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Design of Functional Metalloproteins

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Abstract

Metalloproteins catalyze some of the most difficult and yet important functions in Nature, such as photosynthesis and water oxidation. An ultimate test of our knowledge of how metalloproteins work is by designing novel metalloproteins. Such design can not only reveal hidden structural features that may be missing from studies of native metalloproteins and their variants, but also result in new metalloenzymes for biotechnological and pharmaceutical applications. While it is much more challenging to design metalloproteins than non-metalloproteins, much progress has been made in this area, particularly toward functional design, thanks to recent progress in areas such as computational and structural biology.

Metalloproteins account for nearly half of all proteins in biology. Protein metal-binding sites are responsible for catalyzing some of the most difficult and yet important functions, including photosynthesis, respiration, water oxidation, molecular oxygen reduction, and nitrogen fixation. Much effort has been devoted toward understanding the structure and function of metalloproteins, as summarized in other reviews in this Nature Insight series. The ultimate test is to utilize this knowledge to design new metalloproteins that reproduce structures and functions of native metalloproteins[1-3]. Metalloprotein design is not an intellectual exercise that simply duplicates biochemical and biophysical studies of native metalloproteins. This bottom-up approach can elucidate structural features that may remain hidden in those studies; while the biochemical and biophysical studies can reveal mostly individual features that result in a loss of function, design requires incorporation of all the structural features needed to attain a function. Armed with insights obtained from the process, metalloprotein design may lead to the design of novel metalloproteins with improved properties such as higher stability, greater efficiency, or even unprecedented functions not found in Nature, for use in an even wider range of biotechnological and pharmaceutical applications.

Despite the promise, metalloprotein design has proven to be more challenging than its non-metal containing counterpart. Both fields require addressing the same issue of polypeptide design, but metalloprotein design involves metal ions that are much larger in number than amino acids, and geometries that are much more variable. On the other hand, most metal-binding sites are highly chromatic and display distinctive magnetic properties, making it easier to characterize the metalloprotein design using metal-based spectroscopic techniques rather than x-ray crystallography or NMR, thus shortening design cycles. Therefore, the field of metalloprotein design has enjoyed much success recently, thanks to advances in biophysical, computational, and structural biology. The field is also continually progressing from pure structural to more functional designs, and from primary coordination sphere to secondary coordination sphere design and beyond. These recent progresses will be summarized here, with focus on creating *new* and *functional* metal-binding sites in either *de novo* designed or native protein scaffolds. In addition, an emerging area of designing novel metalloproteins with expanded functions beyond those available in Nature through the introduction of unnatural

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amino acids or non-native metal cofactors will be covered. The methodologies used in achieving the goals will be summarized.

Metalloprotein design in *de novo* designed scaffolds

The purest and most challenging form of metalloprotein design is *de novo* design or design "from scratch". *De novo* design of a metalloprotein involves constructing a polypeptide sequence not directly related any natural protein, to precisely fold into a defined three-dimensional structure that binds a metal ion[4]. Most work in this area has focused on introducing metal-binding sites into designed α-helical bundles (Fig. 1), which are among the first *de novo* designed proteins. These α-helical bundles are a common scaffold for a number of heme proteins. Therefore, not surprisingly, heme centers have been among the first and most common metal centers incorporated into *de novo* α-helical bundles[5], from mono heme binding (Fig. 1a) [6] to four heme maquettes (Fig. 1b)[7] mimicking long-range electron transfer proteins. These proteins were rationally designed by direct comparison to native proteins, such as cytochrome bc_1, for clues as to the placement of essential heme binding and structure-stabilizing residues. In addition to electron transfer[8] and proton-coupled exchange[9] functions, a functional aniline hydroxylase[10], peroxidase[11], and oxygenase[12] have also been engineered into *de novo* proteins. An exciting development in this field is the design of a di-heme containing four-helix bundle with oxygen transport properties, whose O_2 affinities and exchange timescales match those of natural globins[13]. Remarkably, this designed protein binds O_2 even tighter than CO. A key to the success is reduction of helical-interface mobility to exclude water from the heme binding site, thereby reducing heme oxidation and increasing O_2 binding stability.

In addition to heme, other metal ions/cofactors have been engineered into *de novo* designed helical bundles by introducing metal-binding ligands at specific locations to mimic those in native proteins. Examples include the His_2Cys_2 ligand set found in Zn-finger proteins (Fig. 1c)[14] and the His_3 set in carbonic anhydrase[15] The x-ray crystal structure of an arsenic bound three-stranded coiled-coil protein (Fig. 1d), resembling the putative tris Cys metal-binding environment of the natural arsenic binding protein, ArsR, has been reported, affording the first biologically relevant small molecule mimic of an AsS_3 coordination[16]. Metal-binding ligands Glu and His have also been introduced by retrostructural analysis, whereby the active site of a protein can be separated into idealized secondary structural components (e.g., an α-helix and a β-hairpin), and mathematically described as a root-mean-square deviation from the idealized structure. This strategy led to the construction of dinuclear metalloproteins in α-helical bundles that can bind two Zn(II), Co(II), or Fe(II), with the Zn(II) crystal structure (Fig. 1e) shown to be structurally analogous to the diiron site of ribonuclease reductase and ferritin[17]. One of these diiron proteins was reported to catalyze the two electron oxidation of 4-aminophenol, similar to the reaction catalyzed by diiron phenol oxidases[18,19].

