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Metalloenzyme Design and Engineering through Strategic Modifications of Native Protein Scaffolds

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Abstract

Metalloenzymes are among the major targets of protein design and engineering efforts aimed at attaining novel and efficient catalysis for biochemical transformation and biomedical applications, due to the diversity of functions imparted by the metallo-cofactors along with the versatility of the protein environment. Naturally evolved protein scaffolds can often serve as robust foundations for sustaining artificial active sites constructed by rational design, directed evolution, or a combination of the two strategies. Accumulated knowledge of structure-function relationship and advancement of tools such as computational algorithms and unnatural amino acids incorporation all contribute to the design of better metalloenzymes with catalytic properties approaching the needs of practical applications.

Introduction

Metalloenzymes catalyze a wide variety of reactions with high efficiency, selectivity and under mild conditions, by combining the powerful reactivities of metal ions with the exquisite control of electronic and steric properties achievable with proteins. These catalysts perform vital reactions in biochemical processes such as photosynthesis, respiration, and natural product biosynthesis and metabolism. Therefore, elucidation of the structural features responsible for their extraordinary efficiency and versatility is a central goal of both chemistry and biochemistry. Using a “bottom up” approach of designing and engineering proteins with predictable structures and activities is an effective way to achieve this goal, as it tests our knowledge, reveals important structural features that may be concealed in native metalloenzymes, and promotes development of enzymes with novel activities.

A recent survey of >38,000 protein crystal structures in the Protein Data Bank revealed that all of these proteins belong to only ~1200 different scaffolds (Scop Classification Statistics; URL: <http://scop.mrc-lmb.cam.ac.uk/scop/count.html>), and many folds, such as the Greek key β barrel, are used by hundreds of proteins with different activities. The observation that

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Nature achieves almost unlimited functional diversity using a limited number of scaffolds suggests that, instead of designing a new scaffold for every new function, it is possible to use naturally evolved proteins as scaffolds to design and engineer various new structures and functions.

There are generally two approaches for protein design and engineering. One is rational design based on knowledge of the desired chemical reaction, the original protein scaffold, and possible structure-function relationships from either previous experiments or computational modeling [1, 2, 3 and 4•]. The other is directed evolution, a mimic of natural “Darwin evolution,” in which desired properties of proteins are obtained by *in vitro* or *in vivo* screening of mutant libraries constructed by random mutations, saturation mutagenesis at certain sites or gene shuffling [5, 6, 7 and 8]. As the two approaches have unique strengths and weaknesses, the strategy of combining them to take advantage of the precision of human knowledge as well as the power of evolution has grown in popularity [9, 10 and 11]. Moreover, recent developments in incorporation of unnatural amino acids (UAA) [12] and engineering of unnatural metallo-cofactors into protein scaffolds [11,13, 14 and 15] have further expanded the chemical versatility of engineered proteins. Here we highlight recent achievements in this area, with focus on publications in the last 2–3 years.

Rational design

Design guided by prior knowledge

The most straightforward form of metalloenzyme engineering is the use of general inorganic and biochemical insights, and knowledge derived from prior studies to guide mutagenesis of native proteins, with the goal of creating metalloenzymes having new structural features to achieve novel or improved function. Because this approach seeks a gain of function instead of perturbation or loss of the function, it can test our knowledge and in some cases reveal the importance of subtle structural features, such as hydrogen bonding interactions, on enzymatic function, which may not be obvious from studies of native enzymes.

A primary example of the success of this approach is the design of a non-heme Fe_B site in the distal heme pocket of myoglobin (called Fe_BMb) to mimic the heme-non-heme di-iron catalytic center of bacterial nitric oxide reductase (NOR) [16 and 17]. In the absence of a structure of NOR, due to inherent difficulties of crystallizing large membrane proteins, the design was guided by the known structure of heme-copper oxidase (HCO) and its sequence homology with NOR. A glutamate and two histidines known to be conserved in the NOR Fe_B site were introduced into the distal site of Mb, resulting in the Fe_BMb, which binds Fe(II) and promotes NOR activity [16••]. Encouraged by this success and based on the hypothesis that at least one more conserved Glu may play a role in NOR, an additional Glu, I107E, was introduced into Fe_BMb, as a potential hydrogen-bonding donor to the NO substrate (Figure 1a). Such a design of the non-covalent interaction increased the NOR activity by ~100% [17]. Additionally, investigation of the effect of different metals in I107E Fe_BMb revealed the critical role of the metal in the Fe_B site in weakening the heme Fe-His bond and raising the heme reduction potential by up to 70 mV. An alternative model with one of the His ligands of Fe_BMb replaced with a Glu, mimicking the proposed 2-His-1-Asp active site of gNORs, was also investigated and found to successfully reduce NO to N₂O

with Fe or Cu in the non-heme metal site [18]. Even though these studies were conducted before the 3D structures of NOR was available, using knowledge and activity-guided design, the designed proteins very closely mimic the structure of the native Fe_B site (Figure 1a) [19].

