Rational design of metalloenzymes: From single to multiple active sites

Ying-Wu Lin ^{a,b*}

Abstract

Artificial metalloenzymes combine the advantages of both natural enzymes and chemically synthesized models, which have achieved significant progress in the last decade. This review summarizes the recent achievements in rational design of metalloenzymes from single to multiple active sites in natural or *de novo* protein scaffolds, with a diverse range of functionalities, even beyond those of natural metalloenzymes. These achievements include construction of mononuclear active site by metal substitution or incorporation, design of homo- or hetero-dinuclear site, introduction of Fe-sulfur or other metal clusters, reconstitution of metallo-porphyrins or other metal complexes, and design of dual or multiple active sites in single, dimeric proteins, *de novo* proteins, protein-protein interfaces, as well as protein oligomers and polymers. Other new trends in recent designs are also discussed. The progress not only elucidates the structural and functional relationship of natural metalloenzymes, but also provides practical clues for design of advanced artificial metalloenzymes with potential applications.

Keywords: Metalloproteins; Metalloenzymes; Protein design; Multiple active sites; Biocatalysts

Abbreviations: UAAs, unnatural amino acids; Rd, rubredoxin; EPR, electron paramagnetic resonance; Az, azurin; SOR, superoxide reductase; PDB, protein data

bank; 6-PGLac, 6-phosphogluconolactonase; H-bond, hydrogen-bond; NIR, nitrite reductase; ET, electron transfer; 3SCC, three stranded coiled coil; Bpy-Ala, (2,2'-bipyridinine-5-yl)alanine; CcO, cytochrome *c* oxidase; N₂OR, nitrous oxidase reductase; XANES, X-ray absorption near-edge structure; DF, Due Ferri; 4-AP, 4-amino-phenol; POP, prolyl oligopeptidase; ee, enantiomeric excess; HCO, heme-copper oxidase; NOR, nitric oxide reductase; MnP, manganese peroxidase; Mb, myoglobin; RFQ-rR, freeze-quench resonance Raman; CcP, cyt *c* peroxidase; BMC, microcompartment; CYP, cytochrome P450; TON, turnover number; TDHC, tetrahydrocorrins; NB, nitrobindin; RCM, ring-closing metathesis; ROMP, ring-opening metathesis polymerization; SAV, steptavidin; NCS, neocarzinostatin; BLG, β -Lactoglobulin; HSA, human serum albumin; BSA, bovine serum albumin; ITC, isothermal titration calorimetry; AldO, alditol oxidase; FAD, flavin adenine dinucleotide; MP, microperoxidase; HB, heme-binding; GTL, *Geobacillus thermocatenulatus* lipase; 4NPA, 4-nitrophenyl acetate; LmrR, Lactococcal multidrug resistance Regulator; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; *m*CPBA, meta-chloroperbenzoic acid; Fd, ferredoxin; Fld, flavodoxin; HTHP, hexameric Tyr-coordinated heme protein; AFM, atomic force microscopy; TEM, transmission electron microscopy; MRI, magnetic resonance imaging.

1. Introduction

Metalloenzymes are a major class of metalloproteins in biological systems, catalyzing fundamental reactions, such as O₂ reduction and N₂ fixation, with high reactivity and selectivity. A prerequisite for understanding the biological systems is to understand the structural and functional relationship of metalloenzymes. To achieve this goal, it is required not only the study of natural metalloenzymes, but also the ability to create artificial metalloenzymes [1, 2]. With the relative high abundance of the first row transition metals in living systems, natural metalloenzymes usually choose to use these metals for biological catalysis [3]. Not only metal ions, but also the type of metal cofactors, such as heme and iron-sulfur clusters, are limited for natural metalloenzymes [4]. Additionally, there are only twenty-two naturally occurring amino acids including selenocysteine and pyrrolysine, and half of them can serve as ligands for the metal center. These limitations thus narrow the scope of functionalities for natural metalloenzymes.

Alternatively, chemical catalysts with diverse reactivities can be developed by using the transition metals ions from the first row to the third row in the periodical table. These catalysts can be designed and synthesized to mimic the catalytic site of natural metalloenzymes [5-7]. Meanwhile, it is difficult to incorporate secondary coordination sphere interactions into the models, which has achieved progress recently, such as the use of intramolecular hydrogen bonds and other non-covalent interactions to regulate the function [8-15]. Moreover, chemically synthesized models are lack of long-range interactions that are also critical for natural metalloenzymes to achieve high reactivity and selectivity [16]. The synthesis of chemical enzyme models is time-consuming. In addition, chemically synthesized models usually work only in

organic solvents, which is thus not environmental benign.

To overcome the limitations of both natural metalloenzymes and chemically synthesized models, many efforts were made to combine their favourable features by design of artificial metalloenzymes in the last decade (Fig. 1) [2, 17-43]. During this course, many approaches were developed, including metal substitution, metal cofactors replacement, and unnatural amino acids (UAAs) incorporation in native or *de novo* protein scaffolds, as well as with the help of computational designs [44-51]. In addition, a unique feature of artificial metalloenzymes is the possibility of random mutagenesis followed by high-throughput screening [52-55]. The achievements not only deepened our understanding of the structural and functional relationship of metalloenzymes, but also expanded the reaction scope of natural metalloenzymes. Recently, in addition to design of mononuclear metal center, new achievements have been made in design of artificial metalloenzymes with dual and multiple active sites, not only to mimic those of more complicated complexes of natural metalloenzymes, but also to create more advanced metalloenzymes with functions beyond those of natural enzymes.

In this review, the recent achievements in rational design of metalloenzymes from single to multiple active sites were summarized, especially from the past three years (2014-2016), by either redesign of natural metalloenzymes or design of *de novo* metalloenzymes. Some other new trends in recent designs were also discussed. These achievements provide not only deep insight into the structure-function relationship of natural metalloenzymes, but also practical clues for creating metalloenzymes with advanced functions and potential applications.

Fig. 1 Features and limitations of natural metalloenzymes and chemically synthesized models, in comparison to those of artificial metalloenzymes.

2. Design of metalloenzymes with a mononuclear site

2.1 Metal-substitution

Natural metalloenzymes usually use the first row transition metals for biological catalysis [3], leading to limited functionalities. Therefore, a straightforward approach for design of metalloenzymes with a mononuclear site is to substitute the existing metal ion with a non-native or non-biological metal ion, which may offer new opportunities to design artificial metalloenzymes with advanced functions [1, 2, 56]. A notable example is for Rubredoxin (Rd), an iron-sulfur protein with a single iron coordinated by four Cys residues in a conserved $-CX_2C-X_n-CX_2C-$ binding motif (Fig. 2a) [57]. An apo-form (apo-Rd) can be prepared by acid (such as trichloroacetic acid, TCA) precipitation under reducing conditions. In the past two decades, a series of metal-substituted Rd proteins were prepared by incorporating distinct metal ions, including Fe(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II), Hg(II), Ga(III) and In (III), into

the iron-binding site of apo-Rd [57-61], For example, Shafaat and Slater [61] prepared a Ni(II)-substituted Rd, which is capable of light-initiated and solution-phase H_2 production, representing a minimal metalloenzyme model for native hydrogenase.

Recently, Moura and co-workers [62] successfully incorporated molybdenum (Mo) into apo-Rd (Fig. 2b), which mimics the mononuclear molybdoenzymes that contain a Mo atom coordinated by the *cis*-dithiolene group of one or two pyranopterin cofactors, as well as by other atoms such as oxygen, sulfur or selenium atoms. Electron paramagnetic resonance (EPR) and Raman spectroscopic studies indicated that a stable Mo(VI)-(S-Cys₄)(=O)(-OH or -SR) center was formed in the Mo-Rd, which can be reduced to Mo(IV) by dithionite *via* a Mo(V) species. Importantly, the constructed Mo(VI)-Rd promotes oxidation of arsenite ($As^{III}O_3^{3-}$) to arsenate ($As^VO_4^{3-}$) (Fig. 2c), although it has a less efficiency compared to native arsenite oxidase. Therefore, this study presents a novel example for conversion of one-electron transfer protein (Fe-Rd) into an oxygen atom/two electrons transfer metalloenzyme (Mo-Rd) by simple metal-substitution.

Fig. 2 Design of mononuclear metalloenzyme by metal-substitution in Rd. (a) X-ray crystal structure of Rd showing the Fe(III)-binding site (PDB code 1IRO) [57]; (b) Procedure for preparation of oxidized oxo-Mo^{VI}-Rd and of reduced Mo^{IV}-Rd [62]; (c) Spectral change of oxo-Mo^{VI}-Rd reacted with 1 mM arsenite. *Inset*, absorbance change at 360 nm *versus* time in presence of 0.1-1.0 mM arsenite. Reprinted with permission from ref. [62], Copyright 2015 Springer.

Since different metal ions may prefer different ligands as well as coordination structure, the existing metal-binding site in natural metalloenzymes may not have suitable ligands and conformation for binding other non-native metal ions, which is thus a limitation in design of metalloenzymes by direct metal-substitution. Therefore, redesign of the active site may help to improve the metal binding affinity, as well as the reactivity. For example, Azurin (Az), an electron protein, prefers to bind both Cu(I) and Cu(II) with a binding motif of 2-His-1-Cys (His46, His117 and Cys112) in

the trigonal plane, with Met121 and the backbone carbonyl of Gly45 in the axial positions (Fig. 3a) [63]. Fe(II) can also be incorporated into the copper-binding site whereas with a low occupancy of 35%, and Fe(II)-Az was redox-inactive [64]. Lu and co-workers [65] made an effort to redesign the metal-binding site by replacing M121 with a Glu, which increases the binding affinity of M121E Az for Fe(II) (100% occupancy). Moreover, the negatively charged Glu121 lowered the reduction potential of Fe(II)-M121E Az relative to that of Fe(II)-Az, making the Fe(II) center readily oxidized by the one-electron oxidant Na_2IrCl_6 . More interestingly, Fe(II)-M121E Az exhibits superoxide scavenging activity, although substantially lower than that of native superoxide reductase (SOR). Furthermore, a mutation of M44K was made in the secondary sphere of the Fe(II) center (Fig. 3b) to closely mimic the key K48 in native SOR that is critical for reactivity by guiding the binding of superoxide, which increases the SOR activity by two orders of magnitude (Fig. 3c). These findings demonstrate that metal-substitution, in combination with redesign of the active center and modification of the secondary sphere, is powerful in design of functional metalloenzymes with an active mononuclear site.

Fig. 3 Design of mononuclear metalloenzyme by metal-substitution in Az. (a) X-ray crystal structure of Az showing the Cu(II)-binding site (PDB code 4AZU) [63]; (b) X-ray crystal structure of Fe(II)-M121E Az (PDB code 4QLW), highlighting the role of E121 as well as of K44 in the secondary sphere [65]; (c) Reactivity of Fe(II)-M121E Az and Fe(II)-M121E/M44K Az toward superoxide. Reprinted with permission from ref. [65], Copyright 2014 American Chemical Society.

2.2 Metal-incorporation

Although more than a half natural proteins are no-metal containing proteins [66], it does not mean that they definitely do not bind metal ions. Ward and co-workers [67] did a systematic search in the protein data bank (PDB) and identified 121 non-metallated proteins with a potential metal-binding motif of 2-His-1-Glu/Asp. Therefore, these proteins offer idea protein scaffolds for directly incorporating metal ions in the latent metal-binding site, which may confer catalytic activity. As

confirmed recently, Fujieda, Ward and co-workers [68] showed that upon Cu(II) binding to 6-phosphogluconolactonase (6-PGLac) (Fig. 4a), Cu(II)-6-PGLac exhibited an impressive peroxidase activity ($k_{\text{cat}}/K_{\text{M}}$, $6.9 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) by using *t*-butyl hydroperoxide (t-BuOOH) as an oxidant. X-ray crystal structure revealed that one bound Cu(II) is coordinated by two His residues (His67 and His104) and a water molecule forming a hydrogen(H)-bond to the carbonyl oxygen of Val101 (Fig. 4b). Docking simulation further showed that Cu(II)-6-PGLac harbors a substrate-binding pocket capable of accommodating *o*-dianisidine (Fig. 4c), which rationalizes the observation of a low K_{M} value ($11 \pm 3 \text{ }\mu\text{M}$), comparable to native horseradish peroxidase ($13 \text{ }\mu\text{M}$). Therefore, this example suggests that incorporation of metal ions in a latent metal-binding site of naturally non-metallated proteins offers a novel strategy for design of metalloenzymes with different activity.

