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Metalloenzyme design and engineering through strategic modifications of native protein scaffolds

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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.

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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 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 structure–function relationships from either previous experiments or computational modeling [1–3,4•]. 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 [5–8]. 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 [9–11]. Moreover, recent developments in incorporation of unnatural amino acids (UAA) [12] and engineering of unnatural metallo-cofactors into protein scaffolds [11,13–15] 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••,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 $\sim$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 N_2O 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 a 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 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 incorporation of 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 $\sim$100% over its non-crosslinked 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 hydroxyquinolinyl 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 metallocofactors 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 [27,28]. For example, an organometallic pianostool complex, $RhCp^{*biotin}Cl_2$ 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 pianostool 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

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