Just as the above study demonstrated the importance of non-covalent interaction in enhancing enzymatic NOR activity, incorporating a Tyr residue next to one of the histidine ligands of a designed Cu_B site in Mb that mimics HCO [20] was shown to be sufficient for imparting HCO-like oxygen reduction activity [21••]. In the absence of this residue, the presence of metal in the designed Cu_B site had surprisingly negligible effect. A crystal structure of one such protein indicates the presence of a water and associated hydrogen-bonding network that may play a role in the imparting function [21••]. More importantly, such a design resulted in a protein with >1000 turnovers. These studies demonstrate the value of the protein design approach in elucidating the subtle roles of structural features in native enzymes, particularly the importance of non-covalent interactions in achieving high enzymatic activity.

Design through incorporation of unnatural amino acids

One advantage of engineering metalloenzymes in natural protein scaffolds is that the small and stable scaffolds can be chosen, allowing incorporating unnatural amino acids into full-length proteins with high yields and low costs. Such an approach can be applied to understanding novel post-translational modifications of native enzymes, such as the Tyr-His crosslink found in HCOs. Although this crosslink has been identified by crystallography and confirmed by biochemical studies, its role remains to be understood, due to the difficulty of generating native HCOs without such a crosslink while maintaining other structural features. To overcome this limitation, an unnatural imidazole-tyrosine residue was genetically incorporated into a HCO model in myoglobin, Cu_BMb, which showed that this unique feature increased the oxidase activity by ~100% over its non-crossed linked counterpart [22]. Similarly an unnatural MeS-tyrosine residue, mimicking the native Cys-Tyr modification of nitrite reductase, was introduced into myoglobin and showed a 4-fold increase in hydroxylamine reduction [23].

One major challenge in the metalloenzyme design field is relatively low metal-binding affinity of designed proteins in comparison with that of native metalloenzymes. This challenge has recently been met by genetic incorporation of metal-chelating unnatural amino acids, such as hydroxyquinoliny alanine (HQAla) [24] and bipyridyl alanine (BpyAla) [25], into proteins. In the latter work, the incorporation was aided by computational design with atomic level accuracy using RosettaMatch and RosettaEnzyme programs, resulting in a novel metal site having picomolar affinity (Figure 1c) [26••]. The ability to precisely design sites with such a high affinity, which is in the range of native enzymes, opens many opportunities for their use as biophysical probes, and catalytic centers for enhanced or novel enzymatic activities. In addition, such new metallo-cofactors could be implemented under *in vivo* conditions, opening opportunities for high throughput screening without tedious protein purification [5].

Design through incorporation of unnatural metal-containing cofactors

In addition to unnatural amino acids, unnatural metal-containing cofactors have also been incorporated into proteins in efforts to expand the functions of metalloenzymes [14]. Anchoring of biotin conjugated metal complexes to streptavidin has proven to be a general and effective strategy [27 and 28]. For example, an organometallic piano-stool complex, $\text{RhCp}^*\text{biotinCl}_2$ was recently incorporated into streptavidin, enabling coupling of benzhydroxamic acid and methyl acrylate (Figure 1b) [29••]. When combined with mutations around the cofactor-binding site, the approach resulted in high yield (95%), regio- (19:1) and enantioselectivity (91:9). This attachment strategy was more thoroughly investigated by docking and molecular dynamics to enhance artificial transfer hydrogenase (ATHase) activity of Rh and Ir piano-stool complexes by optimizing linker length and secondary anchoring [30]. 100% ATH activity of the Rh complex could be achieved by positioning the second His anchor at either position 112 or 121, and enantiomeric excess of up to 55% (S) or 79% (R), respectively, was observed (Figure 1b). Remarkably, the group has demonstrated that isolation of these organometallic cofactors from each other using a protein scaffold imparts biocompatibility and enables them to be used in synthetic cascades with native enzymes that would typically be mutually inhibited with the catalyst [31•]. An Ir-Cp* based ATHase was compatible with a monoamine oxidase, catalase, and horseradish peroxidase, and could achieve high conversion for enantioselective reduction and enantio-enrichment (86–99% ee) of a number of important substances.