Fig. 4 Design of mononuclear metalloenzymes by incorporating metal ions in native protein scaffolds. (a) The binding of Cu(II) to the putative metal-binding site of 6-PGLac (PDB code 4TM8), and (b) confirmed by X-ray crystal structure of Cu(II)-6-PGLac (PDB code 4TM7), (c) docking simulation of *o*-dianisidine binding to pocket of Cu(II)-6-PGLac. Reprinted with permission from ref. [68], Copyright 2015 The Royal Society of Chemistry.

Alternative to native protein scaffolds, *de novo* designed protein scaffolds such as coiled coils and helical bundles [33, 69] are convenient for design of metalloenzymes by incorporation of metal ions, since they have regular repeating structure and thus the metal-binding site can be precisely designed. By taking the advantages of three α -helical coiled coils, Pecoraro and co-workers [70] designed a functional Cu site with three His ligands (Cu-His₃), which closely mimics the type-2 Cu site of native copper nitrite reductase (NIR), and exhibits considerable NIR activity (Fig. 5a). Ideally, the reduction potential and NIR activity are tunable by modifying the charged residues in the secondary sphere of the type-2 Cu center.

Moreover, the asymmetric type-1 Cu site of native copper NIR, Cu-His₂Cys(Met), can also be engineered in the three α -helical coiled coils [71]. Although the designed protein did not fully reproduce the spectroscopic features of native cupredoxins, an intermolecular electron transfer activity was observed (Fig. 5b), with the first-order and bimolecular ET rate constants of 10^5 s^{-1} and $10^8 \text{ M}^{-1}\text{s}^{-1}$, respectively. Since both type-1 and type-2 Cu sites were independently designed in three α -helical coiled coils, one might expect that both the sites can be jointly engineered in the same protein scaffold, which will more closely mimic the structure and function of native copper NIR.

In addition, Pecoraro and co-workers [72] successfully designed a Zn(II) binding site, Zn(II)-His₃O, in a three stranded coiled coil (3SCC) and in a single stranded three-helical bundle ($\alpha_3\text{H}_3$) (Fig. 5c), respectively, which exhibits expressive hydrolase activity by hydration of CO₂, with an efficiency comparable to or within 1-3 orders of magnitude of native carbonic anhydrases.

Fig. 5 Design of mononuclear metalloenzymes by incorporating metal ions in *de novo* protein scaffolds. (a) A model of Cu(I/II)-His₃O site designed in 3SCC exhibiting NIR activity. Reprinted with permission from ref. [70], Copyright 2012 National Academy of Sciences; (b) A model of type-1 Cu site designed in α₃D-CH₃ showing ET activity from the copper center to the photo-oxidant, Ru(III)(bipy)₃. Reprinted with permission from ref. [71], Copyright 2015 Elsevier; (c) A model of Zn(II)-His₃O site based on the NMR solution structure of α₃H₃, catalyzing the hydration of CO₂. Reprinted with permission from ref. [72], Copyright 2014 Wiley-VCH.

To facilitate metal ions binding to native or *de novo* protein scaffolds, Schultz and co-workers developed an efficient approach by genetic incorporation of metal-chelating UAAs, such as (2,2'-bipyridine-5-yl)alanine (Bpy-Ala), into the polypeptide chain. They showed that genetic incorporation of Bpy-Ala into catabolite activator protein can convert the protein from a DNA binding protein to a Cu(II)-dependent site-specific DNA cleaving enzyme [73]. Bpy-Ala was also used to design artificial metalloproteins with an ability to bind divalent cations such as Co(II), Zn(II), Fe(II) and Ni(II) with high affinities [74]. In a recent study, Kang *et al.* [75] engineered zinc-finger-like peptides to contain a Bpy-Ala, and found that the fingers

bind low-spin Fe(II) rather than Zn(II). More recently, Schultz and co-workers [76] showed that trimeric coiled-coil can be stabilized by introducing Bpy-Ala at its N-terminus upon Fe(II)/Zn(II) binding. More importantly, the stable trimeric coiled-coil resembles the intermediate during host cell-viral fusion, which thus provides the possibility to serve as an anti-viral reagent. For more designs of metalloproteins using metal-chelating UAAs, readers are referred to recent excellent reviews [77-79].

3. Design of metalloenzymes with a dinuclear site

3.1 Homo-dinuclear site

In addition to mononuclear site, many natural metalloenzymes use multi-metal sites as catalytic sites, as well as electron- or proton-delivery systems. Examples for a homo-dinuclear site in natural metalloenzymes include the Cu_A center in cytochrome *c* oxidase (CcO) [80] and nitrous oxidase reductase (N₂OR) [81], and di-iron center in O₂ carrier hemerythrin [82] and methane mono-oxygenase hydroxylase [83], *etc.* These dinuclear sites thus represent an challenge in artificial metalloenzyme design. Based on sequence alignment of the type-1 Cu center and the Cu_A center, Lu and co-workers [84] designed a Cu_A center in Az by replacing the type-1 Cu binding loop with the corresponding Cu_A binding loop in native CcO, which closely reproduces the spectroscopic and geometric features of native Cu_A center. As well-reviewed by Wilson *et al.* [85], much insight such as the assembly mechanism of the Cu_A center and the functional advantage of Cu_A over type-1 Cu center, have been obtained for native Cu_A proteins by studying the simple model of Cu_A-Az.

Aside from native protein scaffolds, a purple Cu_A center was also engineered in *de novo* protein scaffolds such as a four stranded coiled coil, bi-AM2C, in which the two

copper ions were bridged by two Cys residues, with each copper coordinated by two His residues (Fig. 6a) [86]. Cu(II)-bi-AM2C showed the typical absorption bands of Cu_A site at 488 nm and 530 nm. In addition, both EPR spectrum and X-ray absorption near-edge structure (XANES) analysis confirmed that the Cu_A site was successfully constructed. In another study, Allen and co-workers [87] reported the formation of di-copper site in a four-helix bundle upon the binding of Cu(II). As designed in the sequence and characterized by EPR studies, the two copper ions were presumably bridged by two Glu residues, with imidazole and carboxylates as additional ligands (Fig. 6b). While no NMR or X-ray structure of Cu(II)-bound four-helix bundle was reported, indirect structural information could be obtained from other divalent metal ions such as Cd(II), Co(II), Mn(II) and Zn(II) binding to the four-helix bundle [88]. For instance, each metal ion of these dinuclear centers was five-coordinated by three carboxylates and one histidine. Recently, Allen and co-workers [89] showed that the artificial Mn(II)-bound protein is capable of transferring electrons to a natural system, bacterial reaction centers. Meanwhile, the function of a di-copper site in the four-helix bundle remained to be elucidated.

Comparatively, it was well-studied for the function of a di-iron site in four-helix bundles, the "Due Ferri" (DF) family, as pioneered by DeGrado and co-workers [88, 90]. For example, di-Fe(II)-DF3, a helix-loop-helix dimer binding two iron ions, is capable of catalyzing O₂-dependent oxidation of a larger catechol derivative, 3,5-di-*tert*-butyl-catechol (3,5-DTBC), to the corresponding quinone (3,5-DTBQ) (k_{cat} 13.2 min⁻¹, $k_{\text{cat}}/K_{\text{M}}$ 6315 M⁻¹min⁻¹) (Fig. 6c), with a catalytic efficiency ~4.6-fold higher than that for a small substrate, 4-amino-phenol (4-AP) (k_{cat} 27.2 min⁻¹, $k_{\text{cat}}/K_{\text{M}}$ 1380 M⁻¹min⁻¹) [91]. The four-helix bundle can be redesigned into a single polypeptide

chain, with three computationally designed loops linking each helices (DFsc). By introduction of four Gly residues (G4DFsc), a substrate channel can be created in the protein scaffold, which affords an ability of di-Fe(II)-G4DFsc to catalyze two-electron oxidation of 4AP to the corresponding quinone imine [92]. Moreover, by introduction of an additional His in the di-iron center to mimic the active site of natural *p*-aminobenzoate *N*-oxygenase, the constructed di-Fe(II)-3His-G4DFsc efficiently converts *p*-anisidine to the corresponding hydroxylamine (Fig. 6d) [93]. These studies demonstrate that the DF family proteins are ideal *de novo* protein scaffolds for design of metalloenzymes with a dinuclear site, with function tunable from phenol oxidase to *N*-hydroxylase.

Fig. 6 Design of metalloenzymes with a homo-dinuclear site of biological metal ions:

(a) A computer model of a designed Cu_A center in bi-AM2C scaffold. Reprinted with permission from ref. [86], Copyright 2012 American Chemical Society; (b) A computer model of di-copper center in P_0 scaffold designed from the DF protein

family. Reprinted with permission from ref. [87], Copyright 2015 American Chemical Society; (c) Solution of di-Zn(II)-DF3 (PDB code 2KIK), and its iron derivative showing catechol oxidase activity [92]; (d) Solution of di-Zn(II)-3His-DFsc (PDB code 2LFD), and its iron derivative showing *N*-hydroxylase activity [93].

Nonbiological metal ions such as rhodium can also form a dinuclear complex and catalyze a broad range of reactions including cyclopropanation [94]. To improve the catalytic selectivity, Lewis and co-workers [95] chose a thermostable prolyl oligopeptidase (POP), a protease with a large internal cavity, as a scaffold to fully encapsulate a dirhodium tetracarboxylate complex (Fig. 7). They genetically introduced an UAA, *p*-azido-Phe, in the protein cavity at position 477 to covalently link the cofactor by a strain-promoted click reaction. Moreover, they replaced four residues (Glu104, Phe146, Lys199 and Asp202) in the active site with Ala to expand the cavity size. The constructed metalloenzyme catalyzes enantioselective cyclopropanation of a range of styrenes and donor-accepter carbene precursors (Fig. 7). In addition, the yield and enantioselectivity could be improved by introduction of His and Phe residues (His328, Phe97 and Phe99) in the active site, increased to be 74% and 92% enantiomeric excess (ee), respectively. These results indicate the importance of scaffold selection as well as active site modification in artificial metalloenzyme design, especially for insertion of cofactors with a large volume.

Fig. 7 Design of metalloenzymes with a homo-dinuclear site of nonbiological metal ions: A modeling structure of prolyl oligopeptidase covalently linked a dirhodium tetracarboxylate complex, with the chemical structure shown in box, and the representative reactions catalyzed by the artificial enzyme. Reprinted with permission from ref. [95], Copyright 2015 Macmillan publishers.

3.2 Hetero-dinuclear site

Examples for a hetero-dinuclear site in natural metalloenzymes include the heme-copper center in heme-copper oxidase (HCO) (Fig. 8a) [96] and CcO [80], the heme-nonheme iron center in nitric oxide reductase (NOR) (Fig. 8b) [97], and the heme-manganese center in manganese peroxidase (MnP) (Fig. 10a) [98], *etc.* These enzymes play crucial roles in biological systems, catalyzing fundamental whereas critical reactions such as reduction of O₂ to water, reduction of NO to N₂O, and oxidation of Mn²⁺ to Mn³⁺, *etc.* Despite their functional importance, these native enzymes are difficult to study because many of them (e.g. HCO and NOR) are

membrane proteins and contain other metal-binding sites, which makes it difficult to prepare isolated enzymes with high purity and to study their property and function by spectroscopic methods.

