Engineering Novel Metalloproteins: Design of Metal-Binding Sites into Native Protein Scaffolds

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I. Introduction

A. Two Steps of Protein Design

Proteins play an essential role in biology. Whether it is in catalysis or molecular recognition, proteins set a golden standard of efficiency and selectivity that few other natural or artificial molecules can match. For this reason, the study of protein structure and function has been the focus of many years of research. Given the amount of time and effort devoted to this endeavor and the accomplishments achieved, it is fair to ask how much we have learned from these studies. The best way to test our knowledge is to design a protein with a desired structure and function.

The protein design process can be divided into two steps: the design of overall scaffold and the design of active sites (Figure 1). One of the most exciting advances in the protein design field is the successful de novo design of α-helical bundles.[1−4] Recently, important progress has also been made in the de novo design of other scaffolds.[5−7] Equally exciting results have been obtained in engineering active sites into those de novo designed scaffolds, such as the α-helical bundles.[8−12] These works are reviewed elsewhere in this issue. Here, we focus on the creation of metal-binding sites in existing proteins with characteristic scaffolds by de novo design or by the redesign of existing sites.

B. Choice of Scaffolds in Protein Design

Although the design of the overall scaffold and the active sites are linked, it is helpful to distinguish these two steps because our knowledge about each step is limited at this early stage of protein design. For example, when learning how to design protein active sites, we may choose to use de novo designed scaffolds made in the laboratory or native scaffolds provided by nature. While the design of active sites in de novo designed scaffolds represents the ultimate goal of protein design and is considered de novo design in its purest and most challenging term, the

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Steven Berry was born in 1975. He received his B.Sc. degree in Biochemistry and Molecular Biology from the University of Minnesota, Duluth, in 1997. He is currently working on his Ph.D. degree in Chemistry with Professor Yi Lu at the University of Illinois at Urbana–Champaign. He is currently studying new and redesigned metal-binding sites in the blue copper protein azurin.

following reasons make the design of active sites into native scaffolds equally attractive.

A recent evaluation of more than 11 000 structures in the Protein Data Bank indicates that they can be classified into ~560 different scaffolds.[13] This analysis is consistent with the observation that the same protein scaffolds, such as triosephosphate isomerase (TIM) barrels, α helical bundles, and Greek key β barrels, are used many times in different proteins, with different active sites crafted into the same scaffold. Therefore, nature is good at using a limited number of scaffolds to design proteins for various functions. Learning this "trick" is an important part of the protein design process.

While de novo designed helical bundles are one of the most common scaffolds and thus an excellent choice for active site design, few successful examples of other de novo designed scaffolds have been reported. The design of active sites into native scaffolds offers more scaffold choices and thus presents more opportunity to test our knowledge and to build proteins with novel properties.

Thomas Daniel Pfister was born in 1973 in Montreal, Canada. He received his B.Sc. degree in Biochemistry from McGill University, Montreal, in 1996. He obtained his M.Sc. degree in Chemistry from Carleton University, Ottawa, in 1999, where he studied phosphorylation-regulating enzymes from cold-hardy gall insects with Professor Kenneth B. Storey. In 2000, he started his Ph.D. degree with Professor Yi Lu at the University of Illinois at Urbana–Champaign, where he studies cytochrome *c* peroxidase (CcP) and works on engineering and redesigning heme peroxidases using CcP as the scaffold.

Most native scaffolds have high thermodynamic stability and extraordinary tolerance for residue substitution, deletion, and insertion. These properties allow more drastic changes of the native scaffolds and make active site design more likely to succeed. The resulting designed protein can more often assume globular rather than molten globular forms and thus is more amenable to spectroscopic and X-ray crystallographic characterization. Moreover, years of research in molecular biology, biochemistry, and biophysics make the construction, purification, and characterization of proteins with characteristic scaffolds a common laboratory practice. As the understanding of protein structure improves, de novo designed proteins will become more attractive as scaffolds. Until then, working with existing native scaffolds affords equal advantages.

C. Design and Engineering Novel Metalloproteins Based on Native Protein Scaffolds

An important branch of protein active site design is the design and engineering of new metal-binding sites into native proteins with characteristic scaffolds. Metal ions add new functionality to proteins and help catalyze some of the most difficult biological reactions. Protein reactivity is finely tuned by using different metal ions, different redox states of the same metal ion, or different ligands and geometric arrangements. Probably for these reasons, metal-binding sites are found in about 1/3 of structurally characterized proteins and in about 1/2 of all proteins.[14] Therefore, designing and engineering novel binding sites in proteins is an important test of our ability to design proteins.

Another advantage of choosing metal-binding sites as targets for protein design is the rich spectroscopy available for evaluating the design process. At this early stage of protein design, one can learn just as

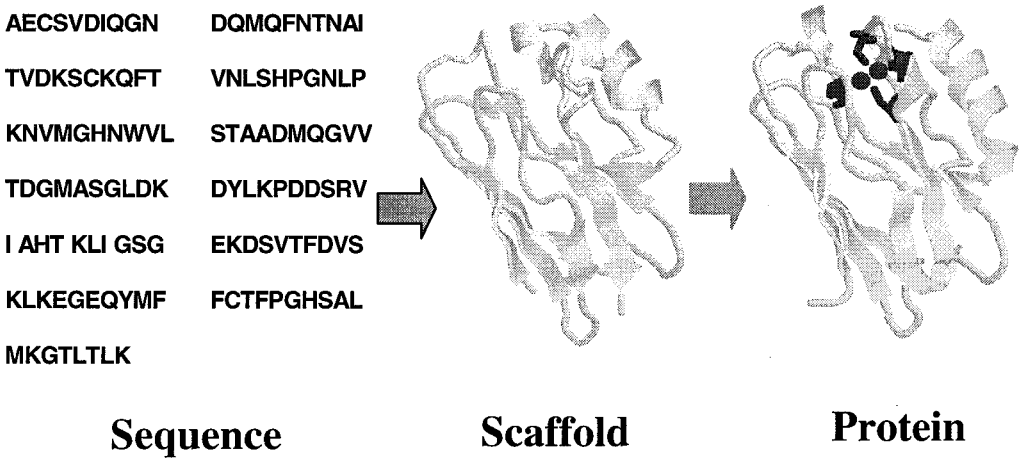

Figure 1. Two steps in protein design.

much from a failed design as from a successful one, as long as the site is properly analyzed. The rich color and magnetic properties of metal ions serve nicely as in situ probes where one can obtain immediate feedback from solution spectroscopy, without having to rely heavily on the more elaborate techniques such as X-ray crystallography and NMR.