Similarly, the hemes in heme proteins have regularly been replaced with heme-like unnatural cofactors, such as metal substituted porphyrins and other macrocycles [14 and 15]. One recent example is the efficient hydroxylation of the sp^3 C-H bond in ethylbenzene by a Mn-porphycene substituted myoglobin, with 13 turnovers and an initial TOF of 33 h^{-1} [32]. In addition, other non-macrocyclic cofactors, such as metal-salen (N,N'-Ethylenebis(salicylimine)) complexes have been frequently used to replace heme in proteins due to their similar planar geometry to heme and their high activity and versatility in carrying out a number of reactions [14, 33, 34• and 35]. A systematic investigation of multiple covalent anchor positions for binding a double anchored Mn-salen into myoglobin based on computational modeling has revealed that the effect of each anchor position on both rate and enantioselectivity is independent and additive, and rate and enantiomeric excess (ee value) could be boosted 75% and 15% toward the S enantiomer, respectively, by moving the left anchor position [34•]. These effects are attributed to steric interactions in the active site, and highlight the delicate influence that attachment strategies may have on the activities of unnatural cofactors.

Library screening and directed evolution

While rational design can be effective if there is sufficient knowledge about the enzymes to be designed, this approach will have limited success in situations where less is known. Just as natural enzymes have evolved for efficiency and selectivity, using nature's strategy of random mutagenesis and directed evolution has proven to be an effective alternative strategy to make natural or engineered enzymes more general catalysts, or tailored to give a product of interest. A primary example is directed evolution of cytochrome P450s, which are the

workhorses for catalyzing hydroxylation of C-H bonds with high efficacy and selectivity in biology. This transformation is of great importance in both biological processes and synthetic chemistry. Therefore, it is not surprising that P450s have been among the major targets for protein engineering efforts for more than a decade, and huge libraries of P450s with unprecedented activities have been amassed [36 and 37]. Recently, saccharide demethylase activity was successfully engineered into a P450 from *Bacillus megaterium* (P450_{BM3}) by screening a library of P450_{BM3} variants followed by combination of random and site directed mutagenesis (Figure 2a, path I). Mutants with high demethylase activity and regioselectivity for a series of specific substrates were successfully identified [38].

Similarly, the previously characterized F87A mutant of P450_{BM3} was engineered into two sets of biocatalysts for hydroxylation of complex steroids with high regio- and stereoselectivity using two library-generation strategies (Figure 2a, path II) [39•]. In a procedure known as combinatorial active-site saturation testing (CASTing), a library of 8,700 active site mutants was produced by randomizing groups of active site residues selected by crystal structure analysis using reduced amino acid alphabet NDC codon degeneracy for groups A and C or NNK codon degeneracy for group B. Screening of this library afforded efficient catalysts for both 2 α - and 15 α - products with 96–97% regio-selectivities. In an alternative strategy, screening a library generated by iterative saturation mutagenesis (ISM) of an inferior 15 α - hydroxylation catalyst boosted regioselectivity from 62% to 96%. The CASTing strategy was also successfully applied in engineering alcohol dehydrogenases (ASHs) with either R- or S- stereoselectivity for reduction of a prochiral ketone [40•]. While the initial ADH from *Thermoethanolicus Brockii* (TbSADH) had only moderate R selectivity (66% ee), directed evolution using CASTing of six sites chosen based on crystal structure analysis afforded both R and S selective enzymes with ee value >97%, after just one round of screening. On the other hand, one of the most efficient chiral Ru-based transition metal catalysts ((TsDPEN)(p - cymene)RuCl₂) failed to impart any chiral selectivity in this reaction.

In addition to changing the substrate scope and improving stereoselectivity of the native metalloenzymes, the catalytic capabilities of P450s have been successfully expanded to abiological synthetic reactions, such as olefin cyclopropanation via carbene transfer (Figure 2a, path III) [41••], a process that is isoelectronic to the native oxygen insertion reaction. Initial active mutants were obtained by screening of an existing 92-member P450_{BM3} library with diversified activity and protein sequence. Directed evolution was used subsequently to yield the desired catalyst with synthetically useful stereoselectivities. It is noteworthy that even a *cis*-selective catalysis with high activities and selectivities (*cis*:*trans*, 92:8; ee, –97%; total turnover number, 293), a challenge in synthetic chemistry, could be achieved by this method. Moreover, a combination of directed evolution with site directed mutagenesis of this catalyst produced an enzyme P411_{BM3}-CIS that could perform cyclopropanation in *E. coli*, using the cells' own reductive machinery [42••]. The major mutation of the primary coordinating proximal Cys ligand to a Ser (C400S) raised the reduction potential of the heme cofactor to a range accessible by the physiological reductants NAD(P)H. Remarkably, such an *in vivo* catalysis system could be efficiently scaled up, achieving 27 g L⁻¹, with 48,800 turnovers, ~90% *cis*/*trans* ratio, and 99% ee_{*cis*}. It was later reported that this variant

