
- Yeung N, Lin YW, Gao YG, Zhao X, Russell BS, Lei L, et al. Rational design of a structural and functional nitric oxide reductase. *Nature (London, U K).* 2009; 462:1079–82. [PubMed: 19940850]
- Yeung N, Lin YW, Gao YG, Zhao X, Russell BS, Lei L, et al. Rational design of a structural and functional nitric oxide reductase. *Nature.* 2009; 462:1079–82. [PubMed: 19940850]
- Yeung N, Lu Y. One heme, diverse functions: using biosynthetic myoglobin models to gain insights into heme-copper oxidases and nitric oxide reductases. *Chemistry & Biodiversity.* 2008; 5:1437–54. [PubMed: 18729107]
- Yikilmaz E, Xie J, Brunold TC, Miller AF. Hydrogen-bond-mediated tuning of the redox potential of the non-heme Fe site of superoxide dismutase. *J Am Chem Soc.* 2002; 124:3482–3. [PubMed: 11929218]
- Yu Y, Cui C, Liu X, Petrik ID, Wang J, Lu Y. A designed metalloenzyme achieving the catalytic rate of a native enzyme. *J Am Chem Soc.* 2015; 137:11570–3. [PubMed: 26318313]
- Yu Y, Lv X, Li J, Zhou Q, Cui C, Mukherjee A, et al. Defining the role of tyrosine and rational tuning of oxidase activity by genetic incorporation of unnatural tyrosine analogs. *Proc Natl Acad Sci USA.* 2014 acceptance pending revisions.

- Yu Y, Mukherjee A, Nilges MJ, Hosseinzadeh P, Miner KD, Lu Y. Direct observation of a Tyrosyl radical in a functional oxidase model in myoglobin during both H₂O₂ and O₂ reactions by EPR. *Journal of the American Chemical Society*. 2014; 138:3869–75.
- Zanghellini A, Jiang L, Wollacott AM, Cheng G, Meiler J, Althoff EA, et al. New algorithms and an in silico benchmark for computational enzyme design. *Protein Sci*. 2006; 15:2785–94. [PubMed: 17132862]
- Zastrow ML, Pecoraro VL. Designing functional metalloproteins: From structural to catalytic metal sites. *Coordination Chemistry Reviews*. 2013
- Zeng J, Geng M, Jiang H, Liu Y, Liu J, Qiu G. The IscA from *Acidithiobacillus ferrooxidans* is an iron-sulfur protein which assemble the [Fe₄S₄] cluster with intracellular iron and sulfur. *Arch Biochem Biophys*. 2007; 463:237–44. [PubMed: 17470358]
- Zhang Y. I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics*. 2008; 9:40. [PubMed: 18215316]
- Zhao X, Yeung N, Russell BS, Garner DK, Lu Y. Catalytic reduction of NO to N₂O by a designed heme copper center in myoglobin: implications for the role of metal ions. *Journal of the American Chemical Society*. 2006; 128:6766–7. [PubMed: 16719438]
- Zhao X, Yeung N, Wang Z, Guo Z, Lu Y. Effects of metal ions in the Cu_B center on the redox properties of heme in heme-copper oxidases: Spectroelectrochemical studies of an engineered heme-copper center in myoglobin. *Biochemistry*. 2005; 44:1210–4. [PubMed: 15667214]

Figure 1.

Crystal structures of the active sites of *cbb*₃ oxidase (PDB ID 3MK7), *cNOR* (PDB ID 3O0R) and *MnP* (PDB ID: 1MNP) and the reaction catalyzed by each enzyme.

Figure 2.
General process of computational design of heteronuclear metalloproteins.

Figure 3.

X-ray crystal structures of Mb-based models (a) The histidine coordinated heme center of WT-swMb (PDB ID 1MBO) that already contains one of the histidines (H64) required to coordinate copper (b) The Cu bound structure of a Cu_BMb mutant (PDB ID 4FWY) that was obtained by mutating L29 and F43 to histidines. The three histidines coordinate copper in triagonal geometry. (c) The nonheme Fe added Fe_BMb (PDB ID 3M38) that was obtained by incorporating a glutamate (68E) in the nonheme metal binding center of Cu_BMb. The three histidines and a glutamate bind nonheme Fe in distorted octahedral geometry.

Figure 4.

(a) Structural alignment of CcP (PDB ID 2CYP, green) and MnP (PDB ID 1MNP, gray) indicates the possible position of primary ligands to be designed in CcP for Mn binding site. (b) Final MnCcP.1 model binds Mn(II) (PDB ID 5D6M, orange).

Figure 5. Titration of Fe(II) in Fe_B Mb variants as observed by UV-Vis (a) and EPR (b) spectroscopy. Figure 4a and 4b is adapted with permission from ref. (Chakraborty et al., 2014) and (Lin et al., 2010) respectively.