Designing and engineering new metal-binding sites into proteins uses the same biochemical techniques as studies of proteins and their mutant forms (e.g., site-directed mutagenesis). However, there are importance differences. First, like de novo protein design, metal site design is a minimalist approach focusing on determining if the *necessary* structural features identified from the study of native enzymes are *sufficient* to confer the structure and function of the enzyme.[15] In other words, rather than making mutations in existing metal-binding sites and looking for loss of structure and activity, protein redesign stresses the mutations that produce new structure and reactivity. Second, metal site design may offer *extra* advantages because it allows the comparison of different metal-binding sites in the same protein framework. Therefore, a direct comparison of their structural and functional properties can be made without complications arising from other residues or secondary structure around the metal-binding sites. An example is given in section III.A.2.iii.c.

Many excellent reviews on metalloprotein design have appeared previously,[15−25] including two reviews in this issue. Here we attempt to focus on progress made and methodology used in the design and engineering of novel metal-binding sites in native proteins with characteristic scaffolds. Because the focus of the review is on new metal-binding sites, many interesting works on refining the properties of the existing sites (such as binding affinity or substrate-binding specificity) will not be covered here.

II. Redesign of an Existing Metal-Binding Site to a New Site with Dramatically Different Structure and Function

One of the most effective approaches in the design and engineering of novel metalloproteins is the redesign of an existing metal-binding site to a new site possessing dramatically different structural and functional properties. This approach bypasses not only the design of the overall scaffold, but also the initial creation of the metal-binding site. In addition to being more technically feasible with a better chance of success than other methods, this approach best reveals the role of specific residues responsible for a particular structural or functional feature of the metal-binding site of interest.

A. Redesign of Heme Proteins

Heme proteins catalyze a variety of reactions ranging from electron transfer, small molecule transport and sensing, to oxygen activation.[26−28] It is amazing that proteins often utilize the same type of heme to accomplish this diverse range of reactions. Biochemical and biophysical studies of heme proteins and their variants indicated that the type of reaction catalyzed is controlled primarily by (a) the proximal axial ligand and to a lesser extent by the residues surrounding it, (b) the architecture of the distal site, and (c) the type of heme. Redesign of heme proteins, from one type into another, provides a test of the known factors governing the structure and function of a heme protein and allows the direct comparison of two different heme proteins in the same framework. This knowledge is invaluable for de novo heme protein design.

In this section we attempt to cover successful examples of engineering novel metalloproteins through structural changes at the proximal and distal sides of the heme and to the heme itself. The design and creation of new metal-binding sites next to the heme is covered in section III.A.2.ii.b,c and III.A.3.ii.

1. Variation of the Proximal Ligand of Heme Proteins

Several amino acids are known to serve as axial or proximal ligands to heme proteins.[26−28] In cytochromes (cyt), histidine, methionine, lysine, and cysteine are common axial ligands that coordinate the heme. Bis-His and His-Met coordination, found in cyt b_5 and cyt *c*, respectively, are by far the most common ligation states of the cytochromes (Figure 2). Other ligation states, such as bis-Met and His-Lys, have also been found. Interesting ligation from the terminal α-amino group of a peptide bond has been observed in cyt *f* (Figure 2D).[29] Heme-based

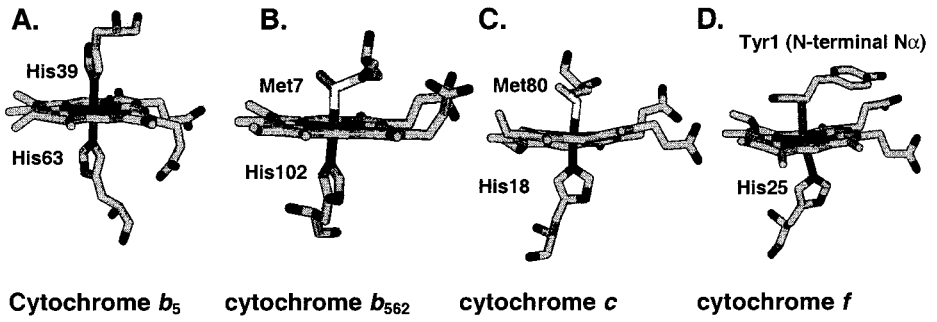

Figure 2. Cytochromes with different axial ligand coordination: (a) cytochrome b_5, (b) cytochrome b_{562}, (c) cytochrome *c*, and (d) cytochrome *f*.

