

Engineering metal-binding sites in proteins

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Metal-binding sites have been engineered into both *de novo* designed and naturally occurring proteins. Although the redesign of existing metal-binding sites in naturally occurring proteins still offers the most promise for a successful design, the more challenging goal of engineering metal-binding sites in *de novo* designed proteins and peptides is being achieved with increasing frequency. Creating new metal-binding sites in naturally occurring proteins combines the strength of both approaches. Currently, all three approaches are being used effectively in elucidating the structure and function of naturally occurring metalloproteins.

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Abbreviations

CcP	cytochrome <i>c</i> peroxidase
EPR	electron paramagnetic resonance
HRP	horseradish peroxidase
Mb	myoglobin
MnP	manganese peroxidase

Introduction

Metal ions play important roles in many biological systems. For example, currently, at least one-third of all proteins appear to contain metal ions and all ribozymes (RNA enzymes) appear to be metalloenzymes. Naturally occurring metal ions add extra dimensions to the properties of proteins and ribozymes, which otherwise are constrained by the finite number of building blocks that make up their primary structures: twenty natural amino acids for proteins and four natural nucleotides for ribozymes.

Metal-binding sites in proteins and peptides are attractive targets in protein engineering and design because the factors required for metal binding are relatively well understood, and metal ion binding frequently stabilizes folded peptides and proteins, thus aiding in the design. The degree of success of a particular design can often be measured with relative ease using the physical properties of the metal ion as an *in situ* probe. New and interesting properties are often conferred on the folded peptide or protein which can be fine-tuned at a level that often surpasses by far that achievable by metal-free proteins. Such tuning can be accomplished by employing different metal ions, by varying the oxidation

state and electronic structure of the same metal ion, or by altering the geometry and ligand-binding properties of the metal-binding sites; all of these changes can be made without changing the sequence and structure of the macromolecular backbone.

The rate of publication in the area of design and redesign of metal ion containing biological macromolecules has been rapidly increasing during the past several years, and several distinct themes have emerged. Some of these themes are by necessity under-represented in this review because of the limited time period that it covers, that is, early 1996 through to the first quarter of 1997. Recent reviews should therefore be consulted in order to obtain a more representative overview [1*,2*,3,4].

Metal-binding sites in designed proteins

Successful *de novo* design of large proteins or enzymes will ultimately require a detailed understanding of, first, how amino acid sequences determine the ultimate 3D structures of folded proteins, second, the factors that determine stability and flexibility, and third, the factors that determine reactivity and substrate specificity. Such large-scale design is not yet possible, but the increasing success in the design of peptides and small proteins gives great promise for the future. During the past year, several successful examples of such design have contained bound metal ions or metal ion containing cofactors, which are the subject of this section.

Designed zinc finger proteins

Zinc finger DNA-binding proteins typically contain multiple domains whose folded structures are individually stabilized by bound Zn²⁺ ions. The modular design of these domains makes this class of proteins a particularly interesting target for design, the success of which can be measured by their DNA-binding behavior, in other words, their relative affinities and sequence specificities [5**]. Two different approaches to the preparation of artificial zinc finger proteins—rational design and selection—provide an interesting contrast. A rational design approach tests whether the investigator can predict which amino acid substitutions will improve affinity, selectivity, or both in DNA binding [5**,6*]. A recently reported sequential selection strategy using phage display methodology has provided another approach to preparing artificial zinc finger proteins that recognize desired DNA sequences [7,8**]. Comparing the results of these two approaches, that is, rational design versus selection, is expected to provide valuable information about the factors that determine affinity and sequence selectivity in protein–DNA binding.

Other metal-binding sites in *de novo* designed proteins or peptides

The ability to design peptide sequences that fold into predictable structures without incorporation of stabilizing elements such as disulfide bridges or metal-binding sites is an important goal of *de novo* design. Metal-binding peptides are excellent systems for such studies as some metalloproteins fold only as a consequence of metal binding whereas others adopt a native-like folded structure in the absence of metal ions, thus preforming their metal-binding sites. Imperiali and coworkers [9,10**] have started with a peptide that folds only as a consequence of metal ion binding and have improved its ability to bind metal ions by a process of iterative amino acid substitution, finally arriving at a 23-residue peptide that folds without the assistance of metal binding. This research group has also evaluated several separate strategies for increasing the metal-binding affinities of designed peptides [11*] and has used a similar methodology to design a peptidyl fluorescent sensor for zinc [12].

