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Artificial metalloenzymes derived from three-helix bundles

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Three-helix bundles and coiled-coil motifs are well-established *de novo* designed scaffolds that have been investigated for their metal-binding and catalytic properties. Satisfaction of the primary coordination sphere for a given metal is sufficient to introduce catalytic activity and a given structure may catalyze different reactions dependent on the identity of the incorporated metal. Here we describe recent contributions in the *de novo* design of metalloenzymes based on three-helix bundles and coiled-coil motifs, focusing on non-heme systems for hydrolytic and redox chemistry.

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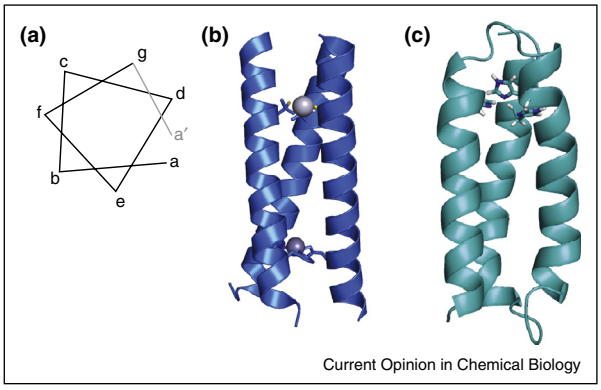
Introduction

The relationship between the structure and function of proteins has been a subject of great interest in protein biochemistry since its inception. The interplay between primary, secondary, and tertiary structure has been studied by the likes of chemists such as Linus Pauling, who famously predicted with great accuracy the α helix and β sheet based only on idealized hydrogen bonding situations. Essentially, there are two ways to approach the fundamental question of, 'how do proteins work?' One approach is the top-down method of classic protein biochemistry — using mutations, inhibitors, and other tools to perturb or abrogate function to understand key structural features that are required for folding, a specific interaction, or catalysis. The second, bottom-up approach is protein design, where features are incorporated into a design with the goal of understanding to what extent a certain structural element may contribute to a feature found in native proteins.

An estimated one-half to one-third of native proteins require a metal ion for proper folding or catalytic function [1]. Consequently, the field of metalloprotein design has evolved alongside the greater protein design field. Much of this metalloprotein design field has been reviewed extensively in recent articles [2–6]. Protein design can be broadly classified into protein redesign and *de novo* protein design. Protein redesign uses native proteins as scaffolds and then builds new functions or binding sites into the pre-existing scaffold. *De novo* protein design seeks to generate a protein 'from scratch' and features scaffolds whose primary sequence bears no relation to native proteins, thus identifying the basic, minimal features for function. One significant advantage of *de novo* design is that a single domain may be explored without the complications of allostery or multiple metal sites. From the perspective of bioinorganic chemistry, these approaches allow researchers to evaluate the extent to which the primary and secondary coordination spheres of a metal affect a metalloprotein's activity and function. Recent papers from our lab and others have indicated that reproducing the primary coordination sphere alone can confer a certain level of activity to catalytic models.

To date, most established *de novo* designed protein scaffolds consist of α helical secondary structures, which either self-assemble to form coiled coils or fold as helix-loop-helix motifs into a helical bundle [2], although catalytic metalloenzymes have recently been reported using b-sheet constructs [7•]. The α helical regions of the *de novo* designed a-helical proteins are based on the heptad repeat strategy: where seven amino acids form repeats (with residues **a**–**g** see Figure 1a) in which the **a** and **d** positions are occupied by hydrophobic residues that face inwards in the coiled coil or helix bundle, driving the folding and association of the peptides. Substitution of a residue in the **a** or **d** position with a coordinating amino acid provides a metal-binding site. The **TRI** family of peptides consists of three heptad repeats with the sequence LKALEEK, which self-assemble into three-stranded coiled-coils above pH 5.5 (Figure 1b) [8,9]. A related three-helix bundle protein, designated α_3D, was designed by adding loop regions and varying the sequence of the heptads to create a protein that exhibits native-like folding and conformational specificity (Figure 1c) [10,11]. Much of the early effort to design non-heme metal centers focused on developing stable scaffolds for transitions metals, heavy metalloids and lanthanides. In this Current Opinion we will outline recent efforts to build efficient non-heme redox and

Figure 1



(a) Helix wheel schematic showing the relative positions of amino acids **a**–**g** in a heptad repeat. Residues **a** and **d** point in the same direction such that when occupied by hydrophobic residues, they will drive the association of two or more helices. **(b)** The **TRI** scaffold self assembles into three stranded coiled coils. Substitution of two leucine layers by cysteine and histidine creates a bimetallic construct, $Hg^{II}_{S}Zn^{II}_{N}$(**TRI**L9CL23H)$_3$, the fasted reported CA mimic for CO_2 hydration. Shown is the crystal structure for $Hg^{II}_{S}Zn^{II}_{N}$(**CS**L9CL23H)$_3$, a crystallographic analogue for the **TRI** system (PDB: 3PBJ). **(c)** Model of α_3DH_3 based on the solution structure of α_3D (PDB: 2DSX). α_3DH_3 incorporates three histidine residues for metal binding and has been shown to be capable of CO_2 hydration.

hydrolytic enzymes in *de novo* designed three-stranded coiled-coils and three-helix bundle proteins.