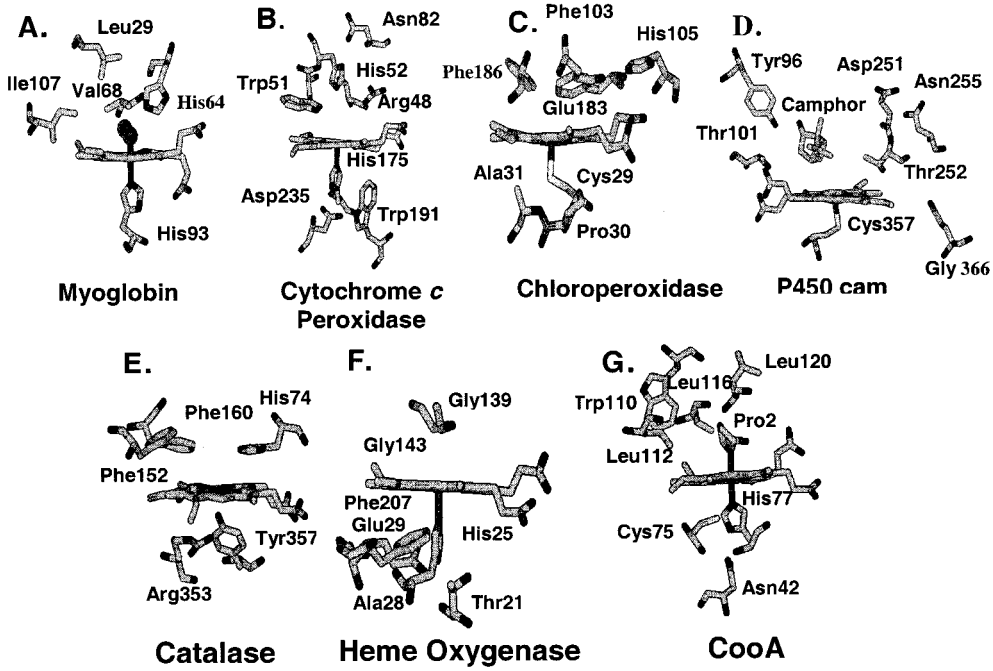

Figure 3. Heme proteins with one open coordination site: (a) myoglobin, (b) cytochrome *c* peroxidase, (c) chloroperoxidase, (d) cytochrome P450, (e) catalase, (f) heme oxygenase, and (g) CO sensing transcription factor CooA.

sensors and enzymes are mostly five coordinate with an open site for binding small molecules such as O_2 and CO. In those proteins, histidine is by far the most common axial ligand, with cysteine and tyrosine found in a few classes of heme enzymes such as cyt P450, chloroperoxidase (CPO), and catalase (Figure 3). Recently, a new axial ligand, the N-terminal nitrogen of proline, was discovered in the CO sensing heme protein called CooA (Figure 3G).[30] It has been well recognized that the axial ligands play important roles in modulation of structure and function of heme proteins, such as redox potentials, electronic structure, spin states, electron-transfer rates, and catalytic properties.[26−28,31−36] In enzymes, such as cyt cd_1 nitrite reductase, axial ligand changes occur during the catalytic cycle.[37] Therefore, redesigning heme proteins by changing their axial ligands can test our knowledge and help us learn principles for designing novel heme proteins.

i. From One Cytochrome to Another Cytochrome with a New Set of Axial Ligands. The first replacement of a heme axial ligand was reported by Sligar et al. in cyt b_5 using site-directed mutagenesis.[38] Cyt b_5 contains a heme iron coordinated by a pair of axial histidines (His39 and His63, see Figure 2A). To redesign this bis-His cytochrome to the His-Met cytochrome, such as cyt b_{562} and cyt *c* (Figure 2B, C), the authors mutated the His39 to Met. UV−vis, EPR, and RR studies indicated that the ferric protein contained a mostly high-spin, six-coordinate heme center instead of the low-spin, His-Met heme in cyt *c* or cyt b_{562}. The sixth ligand was proposed to be either the methionine or a water molecule. Interestingly, the His-to-Met mutation resulted in ∼240 mV decrease in reduction potential, from ∼6 mV (vs SHE) for the wild-type cyt b_5 to −240 mV for the variant protein. Normally His-Met cytochromes have a higher reduction potential than bis-His cytochromes (for example, *c* type cytochromes with His-Met ligation usually have reduction potentials in the range of 0 to + 400 mV, while those with bis-His ligation have potentials in the range of −400 to −100 mV).[26,27] This opposite change in reduction potential for the His-to-Met mutation may be explained by the change in spin state, from low-spin in the native protein to high-spin in the mutant protein. The high-spin state of the redesigned protein may also responsible for its ability to bind small ligands such as CO and to exhibit peroxidase activity. Similar His-to-Met mutations were carried out in cyt c_3, a multiheme protein

with four hemes in the bis-His coordination mode.[39,40] Out of the four variant proteins with His-to-Met mutations, methionine was shown to coordinate to the heme iron in two of them. Not surprisingly, the two variants with the Met-His ligation possess a reduction potential that is 160−180 mV higher than those of wild-type cyt c_3 (∼−250 mV vs SHE).[39,40] A still further increase in reduction potential was observed when the His-Met-coordinated cyt b_{562} (Figure 2B), normally with a reduction potential ranging from 150 to 275 mV (vs SHE) depending on pH, was changed to bis-Met,[41,42] again consistent with the trend of the axial ligand-dependent reduction potentials.