Another approach to rational peptide design is to construct a synthetic peptide to mimic structurally the functional portion of an otherwise unrelated, biologically active peptide and then to compare its activity with that of the natural peptide. Tian and Bartlett [13*] have used this approach successfully in the design of tetrapeptides that mimic the proteinaceous inhibitor tendamistat—an inhibitor of β -amylase—when bound to Cu^{2+} ions.

Designing peptides to bind heme groups without covalent attachment of the peptide and the porphyrin presents a different kind of challenge because only one or at the most two metal–ligand interactions are likely to occur, and the remainder of the interactions are via noncovalent heme–peptide interactions. This type of heme binding can be very strong in heme-containing proteins, which frequently bind heme tightly but without covalent attachment to the porphyrin ligand. Recently, Benson and coworkers [14*] have reported a designed peptide in which the complexation of a Co(III) porphyrin causes induction of a high helix content. A related peptide–porphyrin system has recently been reported by Pavone and coworkers [15*].

A promising approach to the *de novo* design of large and complex proteins is to assemble them from smaller peptide motifs or ‘maquettes’ whose individual sequences cause them to fold into small domains of predictable structure. Metal-binding maquettes are particularly attractive because the metal ion may stabilize the desired folded structure and may often provide a useful probe. Some notable successes have recently been achieved using this approach in the design of α -helical bundle proteins that incorporate hemes or iron–sulfur clusters [16**,17*,18,19*].

An interesting use of designed metal-binding peptides is in the control of membrane permeability: the membrane-

spanning ability of the peptide can be altered upon the reversible complexation of a metal ion [20*].

New metal sites in naturally occurring proteins

Our limited ability to predict how large proteins will fold and our incomplete understanding of the factors that stabilize large folded proteins continue to hinder the design of large proteins *de novo*. Protein redesign bypasses this problem by starting with a naturally occurring, stable, folded protein and then modifying local portions of it without causing major perturbations to its natural folding pattern and stability. This approach allows investigators to redesign small portions of a protein that are held in place by stable scaffolding provided by the folded protein. Nature seems to have used the same approach. For example, thousands of 3D structures of proteins determined from X-ray crystallography and NMR spectroscopy can be classified into only a limited number of stable scaffoldings, and the active sites of the proteins, including the metal-binding sites in metalloproteins, are achieved by evolutionary fine tuning. The results of efforts to redesign metal-binding sites, therefore, help to provide insight into the natural process.

To act as a switch or a probe

Metal ion binding sites have been designed into naturally occurring proteins in such a fashion that reversible metal ion binding to a site may act as a switch to turn reactivities or other properties of the protein on or off. An interesting new example of an engineered metal ion binding switch for trypsin has been recently reported [21**]. Metal ion binding occurs with unusually high affinity, inhibiting proteolytic activity by involving the catalytic histidine in the metal complex, and the inhibition is fully reversed when the bound metal ion is removed. Another interesting use of engineered metal-binding sites as switches is in altering the membrane-spanning ability of a pore-forming protein to regulate membrane permeability (as mentioned above), and a recent study demonstrates the ability of such a switch to operate in intact fibroblasts [22*].

To aid in purification

Engineering metal-binding sites into proteins for use in immobilized metal affinity chromatography is a well-established method for protein purification. A particularly successful application of this approach has been published recently, in which a rigid, high-affinity zinc site, based on the structure of carbonic anhydrase, has been engineered into recombinant serum retinol-binding protein [23].

To probe structure-function relationships in a particular metalloprotein

A common approach to testing the relationship between the structure of a particular enzyme or other protein and its biological mode of action is to study the effects of site-directed mutations. This approach has often been highly successful when the region of interest is altered

by the introduction or modification of a metal ion binding site. Some interesting examples have appeared recently. Metal ion binding sites have been engineered between transmembrane helices to test the effects of restraining specific conformational changes or to block binding of agonist and antagonist ligands [24–26]. A cation-binding site has been engineered into ascorbate peroxidase—an enzyme whose structure is very similar to that of cytochrome *c* peroxidase with the exception that it lacks the cation site—and the effect of its presence has been investigated [27•]. A manganese-binding site has also been engineered into cytochrome *c* peroxidase [28•]. Lastly, a novel approach to protein cross-linking has been published in which a His₆ tag, usually engineered into proteins to aid in their purification, is used as an internal receptor for a reagent containing nickel that, upon addition of peracid, leads to oxidative cross-linking to nearby proteins at the His₆ tag location [29].