Taking a cue from naturally occurring Cys rich coordination sites in proteins, the introduction of Cys ligands led to the development of α-helical coiled-coil metalloproteins capable of binding Cu(II), Zn(II), Cd(II), Hg(II), and As(III)[20-22]. One exciting aspect of *de novo* design is the demonstration that these *de novo* proteins can stabilize unusual metal coordination states, such as three-coordinate Hg(II) by a tri-helical bundle over the bis-coordination often preferred by Hg(II)[23,24]. In other cases, metal-binding can actually direct the folding and assembly of an α-helical bundle from either a random coil, less folded, or misfolded state, as was the case of Cd(II)[25] and Hg(II) binding[26] to Cys containing peptides predisposed to form helices. Interestingly, geometry-selective binding of two different Cd(II) in one designed protein has been achieved and confirmed by the correlation of ^{113}Cd NMR and ^{111m}Cd Perturbed Angular Correlation spectroscopy[27,28].

In stark contrast to the design of α-helical proteins, *de novo* design of β-structure proteins is still in its infancy, because there were few model systems with which to study β-sheets in isolation from other protein structures. The *de novo* design of a redox active rubredoxin mimic, RM1, is a rare example of a structural and functional metallo-β-sheet protein[29]. RM1 was shown to bind iron and reversibly cycle between the Fe(II/III) oxidation states, mimicking native rubredoxin. The RM1 β-hairpin structure was designed computationally by constraining the positions of the peptide that were strategic in mimicking and/or stabilizing the β-structure and subsequent use of another computer program, SCADS[30] to select the most probable amino acids for the remaining positions.

Metalloprotein design in native protein scaffolds

While designing metalloproteins using *de novo* designed scaffolds offers the prospect of complete control over a protein's structure and metal-binding properties, our current knowledge of protein folding limits the number of *de novo* designed scaffolds to only a few types, such as the α-helical bundles discussed above. It has been observed that β-sheet containing proteins tend to support more rigid and more pre-organized metal-binding sites than α-helical proteins, which are inherently more flexible[31]. Unlike *de novo* designed scaffolds, the Protein Data Bank contains ~1000 natural protein scaffold types (http://scop.mrc-lmb.cam.ac.uk/scop/count.html#scop-1.73), most of which maintain the same fold and similar stability even after numerous mutations. Therefore, metalloprotein design using native protein scaffolds provides many more scaffold choices that are more tolerant to mutations, allowing for the incorporation of metal-binding sites into proteins with less concern over decreased protein stability. When chosen carefully, native proteins are also relatively easier to crystallize than *de novo* designed scaffolds, making 3D characterization possible, an objective that is critical to success. Perhaps the strongest argument for designing metalloproteins using native scaffolds is that Nature employs the same approach since the same scaffold is often seen in numerous proteins with diverse metal-binding motifs and functions, suggesting that these native scaffolds are robust and modifiable. For example, the Greek key β-barrel fold has been shown to be used by ~600 different types of proteins with diverse functions, ranging from oxidase, reductase, amylase, and dismutase activities, for instance. Unearthing the secret of how Nature is able to use the same scaffold to design a variety of metal-binding sites is an important goal of metalloprotein design[1].

Biochemical techniques, such as site-directed mutagenesis, have been used extensively to study the function of metalloproteins. The loss of function accompanied by certain mutations (usually to highly conserved residues) allows identification of residues essential for function. Although serving a different purpose, the same mutagenesis techniques can be used for metalloprotein design to impart new function into a protein scaffold by the introduction of residues that bind metal ions. This approach can be in the form of redesigning existing metal-binding sites to introduce novel functionality, introducing mononuclear metal-binding sites to proteins that do not natively bind metal ions, or by introducing homonuclear or heteronuclear metal-binding sites into proteins. The designs are often aided by empirical approaches through knowledge/experience, rational design using computer programs, or combinatorial selections.

The redesign of an existing metal-binding site to introduce novel function or metal specificity is the simplest form of metalloprotein design, as this approach relies on the structural differences between template and target proteins, and is amenable to empirical designs through knowledge/experience. Despite its simple form, this approach is still quite powerful in elucidating structural features important for the change, or gain of function by redesign. Heme proteins have been extensively redesigned in this way. They are one of the most diverse classes of proteins, with functions ranging from electron transfer, small molecule (e.g., oxygen and NO) transport, sensing, and activation[5,32,33]. By systematically changing these characteristics

of heme proteins, researchers have been able to convert one type of heme protein into another, as well as introduce novel function or substrate specificity into a heme protein[32,34].

The selectivity of designed metalloproteins for specific metals has also been utilized for the purpose of metal-sensing applications. Ratiometric Zn(II) sensors have been created by designing Zn(II) binding sites into green fluorescent proteins and by taking advantage of the resulting fluorescent signal variations due to Zn(II)-dependent conformational changes[35-37]. The metal-binding site of NikR, a DNA binding protein, has been redesigned to bind UO_2^{2+} rather than its native Ni^{2+} cofactor (Fig. 2a)[38], resulting in an artificial protein that binds DNA only in the presence of UO_2^{2+}. In addition to sensors, designed metal-binding sites have allowed modulation of protein-protein interactions[39,40], which are at the heart of many diverse biological functions. Because of complicated non-covalent interactions, it has been difficult to control the interactions well. These designed metal-binding sites allowed specific control of these interactions.

More complex metal-binding site redesign may require rational design using computational biology, such as the simultaneous incorporation and adjustment of functional elements to convert the GlyII enzyme from a glyoxalase, which hydrolyzes thioester bonds, to a β-lactamase[41]. In order to impart the new functionality, the metal-binding site had to be significantly redesigned in order to change the metal-binding geometry, as well as the substrate binding pocket.

While redesign of metal-binding sites can offer insight into the different structural features between the template and target proteins, those structural features common to the metal-binding sites of both proteins will remain hidden. Introducing metal-binding sites into a protein location where no native metal-binding site is found, can overcome this limitation, although this approach requires a higher level of complexity. A common strategy is to base the design on structural homology between the template protein which contains no metal ions and the target metalloprotein, to introduce metal-binding sites at the corresponding positions. Using this strategy, new Zn(II) binding sites have been introduced into charybdotoxin[42] and retinal binding protein[43] to mimic carbonic anhydrase (CA).

