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Design and engineering of metalloproteins containing unnatural amino acids or non-native metal-containing cofactors

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An emerging branch of metalloprotein design and engineering is on the horizon, where unnatural amino acids or non-native metal-containing cofactors are employed in the design and engineering process. These endeavors have been shown to be quite effective in elucidating the precise roles of key residues in protein structures and functions, in providing guiding principles on protein design, in fine-tuning the protein properties to an unprecedented level, and in expanding the repertoire of protein functionalities, and thus its range of applications.

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Introduction

Metalloproteins are among the most efficient and diverse biocatalysts as they employ metal ions such as copper or metal-containing cofactors or prosthetic groups such as heme. Exciting progress has been made in the design and engineering of metalloproteins that closely mimic their native counterparts [1–10]. Protein design is an excellent way to test our knowledge on structure and function of metalloproteins, to reveal new principles that may not be apparent from studies of native proteins, and to obtain small, stable and cost-effective model proteins for biochemical, biotechnological and pharmaceutical applications. However, a closer look at native metalloproteins reveals that they use only a small sub-set of ligands and of metal-containing prosthetic groups synthesized by inorganic chemists. For example, there are only 20 natural amino acids, less than half of which are capable of coordinating to metal ions, and the number of metal-containing cofactors is also limited. Therefore, an emerging branch in the field of metalloprotein design and engineering is the introduction of unnatural amino acids or non-native metal-containing cofactors into proteins. A major benefit of such an endeavor is elucidating the precise roles of key residues important in protein structure and function. By replacing natural amino acids or metal-containing cofactors with their unnatural analogs, it is possible to replace one specific structural element with another without grossly changing the overall structure of the site it replaces. In addition, this endeavor allows fine-tuning of protein functional properties at an unprecedented level. Therefore, the repertoire of protein functionalities and its range of applications can be expanded significantly. This review covers recent progress in this field, focusing mainly on work published since 2002.

Introducing unnatural amino acids into metalloproteins

Methods

Several methods have been developed to incorporate unnatural amino acids into proteins. A straightforward method is the total synthesis of proteins using solid-state peptide synthesizers [11]. This method allows incorporation of any unnatural amino acid at any specific location during the synthesis. Some limitations of this method include limited size (currently ~60–100 amino acids), requirement of appropriate protecting groups on reactive side chains, and relatively high costs. To extend the size range of the solid-state synthesis, a method was developed called native chemical ligation, where the protein is synthesized by the covalent attachment of two synthetic peptides with unprotected side chains via a peptide linkage [12]. This involves the nucleophilic attack on a C-terminal thioester of the N-terminal peptide by the N-terminal cysteine thiol of the C-terminal peptide, and is followed by an S→N acyl rearrangement that forms a native peptide bond.

For large proteins, methods free of size limitations are required. A classic method is the replacement of one type of amino acid in the protein with an unnatural amino acid by expression of the protein in cells lacking the capability to produce a particular amino acid (i.e. an auxotroph) and supplementing the growth media with an analog of the natural amino acid [13]. However, this method can only replace a limited number of amino acids, such as methionine, for which an auxotroph is readily available. Because the method replaces the entire complement of the given amino acid with the unnatural ones, it is often not position-specific. Another method is chemical modification of an amino acid residue, such as cysteine, located at the desired position [14,15]. Like the auxotrophic method, this technique is limited by the type of amino acid it can modify (mostly cysteines and lysines), and the

unnatural amino acid it can be converted into. A more specific way of introducing unnatural amino acids is by cavity complementation [16]. This approach involves replacing one of the metal ion ligands via site directed mutagenesis with small amino acids (Gly or Ala) to create a cavity. Addition of exogenous ligands of the appropriate size and character complement the cavity created by the replacement. The cavity complementation approach is useful only when a cavity can be generated with enough rigidity and the added unnatural ligand has strong enough affinity to the cavity, since the ligand is not covalently linked. The most specific method of placing unnatural amino acids into metalloproteins is the use of tRNA molecules, charged with unnatural amino acids that recognize a stop (amber) codon during *in vitro* translation [17–21]. Extension of this system *in vivo* in some cases has resulted in cost reduction and increase in yield [20–22]. A major limitation of this method remains the charging of the tRNAs with the unnatural amino acids. Expansion of unnatural amino acids beyond structural analogs of natural amino acids is also desirable.