To provide insight into the structural and functional relationship of native HCO, Lu and co-workers [99] employed a biosynthetic approach to design metalloenzymes with a hetero-dinuclear center using easy-to-purify and easy-to-characterize protein myoglobin (Mb) as a model system. By mutating two distal residues (Leu29 and Phe43) to His in sperm whale Mb, together with the native His64, a copper-binding site of three histidine residues (3-His) was formed in the heme pocket (termed Cu_BMb). Later on, a Tyr33 residue was introduced in the secondary sphere to closely mimic the heme-copper center of native HCO (Fig. 8c) [100]. Meanwhile, the binding of Cu^{II} to the Cu_B site has little influence on the oxidase activity F33Y Cu_BMb, as for Cu_B-independent Cyt *bd* oxidase [101]. Interestingly, Cu^{II} binding to the Cu_B site inhibits the peroxidase activity of Cu_BMb, by blocking the essential role of distal histidines in activation of H₂O₂ [102]. However, the copper ion in the Cu_B site enhanced the NIR activity of Cu_BMb, likely facilitating the binding of nitrite and/or cleavage of N-O bond [103]. These observations provide valuable insight into the role of Cu_B in the cross-reactivity of native CcO, which is otherwise difficult to obtain by studying the native enzyme.

To probe the role of a conserved Tyr in the heme-copper center of HCO, Wang, Lu and co-workers [104, 105] synthesized a series of UAA analogs of Tyr with decreasing *pK_a* and increasing reduction potential, and genetically encoded into the Tyr33 position of Cu_BMb. It was interesting to observe a linear dependence of both the O₂ reduction activity and the fraction of H₂O formation with the *pK_a* of these Tyr

analogs. Both the proton and electron donating abilities of the conserved Tyr in the active site are crucial for the native function of HCO. Moreover, to probe the role of Tyr-His cross-link in the heme-copper center of native HCO, Wang, Lu and co-workers [106] genetically encoded an UAA, ImiTyr (2-amino-3-(4-hydroxy-3-(1*H*-imidazol-1-yl)phenyl) propionic acid), into the Tyr33 position of Cu_BMb, which confers an activity of O₂ reduction to water 2.5-fold that of F33Y Cu_BMb, with more than 1000 turnovers. For more details in fine-tuning the activity of Cu_BMb using a variety of UAAs, the readers are referred to an excellent review by Wang, Lu and co-workers [77, 79].

To further mimic the heme-nonheme dinuclear center of native NOR, a Glu68 was introduced in Cu_BMb to create a nonheme iron binding site of 3-His-1-Glu (named Fe_BMb) [107]. To improve the NOR activity of Fe_BMb, an additional Glu107 was introduced in the secondary sphere of the dinuclear center (Fig. 8d) [108], which more closely mimics the native NOR that contains several conserved Glu residues in the active center. Then, the roles of the conserved Glu and the iron in the Fe_B site were well-addressed by studying I107E Fe_BMb. The two Glu residues play different roles in NO reduction activity, i.e, one serves as a ligand to Fe_B (E68) and the other acts as a proton transfer group (E107). The two electrons required for NO reduction were demonstrated to come from both the ferrous heme and nonheme Fe_B.

Fig. 8 Design of metalloenzymes with a hetero-dinuclear site using Mb as a model system: (a) X-ray structure of the heme-copper center of native HCO from *P. stutzeri* *cbb3* oxidase (PDB code 5DJQ) [96]; (b) X-ray structure of the heme-nonheme di-iron center of native NOR from *P. aeruginosa* (PDB code 3O0R) [97]; (c) X-ray structure of Cu(II)-F33Y Cu_BMb (PDB code 4FWY) [100]; (d) X-ray structure of Fe(II)-I107E Fe_BMb (PDB code 3M39) [108].

In NO reduction, stopped-flow UV-Vis spectroscopy and rapid freeze-quench resonance Raman (RFQ-rR) studies revealed the formation of a transient dinitrosyl complex, six-coordinate low-spin heme-nitrosyl/Fe_B-nitrosyl adduct, 6cLS[FeNO]⁷/[Fe_BNO]⁷, which produces N₂O at a rate of 0.7 s⁻¹ in I107E Fe_B Mb (Fig. 9) [109]. Meanwhile, an Ile (Fe_BMb) or Phe (I107F Fe_B Mb) in the secondary sphere increases the rate of the formation of a dead-end dinitrosyl complex, five-coordinate low-spin heme-nitrosyl complex, 5cLS[FeNO]⁷ [110]. These observations demonstrate that the

(called MnCcP). The Mn(II)-binding site was further optimized by reverting E41 back to Gly, replacing D181 with Glu to elongate the side chain, and making the triple mutation, D37E (called MnCcP.1) (Fig. 10b, inset) [113].

Recently, new achievements were obtained for enhancing the binding affinity of Mn(II) and the Mn(II) peroxidase activity by fine-tuning the secondary sphere interactions (Fig. 10b) [114]. First, removal of the H-bonding interaction of Y36 and E45 by Y36F mutation resulted in a 2.8-fold decrease in K_M value of Mn(II) oxidation; Second, introduction of a salt-bridge between residue K179 and E37 by K179R mutation improved the coordination of both E35 and E181, with a 2.6-fold decreased K_M ; Third, elimination of the steric effect of Ile40 by I40G mutation allowed E37 coordinating to Mn(II) in a suitable orientation, resulting in a K_M value comparable to that of native MnPs. Interestingly, the effects of Y36F and K179R mutations are additive, although the combinations of other mutations are not. Importantly, these MnCcP.1 derivatives are active toward both oxidation of Mn(II) and degradation of substrates, such as both alkali treated lignin and a lignin model compound, which demonstrates that these artificial metalloenzymes are functional models of native MnP.

Fig. 10 Design of metalloenzymes with a hetero-dinuclear site using CcP as a model system: (a) X-ray structure of the heme-manganese center in native MnP (PDB center 1MNP) [98]; (b) Effects of secondary coordination shell residues mutation on the catalytic performance of designed MnCcP.1. Reprinted with permission from ref. [114], Copyright 2016 American Chemical Society.

4. Design of metalloenzymes with a multinuclear site

4.1 Fe-sulfur clusters

While it is of difficulties in design of a functional dinuclear site, the design of metalloenzymes with a multinuclear site has more challenges, due to the combination of effective design of host proteins, synthesis and assembly of metal-clusters in protein, and characterization of structure and activity [51]. Fe-sulfur clusters, such as [2Fe-2S], [3Fe-4S] and [4Fe-4S] clusters, are one of the most ubiquitous cofactors in nature, with diverse functions ranging from electron transfer, catalysis, O₂-sensing, to structural roles [115]. Due to their importance in biology, Fe-sulfur clusters are of considerable interests for design of metalloenzymes [51]. Goldren *et al.* [116] first

incorporated a [4Fe-4S] cluster into the hydrophobic core of thioredoxin, a natural protein in the absence of any cofactors, by introducing four Cys residues with computational design. Ghirlanda and co-workers [117] first reported the design of two identical [4Fe-4S] clusters in a *de novo* designed three-helical bundle, which are separated by >30 Å and far from efficient electron transfer. In following study, these authors performed a second generation design and shortened the intercluster distance to 12 Å, which is comparable to native systems for effective electron transfer [118]. The designed protein, called DSD-Fdm, exhibits a redox potential of -479 mV ([4Fe-4S]^{2+/1+}), within the lower range of natural ferredoxins. Importantly, the reduced DSD-Fdm could transfer electrons to ferric Cyt *c*₅₅₀ (Fig. 11a), a natural ferredoxin substrate, and generate reduced Cyt *c*₅₅₀ in a stoichiometrical manner (DSD-Fdm 2[4Fe-4S]⁺ + 2Cyt *c*₅₅₀ [Fe³⁺] → DSD-Fdm 2[4Fe-4S]²⁺ + 2Cyt *c*₅₅₀ [Fe²⁺]), mimicking the functional role of natural ferredoxins. Moreover, DSD-Fdm could transfer energy to non-native system such as photoexcited dye, suggesting potential applications in design of artificial light-driven devices. The three-helical bundle also offers an ideal scaffold for fine-tuning the redox potential as well as the form of the clusters. For example, replacement of one of the Cys ligands of [4Fe-4S] cluster with either Leu or Ser (DSD-3CLeu or DSD-3CSer), resulted in a more stable [3Fe-4S] cluster, with a redox potential of 12 mV and -4 mV, respectively [119].

More recently, Kerfeld and co-workers [120] took the structural advantage of a trimeric bacterial microcompartment (BMC) shell protein, BMC-T1, and designed a [4Fe-4S] cluster at the three-fold symmetry axis of the trimer by introducing a single mutation, BMC-T1-S55C. The three Cys55 residues provide a binding-site of [4Fe-4S] cluster, with a water molecule acting as the fourth ligand. The X-ray

structure of the designed protein was determined to 1.8 Å resolution (Fig. 11b), showing the first structural evidence for the success of the [4Fe-4S] cluster design. Spectroscopic studies showed that the cluster is redox active, with a redox potential of -370 mV, which confers an electron-transfer function to the shell protein. With both structural information and excellent redox property, in combination of the ability of BMC to enclose other enzymes, the designed BMC-T1-S55C has potential applications in synthetic biology and offers an ideal scaffold for creating multiple enzyme systems.

Nitrogenase catalyzes the reduction of various substrates (N_2 , C_2H_2 , CN^- , *etc*) using its cofactor, $[(\text{Cit})\text{MoFe}_7\text{S}_9\text{C}]^{n-}$, namely M-cluster, which can be viewed as the combination of $[\text{Mo}-3\text{Fe}-3\text{S}]$ and $[4\text{Fe}-3\text{S}]$ subclusters by three S atoms and one carbide (C^{4-}) atom (Fig. 11c) [121]. This unique high-nuclearity metallocluster has attracted much attention for several decades. As early as in 1981, Holm and co-workers [122] synthesized a hexanuclear, dithiolate Fe-sulfur cluster, $[\text{Fe}_6\text{S}_9(\text{SEt})_2]^{4-}$, designated Fe_6^{RHH} (Fig. 11c), which closely mimics the geometry, as well as the anionic nature, of the M-cluster. Recently, Hu, Ribbe and co-workers [123] showed that after removal of the native M-cluster in the catalytic component of nitrogenase (designed Apo-NifDK), the Fe_6^{RHH} cluster can be inserted into the apo-protein (designed NifDK^{Fe}) (Fig. 11c). The reconstituted artificial enzyme, NifDK^{Fe}, catalyzes the reduction of C_2H_2 to C_2H_4 in presence of the reductase component of nitrogenase and ATP, with an activity comparable to that of native enzyme. Moreover, NifDK^{Fe} was capable of reducing CN^- to C_1 - C_3 hydrocarbons in absence of ATP and presence of Eu^{II} -DTPA, with CH_4 being the major product. This study demonstrates that functional artificial enzyme with a high-nuclearity center can be designed by

consideration of the suitable geometry of the cluster, the electrostatic interactions between the cluster and protein scaffold, as well as the appropriate ligands provided by the protein scaffold.

Fig. 11 Design of multinuclear metalloenzymes with an Fe-sulfur cluster: (a) DSD-Fdm designed with two [4Fe-4S] clusters transfers electrons to ferric Cyt *c*, generating reduced Cyt *c*. Reprinted with permission from ref. [118], Copyright 2014 American Chemical Society; (b) Side view of the structure of BMC-T1-S55C, showing the [4Fe-4S] cluster (PDB code 5DII). Reprinted with permission from ref. [120], Copyright 2014 American Chemical Society; (c) Construction of artificial metalloenzyme by removal of the M-cluster from a native nitrogenase scaffold, followed by insertion of a synthesized [Fe₆S₉(SEt)₂]⁴⁻ cluster. Reprinted with permission from ref. [123], Copyright 2015 Wiley-VCH.