The methionine ligand (Met80) of the His-Met cyt *c* (Figure 2C) was replaced by histidine, cysteine, selenomethionine, and several other unnatural amino acids using protein semisynthesis.[43−47] These studies indicated that replacement of the axial Met80 could lead to a dramatic change in the heme reduction potential. The His-to-Cys change resulted in ∼650 mV decrease in reduction potential, from 262 mV for the wild-type cyt *c* to − 390 mV (vs SHE) for the cyt *c*-Cys80. It is by far the largest change of reduction potentials from axial ligand mutations. A more than 240 mV decrease in reduction potential was also observed when the bis-His-coordinated flavocytochrome b_2, normally with a reduction potential of −17 mV (vs SHE), was changed to His-Cys ligation.[48]

A novel, functional variant of cytochrome *c* was created by replacing its axial His with Arg (His18Arg). The formal potential of the mutant was not affected, thereby maintaining the electron transport function of the protein. However, its rate of electron transfer was 2−10 times slower.[49] The Arg residue was proposed to adopt a torsional geometry similar to His.[50,51] However, the Arg ligand has more flexibility than His and may have caused fluctuations in the Fe−N bond length affecting the electron-transfer rate. Similarly, the His-Met ligation of cyt c_{550}[52] and cyt c_1[53,54] was changed to His-Lys. In addition to a decrease of reduction potential by 329 mV, the variant displayed spectroscopic properties similar to those of the alkaline form of cyt *c*, which was shown to contain His-Lys ligation.[55]

ii. From Cytochrome *c* to Myoglobin and from Myoglobin to a Cytochrome. The major difference between cytochromes and other heme proteins is that cytochromes generally contain a low-spin six-coordinate heme while other heme proteins are generally high-spin and five-coordinate with an open coordination site (sometimes occupied by an exchangeable water molecule). The first step in redesigning cytochromes to a heme enzyme for catalysis is mutation of one axial ligand to a noncoordinating amino acid. This step has been accomplished in cyt *c* by changing the Met80 ligand to an alanine using either protein semisynthesis[46,56−58] or site-directed mutagenesis.[59] The latter method used a novel dual gene/protein system with a designed His-X_3-His chelation site on the surface for ease of purification (see section III.A.2.i.d). It resulted in a much larger amount of protein at a fraction of the cost of protein semisynthesis. Electronic absorption spectra of both the resting state and its deoxy, oxy, and CO derivatives showed striking similarities to those of corresponding derivatives of myoglobin (Mb). Therefore, the Met80-Ala-cyt *c* variant possessed a similar binding site for dioxygen and other exogenous ligands as in Mb. Similarly, cyt b_{562}, a low-spin His-Met cytochrome (Figure 2B), was redesigned to a heme protein that binds exogenous ligands such as CO.[60] In this study, the methionine ligand was changed to either glycine or alanine. Interestingly, the resulting protein was not stable and lost heme during purification. A careful inspection of the crystal structure of the protein revealed that two glutamate residues were in close proximity to the heme propionate oxygen atoms. The authors argued that electrostatic repulsion between the negatively charged glutamates and propionates may have caused the heme loss. When these glutamates were mutated to serine, a stable CO derivative of the mutant protein was obtained.

Myoglobin has also been redesigned into a cytochrome like the bis-His cyt b_5.[61−63] Myoglobin contains a high-spin heme coordinated by a proximal histidine at one axial position and an exogenous ligand, such as O_2 and CO, at the other axial position (Figure 3A). In the distal pocket, two highly conserved residues, His and Val, are present to interact with the bound exogenous ligands. The distal histidine does not coordinate to the heme iron directly. When the distal valine was replaced with a histidine, a low-spin bis-His-coordinated heme variant was obtained, as demonstrated by electronic absorption, EPR, IR, MCD, and NMR spectroscopic techniques as well as X-ray crystallography.[61−63] Interestingly, the mutation resulted in ∼170 mV decrease in reduction potential of myoglobin, which normally is ∼60 mV (vs SHE).

iii. From Cytochromes/Myoglobins to Heme Oxygenase. Oxidative degradation of heme is an important catabolic step in biology. The reaction is catalyzed by heme oxygenase (HO) in the presence of O_2 and NADPH-dependent cytochrome P450 reductase.[64−66] The heme is degraded first to hydroxyheme, then to verdoheme, and finally to biliverdin. For a long time heme degradation activity was also observed in myoglobin upon exposure to O_2 and a reductant such as ascorbate.[67] This process was termed "coupled oxidation" and has been used as a model for HO because it shares several features, including the product formation and, in certain cases, regioselectivity.[64,67] Several mutations in the distal pocket of myoglobin, including Val67Ala and Val68Ser in horse heart Mb[68] and Leu29His/His64Leu double mutation in sperm whale Mb,[69,70] showed much enhanced coupled oxidation activity.

Both cyt b_5 and b_{562} were redesigned to gain new coupled oxidation activity. In cyt b_5, this task was accomplished first by replacing one of the two axial histidines (in fact, it is the histidine opposite to the histidine mutated by Sligar et al.,[38] see section II.A.1.i) by a methionine.[71,72] Electronic absorption, EPR, and NMR spectroscopic studies indicated that the ferric form of the protein contained a high-spin heme with histidine as the proximal ligand and no methionine coordination. Interestingly, this mutation

resulted in the efficient and regioselective coupled oxidation of heme and produced >90% of the α-isomer of verdoheme, which was a known reaction intermediate of HO. Similar results were also obtained when the axial histidine of the His-Met cyt b_{562} was replaced with a methionine.[73] As discussed in section II.A.1.i, one of the methionines in the variant was labile in the oxidized state and new coupled oxidation activity was observed in this variant. This variant was unique in that an additional Arg to Cys mutation resulted in conversion of this variant from a *b*-type to a pseudo-*c*-type heme protein with one covalently attached heme through a single thioether linkage (see section II.A.3). Therefore, this was the first example of a pseudo-*c*-type heme protein that could carry out coupled oxidation reactions.

In an effort to redesign cyt b_5 to a protein that can perform coupled oxidation beyond verdoheme to biliverdin, the final product of HO activity, Avila et al. investigated a mutation of one of the axial histidine ligands (this time it is the same histidine mutated by Sligar et al.,[38] as discussed in section II.A.1.i) to valine.[74] Spectroscopic evidence strongly suggested that the new variant could carry out coupled oxidation to give biliverdin.