When there is no structural homology, computer aided design using programs such as Metal-Search[44] and Dezymer[45], has become indispensable. A Ca(II)[46], Pb(II)[47], and $[Fe(Cys)_4]$ rubredoxin center[45] have all been designed into scaffold proteins using computer aided design. In the case of an artificial rubredoxin designed into the non-metalloprotein thioredoxin, it was found to undergo several rounds of air oxidation and reduction by β-mercaptoethanol, similar to native rubredoxins[45].

An even higher level of difficulty in metalloenzyme design is the introduction of dinuclear metal-binding sites or metal clusters into proteins. An example is the binuclear Cu_A center, consisting of two copper ions bridged by two cysteine thiolates, with each copper ion also coordinated to a histidine nitrogen (Fig. 2b). New Cu_A centers were designed into the cupredoxins azurin (Az)[48,49] and amicyanin[50,51] using a technique called loop directed mutagenesis (LDM), in which the copper binding loop of cupredoxins is replaced by the corresponding loop in C*c*O that share similar structural homology. LDM has also been used to introduce lanthanide binding sites into a helix-turn-helix motif by drafting a calcium binding EF-hand loop, and such a designed protein has been shown to promote DNA and phosphate hydrolysis[52].

Heteronuclear bimetallic centers are also found in many natural proteins and they perform important biological functions such as oxygen reduction, lignin degradation and nitric oxide reduction. Designing metalloproteins to mimic the enzymes that carry out these complex reactions is even more challenging than designing homonuclear metal-binding sites because

of the selective placement and binding of two or more different metal centers. An example is the heme-Cu_B center in C*c*O where three histidines bind to a Cu(II) ion that is ~4.5 Å away from the heme iron. Computer-aided design was used to introduce the histidines above the heme of myoglobin (Mb) at positions corresponding to C*c*O (Fig. 2c)[53]. Copper binding was confirmed by multiple spectroscopic techniques. Further studies showed the importance of the Cu_B site in O_2 binding and reduction, as well as the significance of a proton network[54]. This designed protein also displays interesting functional properties, such as nitric oxide reduction[55], similar to certain members of C*c*Os. A Mn(II) binding site has also been designed into cytochrome *c* peroxidase, resulting in a heme-Mn(II) protein with Mn(II) oxidation activity, as in native manganese peroxidase (MnP)[56], a protein involved in lignin biodegradation, which is a critical step in biomass conversions from plant materials to biofuels.

In addition to discrete metal-binding sites, metal nanoparticles have been introduced into proteins[57], including Pt nanoparticles in ferritin[58] and a small heat shock protein[59]. The former catalyzes size-selective olefin hydrogenation while the latter catalyzes hydrogen production.

Even though empirical approaches through knowledge/experience and rational design using computer programs have been quite successful in designing metalloproteins, it is still difficult to design certain metalloproteins, especially functional ones, due to the large number of potential interactions within the active site of a metalloenzyme. In this case, combinatorial design and directed evolution of novel metalloenzymes is another powerful choice. For example, directed evolution has been extensively applied to antibodies to develop novel metalloenzymes. Utilizing phage display, the 7G12 antibody protein was evolved to bind heme, with which the oxidation of tyramine could be catalyzed[60]. A similar technique has recently been expanded to find antibodies for inorganic ligands, such as BINAP and BINOL, with the goal to develop completely novel metalloenzymes[61]. A striking example of this approach is the use of directed evolution to obtain a new cyt P450 with the ability to selectively hydroxylate propane, a function that has not yet been achieved by small molecule catalysts[62].

Designing novel metalloproteins containing unnatural amino acids or non-native metal cofactors

Metalloprotein design using *de novo* designed or native protein scaffolds has succeeded in providing valuable insights into the fundamental rules that govern protein structure and function. Replicating native protein structure and function, although quite challenging, is not the only goal of protein designers; they want build proteins with properties beyond those found in Nature[63]. In recent years, the repertoire of functional groups such as amino acids or cofactors available to protein designers has been expanded beyond those available in biological systems.

A myriad of techniques have been developed for the incorporation of unnatural amino acids into a protein scaffold, each with its own advantages and limitations[63]. Total synthesis of proteins using solid phase peptide synthesis enables incorporation of any synthetic amino acid for which successful protection/deprotection techniques can be applied[64], but cost and size constraints limits the use of total synthesis to smaller proteins (~60–100 amino acids). Native chemical ligation (NCL), involving covalent attachment of two synthetic peptides, has expanded this size regime[65], but cost is still an issue, at least for large proteins. Expressed protein ligation (EPL), the coupling of a bacterially expressed peptide fragment with a synthetic peptide fragment containing unnatural amino acids, has reduced cost and extended the protein size even further, but it often requires a cysteine residue at the point of ligation to maximize ligation efficiency[66], although methods have been developed to use other residues at the point of ligation, with less efficiency. Methods not restricted by protein size include auxotroph generation[67], chemical residue modification[68], and cavity complementation[69], but these methods have limited versatility in terms of the variety of unnatural amino acids that can be

incorporated. A promising and versatile technique is the use of custom evolved tRNA synthetase molecules for *in vitro* and *in vivo* translation of new residues through recognition of the amber stop codon[70]. A seminal contribution from this approach was the design of a DNA cleaving protein through site-specific genetic incorporation of the DNA cleaving agent (2,2′-bipyridin-5-yl)alanine into a DNA binding protein[71]. Upon addition of Cu(II) or Fe(II) and reducing agent to the protein, a double stranded DNA substrate was cleaved at its consensus sequence. The variety of unnatural amino acids this technique can incorporate is higher than those of auxotroph generation, chemical residue modification, or cavity complementation, but lower than those of total synthesis, NCL or EPL.