also catalyzes intramolecular C-H amination *in vitro* and *in vivo*, by activation of a sulfonylazide (Figure 2a, path IV), and the T268A and C400S were identified to be the major contributors. Furthermore, a previously characterized T438S mutation increased the enantioselectivity from 67% to 73% [43]. Recently, the first enzyme catalyzing carbenoid insertion into N-H bonds (Figure 2a, path V) was achieved similarly by screening of ten P450_{BM3} variants. The selected catalyst named H2-5-F10 promoted the reaction in water with high selectivity for single insertion even in the presence of competing substrates such as styrene [44].

In certain cases when the desired substrates possess some similarity to the native one, an alternative substrate engineering method has been shown to achieve reactivity with non-native substrates. One such example is the oxidation of small volatile alkanes and arenes, which are too small to be a good fit for the active site of P450s for hydroxylation. Rather than rational engineering of the protein scaffold, which has shown limited success, two groups have independently achieved the catalytic hydroxylation of these classes of molecules by screening inert substrate mimics, which act as regulators to activate the enzyme, and reduce the size of the active site pocket (Figure 2a, path VI) [45•, 46• and 47•]. Kawakami *et al.* reported oxidation of small alkanes, with rates up to $\sim 110 \text{ min}^{-1}$ and up to $\sim 55\%$ coupling [45•]. Similarly, Zilly *et al.* showed oxidation of alkanes from propane to octane with TONs from ~ 500 to ~ 3600 [46•]. Most remarkably, the latter group was able to achieve monooxygenation of methane, the strongest C-H bond, with TONs up to ~ 2500 , using a perfluorodecanoic acid activator [46•]. Benzene hydroxylation was also achieved with controlled selectivity in the presence of decoy molecules, the coupling efficiency and turnover rate of which were significantly greater than P450 variants developed by directed evolution [47•]. This strategy of using native substrate mimicking activators deserves further attention in future metalloenzyme engineering investigations.

Although native metalloenzymes are generally reliable and robust starting points for directed evolution, it is noteworthy that significant structural changes may occur after engineering in some cases. An artificial RNA ligase 10C with multiple turnovers and rate enhancement of more than two-million-fold has been developed from the zinc-finger DNA-binding domain of human retinoid X receptor (hRXR α), by selection from an *in vitro* protein library of $> 10^{12}$ independent sequences followed by *in vivo* evolution [48]. NMR studies of ligase 10C demonstrated that only two cysteines of each zinc finger in hRXR α were still coordinating the metal center, and the other two were replaced by N/O ligands. Moreover, the helix structure in original scaffold was replaced by unstructured loops in ligase 10C (Figure 2b) [49]. The completely reorganized protein fold of ligase 10C led to increased flexibility, which was possibly required in the transient interaction with target molecules in catalysis. Although this discovery is serendipitous from activity-directed evolution, it opens up a new possibility for developing artificial folds by protein engineering.

Combination of rational design and directed evolution

The rational design and directed evolution approaches described in the above sections are nearly orthogonal and can serve complementary roles. Rational design offers a direct way to achieve desired activities by artificial construction of corresponding active sites [1 and 3],

but the activity is often weak, due to the absence of global compensatory mutations to accommodate the new activity, which can be difficult to predict. Directed evolution, on the other hand, allows many possible combinations of compensatory mutations to be screened rapidly to enhance activity and often stability, but it is relatively difficult to obtain new activity due to limitations such as the extent of libraries necessary for screening and the likelihood of being trapped in local minima during iterative rounds of evolution [5]. Therefore, combining the two approaches is an effective way to achieve more active or novel enzymes. The flowchart in Figure 3a demonstrates the successful implementation of such a combined strategy for the conversion of a class of NADPH-dependent oxidoreductase, ketol-acid reductoisomerases (KARIs), to NADH-dependent oxidoreductases [50•]. A detailed analysis of available crystal structures of KARIs and an extensive sequence alignment of a myriad of KARIs identified a loop $\alpha_2\alpha\beta$ as the determinant for the cofactor specificity. Modification of the loop based on rational design successfully imparted NADH dependence. Subsequently directed evolution was utilized to overcome the low activity accompanying the cofactor switch, and two enzymes with catalytic efficiencies higher than wild type were obtained.

