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Incorporating metals into *de novo* proteins

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The *de novo* design of artificial metalloproteins from first-principles is a powerful strategy with which to establish the minimum structure required for function, as well as to identify the important design features for tuning the chemistry of the coordinated metal ion. Herein we describe recent contributions to this field, covering metallo-porphyrin, mononuclear and multinuclear metal ion sites engineered into *de novo* proteins. Using miniature artificial scaffolds these examples demonstrate that complex natural protein folds are not required to mimic naturally occurring metal ion sites in proteins. More importantly progress is being made to engineer *de novo* metalloproteins capable of performing functions not in the repertoire of biology.

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Introduction

Metal ions are found in one-third of all proteins and play important structural and functional roles. Significant effort has been directed towards understanding the role of the protein matrix in tuning the metal ion properties, in an effort to elucidate the underlying design requirements. The ultimate goal is to utilise these design principles so as to generate functional artificial metalloproteins. Mutagenesis studies of native protein scaffolds, or re-engineering of metal ion sites into other protein scaffolds, are often hampered by the complexity of the natural scaffold and can be heavily biased by the 'evolutionary baggage' they contain. An attractive approach therefore involves the *de novo* (from scratch) design of both an artificial miniature protein fold and at the same time a metal ion binding site. These would allow one to address, without bias, what features of the protein matrix are important in tuning the metal ion properties. Though various *de novo* protein folds have been prepared including β-sheets and mixed α/β-motifs, the introduction of metal ion binding sites has generally focussed on α-helices and bundles thereof (see Figure 1). These scaffolds are easier to design, relying primarily on the heptad repeat approach *abcdefg* and the population of the *a* and *d* sites with hydrophobic residues which form a hydrophobic core, and as such represent an attractive starting point for metalloprotein engineers. This short review has focused on the *de novo* design of metalloproteins which have been reported in the last couple of years. Readers are directed to some excellent reviews covering earlier findings [1–3].

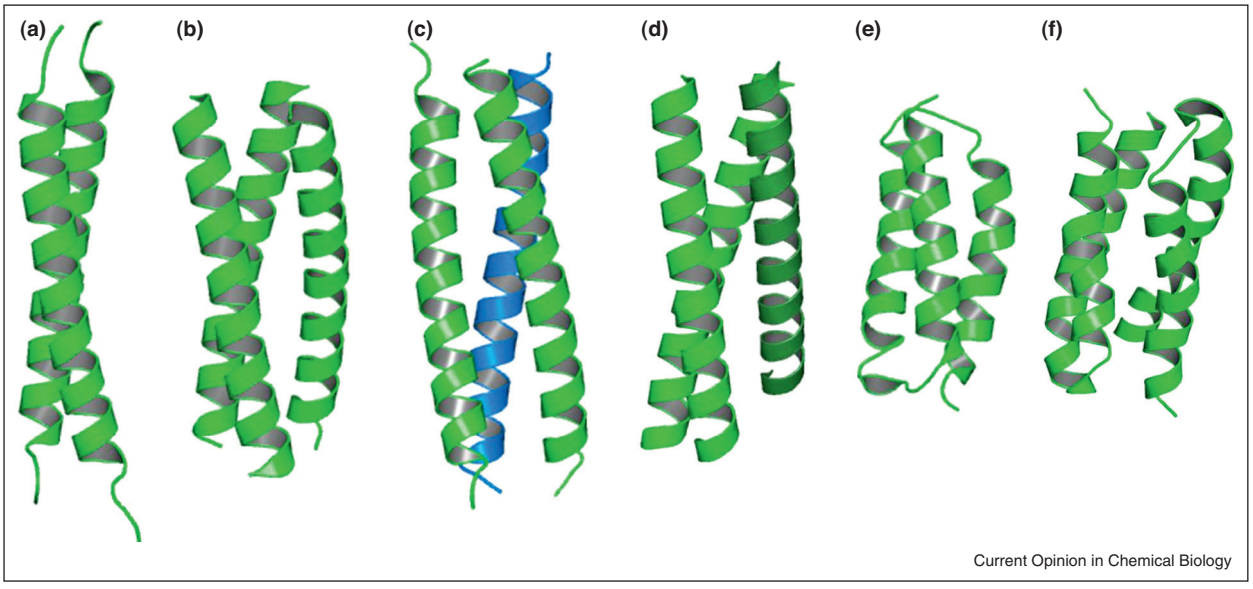
Metallo-porphyrins

The introduction of metallo-porphyrins into designed proteins has received significant attention as hemeproteins are capable of performing a large range of functions including oxygen transport, electron transfer/transport and catalysis. Recently the design of a mini helix–heme–helix architecture named mimochrome VI has been reported, capable of forming an asymmetric 5-coordinate iron-porphyrin with a cavity on the distal face for small molecule access. This was immobilised on a self-assembled monolayer coated gold electrode and found to electrocatalytically turn over dioxygen [4], and in solution reported to be capable of peroxidise-like catalytic activity [5]. An attractive advantage of mimochrome VI is that unlike native peroxidises, it is catalytically active in the presence of an organic co-solvent, broadening the scope of where it could be applied. A similar asymmetric 5-coordinate iron-porphyrin was introduced into a larger four-helix bundle as mimochrome VI was too small to engineer an Arg residue on the distal face, which enhanced hydrogen peroxide activation and improved catalytic activity [6].