The expansion of the amino acid regime has made significant contributions toward determining systematically the role of each amino acid in tuning the activity of the metal center toward a given reactivity, and in increasing the breadth of chemical transformations performed by proteins. For instance, replacement of heme binding His residues in *de novo* designed four-α-helical bundles with 4-β-(pyridyl)-L-alanine and 1-methyl-L-His has resulted in an increase in the reduction potential of the metal site for the pyridyl system and a preference for five-coordinate low-spin heme in the 1-methyl-L-His system[72]. In another system, introduction of bulky penicillamine ligands in place of cysteines led to the creation of an open metal-binding site in a ferredoxin model peptide[73]. This methodology resulted in the isolation of the first water-stable CdS_3 complex through subtle manipulation of steric crowding around the metal center[74]. Furthermore substitution of L-amino acids for their D-enantiomers resulted in fine control between 3- and 4-coordinate Cd(II) complexes in a *de novo* designed metallopeptide[75]. Finally, the role of a conserved Tyr residue near the active site of rubredoxin was probed via a set of unnatural Tyr analogs in which the para -OH group was replaced with -H (native Phe), -F, $-NO_2$, and -CN, spanning a range of electron withdrawing strengths (Fig. 3a)[76]. Interestingly, a linear correlation was found between the reduction potential of the nearby heme and the Hammett σ_p value, but no such trend is observed with dipole moment or residue size.

A powerful advantage to the introduction of unnatural amino acids is the ability to probe the role of the amide backbone, since site-directed mutagenesis using natural amino acids cannot modify the protein backbone. Both the carbonyl and nitrogen functionalities of the protein backbone have been implicated in critical and specific hydrogen bonding interactions, either directly to the metal, or to the metal ligands. For example, the Fe_4S_4 cluster of high potential iron proteins (HiPIPs) has such an amide interaction, which was found to be responsible for stabilizing the reduced form through attenuation of a metal bound sulfur[77]. Replacement of this amide linkage with an ester linkage indeed resulted in a lowering of the reduction potential by ~100 mV.

Through the use of EPL, the metal bound Cys and Met residues in the electron transfer protein azurin were replaced with isostructurally similar unnatural amino acids (Fig. 3b)[78,79]. Replacing Cys with selenocysteine resulted in drastically different spectral features, but did little to affect the reduction potential. In contrast, replacing methionine with selenomethione and other isostructural variants resulted in spectral features similar to the wild type protein, but with drastically different reduction potentials. The use of unnatural amino acids helped to identify the hydrophobicity of the residues at this position as the major factor in tuning the reduction potential of the protein.

The repertoire of non-native functionalities has also been expanded through the incorporation of non-native metal containing cofactors into the protein scaffold. Biological systems incorporate metal cofactors such as heme through both non-covalent and covalent means. Among the covalent approach, Nature uses both single and dual point anchoring strategies to position the prosthetic group within the protein scaffold. These strategies have all been utilized

for incorporation of non-native metal cofactors, with the non-covalent approach being the most extensively used strategy. For example, replacement of the native heme cofactors of myoglobin and horseradish peroxidase (HRP) with the less symmetrical structural isomer, porphycene (Fig. 4b) results in enhanced peroxidase activity, highlighted by a twelve-fold increase in oxidation of thioanisole when compared to native HRP[80-82]. In addition to native protein scaffolds, *de novo* designed α-helical bundles have also been engineered to bind non-biological heme cofactors preferentially over biological hemes[83,84].

In addition to heme analogs, other planar moieties similar to heme have also been substituted into heme proteins. For example, strong binding of Mn, Fe, and Cr Schiff base complexes into a myoglobin scaffold was observed after careful design of the protein to relieve steric repulsion in the active site (Fig. 4b)[57,85,86]. Such rational design has resulted in novel asymmetric catalysts capable of oxidation rate enhancement and chiral induction.

To incorporate non-planar metal-containing cofactors into proteins where there is no native binding site is much more challenging. One way to meet such a challenge is through derivatization of the catalyst with a moiety exhibiting natural bio-affinity for a protein target, through, for example, the use of the high-affinity biotin-streptavidin couple ($K_a = 1 \times 10^{15}$ M^{-1}). In this way, metal cofactors have been incorporated into the streptavidin scaffold through covalent attachment to a biotin moiety (Fig. 4c)[87-89] resulting in artificial biocatalysts with new activities including hydrogenation of alkenes, transfer-hydrogenation of ketones, and C-C bond formation through allylic alkylation, and up to 99% ee[88-91].

While the use of biotin and avidin/streptavidin to incorporate non-native metal cofactors is very powerful, not all cofactors are amenable to such a strategy, as binding affinity and location may not be readily controlled. Covalent attachment is an effective and versatile approach to attach a cofactor to the protein at a defined location through selective conjugation techniques involving engineered or native Cys or Lys residues. This strategy has been utilized to attach DNA cleaving agents such as 1,10-phenanthroline-copper and iron-EDTA to DNA binding proteins for the generation of artificial nucleases, [92], and 1,10-phenanthroline-copper (Fig. 4d) for enantioselective hydrolysis[93,94].