In more complicated cases, implementing completely new activities in a protein scaffold from scratch often requires multiple iterative rounds of rational design and directed evolution. The RosettaMatch and RosettaEnzyme algorithms enable rapid, thorough, and systematic searching of the PDB for scaffolds that can accommodate catalytic restraints, such as hydrogen-bonds, with the transition state of the desired reaction, and optimization of non-catalytic interactions like shape complementarity [3, 4, 51 and 52]. This methodology has recently been applied to metalloenzymes with the redesign of a Zn-dependent adenosine deaminase into an organophosphate hydrolase (Figure 3b) [53••]. The first generation protein by computational design gave modest catalytic efficiency of $4 \text{ M}^{-1} \text{ s}^{-1}$ toward diethyl 7-hydroxycoumarinyl phosphate. This activity was further improved to $10^4 \text{ M}^{-1} \text{ s}^{-1}$ by several rounds of saturation mutagenesis and random PCR directed evolution, while native adenosine deaminase activity was abolished. Analysis of the interactions that were achieved only by directed evolution reveals features that should be accounted for in computational design approaches, such as pK_a and backbone flexibility. This emerging and developing strategy holds significant promise for the future of metalloenzyme engineering.

Outlook

Though tremendous successes have been achieved in protein design with respect to understanding and mimicking native metalloenzymes, as well as realizing unprecedented activities [1, 5, 9 and 10], the activities achievable by artificial design are generally still far behind those of natural evolution. The best protein catalysts obtained by complementary usage of rational design and directed evolution could only enhance the reaction rate by 10^6 fold, while native enzymes often achieve rate enhancements of more than 10^{12} [54 and 55], demonstrating the challenging road ahead in protein design and engineering. Moreover, application of metalloenzymes in organic catalysis is still limited in comparison to small molecule catalysts, even though reported artificial metalloenzymes have displayed promising capabilities to adopt new functions.

Advancing the field of metalloenzyme engineering even further will require growing knowledge of protein structure-function relationships, advanced computational design capabilities, and active development of new technologies, such as unnatural amino acids synthesis and incorporation, with particular attention to increasing metal-binding affinity and integrating non-covalent interactions around the metal centers. Conversely, progress in metalloenzyme design and engineering will also advance methodological developments in these areas. Given the accelerated progress made in the past few years, we are confident that the near future will bring more exciting designs, and engineered metalloenzymes with high activities, selectivities approaching those of native enzymes, and reaction or substrate scopes unprecedented in nature.

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Highlights

Design in native protein scaffolds is powerful method to understand metalloenzymes.

Rational design using knowledge and computation gave novel metalloenzyme activities.

Unnatural amino acid methods open new opportunities for non-native functions.

Directed evolution has tailored metalloenzyme reactivity toward products of interest.

Combination takes advantage of both methodologies and is becoming method of choice.

Figure 1.

Recent examples of rational metalloenzyme design. (a) $\text{Fe}_\text{B}\text{Mb}$, designed prior to structure elucidation of NOR, is structurally similar to NOR and displays NOR activity [16]. Incorporation of I107E mutation doubles nitric oxide reduction activity [17]. (b) Biotin conjugated $\text{RhCp}^*\text{biotinCl}_2$ catalyst anchored in streptavidin, with additional secondary coordination sphere interactions catalyzes C-H bond activation (From reference [29]. Reprinted with permission from AAAS) and asymmetric transfer hydrogenation (Reprinted with permission from reference [30]. Copyright (2012) American Chemical Society). (c) Design strategy for RosettaMatch design of BpyAla-based metal binding site. Crystal structure of Co (pink) bound form of the designed

protein, compared with the predicted model (gray). Reprinted with permission from reference [26], Copyright (2013) American Chemical Society.

Figure 2.

(a) Reactions catalyzed by cytochrome P450 variants. (b) 3D structure of hRXRα (left, PDB 1RXR) and ligase 10C (right, PDB 2LZE, flexible termini omitted for clarity).

Figure 3.

(a) Flowchart of cofactor switch guide for the KARI enzyme family [50]. (b) Top: the designed organophosphate hydrolysis reaction; Bottom: transition state of the reaction and designed complementary hydrogen bonding interactions by RosettaMatch algorithm or shape complementarity by RosettaDesign algorithm. Reprinted by permission from Macmillan Publishers Ltd: [Nature Chemical Biology] (reference [53]), copyright (2012).