A compromise between the synthetic and biological methods mentioned above is semisynthesis, where bacterially expressed peptides and/or synthetic peptides are coupled [23]. This method is advantageous because long peptides can be expressed in bacteria at low cost and in large quantities. Recent progress with the expressed protein ligation (EPL) method enables coupling of a bacterially expressed peptide with a synthetic peptide without the requirement of protecting groups, which increases overall yields [24]. Limitations of this method include the requirement of a cysteine residue at the junction of the expressed protein and the synthetic peptide, and difficulty in introducing unnatural amino acids in peptides not close to either the C- or N-terminus of the protein. Innovative methods are being developed to overcome these limitations [23]. Therefore, EPL is one of the most promising methods for efficient and cost-effective production of large proteins containing unnatural amino acids at specific positions with quantities sufficient for spectroscopic and X-ray crystallographic studies.

Recent examples

The solid state total synthesis method has been applied to the introduction of unnatural amino acids into *de novo* designed proteins. For example, two His residues in a *de novo* designed four-α-helix bundle heme protein have been replaced by 4-β-(pyridyl)-L-alanines (Figure 1a) [25••]. This change from a bis-His to bis-pyridyl ligation resulted in ~60 000-fold decrease in protein binding affinity to ferric heme and a 287 mV increase in heme reduction potential. Similarly, replacement of the His ligands in a *de novo* designed four-α-helix bundle heme protein with 1-methyl-L-His resulted in > 700 000-fold weaker affinity for the ferric heme (Figure 1a), but only a 125-fold decrease affinity for the ferrous heme [26]. Interestingly, methylation of the N^{ε} position of the His residue prevented the favored coordination mode and resulted in a five-coordinated high-spin ferrous heme protein similar to deoxymyoglobin. The resulting protein forms a stable CO-adduct, but not the O_2 complex.

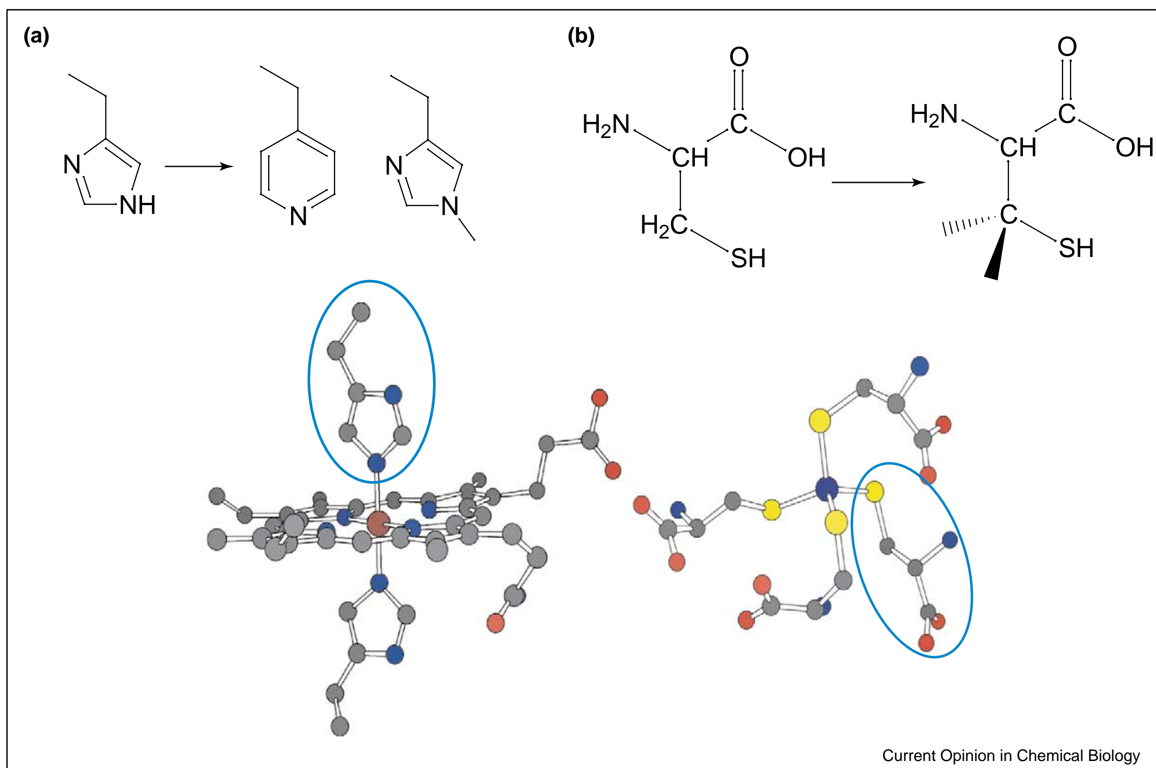
Creation of an open-binding site for substrate binding and reaction in *de novo* designed proteins has been a long sought-after goal. Introduction of steric bulk using unnatural amino acids has now been shown to be an effective strategy to achieve this goal [26]. This principle has also been demonstrated by replacement of four cysteines in a ferredoxin model maquette with penicillamines (Pen) (Figure 1b). The added steric bulk of the Pen ligands transformed the designed metalloprotein from a symmetrical $(S\bullet Cys)_4$ ligation to an asymmetrical $(S\bullet Pen)_3$-$(H_2O)_1$ ligation [27].