While the single-point covalent approach mentioned above makes it possible to attach a non-native cofactor at a defined location, it does not necessarily fix the conformation of the cofactor inside the protein. For example, single covalent attachment of a 4-(2-methanesulfonylthio-ethoxy)salicylidene-1,2-ethanediamino-manganese(III) (Mn-Salen) cofactor to apo myoglobin through a single cysteine-selective linker resulted in only 12% ee toward sulfoxidation of thioanisole[95], suggesting that the Mn-Salen conformation may be too flexible inside Mb to facilitate high enantioselectivity. Addition of a second linker (Fig. 4e) to the cofactor and selective cavity positioning led to a significant increase in catalytic efficiency (over 7-fold increase) and selectivity (from 12% to 51%)[95]. Furthermore, it has been shown that such incorporation of Mn-Salen into a myoglobin scaffold enhances significantly the chemoselectivity in oxidation of thioanisole with H_2O_2, virtually eliminating over oxidation to the sulfone product through electrostatic exclusion of the sulfoxide product from the site[96].

Measures of success, lessons learned, and future challenges

Tremendous progress has been made in this exciting field of synthetic biology. Success is measured not only by how close the designed protein is to the native protein, but also by the insight gained from the process. For example, the crystal structure of a designed Cu_A site is almost identical to that of native Cu_A, including both primary and secondary coordination spheres (Fig. 5)[49], indicating that metalloprotein design can lead to structures closely resembling those in Nature. More importantly, two metal-binding sites, native blue copper and

the designed Cu_A center, were placed in the same azurin scaffold, allowing the electron transfer properties of two different metal centers to be compared in the same protein[97], a task that is difficult, if not impossible to do by studying native proteins. Such a comparison provided deeper insight that the Cu_A center is a more efficient ET center than blue copper[97].

Given the initial successes, it is a good time to summarize the lessons learned and to recognize future challenges. Essential to success is the recognition that structural biology must play a major role in the design process. Some early metalloprotein designs have generated much excitement in the field, only to be shown by x-ray crystallography to contain a different metal-binding site than originally designed. While spectroscopy is invaluable in providing support for the design, more concrete evidence from 3D structural characterization is essential. In addition to confirming the initial design, it provides detailed information on what has or has not been designed correctly, thus providing insights on the next level of design.

Furthermore, primary coordination sphere design is not enough in many cases, to confer structure and/or function. Consideration of the environment surrounding the primary coordination sphere is critical to successful design. One primary example is water inside the protein, where the associated hydrogen bonding interactions play essential roles in many metalloproteins, including Cyt P450, heme oxygenase (HO), and C*c*O[33]. For example, even though a Cu_B center was designed into myoglobin that structurally mimics the heme-copper center in C*c*O[53], its function mimics HO rather than C*c*O when O_2 is used as an oxidant[54]. When H_2O_2 is used as an oxidant, a ferryl species, a putative intermediate in C*c*O activity, was observed. These results suggest that the extra protons introduced through H_2O_2 are important for function[54]. The introduction of an additional hydrogen bonding donor through the replacement of the *b*-type heme in Mb with an *o*-type heme, resembling the heme found in C*c*O, allowed suppression of HO activity and promotion of C*c*O activity[98].

Metalloprotein design has focused on the positive design of structural features responsible for correct metal-site geometry and function. However, negative design to prevent competing metal-site geometries and unwanted functions is also critical. For example, in designing dinuclear metalloproteins in a helical bundle, there was a possibility that undesirable heterotetrameric motifs and homooligomers would form since the protein was comprised of different peptides. To circumvent this problem, a computational algorithm was included to destabilize these interactions[99].

The final lesson is that metalloprotein design often results in lower stability because of changes made to the protein scaffold. Sometimes, the stability is so low that even if the design is valid, not enough stable protein can be obtained to prove the design. The stability of the protein must be improved, even if function may not be improved directly. A beautiful example is demonstrated in cytochrome P450 where only after the protein was stabilized, could the highly destabilizing mutations introduced confer novel activity[100].

The majority of the successes in the metalloprotein design field are derived from the construction of a geometrically correct, sterically compatible primary coordination sphere sufficient to reproduce the structure and function of a desired metal center, such as a bis-His heme or His_2Cys_2 tetrahedral Zn(II) site. However, many metal-binding sites in proteins, such as the type 1 copper and Cu_A centers, are not necessarily in their preferred metal ion geometric states. Therefore, one future challenge is to design metal sites and metal clusters with unique geometric requirements. Other future challenges include the design of metal sites at the interface of two or more proteins, which occur often in proteins but have not been designed until recently[39,40], and the design of metal-binding sites from membrane proteins, which has also been rare. An even higher level of challenge is the design of protein metal-binding sites requiring helpers called chaperones. Consideration of the interactions between metal ions and

protein hosts is not enough to confer function, making their interactions with chaperones necessary in the design process. In addition to metal-binding site design, functional metalloproteins also require the design of a substrate-binding site for catalysis. An even higher level of complexity includes coupling the redox reaction of a designed metal-binding site to proton transfer, conformational change, or charge separation.

To meet these challenges, advances in a number of fields are required. The most important requirement is the development of better computer programs for designing metal-binding sites in proteins, including consideration of structural features beyond the primary coordination sphere. In contrast to well-defined force fields for peptide bonds and amino acid side chains, most force fields for metal-binding sites are ill-defined because there are many more variables involved in defining a metal-binding site. These variables include variations on the identity of the metal ion, different oxidation states of the same metal ions, and different ligand and geometric preferences of different metal ions. Density functional theory has been excellent in defining small metal complexes. Extending its success to metal-binding sites in proteins, together with other leading algorithms, such as QM/MM (quantum mechanics/molecular mechanics), is required for the successful rational design of metalloproteins. Despite these challenges, metalloprotein design will remain a vibrant field where exciting discoveries of new insights and novel enzymes will occur every day.