Zinc hydrolytic enzymes

While enzymes of all classes use zinc as part of the active site or for structural stabilization, perhaps the most well known role for Zn^{II} is in the active site of carbonic anhydrase (CA). Carbonic anhydrase is a hydrolase enzyme that catalyzes the reversible hydration of carbon dioxide. With the exception of cadmium carbonic anhydrase from marine diatoms [12] all carbonic anhydrase enzymes rely on Zn^{II} for their *in vivo* activity. In α-CAs, the most extensively studied family of CA, Zn^{II} is tetrahedrally coordinated to three histidine residues and an exogenous water ligand, which is deprotonated to form the catalytically competent species [13]. CA-II of this family is one of the fastest known enzymes, with a catalytic efficiency approaching the diffusion limit in water. The combined simplicity of the primary coordination sphere and the extraordinarily high efficiencies achieved by enzymes make this an attractive design target to understand the relative contributions of the metal site and the protein environment to the rate enhancement of this reaction.

The fastest CA mimic for CO_2 hydration was designed in the **TRI** scaffold as a bifunctional construct with a structurally stabilizing Hg-thiolate site and a catalytic Zn-histidine site [14•]. The construct, termed $Hg^{II}_{S}Zn^{II}_{N}$(-**TRI**L9CL23H)$_3$ was characterized by X-ray crystallography and its activity towards the non-native substrate *p*-nitrophenylacetate and CO_2 was evaluated (Figure 1b). The catalytic efficiency at pH 9.5 towards pNPA was 23.3 M^{-1} s^{-1}, or 100-fold slower than CA-II, and its catalytic efficiency towards CO_2 hydration was 1.8×10^5 M^{-1} s^{-1}, or 500-fold slower than CA-II (see Table 1). The kinetic pK_a was 8.82 as determined by kinetic assays of pNPA hydrolysis. However, native CA has several features, including a hydrogen-bonded threonine near the active site and an extensive hydrogen bond network, which contribute to large rate enhancements and the lowering of the pK_a of Zn bound water [15,16]. Removal of Thr199 lowers the catalytic efficiency by ~100-fold for both pNPA hydrolysis and CO_2 hydration and raises the pK_a of the bound hydroxide from 6.5 to

Table 1

Zn^{II} affinity and kinetic data for CA isoforms and *de novo* designed peptides

Molecule	K_d Zn^{II} [M^{-1}][a]	Kinetic pK_a	CO_2 k_{cat}/K_m [$M^{-1}s^{-1}$][b]	pNPA k_{cat}/K_m [M^{-1} s^{-1}][b]
CA-II	$(4 \pm 1) \times 10^{-12}$ [c]	6.8 ± 0.1[d]	$(9.2 \pm 0.4) \times 10^7$ [e]	3640 ± 150[e]
CA-III[f]	–	8.5 ± 0.1	$(2.9 \pm 0.5) \times 10^5$	6[g]
TRIL9CL23H[h]	$(2.2 \pm .6) \times 10^{-7}$	9.0 ± 0.1	$(1.58 \pm 0.3) \times 10^5$	23.3 ± 0.3
TRIL2WL23H[i]	$(2.4 \pm 0.2) \times 10^{-7}$	9.2 ± 0.1	–	15.8 ± 1.1
TRILH9L23C[i]	$(8 \pm 3) \times 10^{-7}$	9.2 ± 0.1	–	15.5 ± 0.4
TRIL9CL19H[i]	$(4 \pm 2) \times 10^{-7}$	9.6 ± 0.1	–	13.9 ± 1.1
α_3D**H$_3$**[j]	$(5.9 \pm 0.9) \times 10^{-8}$	9.4	$(3.8 \pm 0.5) \times 10^4$	–

[a] Measured at pH 9.0 unless otherwise noted.
[b] Measured at pH 9.5 unless otherwise noted.
[c] Measured at pH 7. From Ref. [26].
[d] From Ref. [27].
[e] pH independent value from Ref. [28].
[f] From Ref. [29].
[g] Measured at pH 6.5.
[h] From Ref. [14•].
[i] From Ref. [17••].
[j] From Ref. [18••].