For larger proteins, native chemical ligation has been used to probe the roles of aromatic residues in rubredoxin [28], the backbone amide–ligand interactions in high-potential iron proteins (HiPIPs) [29], and the heme axial ligand in cytochrome b_{562} [30]. All rubredoxins feature a conserved Tyr near one of the Cys ligands (Figure 2a). Replacing the -OH group at the *para* position of the Tyr with -H, -F, $-NO_2$ and -CN groups resulted in a linear relationship between the reduction potentials of the heme and the Hammett σ_p values (a common measure of the electronic effects of the substituents on aromatic molecules), with electron-withdrawing groups displaying more positive potentials [28]. On the other hand, the reduction potentials do not correlate well with the dipole moment of the side chain nor with the size of the substituents.

Perhaps the biggest advance of introducing unnatural amino acids into metalloproteins is to probe the role of backbone amides, as mutagenesis using natural amino acids cannot replace these. For example, several conserved hydrogen-bond networks have been found between the backbone amides and the metal-bound ligands, such as the cysteine ligand of the Fe_4S_4 cluster in HiPIPs (Figure 2b). Such a hydrogen bond was proposed to stabilize the reduced form of the protein by attenuating the charge density on the metal-bond sulfur, and thus raising the reduction potential. Experimental support for such a proposal was obtained through the removal of hydrogen bonds in HiPIP; placing an ester linkage at the corresponding position resulted in lowering the reduction potential of the iron center by ~100 mV [29].

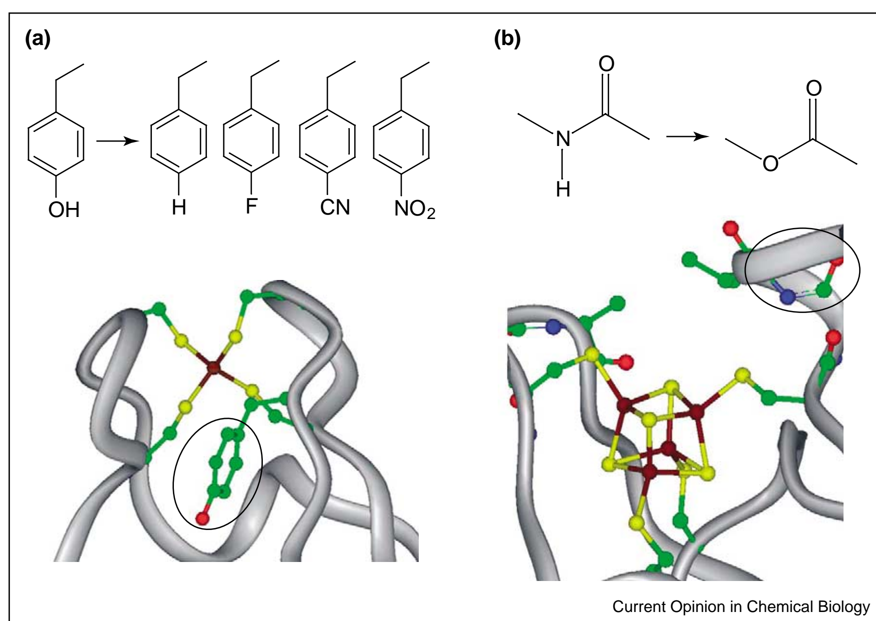
For even larger proteins such as cytochrome P450, the amino acid auxotroph method has been used to replace all of the Met residues with norleucine [31]. This global substitution resulted in a roughly twofold increase in peroxygenase activity (i.e. oxidation of organic substrates