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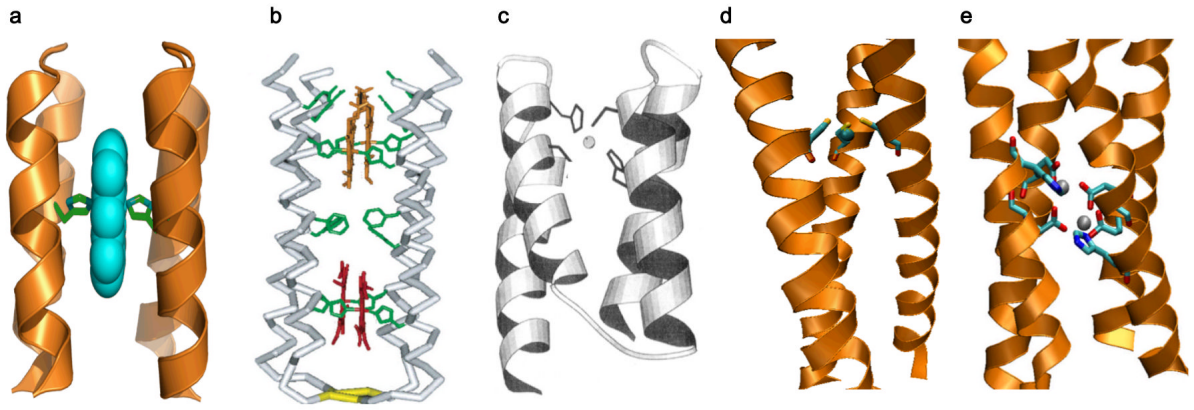

Figure 1. Designed metalloproteins using *de novo* designed scaffolds

a, Computer model of a bis-His ligated mono-heme α-helical bundle. Adapted from ref[6] courtesy of William DeGrado. **b,** Computer model of a bis-His ligated multi-heme four α-helical bundle. Reproduced from ref[5]. **c,** Computer model of Zn(II) bound His_2Cys_2 motif of a Zn-finger mimic. Reproduced from ref[14]. **d,** X-ray crystal structure of As(III) bound three-stranded coiled-coil (PDB 2JGO)[16]. **e,** X-ray crystal structure of di-Zn(II) Due Ferro 1; PDB 1EC5).[17]

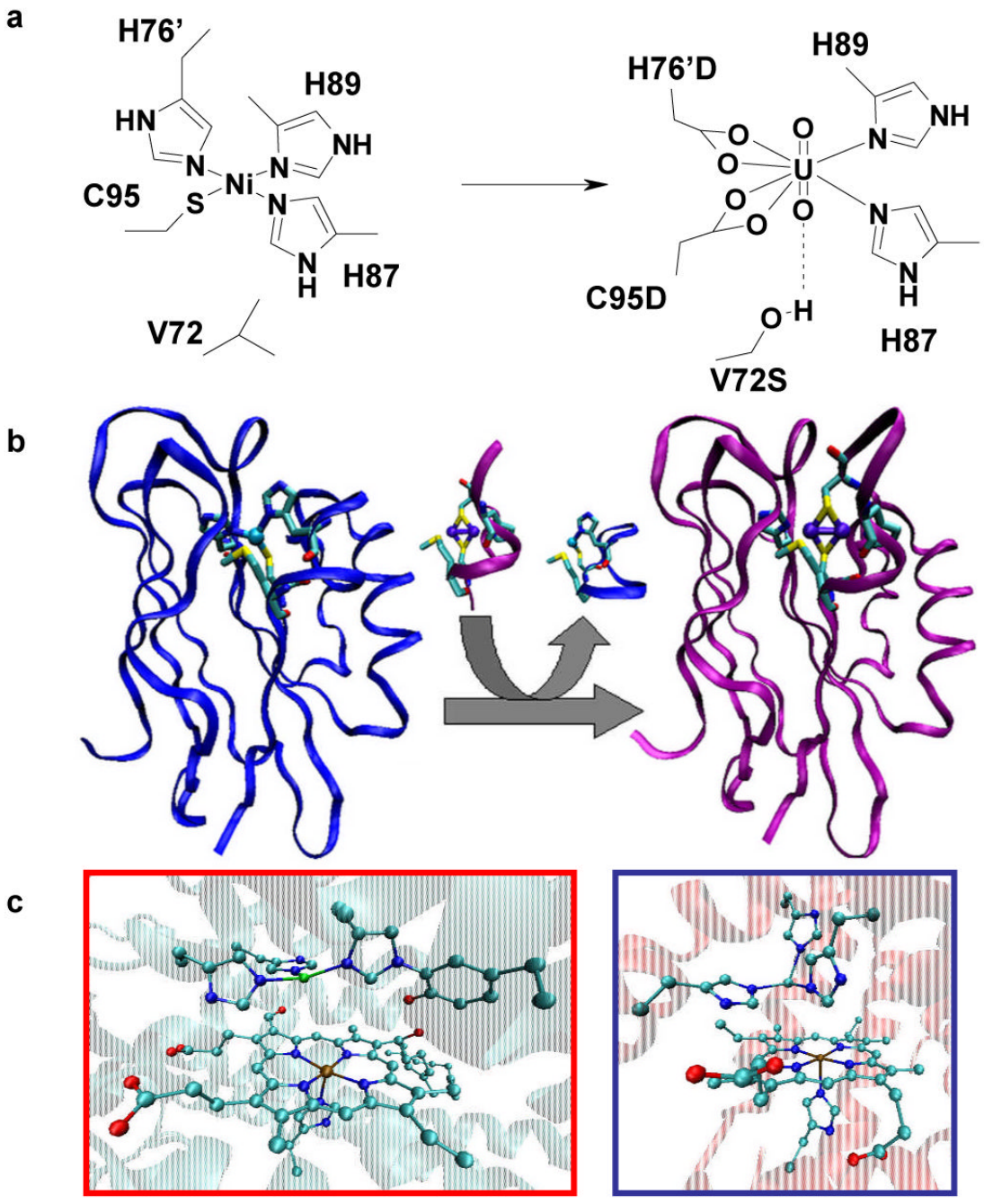

Figure 2. Designed metalloproteins using native scaffolds

a, Native nickel binding site of NikR (left) and the re-engineered UO_2^{2+} binding site of the mutated NikR (right). **b,** Loop directed mutagenesis of blue copper azurin to yield the dinuclear, purple Cu_A azurin construct. **c,** Catalytic heme-copper center in C*c*O (left) and the designed heme-cooper model in sperm whale Mb (right).