4.2 Other metal clusters

In addition to Fe-sulfur clusters, other metal clusters were also used for design of metalloproteins, with functionalities less explored. For examples, metalloproteins with a tetranuclear Cu and Cd site were designed in the interior of a four-stranded and three-stranded coiled coil by using a metal-binding motif of CXXC to form a metal-thiolate cluster [124]. The Cu(I)-binding properties and the structure of $\text{Cd}_4\text{S}_6\text{O}_4$ cluster (Fig. 12a) were well-characterized. By controlling the oligomerization state of helix-bundles, Morozov and Ogawa [125] designed trimers, tetramers and hexamers that uniquely bind 6, 8 and 12 Ag^+ ions, respectively. Upon chemical reduction, a series of Ag^0 nanoclusters were formed within the protein scaffolds, which display strong visible fluorescence with a number-dependent emission energy (Fig. 12b). Recently, Hirota and co-workers [126] observed a novel Zn-SO_4 cluster in the unique cage formed by three-domain swapped Cyt cb_{562} dimers, which consists of 15 Zn^{2+} and 7 SO_4^{2-} ions (Fig. 12c). This observation suggests that domain-swapping may be useful for design of artificial proteins with unique nanostructures, which may be further exploited for introducing desired functions.

Fig. 12 Design of multinuclear metalloenzymes with other metal clusters: (a) X-ray crystal structure of a tetranuclear Cd-thiolate cluster (PDB code 4G1A). Reprinted with permission from ref. [124], Copyright 2013 Elsevier; (b) Fluorescence spectra of helix-bundle oligomers bound with Ag^0 nanoclusters. Reprinted with permission from ref. [125], Copyright 2013 American Chemical Society; (c) Cage structure of three-domain swapped Cyt *cb*₅₆₂ dimers, showing the Zn^{2+} and SO_4^{2-} ions in the internal cavity (PDB code 5AWI). Reprinted with permission from ref. [126], Copyright 2016 The Royal Society of Chemistry.

5. Design of metalloenzymes with a metal complex

5.1 Metallo-porphyrins

Heme proteins are a large class of metalloenzymes crucial for biological systems,

which bind the heme group non-covalently such as heme *b* (Fe-protoporphyrin IX) or covalently such as heme *c* via thioether bonds and other heme-protein cross-links [127]. The heme *b* group can be removed from the protein scaffold under acidic conditions, producing the corresponding apo-proteins [128]. As developed for more than 50 years, a powerful approach for designing artificial heme proteins is to reconstitute the apo-protein with heme *b* derivatives, such as heme analogs by modification with other substituents and replacement of the heme iron with other metal ions [129, 130]. With the advantages of small size (153 amino acids and 1 heme), easy-to-purify and easy-to-characterize, Mb was favored for design of artificial metalloenzymes by this approach [25], as well as other heme proteins such as Cyt P450 [131] and Cyt *b*₅₆₂ [132]. Recently, this old trick has afforded new achievements in design of metalloenzymes in Mb with advanced functions, as exemplified by following case studies.

5.1.1 Heme analogs

The chemical property of heme are strongly affected by its substitutions, especially those at the 2- and 4-positions, as in most post-translational modifications [127]. The modified heme analogs can then be used to fine-tune the property and function of heme enzymes. For example, to increase the heme redox potential required for O₂ reduction by F33Y Cu_BMb designed to mimic native HCO, Lu and co-workers [133] replaced the native heme *b* with heme *a* analogs, diacetyl (DA-) heme, monoformyl (MF-) heme and diformyl (DF-) heme, as well as removing the H-bonding interactions of the proximal His93 (S92A mutation). As shown in Fig. 13, the four F33Y Cu_BMb derivatives exhibit systematically increasing redox potential, which

correlates with their O₂ reduction activity. This study provides a new method for investigation of the relationship between heme redox potential and enzymatic activity.

Fig. 13 Design of metalloenzymes by incorporation of heme analogs in apo-Mb: Heme *b* substitution of F33Y Cu_BMb with heme *a* analogs, and the correlation of heme redox potential and catalytic O₂ reduction activity for the protein derivatives [133].

To provide insight into the structure and NIR activity relationship of globins as regulated by heme 2- and 4-substitutions, Galinato *et al.* [134] prepared several Mb variants containing DA-heme, heme, deuteroheme and mesoheme, respectively, with increasing of the electron releasing capacity of the -R groups at positions 2 and 4. Kinetic studies showed a correlation between the NIR activity and the natural charge on the Fe center in heme analogs (Fig. 14a), with the activity of mesoMb ~3.4-fold that of WT Mb. On the other hand, Lin and coworkers [135] found that the NIR activity of Mb can be regulated by redesign of the heme active center instead of modifying the heme group. By studying a series of Mb distal histidine mutants and the channel mutants, it showed that an enhancement of NIR activity was achieved by creation of a water channel to the heme center (H64A mutation) and relocation of the distal histidine (F43H mutation) (Fig. 14b). The X-ray structure of met F43H/H64A

Mb confirmed the water channel and revealed a unique H-bonding network in heme active center (Fig. 8c, inset). These studies suggest that both the heme group and its micro-environments are potential targets for modifications to improve the function of heme proteins.

Fig. 14 Fine-tuning the NIR activity of Mb: (a) Correlation between the rate constants of nitrite reduction by reconstituted Mbs and the natural charge on the Fe center. Reprinted with permission from ref. [134], Copyright 2016 Elsevier; (b) Relative reactivities of Mb distal histidine mutants and the channel mutants with respect to that of WT Mb. The X-ray structure of met F43H/H64A Mb was shown for clarification [135].

5.1.2 Iron-replaced porphyrins

Although iron is used by natural heme to perform diverse functions, it can be replaced by other metal ions both *in vivo* and *in vitro* [136-138], which can be used to investigate the role of metal center in fine-tuning of heme protein functions. Hartwig and co-workers [54] recently developed a method to replace the heme iron in Mb with a series of other metal ions including abiological noble metals (Co, Cu, Mn, Rh, Ir, Ru and Ag) (Fig. 15a). Moreover, they applied directed evolution strategy to optimize the catalytic performance. The proximal ligand (His93) was first mutated to Gly or Ala, and the distal residues (His64, Phe43, Val68), as well as the surrounding residues (His97 and Ile99), were subsequently mutated to favor substrate binding to the active site (Fig. 15b). The resultant Ir(Me)-porphyrin IX Mb mutants are capable of catalyzing C-H bond insertion reactions with high (-) or (+) enantiomer. Very recently, they also reconstituted the Ir(Me)-porphyrin IX with thermal stable Cyt P450, CYP119, in which the axial Cys317 was replaced with Gly to allow the coordination of the Me group. By further cavity modification using molecular evolution, the artificial metalloenzymes catalyze carbene insertions into C-H bonds with characters of natural enzymes, i.e., fast kinetics, high productivity and high selectivity [55].

By removing the heme under acidic conditions, Zhang and co-workers [139] incorporated Mn-protoporphyrin IX into a double mutant of Mb, L29H/F43H Mb. The three distal histidines (His29, His43 and native His64) facilitates the heterocleavage of O-O bond of an oxidant such as Oxone ($2\text{KHSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$), which generates catalytic species ($\text{Mn}^{\text{IV}}=\text{O}^+$) capable of efficient epoxidation of C=C bond in styrene (48×10^{-3} turnover/min) (Fig. 15c). Control experiments showed that reaction rate is 15×10^{-3} and 5.4×10^{-3} turnover/min for Fe-protoporphyrin IX L29H/

F43H Mb and Mn-protoporphyrin IX WT Mb, respectively, indicating the essential roles of the metal center as well as the multiple distal His residues. In another study, Ghirlanda and co-workers [140] showed that the photo-induced H₂ production activity of Co-protoporphyrin IX was increased ~4-fold by incorporation into the Mb scaffold (Fig. 15d), with a turnover number (TON) of 518 at pH 7.0. The activity was further increased ~2-fold by H64A/H97A double mutation, indicating the importance of the secondary sphere interactions in fine-tuning the activity.

These recent achievements demonstrate that the Mb scaffold is an ideal protein scaffold for design of functional metalloenzymes by heme substitution, due to the advantages such as the hydrophobic pocket protects the cofactor from degradation, the secondary sphere of the pocket allows modifications by single or multiple mutations, and the overall scaffold facilitates studies in aqueous environments instead of organic solvents.

Fig. 15 Design of metalloenzymes by incorporation of iron-replaced porphyrins in apo-Mb: (a) The heme pocket environment of Mb, and the protein solution of apo-Mb reconstituted with various metal-protoporphyrin IX complexes [54]; (b) Directed evolution strategy of Ir(Me)-porphyrin IX Mb to achieve the enantioselectivities in formation of the product shown in the box. Reprinted with permission from ref. [54], Copyright 2015 Macmillan publishers; (c) Styrene epoxidation by Mn-protoporphyrin IX reconstituted L29H/F43H Mb using KHSO_5 as an oxidant. Reprinted with permission from ref. [139], Copyright 2016 The Royal Society of Chemistry; (d) The H_2 production activity of Co-protoporphyrin IX incorporated in Mb using $\text{Ru}(\text{Bby})_3^{2+}$ as a photosensitizer. Reprinted with permission from ref. [140], Copyright 2014 The Royal Society of Chemistry.

5.2 Other metal complexes

5.2.1 In apo-Myoglobin

It has been shown that planar or nearly planar metal complexes, especially those have a similar size to that of porphyrins, such as metal-salophen, salen and phthalocyanine [141-143], can occupy the heme pocket of apo-proteins and afford advanced functions. There are continuous achievements in design of artificial metalloenzymes by incorporation of new metal complexes into the apo-protein of native heme proteins. Take the popular apo-Mb for example, Artero and co-workers reconstituted the apo-protein with two cobaloxime complexes ($\text{Co}(\text{dmgBF}_2)_2$ and $\text{Co}(\text{dmgH})_2$, dmgH_2 = dimethylglyoxime Fig. 16a), and observed a TON of 3.8 and 3, respectively, for H_2 production in presence of a Ru photosensitizer ($[\text{Ru}(\text{bpy})_3]^{2+}$) and ascorbate [144].

In another study, in order to replicate both the coordination behavior and catalytic reaction of cobalamin in native methionine synthase, Hayashi and co-workers reconstituted apo-Mb with a cobalt tetrahydrocorrin, $\text{Co}^{\text{II}}(\text{TDHC})$ (Fig. 16b). X-ray structure revealed that upon reduction, the five-coordinate $\text{Co}^{\text{II}}(\text{TDHC})$ complex was converted to a four-coordinate $\text{Co}^{\text{I}}(\text{TDHC})$ species by cleaving the Co-His93 ligation [145]. This novel species was reactive toward methyl iodide, forming a methylated cobalt complex, $\text{CH}_3\text{-Co}^{\text{III}}(\text{TDHC})$. Subsequently, an intraprotein transmethylation occurred to the N ϵ 2 atom of heme distal His64 (Fig. 16c) [146]. As shown in Fig. 16d, more than 90% His64 in $\text{Co}(\text{TDHC})$ -Mb was transmethyated over a period of 48 h at 25 °C, whereas almost no reaction was occurred in native Mb, which was also supported by theoretical calculations. These observations indicate that protein scaffold can modulate the chemical reaction of $\text{Co}(\text{TDHC})$ complex, offering an ideal protein

model for elucidation of the structure-function relationship of native methionine synthase as well as other cobalamin-dependent enzymes.