Designing new metal-binding sites in catalytic antibodies

An active area of protein design is in the field of catalytic antibodies in which new metal-binding sites have been designed in an effort to expand their functional diversity [30,31]. An interesting variant of this approach has been published recently in which an antibody prepared by selection against an organosilicon compound is observed to perturb reversibly the coordination geometry of a Cu(I) complex [32].

Redesigned metal sites in naturally occurring metalloproteins

There have been many recent successes in efforts to re-engineer existing metal-binding sites in naturally occurring proteins in order to give them new properties. The likelihood of successful design is probably higher in these cases than for either *de novo* design or engineering new metal ion binding sites into existing proteins because a high-affinity metal ion binding site already pre-exists in the target protein. This situation will probably persist until a clearer understanding of the fundamentals of protein folding and stabilization is acquired. In the meantime, all three approaches are providing valuable information.

Redesign to change the specificity of metal sites

Metal-binding specificity is of paramount importance in many cellular processes. A primary example is the Ca²⁺-binding proteins of the EF-hand superfamily and their roles in the regulation of cellular activities. Recent studies of parvalbumin and oncomodulin have determined that the Ca²⁺/Mg²⁺ specificity is an intrinsic property of the metal-binding site [33,34]. In a related study, four different engineered calmodulins with interchanged individual EF-hands have been characterized mechanistically [35]. A switch of metal-binding specificity from Mg²⁺ to Mn²⁺ due to a single Ile→Leu mutation has also been demonstrated in the restriction endonuclease *EcoRV* [36,37•].

Redesign to model other types of metal sites

The mixed-valent binuclear purple Cu_A center, found in cytochrome *c* oxidase and nitrous oxidase reductase, is a new class of copper center. The successful redesign of the classical blue copper proteins azurin and amicyanin by loop-directed mutagenesis into Cu_A proteins has provided new Cu_A-containing proteins for study [38,39•,40,41]. Loop directed mutagenesis technique has also been used to probe the distinguishing characteristics of subclasses of blue copper proteins [42•].

Several successful redesigns of heme proteins have recently been achieved: the proximal histidine in myoglobin (Mb) has been changed into cysteine in an effort to model the coordination configuration of cytochrome *P450* [43]. Sperm whale Mb has also been redesigned into a highly stereospecific peroxygenase [44••], and interesting alterations in reactivity have been found for the distal His→Glu mutant of horseradish peroxidase (HRP) [45].

Redesign to change the properties of existing sites

An interesting outcome of redesigning metal-binding sites is the creation of new metal centers with unprecedented structure or activity as has recently been reported for azurin [46] and copper-zinc superoxide dismutase [47] mutant proteins. A method termed 'cavity complementation' has also been used to redesign metal-binding sites. In this method, one of the ligands is changed to a smaller, noncoordinating ligand such as glycine or alanine, creating a 'cavity' within the metal-binding site. Adding different exogenous ligands can complement the cavity sterically, which often can either restore the activity, or result in a new structure and activity [48–50,51•,52•].

Engineering metal-binding sites in ribozymes

Although the focus of this review is on engineering of metal-binding sites in proteins, the recent advances in the closely related field of ribozymes are also relevant. Like proteins, ribozymes catalyze a variety of different chemical reactions and hold great promise as therapeutic agents against viral diseases. Metal ions play an essential role (both structural and catalytic) in ribozyme catalysis. The understanding of the design and structure of metal-binding sites in ribozymes is critical for its pharmaceutical application. Unlike metalloproteins, most ribozymes are active in Mg²⁺ and have low metal-binding affinity. Recent developments in *in vitro* selection techniques [53••,54] have made it possible to make new ribozymes with altered metal ion specificity [55,56] and with high metal-binding affinity [57,58]. The same technique has also been used to select ribozymes [59••] and deoxyribozymes (DNA enzymes) [60••,61] that can catalyze metalation of mesoporphrins.

Conclusions

The field of metalloprotein, metallopeptide, and ribozyme design and redesign is growing fast and is providing basic information concerning the factors that determine protein folding and stabilization, the nature of protein–metal ion

interactions, and the reactivities of metal ions bound to proteins and ribozymes. It seems only a matter of time until the successful design or redesign of diverse sets of complex metalloproteins and ribozymes will be achieved.

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Creating a cavity in the proximal histidine mutant of horseradish peroxidase (HRP) His170→Ala results in the distal histidine binding to the heme and a dramatically reduced activity. However, the addition of imidazole restores a

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