Figure 1



Use of solid-state peptide synthesis in introducing unnatural amino acids into metalloproteins. **(a)** Substitution of histidine ligands to the heme in a *de novo* designed four-α-helix bundle with 4-β-(pyridyl)-L-alanines or 1-methyl-L-histidine. **(b)** Replacement of cysteine ligands to the $Fe_4(S\bullet Cys)_4$ cluster in designed ferredoxin with penicillamines.

Figure 2



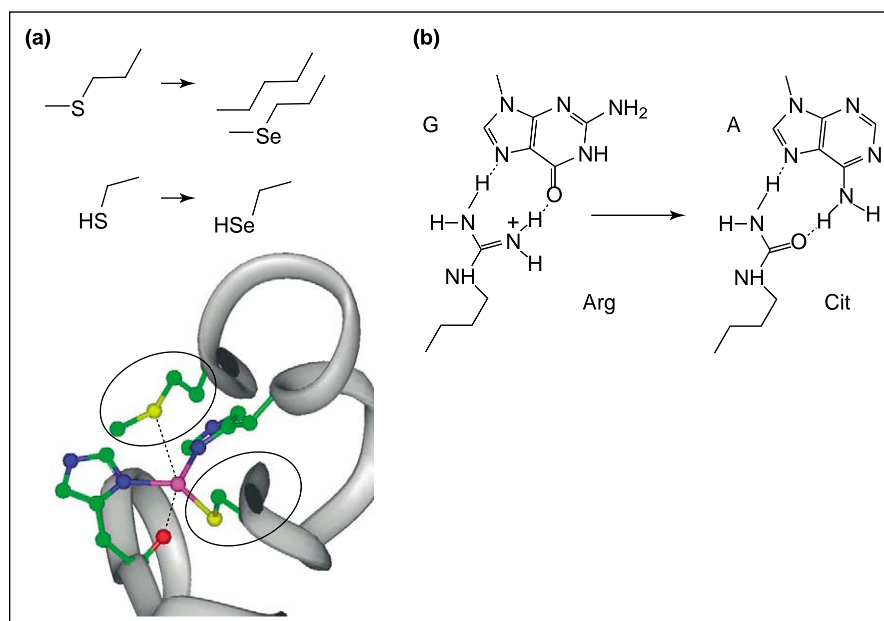
Use of native chemical ligation in introducing unnatural amino acids into metalloproteins. **(a)** Substitution of a tyrosine in a rubredoxin with phenyl alanine and tyrosine analogs. **(b)** Replacement of a backbone amide that is hydrogen-bonded to a cystetine ligand to a $Fe_4(S\bullet Cys)_4$ cluster in HiPIP with an ester.

using hydrogen peroxide, an activity that is normally performed inefficiently by cytochrome P450, a monooxygenase). For specific incorporation of unnatural amino acids, the cavity complementation method has been used to introduce neutral thiol and thioether sulfur donor ligands into a cavity created by replacing the proximal His ligand in myoglobin [32]. This study establishes that neutral cysteine can serve as a ligand in ferrous heme iron proteins, and that ferric cysteinate-ligated heme proteins that fail to retain such ligation on reduction may simply be ligated by neutral cysteine. Finally, recent advances in expanding genetic code through taking advantage of the stop (amber) codon have resulted in specific incorporation of an unnatural amino acid, *p*-aminophenylalanine, into myoglobin [33]. This was accomplished through generation of a completely autonomous bacterium that can biosynthesize the unnatural amino acid from basic carbon sources and incorporate the amino acid with fidelity and efficiency analogous to that of natural amino acids.