While significant progress has been made toward redesigning heme proteins with heme oxygenase-like activity, a more careful mechanistic study of the redesigned proteins is needed to design better heme oxygenase model proteins. For example, a recent finding that the coupled oxidation in myoglobin can be completely inhibited by catalase suggests that exogenous peroxides are involved in the process.[75] This is in contrast to processes in heme oxygenase, which are known to proceed through an internal peroxy intermediate.[64−66] These mechanistically important differences should be considered carefully when coupled oxidation is used as a model for HO reactions.

iv. From Heme-His Proteins to Chloroperoxidase/Cytochrome P450. Among all the axial ligands seen for heme proteins to date, thiolate ligation from Cys has received much attention from researchers. This type of ligation is important to the reactivity and spectral characteristics of P450, NOS, CPO, and CooA.[76,77] The strong electron donation from the thiolate to the iron is proposed to be responsible for heterolytic cleavage of the oxygen−oxygen bond, resulting in the high-valent, active intermediate, compound I, which then can perform a variety of chemical reactions including hydroxylations, epoxidations, and dealkylations.[31,34,35,78] The question of how the same thiolate−heme center performs different functions in various protein systems is extremely important yet remains largely unanswered. Introduction of a thiolate axial ligand into heme proteins that do not naturally possess this type of ligation could offer insight into this important question. This strategy was successfully employed in the human myoglobin system when the proximal histidine was mutated to a cysteine.[79,80] The Cys-to-heme coordination in the resting ferric state of the protein was confirmed by a variety of spectroscopic studies such as electronic absorption, proton NMR, EPR, and RR. A ~280 mV decrease in the reduction potential of the variant from its wild-type Mb potential of 50 mV (vs SHE) was also consistent with thiolate ligation to the heme iron. More importantly, reactions of the variant with cumene hydroperoxide showed that the thiolate ligand enhances heterolytic O−O bond cleavage of the oxidant in comparison with native myoglobin. Furthermore, an up to 5-fold increase in P450-type monooxygenase activity was also observed in the variant.

Interestingly, similar mutation of the proximal histidine to a cysteine in horse heart myoglobin did not result in observable Cys to heme iron ligation as in human myoglobin.[81] However, the investigators found that simultaneous mutation of the distal histidine to either a valine or isoleucine resulted in a P450-like protein in its resting ferric state, as judged by the similarity of the electronic absorption, EPR, and MCD spectra as well as by the ~280 mV decrease in reduction potential.[81] This effect, termed the *trans* effect by the authors, makes a significant contribution to the axial ligand binding and stability. This conclusion was further supported by the work of Matsui et al.,[82] who changed both the proximal histidine (to cysteine) and the distal histidine (to either glycine or valine) in human myoglobin. They showed that while elimination of the distal histidine did not influence the cysteine's ability to promote O−O cleavage, it decreased the H_2O_2-supported oxidation activities, probably because the lack of hydrogen bonding from the distal histidine significantly destabilized H_2O_2 binding.

Following the success of the myoglobin variants, it is desirable to introduce thiolate ligation into a peroxidase system. The reasoning behind this choice was 2-fold. First, similar reaction intermediates (e.g., compounds I and II) were proposed in both peroxidases and P450 proteins;[31,34,35,78] however, the lifetime and reactivity of those intermediates vary. Second, both the peroxidases and P450 proteins were capable of the peroxidase-type electron-transfer reactions and the oxygen insertion reactions of P450. The main difference between a peroxidase and a P450 protein was the ratio of electron transfer to oxygenation or the branching ratio.[35] The catalytic differences between the two types of proteins are subtler than between myoglobin and P450. Successful introduction of thiolate ligation into a peroxidase system would allow investigators to probe the role of His and Cys in the catalytic activity of the peroxidase by comparing the structure and function of the two heme ligations in an otherwise similar protein environment.

Initial attempts to mutate the C*c*P axial His to Cys (H175C) resulted in a very unstable ligand that was rapidly oxidized to cysteic acid.[83] Crystallographic studies of C*c*P, P450cam, and CPO revealed several differences in the environment around their proximal ligands.[31,84−86] In both thiolate-coordinated proteins, a nonpolar amino acid (Phe in P450cam and Leu in CPO) and the protein backbone form an enclosure about the proximal Cys ligand. This nonpolar residue is conserved in several P450 proteins and is observed at the same position in the crystal structures of three

other P450 enzymes.[87−89] In contrast, the analogous amino acid in C*c*P is an aspartic acid (D235, see Figure 3B). This residue accepts a hydrogen bond from the proximal His, giving it more imidazolate character and contributing to the more negatively charged proximal environment in C*c*P.[84,90] Previous D235 mutants have changed the spectroscopic and redox properties of C*c*P through interactions with the H175.[90] Therefore, a H175C/D235L C*c*P double mutant was made.[91] Studies by UV−vis, EPR, and MCD indicate a close similarity between the spectra of ferric substrate-bound cyt P450cam and those of the exogenous ligand-free ferric state of the double mutant and support the conclusion that the latter has a pentacoordinate, high-spin heme with thiolate ligation.[91] The study also marked the first time a stable cyanoferric complex of a model P450 was made and demonstrated the importance of the secondary coordination sphere around the primary coordination ligands in stabilizing metal−ligand ligation.

Similarly, mutation of the proximal His to Cys was made in heme oxygenase (HO, Figure 3F). A protein was obtained with similar electronic absorption and RR spectra as P450 in its resting ferric state and with a decreased reduction potential.[92] However, like all the P450 model proteins mentioned above, the cysteine ligand dissociated upon reduction to the ferric state. The loss of the proximal ligand prevented oxygen binding or destabilized the ferrous−dioxy complex. Instead, it underwent an uncoupled turnover from O_2 to H_2O_2.