A rationally designed four-helix bundle containing two iron-porphyrins was the first to bind dioxygen stably at room temperature, by controlling and preventing water access to the iron-porphyrin, and remarkably with a 10-fold higher affinity than carbon monoxide [7••]. The iron-porphyrin affinity of the distal His, and thereby access to the 5-coordinate iron-porphyrin capable of coordinating dioxygen, can be controlled by mutagenesis. Replacing three Glu residues (which can bury in the hydrophobic core) to Ala, resulted in an increase in distal His iron-porphyrin affinity [8]. This mutation affects the oxidised and reduced states differently, highlighting the importance of characterising all oxidation states of a designed metalloprotein.

Iron-porphyrin bound *de novo* helical scaffolds have also been introduced into membranes for potential electron transfer applications. A membrane spanning four-stranded coiled coil has been computationally designed with two iron-porphyrins located in the interior of the

Figure 1



Examples of various α-helical scaffolds for potential metal ion coordination; (A) dimer, pdb 1C94, (B) parallel homotrimer, pdb 3H5G, (C) parallel heterotrimer, pdb 1BB1, (D) antiparallel homotrimer, pdb 1RB4, (E) α-helical bundle, pdb 2A3D, and (F) four-helix bundle (helix–loop–helix dimer), pdb 2KIK. Shown are main chain atoms as ribbons.

structure, sufficiently close so that electron transfer could occur between the two, with the view to achieving transfer across a bilayer [9]. Using a different membrane soluble two-stranded coiled coil with an iron-porphyrin sandwiched in-between, it was demonstrated that when placed at an appropriate location, introduction of a single aromatic residue significantly alters the iron-porphyrin redox properties [10].

Despite the similarities, less effort has been directed towards the design of other metallo-porphyrin binding *de novo* proteins. A hetero four-stranded coiled coil has been computationally designed capable of binding a zinc-porphyrin in its hydrophobic core with a high degree of discrimination over related metallo-porphyrins, using both positive and negative design [11]. A database search has identified that heme and chlorophyll require different His rotamers for binding [12]. Finally, a four-stranded coiled coil capable of binding two self-quenching zinc-substituted bacteriochlorins, was studied in an effort to better understand how the local environment tunes their ground and excited state properties [13].

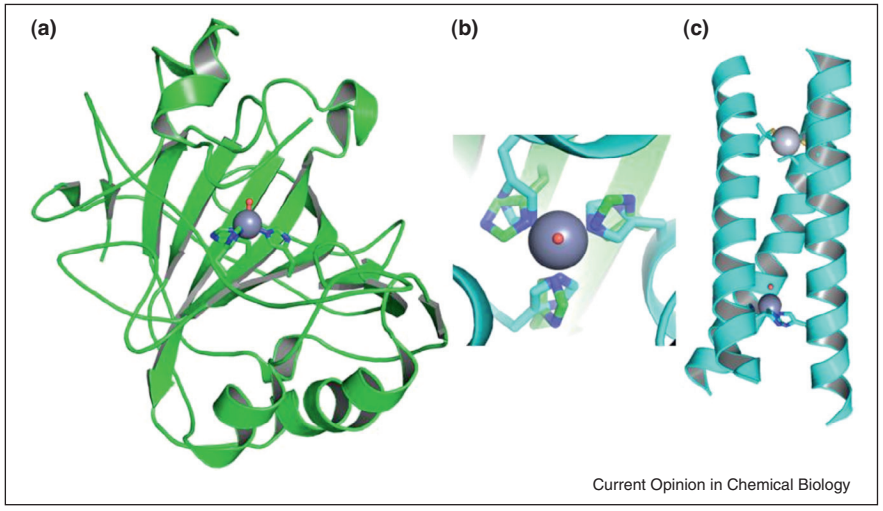
The previous examples all introduce the porphyrins into the interior of the protein; however, cobalt-porphyrins have been used to assemble 'molecular threads' by dimerising coiled coils through ligands on their exterior [14,15].