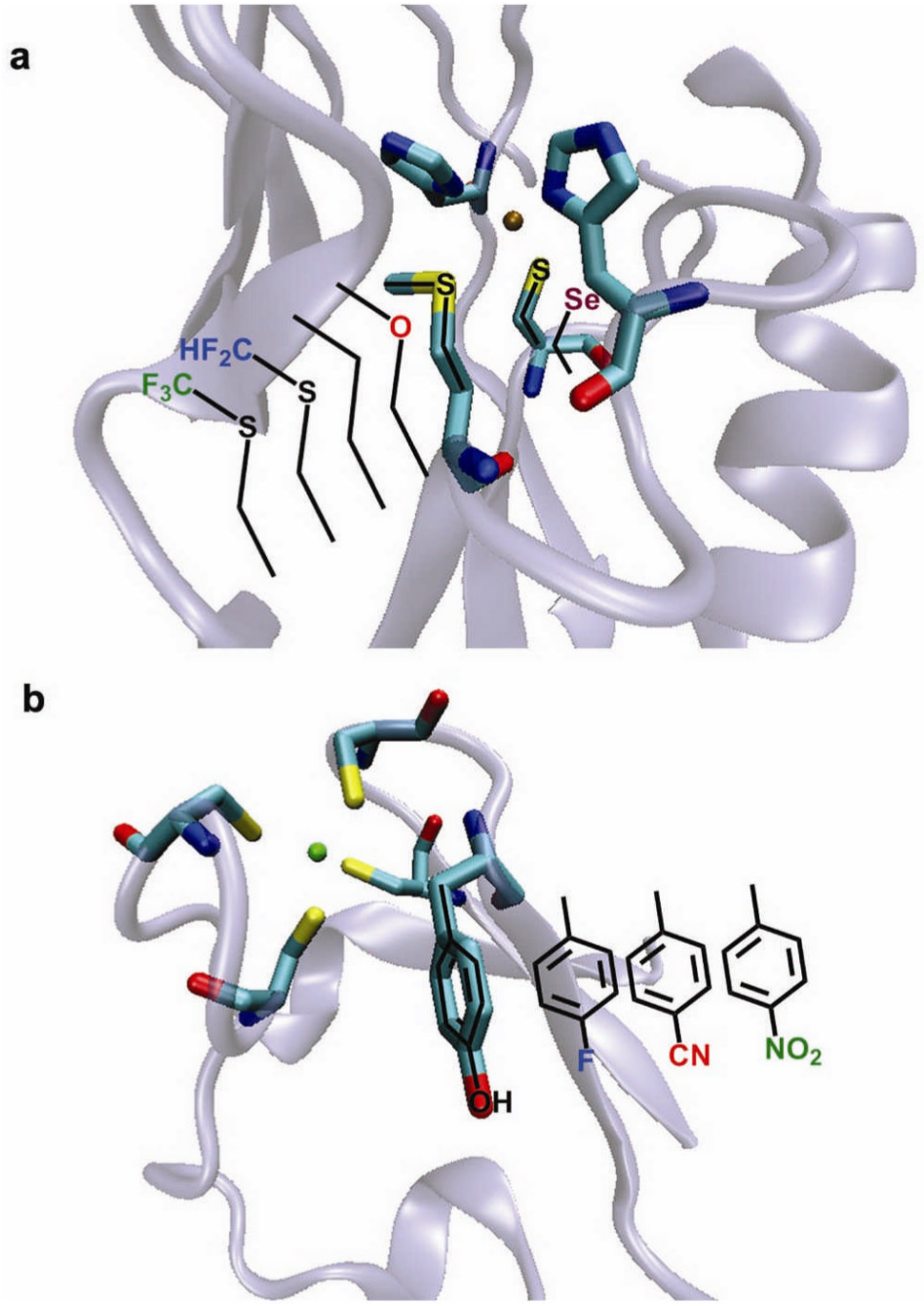

Figure 3. Site specific incorporation of unnatural amino acids into a protein scaffold for tuning of metal properties

Crystal structures of **a,** rubredoxin (PDB: 1CAA) and **b,** azurin (PDB: 4AZU) showing variant residue location for unnatural amino acid incorporation.

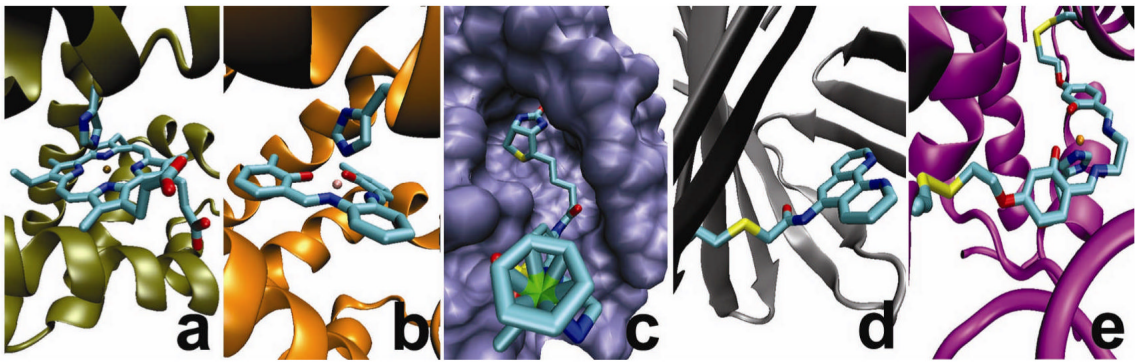

Figure 4. Strategies for non-native cofactor incorporation into a protein scaffold