Stable cysteine thiolate coordination in the ferrous−CO derivative of an engineered protein was demonstrated by Uno et al. using a variant of cyt b_{562}, a heme protein with His-Met coordination.[93] In addition to mutations of the histidine ligand to cysteine (to mimic heme−thiolate ligation) and the methionine ligand to Gly (to create a cavity for exogenous ligands such as CO), the key to success appears to be the recognition by the investigators that two glutamate residues (Glu4 and Glu8) close to the heme make an energetically unfavorable interaction with the heme propionate groups through electrostatic repulsion. Further mutations of both glutamates to serines stabilized the heme−thiolate interaction in the reduction step.

v. From Chloroperoxidase/Cytochrome P450 to Heme-His Proteins. Chloroperoxidase (CPO) catalyzes chlorination of organic substrates. Unlike most peroxidases, CPO contains a cysteine as the proximal ligand (Figure 3C). This ligand was replaced with a histidine using an expression system that involves the replacement of wild-type CPO with the mutant CPO. Surprisingly, this axial ligand replacement resulted in loss of only ∼20% of CPO activity, which led to the conclusion that Cys coordination was not required.[94] Similar attempts to produce an axial Cys to His mutation in P450 were not successful until the development of new procedures involving in vitro refolding and heme reconstitution.[95,96] In contrast to the results in CPO, replacing the axial Cys with His in cyt $P450_{cam}$ resulted in a greatly decreased camphor oxidation rate, elevated uncoupling rate, slower electron-transfer rate from redox partner putidaredoxin, and much greater peroxidase activity. These results suggest that the proximal cysteine is essential for protein folding, substrate binding, electron transfer, and P450 monooxygenase activity.

vi. From Myoglobin/Oxygenase to Catalase. The proximal histidine of myoglobin has been mutated to tyrosine in order to convert it to a catalase.[79,80,97,98] UV−vis, EPR, RR, and hyperfine-shifted proton NMR spectra of the resulting protein resembled those of catalase and natural mutants of hemoglobin (Hb M Iwate or Hb M Hyde Park), which had been shown to contain heme−Tyr ligation. The spectroscopic results were also confirmed by X-ray structure determination.[98] Furthermore, the structural study showed that introduction of the proximal Tyr ligand is accommodated by a shift of a helix (called F helix) in which this residue resides away from the heme pocket. The position of the introduced proximal Tyr with respect to the heme group is also different from that of the His93 residue normally present and resembles that of the proximal Tyr residue of bovine liver catalase. As expected, replacement of His with Tyr resulted in ∼250 mV decrease in reduction potential. Furthermore, this mutation had little effect on myoglobin's ability to carry out heterolytic or hemolytic O−O cleavage. Finally, substitution of the proximal His with Tyr in heme oxygenase yielded a protein with similar spectroscopic properties to those of catalase but resulted in a loss of the proximal ligand upon reduction.[92] Therefore, like the replacement of the proximal His with Cys, the mutation destabilized the ferrous−dioxy complex and uncoupled the reduction of O_2 from oxidation of the heme group, effectively changing the heme oxygenase function to an oxidase.[92]

vii. From C*c*P to Novel Types of Heme Proteins. Replacement of the proximal His with Gln-(H175Q) in C*c*P had little effect on the activity (K_{cat}) of the enzyme.[99] This unexpected result led to the conclusion that the nature of the proximal ligand does not affect the rate-limiting step, shedding some light onto the reaction mechanism of C*c*P. In fact, addition of the H175Q mutation to the W191F mutation (which left C*c*P deficient in electron transfer) restored function from 0.5% to 20% relative to wild-type C*c*P.[100]

Mutation of the proximal His to Glu (H175E) in C*c*P results in a mutant that is about seven times more active than the native enzyme.[83] The increase in activity has been attributed to the negative charge on the Glu, which could provide electrostatic stabilization to Fe(IV), increasing the thermodynamic driving force, resulting in an increased rate of electron transfer. This type of "hyperactivity" had previously been seen for other nonaxial ligand mutant proteins of C*c*P.[101]

viii. Cavity Complementation by Removal of the Proximal Ligand and Addition of Exogenous Ligands. One effective approach of engineering novel metalloproteins with unprecedented new properties is called cavity complementation. This approach involves replacing one of the metal-ion ligands with either Gly or Ala to create a cavity. Addition of exogenous ligands of the appropriate size

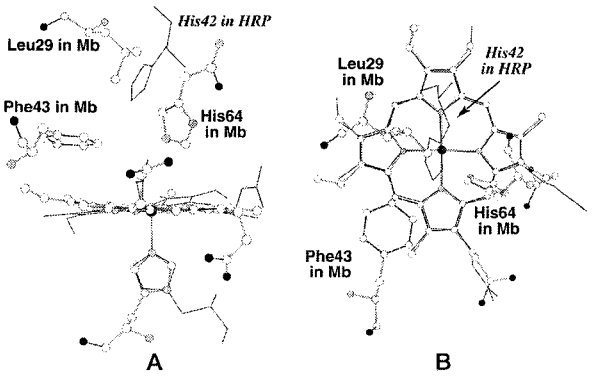

Figure 4. Structural comparison of the distal pocket in myoglobin and horse heart peroxidase. (Reprinted with permission from ref 105. Copyright 1999 American Society for Biochemistry and Molecular Biology.)

and character complement the cavity created by the replacement. In this way, the structure and function of the proteins can be either restored or changed, depending on the exogenous ligand added to the cavity. Since ligands are not limited to the natural amino acid side chains, this approach can be quite versatile in redesigning metalloproteins. A detailed discussion of this exciting field is beyond the scope of this article, and we refer readers to an excellent review.[102]

2. Redesign of the Distal Heme Site

The residues around the distal side of the heme do not coordinate to the iron directly and thus may exert their influence to a lesser degree than the proximal ligands. However, as demonstrated in the examples below, their positions can be very important in determining the function of the protein.