Mononuclear sites

Mononuclear metal ion sites where the majority of ligands are provided by the protein scaffold, have led to some important successes. A tetrahedral $ZnHis_3O$ (where $O{=}OH_2/OH^-$), an excellent model of the carbonic anhydrase active site, and a separate trigonal $HgCys_3$, with a stabilising structural role, have been engineered into the hydrophobic core of a three-stranded coiled coil, see Figure 2. This represents the first example of a *de novo* designed metalloprotein with two different metal ion binding sites with two distinct roles, and displays impressive catalytic activity [16]. Substrate access and metal binding affinity were subsequently found to be sensitive to the relative location of the active site within the coiled coil (e.g., proximity to frayed terminus) [17••]. A similar $ZnHis_3$ site, designed at a protein–protein interface with sufficient space to accommodate a substrate, has also been reported to be catalytic [18].

The type 2 site in copper nitrite reductase was mimicked by generating a $CuHis_3$ site within a three-stranded coiled coil. Both the reduced and oxidised form of the copper coiled coil were fully characterised and the complex found to be catalytically active and robust [19]. Notably this represents the first stable and functional $CuHis_3$ site in aqueous solution. A type 1 copper site has been designed within a four-stranded α-helical bundle (generated from a single peptide strand) with two His, one Cys and an exogenous fourth weakly interacting axial ligand. The nature of this fourth ligand is crucial in establishing a type 1 or 2 site, and so it was necessary to prevent water access. Like type 1 sites in native redox proteins, the mimic displayed fast electron reaction rates [20].

Figure 2



Structure of (A) carbonic anhydrase (pdb 1CA2) and (C) the functional three-stranded coiled coil mimic containing both a $ZnHis_3O$ catalytic site and a $HgCys_3$ structural site (pdb 3PBJ). (B) Overlay of the $ZnHis_3O$ active site from carbonic anhydrase and the *de novo* metalloprotein. Shown are main chain atoms as ribbons, metal ions as grey spheres, coordinating His (nitrogen blue) and Cys (sulfur orange) side-chains in stick form, and the coordinated water/hydroxide as a red ball.

Various studies looking at the binding of heavy metals to thiol rich sites in the hydrophobic interior of coiled coils or helical bundles have been reported [21,22,23], as these provide important insight into heavy metal biochemistry, and have allowed challenging and fundamental questions about metals in biology to be answered using these simplified scaffolds. For example, insight into metal exchange dynamics and the mechanism by which metal ions are sequestered into thiol sites [24]; whether the location of a metal site along a coiled coil alters its chemistry [17••,25]; the importance of ligand preorganisation for metal ion binding to symmetric *a* or *d* substituted sites [26], or an asymmetric equivalent generated in a single chain three-helix bundle [27]; and the importance of stereochemically active lone pairs (demonstrated for As(III) and Pb(II)) and the role second coordination sphere residues play in accommodating these, thereby dictating the binding mode [28]. The recent report of the ^{207}Pb NMR chemical shift of a water soluble $^{207}PbCys_3$ site, is of huge significance considering the importance of these sites in lead toxicity and the wide chemical shift range. Intriguingly ^{207}Pb NMR was shown to be capable of discriminating between similar but not identical $PbCys_3$ sites, and as such could be a very powerful tool in further understanding both metalloprotein design and lead toxicity [29•].