Fig. 16 Design of metalloenzymes by incorporation of metal complexes in apo-Mb: (a) Co(dmgh)₂-apo-Mb adduct shows H₂ production activity. Reprinted with permission from ref. [144], Copyright 2014 American Chemical Society; (b) X-ray structure of apo-Mb with Co^{II}(TDHC) in the heme pocket (PDB code 3WFT) [145]; (c) Proposed mechanism for methylation of His64 in Co(TDHC)-Mb as a methionine synthase model [146]; (d) Time-dependant of formation of methylated His64 in Co(TDHC)-Mb (red) and native Mb (black). Reprinted with permission from ref. [146], Copyright 2016 The Royal Society of Chemistry.

5.2.2 In apo-Nitrobindin

Although much progress was made in design of artificial metalloenzymes using the heme pocket of Mb, there are at least two limitations for achieving advanced

functions. One is that the metal complex is required to be nearly planar with a similar size to that of the heme group, which restricts the choice of ligands for the metal ions; the other is that after binding of the metal complex, there is limited space remaining to bind substrates, especially those with a large molecular size, thereby limiting the repertoire of catalytic reactions. Therefore, it is of importance to employ the cavity of other proteins with a larger volume for design of metalloenzymes with advanced functions. Nitrobindin (NB) is a rigid β -barrel protein with a large hydrophobic pocket harboring a heme for NO transport *in vivo* [147], which retains the unique structure in its apo-form by removal of heme, and is thus favorable for incorporation of other metal complexes.

Hayashi and co-workers [130] have made significant progress recently in design of functional metalloenzymes by incorporating a series of metal complexes into the cavity of apo-NB. For example, to construct an artificial hydrogenase, they tethered a diiron core of native enzyme (H-cluster), $(\mu\text{-S})_2\text{Fe}_2(\text{CO})_6$, to apo-NB by covalently linking a maleimide moiety to Cys96 (Q96C mutation) at the entrance of the cavity (Fig. 17a) [148]. The diiron complex embedded at the side of the cavity retains its activity to evolve H_2 (TON up to 130 for 6 h) under mild conditions in aqueous media (50 mM Tris·HCl, pH 4.0, 25 °C).

More recently, they synthesized a copper terpyridine complex, $[\text{Cu}(\text{typ})]^{2+}$, and covalently linked it to apo-Q96C NB using the same method (Fig. 17b). The constructed metalloenzyme catalyzes a Diels-Alder reaction between azachalcone and cyclopentadiene in 22% yield. This study suggests that after linking a catalytic motif, the cavity of apo-NB still has sufficient space for simultaneous binding of two substrates, dienophile and diene [149]. In a previous study, Lewis and co-workers

[150] constructed a similar metalloenzyme of apo-Q96C NB with a $[\text{Mn}(\text{typ})]^{2+}$ complex, which catalyzes oxidation of benzylic methylene groups into carbonyl groups and styrene into phenyl epoxide, respectively, by using peroxyacetic acid as an oxidant. These studies show that similar to metal-substitution of native enzymes (section 2.1), different metal ions in the identical protein scaffold of artificial metallo-enzymes can afford different catalytic performances.

In another study, Hayashi and co-workers [151] designed a Rh complex with ligation of cyclopentadiene and cyclooctadiene, and covalently linked to apo-Q96C NB, which is capable of catalyzing polymerization of phenylacetylene (Fig. 17c). The secondary sphere of the Rh active site was further modified by triple or quadruple mutations, resulting in a series of derivatives (NB1-NB10) with different sizes and shapes. By doing so, the stereoselectivity of the product can be well-controlled. For example, NB4 (H76L/Q96C/H158L)-Rh produced a *trans:cis* ratio of 82:18 and NB10 (H76L/Q96C/L148W/H158L)-Rh produced a *trans:cis* ratio of 22:88, whereas almost no stereoselectivity was observed for NB Q96C-Rh (*trans:cis* = 54:46). The X-ray structure of NB4-Rh (Fig. 17c, PDB code 3WJC) provided further information and showed that the nonplanar Rh complex was covalently linked at the entrance to the cavity of apo-NB4, with the active site being oriented to favor the access of the monomer.

Furthermore, they prepared a variant NB11 (L75A/H76L/Q96C/M148L/H158A) with a larger cavity, and covalently linked a series of Grubbs-Hoveyda type catalysts with different spacer lengths for olefin metathesis (Fig. 17d). [152] It showed that the metalloenzymes can efficiently catalyze (i) the ring-closing metathesis (RCM) of 4,4-bis(hydroxymethyl)-1,6-heptadiene (TON > 100) and (ii) the ring-opening metathesis

polymerization (ROMP) of 7-oxanorbornene derivative (TON > 9000). More importantly, these high efficiencies were reached in mild water conditions (pH 6.0, 25-40 °C) without any organic cosolvents, which is attributed to the stabilization of catalytic complex in water by embedding in the hydrophobic cavity of apo-NB.

These studies demonstrate that the cavity of apo-NB is attractive for construction of functional metalloenzymes by incorporation of diverse metal complexes with a shape of planar or non-planar, and the size of substrates varies from a single proton to a larger molecule such as cyclopentadiene. In addition to providing a ligand for the catalytic metal complex, the cavity of apo-NB provides a second coordination sphere with a chiral environment that can be further modified to control product stereoselectivity. Furthermore, the good solubility and stability of apo-NB makes it possible to perform the catalytic reactions in mild water conditions. These advantages thus promote future design of new metalloenzymes with more advanced functions.

Fig. 17 Design of metalloenzymes by incorporation of metal complexes in apo-NB: (a) Apo-NB Q96C mutant with covalently linked a diiron hexacarbonyl complex promotes H₂ generation. Reprinted with permission from ref. [148], Copyright 2014 American Chemical Society; (b) Apo-NB Q96C mutant with covalently linked copper terpyridine complex catalyzes a Diels-Alder reaction between azachalcone and cyclopentadiene. Reprinted with permission from ref. [149], Copyright 2015 Elsevier; (c) Apo-NB4 with covalently linked a Rh-complex catalyzes the polymerization of phenylacetylene. Reprinted with permission from ref. [151], Copyright 2014 Wiley-VCH; (d) Apo-NB11 with covalently linked Grubbs-Hoveyda type catalysts catalyzes the ring-closing metathesis (i) and the ring-opening metathesis polymerization (ii) reactions. Reprinted with permission from ref. [152], Copyright 2015 American Chemical Society.

5.2.3 In other protein scaffolds

In addition to the well-exploited protein scaffolds of apo-Mb and apo-NB, there are multiple native and *de novo* protein scaffolds with cavities suitable for generating novel metalloenzymes by incorporation of a metal complex. These native protein scaffolds include the apo-ferritin with a huge inner cavity of 8 nm diameter [17], the biotin-streptavidin system [153], the bacterial pore FhuA with a large β -barrel [154], lipase [155], xylanase [156] and neocarzinostatin [157] with a hydrophobic core, the prolyl oligopeptidase with an enclosed cavity [95], and human carbonic anhydrase with a funnel-shaped binding pocket [47], *etc.* *De novo* protein scaffolds include the designed coiled coils and helical bundles [33], the semi-synthesized β -helical protein [(gp5 β f)₃]₂ with a tubular structure [158], and peptide dendrimers with a flexible

structure [159], *etc.* Since the progress in exploiting these protein scaffolds for metalloenzymes design has been well-reviewed recently by Watanabe [17], Ward [31, 34, 153], Peacock [33], and Ueno [158, 160], in this section we only present the very recent achievements in the biotin-streptavidin system and other protein scaffolds, including neocarzinostatin, lactoglobulin, and serum albumin proteins.

Recently, Ward, Panke and co-workers [53] reported the compartmentalization and *in vivo* evolution of an artificial metalloenzyme based on the biotin-streptavidin system for olefin metathesis (Fig. 18). They first designed a periplasmic expression system for streptavidin (named SAV^{peri}) in *E. coli*, which is capable of assembling the artificial metalloenzyme biot-Ru-SAV^{peri} within the periplasm, endowing the cells with metathesis activity. Then, they used directed evolution to improve the catalytic performance by modification of the secondary coordination sphere. For example, a quintuple (V47A/N49K/T114Q/A119G/K121R) mutant of SAV in complex with biot-Ru (named biot-Ru-SAV^{mut}) displays a cell-specific activity ~5-fold higher than that of biot-Ru-SAV^{peri}. *In vitro* studies further showed that biot-Ru-SAV^{mut} has a catalytic efficiency 2-fold that of biot-Ru-SAV^{peri}, with increasing k_{cat} and decreasing K_M values, respectively. In another study, Wenger, Ward, and co-workers [161] observed a rapid electron transfer (<10 ns) from a biotinylated triarylamine electron donor to a photochemically generated Ru(III) center introduced in SAV, where the photosensitizer [Ru(bpy)₂(phen)]²⁺ was covalently linked to Cys121 in K121C SAV mutant. More recently, Hendrich, Ward, Borovik and co-workers [162] designed several modular artificial cupredoxins by incorporation of a Cu(II) complex of biotinylated di[2-(2-pyridyl)ethyl] amine into S112C SAV mutant with Cys112 acting as a ligand, which reproduces both optical and structural properties of typical type-1

Cu center with an electron transfer function. Together with previous achievements [153], these studies show that the biotin-steptavidin system is well-exploited for design of novel metalloenzymes with diverse functions.

Fig. 18 Design of metalloenzymes by incorporation of metal complexes in protein scaffold of steptavidin: A biotin-Ru complex associated with a quintuple mutant of steptavidin showing enhanced metathesis activity by catalyzing diolefin conversion to umbrelliferone. Reprinted with permission from ref. [53], Copyright 2016 Macmillan publishers.

Neocarzinostatin (NCS) is one member of the enediyne-chromoprotein family with a small size (113 residues). By using the "Trojan horse" strategy, Ricoux, Mahy and co-workers [163] coupled a Cu(II) complex of 1,10-phenanthroline to testosterone, and incorporated into a NCS variant, NCS-3.24 (Fig. 19), which processes a high affinity for testosterone ($K_D = 13 \mu\text{M}$). The hybrid metalloenzyme efficiently catalyzes Diels-Alder cyclization reaction between cyclopentadiene and 2-

azachalcone, with the conversion yield up to 98%, and an *endo/exo* ratio of 84/16, albeit with no enantiomeric excess. This is likely due to the location of the active site at the edge of the pocket, as shown by molecular docking studies, thereby lacking an asymmetric environment to support enantioselectivity. In previous studies [157], it was also showed that an iron(III)-porphyrin-testosterone conjugate associated with NCS-3.24, Fe-Porph-Testo-NCS-3.24, was able to catalyze sulfoxiation of thioanisole by H₂O₂, albeit with a low enantioselectivity (13% ee). Moreover, a Zn(II)-BisPyPol-Testo conjugate associated with NCS-3.24 has RNase-like activity, catalyzing the hydrolysis of *p*-nitrophenyl-2-hydroxypropyl phosphate, the RNA model compound, with a good catalytic efficiency ($k_{\text{cat}}/K_{\text{M}} = 13.6 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.0) [164]. Therefore, these studies suggest that the "Trojan horse" strategy is suitable for the scaffold of neocarzinostatin to design functional metalloenzymes, although the substrate binding site is still needed to be optimized for improving the enantioselectivity.