i. From Myoglobin to Peroxidases. Globins and many peroxidases possess a proximal and distal histidine. Despite this similarity, globins are oxygen carriers and only react with H_2O_2 at a much slower rate ($\sim 10^2$ M^{-1} s^{-1}) than peroxidases (with a rate of $\sim 10^7$ M^{-1} s^{-1}). Furthermore, unlike peroxidases, globins cleave the O−O bond of peroxide both heterolytically and homolytically. To find the structural factors responsible for the differences in reactivity and to redesign globins to function as peroxidases, the Watanabe group carried out a series of experiments based on structural comparisons between sperm whale Mb and horseradish peroxidase/cytochrome *c* peroxidase (Figure 4).[103−105] The distances between the N_ϵ of the distal His and the ferric heme iron are normally 4.1−4.6 Å for globins and 5.5−6.0 Å for peroxidases. Therefore, a series of variants was made that included His64Leu (to eliminate the distal His in Mb), His64Leu/Phe43His (to introduce a distal His at a similar position/distance as in peroxidases), and His64Leu/Leu29His (to introduce a distal His at a farther position/distance than in peroxidases). The positions and distances of the histidines in the variants were confirmed by X-ray crystallography.[105] While His64Leu and His64Leu/Leu29His variants reacted with H_2O_2 ~100 and ~3−6 times slower than the native Mb, the His64Leu/Phe43His variant exhibited ~11-fold higher activity.[103−105] These changes in reactivity were rationalized in terms of the roles of the distal histidines as general acid−base catalysts.[105] The His64 in native Mb functioned only as a general base by enhancing the binding of H_2O_2 through hydrogen bonding to both oxygen atoms of the peroxide, and it was too close to the heme center to support heterolysis of the peroxide bond. In contrast, the His43 in His64Leu/Phe43His variant was at a similar position and distance as in most peroxidases and thus can work both as a general base to enhance the binding of H_2O_2 through deprotonation of the peroxide and as a general acid to facilitate the heterolytic cleavage of the peroxide O−O bond. The His29 in His64Leu/Leu29His was too far away to serve any of these purposes. This work was a beautiful demonstration that proper positioning of the distal histidine is essential for the activation of H_2O_2 by heme enzymes.

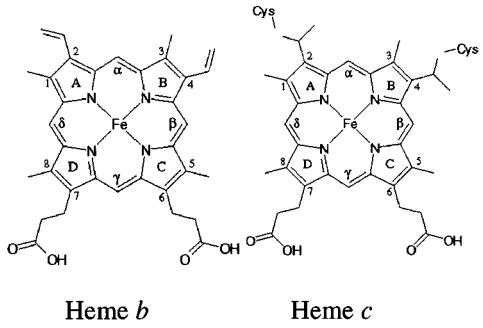

Figure 5. Heme *b* and *c*.

ii. From Heme Oxygenase to Peroxidase. The crystal structure of human heme oxygenase-1 (HO-1) suggests that Gly139 interacts directly with iron-bound ligands (Figure 3F). When this glycine was mutated to Ala, Leu, Phe, Trp, His, or Asp, the resulting protein gained new peroxidase activity by reacting with H_2O_2 to form a ferryl species that catalyzes the peroxidation of guaiacol.[106] The investigators propose that the conversion of heme oxygenase to a peroxidase appears to result from displacement of the distal helix, which shifted due to steric interactions between the larger amino acid side chains and the heme group. Therefore, they concluded that the ferryl species formation responsible for the peroxidase activity was a default reaction and the principal role of the HO active site may be to suppress this unwanted reaction with respect to HO activity. The gain in peroxidase activity in HO-1 was also observed with mutations of Asp140, another distal side residue that may participate in a hydrogen-bonding network involving ligands coordinated to the heme iron.[107,108]

3. Redesign of One Type of Heme Protein into Another Type

Heme is one of the most versatile prosthetic groups in metalloproteins.[28,109,110] Different types of heme have been found in proteins. They include heme *a*, *b*, *c*, *d*, d_1, *o*, chloroheme, heme P460, and siroheme.[28,109,110] They share a common skeleton and differ in structural details due to substitutions at the various positions. The *b*-type heme (or protoporphyrin IX) is the simplest representative (Figure 5). It has methyl groups at positions 1, 3, 5, and 8. Two

propionate groups are present at positions 6 and 7 and two vinyl groups at positions 2 and 4. The *c*-type heme−proteins have a heme covalently bound to the protein by two thioether bonds involving sulfhydryl groups of cysteine residues (Figure 5). One single thioether bond was also found in a few protozoan mitochondrial *c*-type cytochromes. Different types of hemes contain different degrees of conjugation that influence electronic delocalization on the heme plane, while different substituents are designed to optimize heme/protein interactions. Therefore, it is quite interesting to redesign one type of heme protein into another type and study the consequences of the engineering.

The most common way of redesigning one type of heme protein into another is by making the apoprotein, through either heme extraction or protein expression under controlled conditions, and then incorporating different types of hemes or even artificial hemes that are not found in nature (for example, see refs 111−114). However, when this approach is applied to *c*-type heme proteins, additional challenges arise because the thioether bond formation of heme *c* is believed to be assisted by proteins such as heme lyase.[115,116] Several groups have met these challenges.