Figure 6.

(a) Characterization of the O₂ reduction; a) Rates of O₂ reduction to form either water or superoxide/peroxide with 18 μM WTswMb, Cu_BMb, F33Y-Cu_BMb, or G65Y-Cu_BMb; (b) Number of turnovers of O₂ reduction by E-F33Y-Cu_BMb and E-G65Y-Cu_BMb; arrows indicate addition of approximately 28 equivalents O₂. Inset, region of a high number of turnovers. (c) Quantitation of H₂¹⁷O product by ¹⁷O NMR spectroscopy: area of the H₂¹⁷O signal normalized to an external standard for WTswMb, E-F33Y-Cu_BMb and E-G65Y-Cu_BMb at 30, 60, 90, and 120 min. Figures are adapted with permission from ref. (Miner et al., 2012)

Figure 7.

(a) Possible mechanisms for NO reduction in Fe_BMb model. (b) Stopped-flow UV vis absorption spectra of the reaction of Fe_BMb with $60 \mu\text{M}$ NO at 4.0°C . (c) The rR spectra of RFQ samples of the reaction of reduced I107E- Fe_BMb with excess NO. Inset is an overlay of the ^{14}NO ^{15}NO differential signal for the nonheme $\nu(\text{NO})$ modes in the 6-ms RFQ samples of Fe_BMb and I107E- Fe_BMb . Figures are adapted from ref. (Matsumura et al., 2014). © 2014 American Chemical Society

Figure 8.

Further improvement of designed heteronuclear metalloenzymes. (a) Structures of G65Y-Cu_BMb(+6), showing the engineered lysines in blue, and cyt b₅ (PDB IDs 1CYO for cyt b₅ and 4FWY for F33Y-Cu_BMb). (b) Correlation between the heme E°' and catalytic oxygen reduction reactivity of HCO mimics. © 2014 American Chemical Society (c) Enhancement of binding affinity and catalytic efficiency of MnCcP mimics by mutation secondary coordination sphere residues. Figures are adapted with permission from ref (Yu et al., 2015), (Bhagi-Damodaran et al., 2014) and (Hosseinzadeh et al., 2016) respectively.

Table 1

Examples of Heteronuclear metalloenzymes

Enzyme	Metal-binding site
Heme-copper oxidase	Heme-Cu(N _{His}) ₃
Nitric oxide reductase	Heme-Fe(N _{His}) ₃ (O _{Glu})
Sulfite/nitrite reductase	Heme-(μ ₂ -S _{Cys}) ₂ -(Fe ₄ S ₄)
Nitrogenase P-clusters	[Fe ₄ S ₃] ₂ -(μ ₂ -S _{Cys}) ₂ (μ _{4or6} -S)-[Fe ₄ S ₃]
Nitrogenase FeMoCo	[Fe ₄ S ₃] ₂ -(μ ₂ -S) ₃ (μ ₆ -C)-[MoFe ₃ S ₃]
[NiFe]-Hydrogenase	(S _{Cys}) ₂ Ni((μ ₂ -S _{Cys}) ₂ Fe(CN) ₂ CO
CO dehydrogenase	[NiFe ₃ S ₃] ₂ -(μ ₃ -S)-[Fe(N _{His})(S _{Cys})]

Table 2

Techniques for characterization of metalloproteins and their intermediates

Spectroscopy	Obtained Information	Ref.
Electronic Absorption (UV-Vis)	M-Ligand, geometry, stoichiometry, affinity, electronic structure	(Lin et al., 2010, Chakraborty et al., 2014)
Electron Paramagnetic Resonance (EPR)	Ligand, geometry, stoichiometry, spin state	(Lin et al., 2010, Chakraborty et al., 2014, Yu et al., 2014)
Electron-nuclear double resonance (ENDOR)	Ligand and metal interactions	(Shanmugam et al., 2012, Petrik et al., 2016)
Magnetic Circular Dichroism (MCD)	Reaction intermediates (w/cryoreduction) M-Ligand, geometry, stoichiometry, affinity, & electronic structure	(Sarangi et al., 2008, Hadt et al., 2012)
X-ray absorption spectroscopy (XAS)	Ligands, metal-ligand distances (EXAFS) Oxidation States (XANES)	(Sarangi et al., 2008, Hadt et al., 2012)
FTIR/Resonance Raman (rR)	Reaction intermediates of O ₂ /NO reduction	(Hayashi et al., 2007, Lu et al., 2010, Matsumura et al., 2014)
Nuclear resonance vibrational spec (NRVS)	M- ligand vibrations, bond strengths, spin state, coordination	(Chakraborty et al., 2015)
Mössbauer	Oxidation and spin states of iron	(Chakraborty et al., 2014)
Paramagnetic NMR (PNMR)	M-ligand, binding stoichiometry	(Wang & Lu, 1999)