Fig. 19 Design of metalloenzymes by incorporation of metal complexes in the protein scaffold of neocarzinostatin: A Cu(II)-1,10-phenanthroline-testosterone conjugate associated with a neocarzinostatin variant, NCS-3.24, catalyzing the Diels-Alder

reaction between cyclopentadiene and 2-azachalcone. Reprinted with permission from ref. [163], Copyright 2016 Wiley-VCH.

b-Lactoglobulin (BLG) is a small protein folding into a β -barrel structure with eight strands [165], in which five Cys residues form two disulfide bonds, leaving a single Cys121 suitable for chemical modification. Mahy, Banse and co-workers [166] took this advantage and specifically attached a nonheme Fe(II) polyazadentate complex into BLG at Cys121. This biohybrid can oxidize thioanisole into a sole product, phenylmethanesulfoxide, with up to 20 % ee, by using H_2O_2 as an oxidant (Fig. 20). In the absence of substrate, spectroscopic (UV-Vis and EPR) studies revealed that this metalloenzyme generates a side-on peroxo intermediate ($\text{Fe}^{\text{III}}(\eta^2\text{-O}_2)$) upon reaction with H_2O_2 , suggesting an active oxidizing species responsible for the catalytic reaction. This study shows that the protein scaffold of BLG provides not only a groundwork for constructing metalloenzyme with high product selectivities, but also a chance to elucidate the catalytic mechanism, which is essential for further improving catalytic performance.

Fig. 20 Design of metalloenzymes by incorporation of metal complexes in the protein scaffold of **b**-Lactoglobulin: A nonheme Fe(II) polyazadentate complex covalently linked to BLG at Cys121 catalyzing sulfoxidation of thioanisole to phenylmethysulfoxide with H₂O₂. Reprinted with permission from ref. [166], Copyright 2015 Wiley-VCH.

Serum albumin proteins such as human serum albumin (HSA) and bovine serum albumin (BSA) tightly bind metal complexes such as Mn(III)-corrole [167], which can thus be used for design of metalloenzymes [168]. Recently, Gross, Banse, Mahy and co-workers [169] incorporated a bis-sulfonato Mn(III)-corrole into BSA and further associated with a [Ru^{II}(bpy)₃]²⁺ chromophore. As activated by visible light (450 nm), this biohybrid system generated a high-valent Mn^{IV}-oxo species by an oxygen transfer reaction from water. By further using [Co^{III}(NH₃)₅Cl]²⁺ as an electron acceptor, this species selectively oxidizes thioanisole to the corresponding sulfoxide, with 12-16% ee for the *R* isomer (Fig. 21). This study thus represents a clean oxidation catalysis in aqueous medium in the absence of any chemical oxidant by using only visible light. Similarly, Bian, Liang and co-workers [170] incorporated a Co(II)-Schiff base complex into BSA and found that this hybrid catalyzes enantioselective oxidation of a variety of sulfides using H₂O₂ as an oxidant, producing corresponding sulfoxides with up to 100% conversion, 100% chemoselectivity and 87% ee in some cases. In another study, Ménage and co-workers [171] applied the "Trojan horse" strategy to HSA using ibuprofen as a recognition molecule that binds to IIIA domain (drug site 2) with a high affinity. They synthesized an Fe(II)-complex with N₂Py₂ ligand as a derivative of ibuprofen, and associated it with HSA. The

hybrid exhibits a high turnover frequency ($459 \text{ TON} \cdot \text{min}^{-1}$) with moderate selectivity (69%) in oxidation of thioanisole using NaOCl as an oxidant.

Fig. 21 Design of metalloenzymes by incorporation of metal complexes in the protein scaffold of BSA: Visible-light induced enantioselective oxidation of thioanisole to the corresponding sulfoxide by a Mn(III)-corrole complex incorporated in BSA, with [Ru^{II}(bpy)₃]²⁺ as a photosensitizer and [Co^{III}(NH₃)₅Cl]²⁺ as an electron acceptor. Reprinted with permission from ref. [169], Copyright 2016 The Royal Society of Chemistry.

6. Design of metalloenzymes with dual active sites

6.1 In single protein scaffolds

Natural metalloenzymes with dual active sites can effectively perform biological functions, such as CcO uses heme *a* to deliver electrons to the heme *a*₃:Cu_B site for reduction of O₂ to H₂O [4], and copper NIR uses type-1 copper to deliver electrons to type-2 copper where nitrite binds and is reduced [172]. Although many artificial metalloenzymes with a single active site have been created, those with dual active sites in single protein scaffolds are still limited. Lin and co-workers [173] recently

designed dual active sites in a single heme protein scaffold of Mb, by retaining the native heme site and creating a copper-binding site through a single mutation of Arg118 to His or Met with two existing His residues (His24 and His119) (Fig. 22). Both isothermal titration calorimetry (ITC) and EPR studies confirmed copper binding to the designed site of [3-His] and [2-His-1-Met] in R118H Mb and R118M Mb, respectively. The NIR activity of heme active site as well as the copper site was further confirmed by UV-Vis kinetic spectroscopy and EPR studies. Although nitrite was reduced to nitric oxide at two independent sites, the NO produced at the Cu site may rapidly bind to the ferrous heme center. This study presents a new example of artificial metalloenzyme with dual active sites in a single protein scaffold, which also lays the groundwork for further design of functional metalloenzymes by modification of both the heme site and the nonheme metal-binding site.

Fig. 22 Design of metalloenzymes with dual active sites in a single protein scaffold of Mb: Single mutation of R118H/M in Mb generates a copper-binding site of [3-His] or [2-His-1-Met], together with the native heme site, showing NIR activity by reduction of nitrite to nitric oxide at both sites [173].

In a previous study, Fraaije and co-workers [174] constructed an artificial metalloenzyme that combines both oxidase and peroxidase activities in a single protein scaffold (namely oxiperoxidase), by fusing a microperoxidase (MP) to a flavoprotein oxidase, alditol oxidase (AldO) (Fig. 23). Both cofactors, heme and flavin adenine dinucleotide (FAD), were covalently linked to the single polypeptide chain with 1:1 ratio. More importantly, the designed oxiperoxidase, AldO-MP, is capable of catalyzing cascade reactions both *in vivo* and *in vitro*. The product of AldO, H₂O₂, was used as a cosubstrate for MP, converting the peroxidase substrates to the corresponding products. In addition, the authors engineered even smaller oxiperoxidases by constructing a CXXCH heme-binding (HB) motif in different positions in the AldO backbone (HB1-HB4) (Fig. 23). When the CXXCH motif was introduced in the N-terminus of AldO by only three mutations, a functional small oxiperoxidase, AldO-HB1, was created, albeit with only 15% heme occupancy. This study presents a novel example of merging two complementary enzyme activities in a single protein scaffold, making it possible for design of advanced metalloenzymes with dual active sites capable of other cascade reactions.

Fig. 23 Design of oxidase-peroxidase hybrids using AldO as a protein scaffold by fusing a MP or introducing a heme-binding motif (HB1-HB4) in different locations, which are capable of cascade chemistry. Reprinted with permission from ref. [174], Copyright 2012 Wiley-VCH.

As shown recently by Filice *et al.* [175], an artificial metallolipase with dual active sites capable of a cascade reaction was constructed. Based on the structural information of *Geobacillus thermocatenulatus* lipase (GTL), a single Cys was introduced at position 196, with two naturally occurring Cys65 and Cys296 replaced by Ser, which was used to selectively link a phenathroline-based Cu(II) ligand (Fig. 24, box). With the native catalytic site where Ser114 is located, the constructed enzyme can catalyze the hydrolysis of nitroarene ester at its native catalytic site (0.107 min^{-1}), and the product is further reduced to aminoarene at the artificial copper site in presence of NaBH₄ (0.036 min^{-1}) (Fig. 24, left). These results indicate that the comparable native and artificial activities are crucial for an artificial enzyme to perform cascade reactions. Moreover, the artificial copper site can also catalyze Diels-

Alder reaction with excellent performances (98% conversion and 92% ee) (Fig. 24, right), suggesting the diverse functions of the artificial catalytic site. The artificial copper site can also be created at the native site by Ser114 to Cys114 mutation, to catalyze the Diels-Alder reaction, whereas with only 18% ee, revealing the fine-tuning role of micro-environment in enantioselectivity.

Fig. 24 An artificial metallolipase designed by covalently linking a phenanthroline-based Cu(II) ligand to Cys196 in GPL, showing an ability to catalyze both cascade reaction (left) and Diels-Alder reaction (right). Reprinted with permission from ref. [175], Copyright 2015 The Royal Society of Chemistry.

6.2 In dimeric protein scaffolds

Protein dimerization is a common phenomenon [176], as driven by hydrophobic or H-bonding interactions, which might produce protein cavities at the interface suitable for design of catalytic sites. In a previous study, Kuhlman and co-workers [177] designed a zinc-mediated protein homodimer, namely MIDI-zinc, which forms dual zinc sites at the interface coordinated by three His residues with an open site, instead of four designed His residues (Fig. 25a). Later on, they found that the dual zinc sites exhibit an esterase activity, such as hydrolysis of 4-nitrophenyl acetate (4NPA) with

an efficiency ($k_{\text{cat}}/K_{\text{M}}$) of $630 \text{ M}^{-1}\text{s}^{-1}$ ($k_{\text{cat}} = 0.3 \text{ s}^{-1}$, $K_{\text{M}} = 0.42 \text{ mM}$) [178]. Although the activity is 4-fold lower than that of native carbonic anhydrase II ($k_{\text{cat}} = 50 \text{ s}^{-1}$; $K_{\text{M}} = 21 \text{ mM}$; $k_{\text{cat}}/K_{\text{M}} = 2550 \text{ M}^{-1}/\text{s}^{-1}$), MIDI-zinc has a 50-fold lower K_{M} value, indicating that the pocket at the dimer interface is suitable for substrate binding.

In another study, Roelfes and co-workers [179] created dual copper sites at the interface of a transcription factor dimer, the Lactococcal multidrug resistance Regulator (LmrR), by covalently linking a ligand phenanthroline (Fig. 25b, i) or 2,2'-bipyridine (Fig. 25b, ii) to a Cys introduced at position 19 (red) or 89 (orange) (Fig. 25b). The artificial metalloenzyme with dual active sites of Cu(II)-phenanthroline was capable of catalyzing Diels-Alder reaction with excellent enantioselectivities (up to 97% ee) (i), and that with Cu(II)-2,2'-bipyridine capable of catalyzing hydration reactions, e.g. the conjugate addition of water to generate chiral β -hydroxy ketones (up to 84% ee) (ii) [180]. Recently, they further incorporated an unnatural metal-binding amino acid, Bpy-Ala (Fig. 25, iii), into dimeric LmrR at three different positions (19, 89 or 93) using the amber stop codon suppression methodology [181]. Upon Cu(II) binding to the dual sites, the artificial metallo-enzyme can catalyze asymmetric Friedel-Crafts alkylation reactions, which was not catalyzed by Cu(II)-2,2'-bipyridine-LmrR in the previous design, suggesting a distinct structure of the active site. By further optimization of the dual active sites, such as F93W mutation, the dimeric LmrR F93W mutant with Cu(II)-Bpy-Ala at position 89 produced the product with 94% conversion and 83% ee for acylation of 2-methylindole (iii), indicating a crucial role of the aromatic side-chain in the secondary sphere.

A very recent study by Roelfes and co-workers [182] showed that upon binding of

Cu(II) complexes, such as Cu(II)(dmbipy)(NO₃)₂ complex, to a DNA scaffold, the Friedel-Crafts reaction was enhanced >700- to 990-fold. DNA-accelerating effect was also observed for other reactions such as carbene-transfer reactions catalyzed by cationic iron-porphyrin [183], which suggests that DNA scaffolds are alternative to protein scaffolds, with potential applications in design of functional metalloenzymes.

Fig. 25 Design of metalloenzymes with dual active sites in dimeric protein scaffolds: (a) Esterase activity of designed MIDI-zinc by hydrolysis of 4NPA. Reprinted with permission from ref. [178], Copyright 2012 American Chemical Society.; (b) Dual copper active sites created at the interface of LmrR by covalently linking (i) phenanthroline, (ii) 2,2'-bipyridine to a Cys, or (iii) by incorporation of UAA, Bpy-Ala, catalyzing Diels-Alder, hydration, and asymmetric Friedel-Crafts alkylation reactions, respectively. Reprinted with permission from ref. [181], Copyright 2015 The Royal Society of Chemistry.