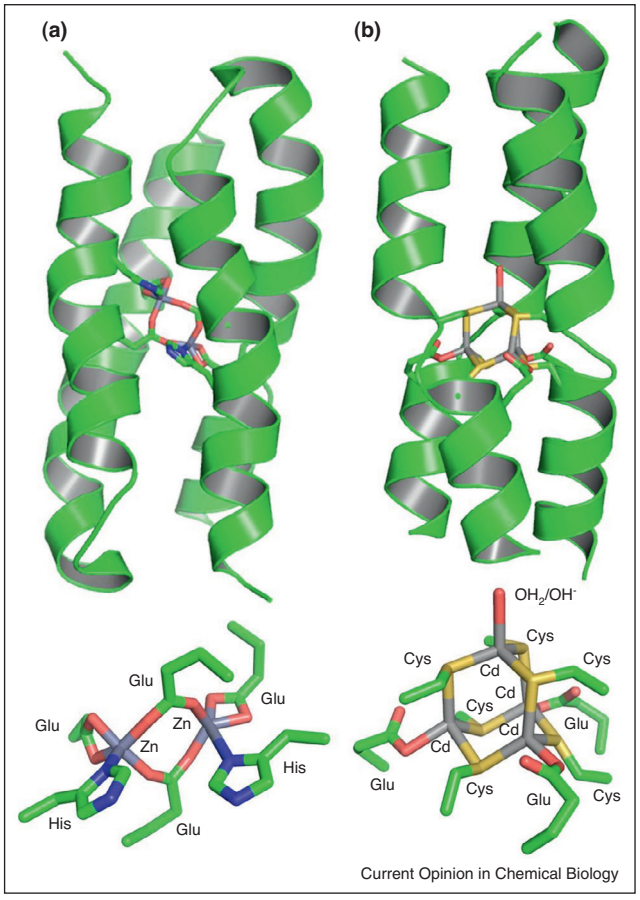
Multinuclear sites

The design of multinuclear metal ion sites can be more challenging. However, an important success is the due ferri (two iron) family of designed proteins [30]. These have been redesigned to introduce O_2-dependent phenol oxidase activity, by engineering an active site cavity in the interior of either a four-stranded heterotetrameric coiled coil [31] or a four-helix bundle (helix-loop-helix dimer) [32] (see Figure 3A). In addition to Fe, the latter was also able to bind Zn, Co or Mn [33]. The activity was then reprogrammed from the oxidation of hydroquinones to the N-hydroxylation of arylamines by four mutations, notably the addition of a His ligand in the active site (inspired by the active site of *AurF*) [34••].

A different dinuclear Fe complex, a mimic of the hydrogenase active site, has been linked to an α-helix through a non-natural residue. The resulting complex performs remarkably well at photo-induced hydrogen production in water when in the presence of both a photosensitizer and a sacrificial electron donor [35]. Importantly not all functional models require multi-helix scaffolds.

Tetranuclear Cu [36] and Cd [37] sites in the interior of a four-stranded and three-stranded coiled coil, respectively, were created using a Cys–Xxx–Xxx–Cys metal binding motif. The X-ray crystal structure of the Cd-thiolate cluster is shown in Figure 3B [37]. A dinuclear Cu site, designed to mimic the unusual Cu_A electron transfer centre (the purple copper site) in subunit II of cytochrome c oxidase, was engineered within a four-helix bundle. Intriguingly this model suggests that the Met residue located in the natural site may not in fact be necessary [38•]. The first report of a tetranuclear iron-sulphur cluster within a coiled coil (other protein folds have previously been used) offers the opportunity to assemble these into extended electron-transfer chains. These could be useful models with which to gain greater

Figure 3



Structures of multinuclear metal sites within *de novo* designed proteins. (A) NMR structure of the dinuclear zinc analogue of due ferri with vacant coordination sites for dioxygen and phenol (pdb 2KIK) and (B) X-ray crystal structure of a tetranuclear cadmium-thiolate cluster (pdb 4G1A). Shown are main chain atoms as green ribbons, metals in grey, His (nitrogen blue), Cys (sulfur orange) and Glu (oxygen red) side-chains, and water/hydroxide in stick form.

understanding of long-range electron-transfer, or could be developed into molecular wires [39].

Miscellaneous metalloproteins

The metalloproteins discussed so far have focused on biologically relevant metal ion sites, which have generally (though not exclusively) been introduced within the interior of the protein scaffold. However, a number of reports exist introducing non-biological metal ions into the design or which take advantage of programmed peptide self-assembly.

For example, dirhodium catalysts have been reported to stabilise α-helices when coordinated through Glu or Asp carboxylate side-chains in the i and either $i+3$ or $i+4$ position [40]. The authors then took advantage of coiled coil assembly to selectively modify an aromatic side-chain by positioning the dirhodium catalyst alongside an aromatic substrate on the adjacent α-helix [41]. They then found that the promiscuous dirhodium catalyst can modify 50% of natural amino acid side-chains due to proximity-driven rate enhancement, achieved by the coiled coil assembly [42••]. Importantly no other modification methods exist for some of these side-chains. A functional biotin affinity tag was also successfully introduced at a specific Trp using this approach [43], and orthogonal modification of proteins has been achieved using coiled coil assembly [44].

Coiled coil assembly has also been used to control the positioning of two chromophores for energy transfer studies. This only occurs in the folded coiled coil and is highly sensitive to the distance separating the two chromophores, being optimal when located in adjacent *e* and *g* sites on opposite α-helices [45].

Metal ions can also be used to induce and promote coiled coil assembly. Introduction of a lanthanide chelator at the N-terminus of a coiled coil, was found to result in cooperative lanthanide binding and coiled coil formation [46]. Metal (Cu, Ni or Zn) induced folding of a coiled coil which was coupled to a native DNA binding domain, was capable of regulating DNA binding [47]. We recently reported coupling a gold triethylphosphine, thought to be the active component of the therapeutic auranofin, to a coiled coil with an exterior which resembles the DNA binding domain of a natural transcription factor [48]. Not all efforts in this field are directed towards mimicking biologically relevant metal ion sites, with potential applications extending from energy transfer to DNA binding.

Conclusions

The use of artificial and miniature protein scaffolds allows the inorganic chemist to answer challenging questions about metal biochemistry, the importance of the protein matrix, and ultimately be able to design new metalloproteins *de novo* capable of performing desired functions not necessarily in the repertoire of biology. The examples discussed herein are making significant progress to these goals and importantly demonstrate that complex protein architectures are not a requirement for tuning the metal ion properties.

Acknowledgements

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