Using the expressed protein ligation method, both Cys and Met ligands in type 1 blue copper azurin have been replaced site-specifically with selenocysteine (SeCys) [34••,35] and selenomethionine (SeMet)/norleucine [36•], respectively (Figure 3a). These isosteric replacements of the ligands to the copper center allowed fine-tuning of the structural and functional properties of the proteins while maintaining its overall metal-binding site characteristics. For example, replacing Cys with SeCys in azurin resulted in marked changes in the electronic absorption (UV-vis) and electron paramagnetic resonance (EPR) spectroscopic features (e.g. 50 nm red shift of the visible charge transfer band, and roughly twofold increases in EPR hyperfine coupling constant). However, the replacement resulted in little change in reduction potential of the copper center. A closer examination of the geometric changes by extended X-ray absorption fine structure (EXAFS) spectroscopy indicate that the Cu–Se bond lengths were found to undergo only minor changes during reduction, suggesting a very similar structure in both redox states and extending the 'rack' hypothesis (i.e. the tertiary structure of the protein creates a preformed site intermediate between those preferred by Cu^{2+} and Cu^{+}) to the Se-substituted protein [35]. On the other hand, replacing Met with SeMet and norleucine resulted in little change in UV-vis and EPR spectral features, but dramatic change (25 and 140 mV, respectively, increases over the native protein) in reduction potentials. The use of isostructural unnatural amino acids at the Met position allowed deconvolution of different factors influencing the reduction potentials of the blue copper azurin by this axial ligand. A linear relationship between the reduction potential and the hydrophobicity of the axial ligand side chains pointed to hydrophobicity as the dominate factor controlling reduction potential [36•].

The expressed protein ligation method has also been used to replace arginine (Arg) with citrulline (Cit) in a zinc-finger protein to expand its DNA recognition

Figure 3



Use of expressed protein ligation in introducing unnatural amino acids into metalloproteins. **(a)** Substitutions of cysteine and methionine ligands to the blue copper center in azurin (shown to left) with selenocysteine and selenomethionine and norleucine, respectively. **(b)** Replacement of arginine in a zinc finger protein with citrulline. Adapted from reference [37••], with permission. Copyright 2003, American Chemical Society.

repertoire [37••]. The DNA-binding specificity of the zinc-finger protein is conferred primarily by three amino acids within the zinc-finger domain [10]. One of the amino acid residues, Arg in position 6 of the carboxy-terminal domain, recognizes the 5′-most guanosine (G) through two hydrogen bonds (Figure 3b). Replacing Arg with other natural amino acids generally resulted in either similar (in the case of Lys) or decreased (in the case of Gln) specificity for G, because Lys also possesses only hydrogen bond donors as in Arg, and Gln is shorter than Arg. Replacing Arg with Cit resulted in the same length as Arg. However, because Cit possesses only one hydrogen-bond donor and one acceptor (rather than two hydrogen bond donors), it can now recognize adenosine (A) with high specificity.

Introducing non-native metal-containing prosthetic groups into proteins

Methods

Native metal-containing prosthetic groups, such as heme, have been incorporated into proteins through either non-covalent attachment (as in myoglobin [9]), single point covalent attachment (as in a few protozoan mitochondrial cytochromes *c* [38]), or dual point covalent attachment strategies (such as in most other cytochromes *c* [39]). Because native proteins are not evolved to bind non-native metal-containing prosthetic groups, such as metal-losalens, strongly and specifically, careful design is required for successful incorporation. Analogous to how nature incorporates native prosthetic groups, three methods, non-covalent, single-point covalent and dual-point covalent attachment, have been demonstrated in introducing non-native metal-containing prosthetic groups into proteins. The non-covalent method is the most challenging as careful design and modifications of both the prosthetic groups and the proteins are required for optimal binding. To restrict the freedom of rotation, single covalent attachment can be used. When the single covalent attachment does not sufficiently lower degrees of freedom of rotation, dual-point covalent attachment can be employed.