Domain-swapping is a common way for protein dimerization, by exchanging a secondary structure such as a helix between two monomers [184-186]. If domain swapping involves a region associated with an active site, it will alter the active site structure in the dimeric protein, and this process may have a possibility to generate novel artificial metalloenzyme. As shown by Hirota and co-workers [187], dimeric horse heart Cyt *c* was formed by domain swapping of C-terminal helix, in which the heme axial Met80 was dissociated from the heme iron, creating a pocket capable binding of substrate (Fig. 26a). Based on this observation, the peroxidase activity of dimeric Cyt *c* was evaluated [188], which revealed an enhancement (~4-fold) of the catalytic efficiency toward both 2-methoxyphenol (guaiacol) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), as compared to the monomer (Fig. 26b). Moreover, reactive species of Compound I (oxyferryl porphyrin π -cation radical) was detected in reaction of dimeric Cyt *c* with meta-chloroperbenzoic acid (*m*CPBA), with a formation rate constant (0.019 s^{-1}) 6-fold higher than that of the monomer (0.0032 s^{-1}), which is thus responsible for the enhancement of peroxidase activity.

Fig. 26 Cyt *c* dimerization by domain-swapping: (a) X-ray structure of dimeric horse heart Cyt *c* (PDB code 3NBS) [187]; (b) Steady-state rates of guaiacol (left) and ABTS (right) oxidation catalyzed by dimeric or monomeric Cyt *c*. Reprinted with permission from ref. [188], Copyright 2015 The Royal Society of Chemistry.

Different from domain-swapped dimer of Cyt *c*, dimeric Mb by domain swapping forms a unique structure with each active site constructed by two protomers almost identical to that of the monomer [189]. X-ray structure revealed that in addition to the hydrophobic interactions, there were four new salt bridges formed between two pairs of the charged amino acids in protomers A and B (K78_A/E85_B, E85_A/K78_B, K79_A/D141_B, D141_A/K79_B). By reversing the charge of these two amino acids, Hirota and co-workers [190] showed that a heterodimeric Mb can be engineered by dimerization of K78E/K79D Mb (namely ED Mb) and E85K/D141K Mb (namely KK Mb). Moreover, with modification of the active site of KK Mb mutant (H64V/V68H E85K/

D141K Mb, namely VHKK Mb), a heterodimeric ED-VHKK Mb with two distinct active sites (one His/H₂O coordination, the other bis-His coordination) can be obtained, as confirmed by X-ray crystallographic study (Fig. 27a). More interestingly, as illustrated in Fig. 27b, the oxidation and coordination states of the two heme sites can be conversed by reduction with either ascorbic acid or dithionite, and subsequent exposure to air, which also indicated that they are two independent active sites. This study demonstrates that protein dimerization by domain-swapping can be well-controlled by rationally modulating interface interactions between the protomers, which can also be applied for design of artificial multiheme proteins.

Fig. 27 Mb dimerization by domain-swapping: (a) X-ray structure of heterodimeric horse ED-VHKK Mb (PDB code 3WYO). Reprinted with permission from ref. [190], Copyright 2015 Wiley-VCH; (b) Conversion of oxidation and coordination states in heterodimeric horse ED-VHKK Mb [190].

Similarly, by constructing two Mb variants with opposing surface charges, D44K/D60K/E85K Mb(+6) and K45E/K63E/K95E Mb(-6), Hoffman and co-workers [191] created a heterodimeric Mb, [Mb(-6), Mb(+6)], with a high affinity ($K_a = 1.0 \times 10^5 \text{ M}^{-1}$) (Fig. 28a). In the absence of X-ray or NMR structural information for the heterodimer, Brownian dynamics simulations showed that the E and F-helices of the protomers are in antiparallel contact with each other, with nearly parallel heme planes at an Fe-Fe distance of $\sim 18\text{-}19 \text{ \AA}$ (Fig. 28b). After substituting the heme in K45E/K63E/K95E Mb(-6) with Zn-deuteroporphyrin, the [ZnMb(-6), $\text{Fe}^{3+}\text{Mb(+6)}$] complex exhibited rapid intracomplex electron transfer, with rate constants increased by $>10^5$ fold those of [ZnMb(WT), $\text{Fe}^{3+}\text{Mb(WT or } \pm 6)$] complexes. Moreover, the charge separated intermediate formed in the [ZnMb(-6), $\text{Fe}^{3+}\text{Mb(+6)}$] upon excitation showed two well-resolved maxima in the progress curve, which was distinct from those of [ZnMb(WT), $\text{Fe}^{3+}\text{Mb(} \pm 6)$] complexes (Fig. 28c). This study indicates that charge-disproportionation is a powerful approach to designing artificial multiheme proteins that can be further functionalized by heme substitution.

Fig. 28 Mb dimerization by charge-disproportionation: (a) A cartoon representation of the design of charge-disproportionation in Mb; (b) A representative configuration for heterodimeric [Mb(-6), Mb(+6)] complex obtained from Brownian dynamics simulations; (c) Progress curves for the charge separated intermediate of [ZnMb(-6), Fe³⁺Mb(+6)] complex, with [ZnMb(WT), Fe³⁺Mb(+6)] and [ZnMb(WT), Fe³⁺Mb(-6)] complexes shown for comparison. Reprinted with permission from ref. [191], Copyright 2016 American Chemical Society.

In a previous study [192], Hoffman and co-workers showed that D44K/D60K/E85K Mb(+6) has more strong electrostatic interactions with its negatively charged redox partner, Cyt *b*₅, and exhibits an electron transfer rate 200-fold that between Mb and Cyt *b*₅. Following this approach, Lu and co-workers [193] introduced the corresponding three Lys residues into G65Y-Cu_BMb, a biosynthetic model of HCOs

[100], called G65Y-Cu_BMb(+6). This mutant exhibited a 400-fold enhancement in electron transfer rate in complex with Cyt *b*₅. Moreover, the G65Y-Cu_BMb(+6)-Cyt *b*₅ enzyme system reduced O₂ at a rate of (52 s⁻¹), which achieves the activity of native Cyt *cbb*₃ oxidase (50 s⁻¹). This study thus represents a remarkable success in artificial metalloenzyme design.

Fig. 29 Design of Mb-Cyt *b*₅ interface to facilitate electron transfer: (a) Structures of G65Y Cu_BMb(+6), showing the engineered Lys residues, and Cyt *b*₅; (b) Oxidase activity of G65Y Cu_BMb(+6) in comparison with those of Cyt *cbb*₃ oxidase and G65Y Cu_BMb under the same optimized conditions [193].

6.3 *In de novo* protein scaffolds

As shown in previous sections (2.2, 3.1 and 4.1), *de novo* proteins such as four-helical bundles are excellent scaffolds for artificial metalloenzymes design by engineering mononuclear or dinuclear metal sites, and incorporating metal complex or

clusters. Moreover, these proteins were designed to be water-soluble for facilitating chemical and physical characterizations. Comparatively, less progress was made in design of functional *de novo* membrane proteins [194]. Recently, DeGrado and co-workers [48] designed a membrane-spanning four-helical bundle capable of transporting first-row transition metal ions such as Zn^{2+} across membranes, with concurrent transport of protons reversely. The four-helical bundle was designed to have two di- Zn^{2+} -binding sites of [2-His-4-Glu] at either end, formed by two pairs of helices (Fig. 30). The Zn^{2+} ion binding to one side of the membrane (the *cis* side) can be transported to the other side (*trans*) by the rocking of the two pairs of helices. The artificial transmembrane transporter was thus named Rocker. Although the activity of Rocker was not comparable to natural transporters, the design of Rocker with its structure was determined at a high-resolution (2.7 Å), indicates a major breakthrough in the field of designing membrane systems.

Fig. 30 A *de novo* designed transmembrane transporter Rocker, with two di- Zn^{2+} -binding sites of [2-His-4-Glu] at either end, transporting Zn^{2+} ions by conformational exchanges of two pairs of helices. Reprinted with permission from ref. [48], Copyright 2014 AAAS.

In case of design of dual heme sites in *de novo* protein scaffolds, it has been more than twenty years from the first report by Dutton and co-workers [195], a two-helical bundle accommodates two bis-histidyl heme groups and assembles into a four-helical bundle dimer. The heme-binding maquettes (robust simplified designs [196]) were optimized recently to be a single-chain, which assemble into four-helical bundles and are capable of binding either two heme groups, or one heme and one heme analogs such as Zn-protoporphyrin and Zn-chlorin, or two of these heme analogs [197]. With the help of the natural cellular machinery for cyt *c* maturation, Anderson *et al.* [198] developed an expression system in *E. coli* to construct an artificial *c*-type maquette by covalently linking a heme to the four-helical bundle through two thioether bonds. The bis-His coordinated heme *c* can undergo ligand exchange between one axial His and external ligands such as CO and O₂ (Fig. 31a). The *c*-type maquette was also designed to possess a second tetrapyrrole binding site for incorporation of heme or heme analogs. For example, heme *b* binding increased the thermal stability of the *c*-type maquette, with T_m value increased from 34 °C to 66 °C (Fig. 31b), and has a redox potential (-250 mV) 60 mV lower than that of heme *c* (-188 mV). Moreover, the *c*-type maquette was converted to a photoactive maquette by replacing the iron in heme *c* with zinc using the hydrofluoric acid and remetallation method. It showed that upon heme *b* binding to the Zn-*c*-type maquette, the fluorescence with an emission at 586 nm was quenched by ~40% (Fig. 31c), suggesting an efficient energy transfer between the two chromophores in the photoactive maquette. With these advances, the artificial metalloenzymes with dual actives can be fulfilled by rationally combining different protein components, such as electron transfer, substrate binding, catalytic

and lighting harvesting components, and the functionalities can thus be integrated comparable to or even beyond those of natural systems [28].

Fig. 31 Design of metalloenzymes with dual active sites in *de novo* protein scaffolds:

(a) A cartoon representation of the designed *c*-type maquette with O₂ reversibly binding to the heme *c*; (b) Temperature dependence of the CD signal at 220 nm upon heme *b* binding the *c*-type maquette [198]; (c) Fluorescence excitation and emission of Zn-*c*-type maquette and the quenching effect of heme *b* binding. Reprinted with permission from ref. [198], Copyright 2014 The Royal Society of Chemistry.

7. Design of metalloenzymes with multiple active sites

In addition to using dual active sites, naturally existing metalloenzymes may form more complicated complexes to perform biological functions, such as the respiratory cyt *bc*₁ complex [199] and the cyt *b₆f* complex of oxygenic photosynthesis [200]. Using chemically synthetic approach, model complexes resembling the catalytic center of the native metalloenzyme may be rationally synthesized. For example, the O₂-evolving center in photosystem II has been successfully mimicked by an artificial Mn₄Ca-cluster [201]. Meanwhile, it is particularly challenging for design of artificial

metalloenzymes to mimic the entire native systems. In recent years, many efforts have been made toward the design of functional metalloenzymes with multiple active sites, by either integrating catalytic components into a single protein scaffold, or exploiting the protein-protein interfaces, protein oligomers and supramolecular assemblies, as reviewed herein.