Native cofactor substitution of heme *b* in myoglobin for **a,** Fe-porphycene and **b,** 3,3-Cr salophen, exploiting the structural and dative-bonding similarities between the native cofactor and the surrogate. Affinity tagging of the catalyst with a protein-selective linker such as the biotin-streptavidin couple, **c,** allows for versatile attachment of non-native cofactors. Covalent strategies include a single attachment strategy as in **d,** showing the adipocyte lipid binding protein (ALBP) linked through Cys117 to a phenanthrolene complex; and dual covalent attachment as in **e,** showing dual anchoring of a Mn-salen complex into the myoglobin scaffold. Description of streptavidin complex obtained from ref[91]. Crystal structure of Fe porphycene, Cr(3,3′-Me_2-salophen)-Mb complex, biotin-streptavidin complex, and phenanthroline-ALBP were obtained from the Protein Databank (PDB codes: 2D6C, 1J3F, 2QCB, and 1A18[94], respectively). Computer model of Mb(L72C/Y103C) generated from an overlay with native heme *b* structure.

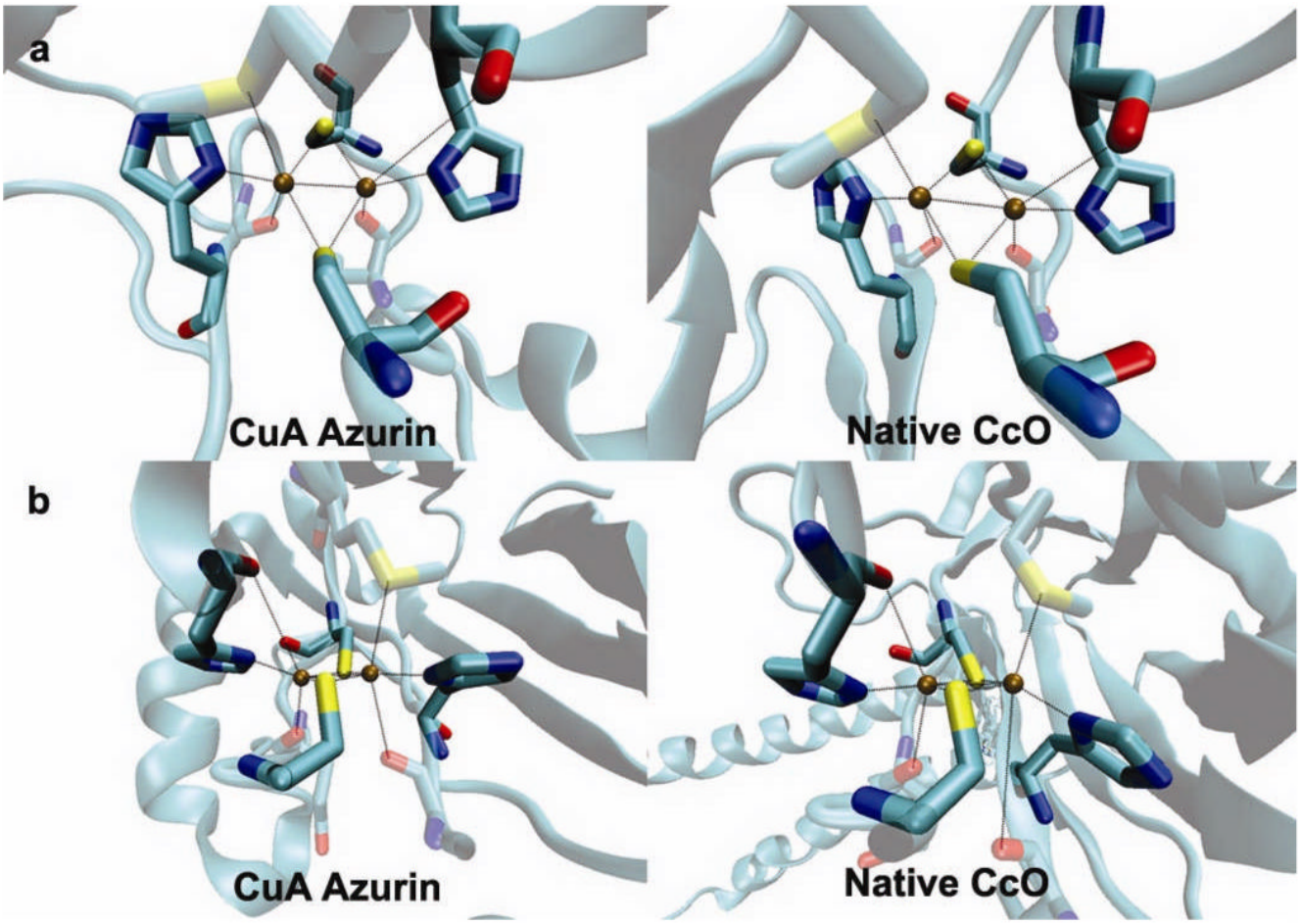

Figure 5. Close match between a designed metalloprotein and its native target protein

Crystal structures of a designed Cu_A site in azurin (PDB code 1CC3)[49] and a Cu_A site in native C*c*O (PDB code: 1AR1) as viewed from above the $Cu_2(S_{cys})_2$ plane **(a)**, and along the $Cu_2(S_{cys})_2$ plane.