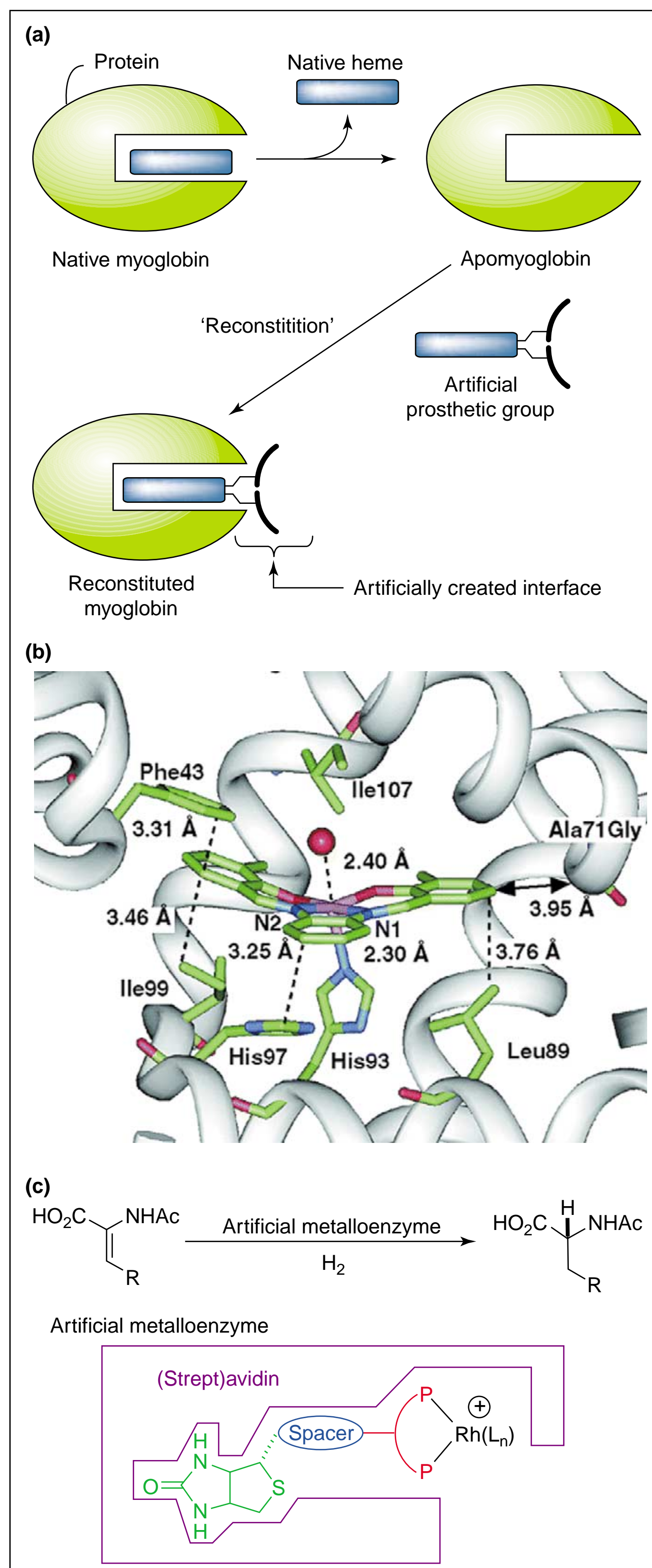
Recent examples

The non-covalent approach has been used to replace heme in heme proteins such as myoglobin with modified metalloporphyrins, such as protoporphyrin IX modified at the two propionate groups (Figure 4a) [7,40]. These modifications allowed covalent attachment of several non-native cofactors close to the heme in the proteins. The non-native cofactors include metal complexes for electron transfer studies and artificial receptors for modulation of heme protein activities in response to specific guest molecules. The work has also created effective binding domains for protein–protein and protein–small-molecule recognition and electron transfer. Extensive review has appeared on these results [7,40] and will not be repeated here. Recently, when the heme has been replaced with iron porphycene [41] or modified by flavin at one of the propionate groups [42••], dramatically enhanced O_2-binding affinity or O_2 activation activities have been observed, respectively. In addition to the heme, heme enzyme substrate (such as camphor for cytochrome P450cam) or ligand (such as histidine) have been modified to attach ruthenium complexes, through a covalent electron tunnelling wire, to the substrate or the ligand so that fast (nanosecond) electron transfer can be achieved, to observe transient intermediates that are otherwise difficult to see using native redox partners [43,44].