7.1 In single protein scaffolds

With the demand of clean and renewable sources of energy, much progress has been made recently in design of artificial photocatalytic systems with multiple active sites for H₂ generation. These systems usually contain the catalyst and photosensitizer, as well as sacrificial electron donors [202]. To increase enzymatic efficiency, the multi-molecular systems are required to be integrated in a single system such as protein-based system for facilitating electron transfer [203]. Recently, Utschig and co-workers [204] designed a series of Ru-protein-Co biohybrids for photocatalytic H₂ production, by directly linking both the photosensitizer, [Ru(bpy)₃]²⁺, and the catalyst, Co(dmgh)₂pyCl (CoPy) or Co(dmghBF₂)₂·H₂O (CoBF₂) (Fig. 32a), to the an electron transfer protein, ferredoxin (Fd) (Fig. 32b) or flavodoxin (Fld). With a native [Fe₂S₂] cluster in Fd acting as an electron relay for delivering electrons from the photosensitizer to the catalyst (Fig. 32b), the Ru-Fd-Co biohybrid produced up to ~650 turnovers of H₂, in which a charge separated state of Ru(III)-Fd-Co(I) was observed lasting for ~6 ms. The native [Fe₂S₂] cluster was required for the H₂ production, as Ru-Fd-Co was inactive by replacement of the cluster with a Ga metal. In the absence of an internal electron relay, the Ru-ApoFld-Co biohybrid showed ~7.6-fold lower efficiency (~85 turnovers), *via* a different charge separated state of

Ru(II)-Fd-Co(I) with a short life ($\sim 810 \mu\text{s}$). These observations suggest that the protein matrix play crucial roles in supporting the functional photocatalytic systems, on one hand it brings together the photosensitizer and the catalyst, and on the other hand, it inhibits back charge recombination, thereby facilitating H_2 production.

Fig. 32 Design of metalloenzymes with multiple active sites in single protein scaffolds: (a) Chemical structure of $\text{Co}(\text{dmgh})_2\text{pyCl}$ (CoPy) (left) and $\text{Co}(\text{dmgbf}_2)_2 \cdot \text{H}_2\text{O}$ (CoBF₂) (right); (B) Biohybrid of Ru-Fd-CoBF₂ showing photocatalytic H_2 production by electron transferring through the native [2Fe-2S] cluster. Reprinted with permission from ref. [204], Copyright 2016 The Royal Society of Chemistry.

7.2 In protein-protein interfaces

Metal ions are usually found not only within protein scaffolds, but also in protein-protein interfaces, where they play crucial roles in supporting quaternary protein structures, mediating protein-protein interactions, or even serving as catalytic centers [205]. An alternative approach for design of metalloenzymes with multiple active

sites is construction of catalytic sites in the interfaces between proteins or protein assemblies. In the past ten years, Tezcan and co-workers [206-208] made many efforts in rational design of protein assemblies by exploiting interfacial metal coordination. Recently, Song and Tezcan [209] designed a tetrameric assembly of cyt *cb*₅₆₂ variant with both structural zinc sites (3-His-1-Asp) and catalytic zinc sites (2-His-1-Glu-1-H₂O) in its interfaces, which plays a role in maintaining the quaternary structure and in supporting the hydrolase activities toward both 4NPA and ampicillin (a β -lactam antibiotic), respectively (Fig. 33). Significantly, this assembly exhibited metallo- β -lactamase function in *E. coli* periplasm, which confers on the bacteria an ability to survive in treatment with ampicillin. This study demonstrates that design of multiple metal sites in protein-protein interfaces is possible for creation of metalloenzymes with new biological structures and *in vivo* enzymatic functions.

Fig. 33 Design of metalloenzymes with multiple active sites in protein-protein interfaces: X-ray structure of Zn-mediated assembly of cyt *cb*₅₆₂ variant with multiple structural Zn sites and catalytic Zn sites (PDB code 4U9E), showing hydrolase activities. The hydrolysis of ampicillin was shown as an example. Reprinted with permission from ref. [209], Copyright 2014 AAAS.

7.3 In protein oligomers and polymers

As shown in 6.1 section, dimeric proteins are suitable for design of metalloenzymes with dual active sites. Then, with the structural units, native protein oligomers and polymers may be suitable for design of metalloenzymes with multiple active sites. As exemplified recently by Hayashi and co-workers [210], the native oligomer of hexameric Tyr-coordinated heme protein (HTHP) was used as a scaffold for design of a light harvesting system. After removal of the native heme group, Zn-protoporphyrin or Zn chlorin e₆ can be fully incorporated into the six heme pockets of apo-HTHP, resulting in a novel array of photosensitizers (Fig. 34). Fluorescence quenching studies further showed that energy migration indeed occurs within the reconstituted HTHP, which makes this system possible for generation of new efficient photocatalysts and devices.

Fig. 34 Design of metalloenzymes with multiple active sites in protein oligomers: Hexameric structure of HTHP and reconstitution by Zn-protoporphyrin or Zn chlorin e₆. Reprinted with permission from ref. [210], Copyright 2015 The Royal Society of Chemistry.

Moreover, the formation of protein oligomers and polymers can be rationally regulated by protein-protein interface interactions [211-213]. Different from Tezcan's approach of metal coordination-initiated oligomerization [206-209], Hayashi and co-workers [214, 215] developed a approach of constructing heme protein assemblies using successive interprotein heme-heme pocket interactions. It has been successfully applied for apo-H63C cyt *b*₅₆₂ [216] and apo-A125C Mb [217] by covalently linking a heme group to the surface Cys, following by successive incorporation of the surface heme group into the native heme pocket, resulting in formation of fibrous assemblies. Cyt *b*₅₆₂ assemblies, ranging from tetramer to hetamer, were also constructed by using Zn-protoporphyrin and immobilized on a gold electrode, which generated cathodic current upon irradiation with white light [218]. Recently, Onoda, Hayashi and co-workers [219] constructed a supramolecular conjugate of Mb and water-soluble poly(acrylate) by covalently linking the heme propionate group to the side-chain carboxyl group of poly(acrylate) (Fig. 35a), followed by incorporation with apoMb or dimeric apo-A125C Mb, which produces Mb-connected frib or microgel, respectively (Fig. 35b). Atomic force microscopy (AFM) image indicated that five or six Mb molecules are directly linked to the polymer chain (Fig. 35c). Based on this observation, a model structure of the conjugate was proposed in Fig. 35d. More importantly, Mb retains its ability to bind exogenous ligands such as imidazole and CO in these conjugates, which suggests that it is possible for further development of supramolecular systems with advanced functions based on other heme-containing enzymes.

Fig. 35 Design of metalloenzymes with multiple active sites in protein polymers: (a) Molecular structure of heme covalently linked to poly(acrylate); (b) Incorporation with apoMb or dimeric apo-A125C Mb to generate protein-polymer conjugate or gel, respectively; (c) AFM image of the apoMb-poly(acrylate) conjugate, and the height profile along the green line; (d) A proposed model structure of the conjugate, with the approximate size of Mb in X-ray structure shown for clarification. Reprinted with permission from ref. [219], Copyright 2016 Wiley-VCH.

In addition to protein assembly, peptides may also assemble to achieve well-defined conformations, such as through the formation of amyloid fibrils [220]. These scaffolds may also be useful for design of artificial metalloenzymes with multiple active sites. Korendovych, DeGrado and co-workers [221] showed that self-assembly of *de novo* designed short peptides (seven residues) generates amyloid-like fibrils, which are capable of efficient hydrolysis of 4NPA in presence of Zn^{2+} (Fig. 36). By

tuning the residue close to the Zn^{2+} -binding site, such as with a glutamine at position 6 of peptide (IHIHIQI), the catalytic efficiency of the fibril can reach $360 \pm 30 \text{ M}^{-1}\text{s}^{-1}$, close to that of MIDI-zinc reported by Kuhlman and co-workers [177]. Moreover, the activity is greater than that of native carbonic anhydrase II on a weight basis (see 6.2 section). This study thus presents a novel nanobioscaffold for creating metalloenzymes with multiple active sites.

Fig. 36 Design of metalloenzymes with multiple active sites in peptide amyloid fibrils: Assembly of *de novo* designed short peptide forms amyloid-like fibrils (TEM image, scale bar, 100 nm), exhibiting ester hydrolysis activity. Reprinted with permission from ref. [221], Copyright 2014 Macmillan Publishers.

8. Conclusions and perspective

As summarized above, tremendous progress has been made in rational design of metalloenzymes from single to multiple active sites in both native and *de novo* protein scaffolds, indicating the coming of age of functional protein design [222], which not only addresses important issues in chemistry and biology, but also offers advanced artificial metalloenzymes with potential applications. Although we organized this article according to the number of the active sites in designed metalloenzymes, there

are several trends in the rational design that can be deduced from the progress.

First, the design is moving from structural to functional models. While it is still challenging to control protein folding and assembly in structural protein design [223], recent efforts have been made to design of functional metalloenzymes, aimed at achieving high efficiency and selectivity [152]. In these attempts, various approaches have been developed, such as substituting the metal ion [65], creating new metal-binding site [173], and exploiting directed evolutions [37, 52, 54, 55, 224, 225]. Heme proteins such as Cyt P450, Mb and Cyt *c* have been evolved to catalyze non-natural carbene-transfer reactions, including N-H, S-H and Si-H insertions [52, 224, 225]. Moreover, rational computational design has contributed significantly to the success [44-51]. In most cases, the combination of two or more of these approaches is more powerful [54, 55], which makes it promising to create metalloenzymes with desired functions.

Second, the design is moving from first to second coordination sphere. In addition to the first coordination sphere, the non-covalent secondary sphere interactions, such as H-bonding interactions, are powerful in fine-tuning the metal-binding affinity of the designed metalloenzymes, as well as the enzymatic efficiency [53, 65, 114, 151]. The protein scaffold of biosynthetic models provides an ideal platform for further design of second coordination sphere interactions, as well as long-rang interactions. Therefore, careful attention needs to be paid in this direction to improve the catalytic performance of metalloenzymes.

Third, the design is moving from using natural to unnatural components, including abiological metals [54] and clusters [124], UAAs [77-79], and *de novo* protein scaffolds [33, 69]. Although only helical bundles have been extensively exploited for

de novo metalloenzymes design, much progress has been made in controlling protein folding and assembly [222, 223, 226, 227], and fully *de novo* designed protein structures, even beyond those found in Nature [228], are expected in design of functional metalloenzymes. The utilization of these diverse unnatural components will certainly overcome the limitations for natural metalloenzymes, and expand the functionalities of designed metalloenzymes beyond the repertoire of natural enzymes [37].

Fourth, the design is moving from simple to complex systems. Although the majority of successful designs are focused on simple models mimicking the active center of natural enzymes, some efforts have been made to design of more complex systems, such as membrane systems [48, 49], photocatalytic systems [204, 229] and multi-enzyme systems [230-232]. These achievements represent the major breakthrough in protein design, and more efforts need to be made in this field, which will bring us closer to the success in mimicking the native systems.

Finally, the design is moving from mechanistic studies to potential applications. With increasing understanding of the mechanisms for natural enzymes, as learned from biosynthetic models [35], it is more desirable to design advanced metalloenzymes for potential applications. To this goal, substantial achievements have been made recently in artificial metalloenzymes, such as those capable of catalyzing reactions beyond native enzymes [54], catalyzing reactions in mild water conditions without any organic cosolvents [152], photocatalytic production of the clean energy of H₂ [204], and working as an electrocatalyst for O₂ reduction [233]. Aside from catalysis, the metalloproteins were designed for other applications. For example, the designed trimeric coiled-coils containing Bpy-Ala stabilized by Fe(II)/Zn(II) could

potentially serve as vaccine candidates or inhibits of viral entry [76]. The designed Ln(III)-binding three-helical coiled coils, Ln(III)-CC, is a potential magnetic resonance imaging (MRI) contrast agent, which is more efficient than the clinically adopted Dotarem [234]. Moreover, the MRI relaxivity of Ln(III)-CC is tunable by the location of the Ln(III)-binding site [235].

Taken together, these achievements, in combination with those ongoing, will ultimately elucidate the structural and functional relationship of natural metalloenzymes. This knowledge, in turn, will endow us with the ability to create novel biocatalysts and artificial biosystems beyond nature for practical applications. It is certain that we will all benefit from the design of metalloenzymes in the future, which is now limited only by our imagination.

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