While the use of modified hemes or substrates takes the advantage of a protein's ability to bind to heme or substrate strongly, non-covalent incorporation of other non-native metal-containing cofactors that bear much less structural similarity to the native cofactor proves to be much more challenging. For example, Mn(III), Fe(III) and Cr(III) Schiff base complexes (such as Mn(III)salen and Cr(III)salophen) are well known to be efficient oxidation catalysts. To incorporate these complexes into the heme-binding site of myoglobin, a structural based design was used to modify both the metal complexes (such as substitution of a tert-butyl group at 5- and 5′-position of salophen) and the protein (such as replacement of Ala71 with Gly) [45••,46]. Crystal structures of an Fe(III) derivative of the complex with myoglobins indicate a highly disordered metal complex inside the myoglobin pocket due to steric repulsion with Ala71 [46]. When this steric conflict is relieved by the Ala71Gly mutation, the metal complex can bind strongly to the myoglobin (Figure 4b). The structure also suggests a possible cavity for small substrates. Indeed, when the distal His64 was changed to Asp, the Cr(III) derivative of this metal-cofactor–protein complex was capable of catalyzing asymmetric sulfoxidation of thioanisole with up to 13% enantiomeric excess (*ee*) [45••].

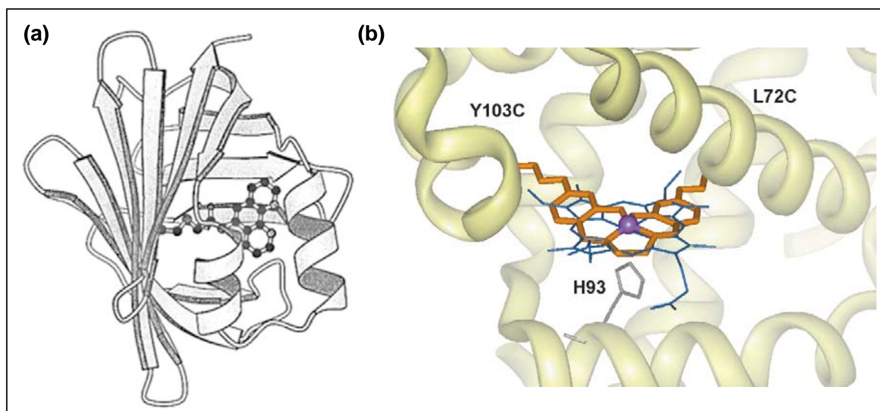
Another method for the non-covalent incorporation of metal complexes that bear little structural similarity to the native metal cofactor (or proteins lacking cofactors) is through covalent attachment of the metal complex to biotin, and thus 'borrowing' biotin's strong binding affinity to the protein avidin (Figure 4c). This concept was first established by Wilson and Whitesides who incorporated a biotinylated diphosphinerhodium(I) complex into avidin and showed that it can catalyze asymmetric hydrogenation of α-acetamidoacrylic acid with up to 44% *ee* [47]. Chan and co-workers have then extended the concept by transforming a different diphosphinerhodium(I) complex into an asymmetric hydrogenation catalyst for itaconic acid with up to 48% *ee* [48]. They observed that the enantioselectivity of the system was significantly influenced by the tertiary conformation within the cavity. Recently, the full advantage of this approach has been demonstrated by Ward and co-workers [49,50••], who

Figure 4



Non-covalent approaches to introducing non-native metal-containing cofactors into proteins. **(a)** Replacement of heme in myoglobin with modified heme. **(b)** Crystal structure of Fe^{III}(3,3′-Me_2-salophen) incorporated into Ala17GlyMb. **(c)** Incorporation of metal complex into proteins through biotin and avidin binding. Parts (a–c) are adapted from references [7,46,50••], respectively, with permission from the American Chemical Society.

Figure 5



Covalent approaches to introducing non-native metal-containing cofactors into proteins. **(a)** A single covalent attachment strategy. A computer model of adipocyte lipid binding protein–phenanthroline complex. **(b)** A dual covalent attachment strategy. A computer model of Mb(L72C/Y103C) with a Mn^{III}-Salen complex covalently attached at two-points and overlayed with heme in myoglobin. Parts (a,b) are adapted from references [53,55••], respectively, with permission from the American Chemical Society.

obtained new diphosphinerhodium(I)-protein complexes that can catalyze asymmetric hydrogenation of acetamidoacrylic acid with up to 96% *ee*. They achieved this goal by first using streptavidin, a protein closely related to avidin but with a deeper binding pocket for biotin, and then through combinatorial chemical modifications of the biotinylated metal complex and site-directed mutations of the protein.

Covalent linkage is an alternative approach for site-specific incorporation of a non-native metal complex that bears little resemblance to the native metal cofactor. Instead of borrowing affinity from strong non-covalent binding between avidin and biotin, this strategy relies on even stronger and more specific bioconjugation methodologies, often through either Cys or Lys. A high yield (~100%) of incorporation can be achieved with minimal structural modifications to either the complex or the protein host, even though the complex itself has minimal affinity to the protein host. For example, 1,10-o-phenanthroline-copper, an effective DNA cleavage agent, has been attached to several DNA-binding proteins [51]. The combination of high cleavage activity of the metal complex with specific DNA recognition selectivity makes the artificial metalloproteins very effective nucleases. Iron-EDTA has been incorporated into proteins for the same reason [52]. Furthermore, Distefano and co-workers have demonstrated that covalent attachment of a Cu(II)/1,10-phenanthroline complex to a single cysteine in an adipocyte lipid-binding protein results in a catalyst that promotes highly enantioselective hydrolysis (with up to 86% *ee*) (Figure 5a) [14,53].

The above successful examples utilize single point covalent attachment methodologies. However, for certain proteins, the single point attachment may not provide enough conformational restriction to the metal complex to confer optimal enantioselectivity. For example, when a Mn-(salen) complex was attached to papain via a single maleimide linker, less than 10% *ee* was observed [54]. Similarly, when another Mn-(salen) complex was attached to the apo-myoglobin through a single methane thiosulfonate linker, only 12% *ee* was obtained. To overcome this limitation, a dual anchoring strategy was used for precise control of the placement of the artificial metallo-complex with specific orientation and limited rotational freedom (Figure 5b) [55••]. This method, when applied to apo-myoglobin, resulted in a significant increase in *ee* (51%). A combination of the dual point attachment strategy with structure-based design, or with combinatorial modifications may be a promising method for designing future efficient asymmetric catalysts.

Conclusions

Important progresses have been made in designing and engineering of metalloproteins that contain unnatural amino acids or non-native metal-containing cofactors. Through the introduction of unnatural amino acids into metalloproteins, subtle roles of key residues around the metal-binding sites have now been elucidated to an unprecedented level of detail. Systematically fine-tuning the metalloprotein's functionality to beyond that of native proteins has also been achieved. By introducing non-native metal-containing cofactors, factors important for binding metal-containing prosthetic groups with high affinity and specificity have been revealed and new metalloproteins with novel structures and functions have been obtained. These accomplishments mark the beginning of an exciting new era of metalloprotein design and engineering. A future direction of this field may be design

and engineering of proteins that incorporate both unnatural amino acids and non-native cofactors in the active site. The clever combination of the advantages of both practices will result in even deeper insight to the fundamental understanding of proteins, and in development of novel proteins for practical applications.

Update

After the review was written, a complete *de novo* design of a native-like four-helix bundle protein that selectively binds a non-biological metalloporphyrin over the native heme was demonstrated [56••]. This work opens a new avenue for computational design of non-native metal-containing cofactors into proteins.

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