Cytochrome b_5 has been redesigned into a *c*-type heme protein through mutation of a surface Asn residue to Cys.[117] This substitution placed the thiol group 3−3.5 Å from the heme 4-vinyl group. The resulting protein was expressed and isolated from *E. coli* as a mixture of four major forms, three of which contained covalent attachment of the introduced Cys with heme 4-vinyl group. The three forms differed in their modes of attachment and degree of oxidation. In the first and second forms, the Cys was attached through the α and β carbon of the heme 4-vinyl group, respectively. The third form is similar to the second form, except that the sulfur involved in the thioether linkage was oxidized to a sulfoxide. Another *b*-type heme protein, cyt b_{562}, was also converted to a *c*-type heme protein by engineering a Cys close to the heme.[118] In this study, the investigators took advantage of the structural homology between the cyt b_{562} and a major class of *c*-type cytochromes that possess the 4-α-helical bundle scaffold. Cysteine residues were then introduced in positions of cyt b_{562} homologous to those in the *c*-type cytochromes. The resulting proteins were expressed and isolated from *E. coli* as either apo- or holo-proteins. All variants produced multiple species with covalently attached heme. NMR structural studies on one of the variants indicated that its overall secondary and tertiary structures were very similar to those of wild type.[119] Furthermore, the redesigned protein with a *c*-type thioether attachment has a significantly increased stability, compared to that of the native *b*-type protein, toward thermal and chemical denaturation.

A *c*-type cytochrome, cyt c_{552}, was converted into a *b*-type cytochrome after both heme-binding cysteines were replaced with alanine, either individually[120] or together.[121] Spectroscopic studies indicated that these replacements did not cause drastic structural changes. Remarkably, the variants with a single thioether attachment have, apart from an altered visible absorption spectrum, almost identical properties, including thermal stability and reduction potential, to the wild-type protein. On the other hand, the variants without any covalent attachment were less stable with the guanidine hydrochloride unfolding midpoint occurring at a 2 M lower concentration than for the wild-type protein. These studies suggest that assembly of certain *c*-type cytochromes may be a consequence of spontaneous thioether bond formation after binding of heme to a prefolded polypeptide.[121,122]

In theory, heme protein redesign through mutations of residues in the proximal and distal side and in variation of heme types should result in similar active site structures, with changes occurring only in the local environment surrounding the residues. While this expectation was met in the majority of cases, several exceptions were observed, particularly in studies involving changes of proximal (or axial) ligands. The most common observations were a lack of coordination when the new axial ligand was introduced and the corresponding spin state changes. For example, in changing bis-His cytochromes to His-Met cytochromes, the proteins were expected to possess the same low-spin, six-coordination as in almost all cytochromes. However, in several cases the protein design resulted in high-spin heme without Met ligation.[38−40] This unexpected result most likely stems from a lack of consideration of the secondary coordination sphere surrounding the mutation. Steric, electrostatic, and hydrogen-bonding interactions may play a role in stabilization (or destabilization) of the coordination mode. This lack of stabilization may be responsible for the large (30−45 °C) decrease in thermostability of cyt c_3 when one of the histidine ligands is changed to methionine.[123] On the other hand, recognition and implementation of secondary coordination sphere stabilization allowed several groups to successfully make stable heme−thiolate proteins that are otherwise difficult to obtain (see section II.A.1.iv).[81,91,93]

Work by Hawkins et al. provided another caveat for heme protein redesign.[124] In their study, the His-Met cytochrome *c* was converted to a bis-His cytochrome by changing a residue (Phe-82) close to Met80 to histidine. The new histidine coordinated the heme iron despite the presence of the original Met80 ligand.[124] This result indicated that the presumption of identical active site topology or metal ligation following isometric amino acid replacement may not always be valid and local structural changes may occur to accommodate new coordination modes.

B. Redesign of Copper Proteins

Most of the early efforts in redesigning metalloproteins focused on heme proteins, probably because heme proteins are the most thoroughly studied metalloproteins. The redesign of heme proteins is easier to control than that of non-heme proteins because the heme prosthetic group largely dictates the active site structure and major protein fold or structural changes are less likely to occur.

While the heme makes a heme protein easy to redesign, it also makes the redesign less versatile

because one can only change the ligands above and below the heme. In addition, the heme often dominates protein spectroscopy, such as electronic absorption spectra, so that subtle changes in other parts of the protein can only be detected indirectly through changes in the heme spectra. Therefore, redesigning a non-heme metalloprotein is a logical extension of protein design.

Among the non-heme metalloproteins, copper proteins are one of the most thoroughly studied groups. Generally they are classified into three types according to their spectroscopic properties.[125−128] Type 1 copper proteins, commonly called "blue copper" proteins, usually have an intense blue color due to the strong absorption around 600 nm. They also have a very small $A_{||}$, which is the hyperfine splitting with the molecular *z* axis (d_{z^2} in the case of Cu(II) proteins) oriented parallel to the external magnetic field, in electronic paramagnetic resonance spectroscopy (EPR). Type 2 copper proteins have spectroscopic properties just like simple copper complexes. Type 3 copper proteins are a group of binuclear copper proteins that have no EPR signal in the oxidized state due to antiferromagnetic coupling of the two neighboring Cu(II) ions. Recently, new classes of copper centers, such as the purple Cu_A[129] and the Cu_z[130] sites, have been identified.

1. From Copper Chaperone Protein to Copper–Zinc Superoxide Dismutase

Metallochaperones are a new class of proteins that are responsible for delivery of metal ions to specific protein targets.[131−133] For the type 2 copper protein copper−zinc superoxide dismutase (CuZnSOD),[134] it was shown that a protein called copper chaperone for SOD (CCS) delivered copper ions to SOD.[131−133] Interestingly, the overall scaffold of one of the domains of CCS is a greek key β barrel, strikingly similar to that of CuZnSOD (Figure 6a).[135,136] In yeast CCS, the residues necessary for the formation of the copper and zinc sites in CuZnSOD are absent, and therefore, no metal-binding site is present in this domain.[135] However, all the zinc site ligands and three of four copper site histidine ligands in CuZnSOD are conserved in human CCS (hCCS). The zinc site in hCCS displayed the same structure as in CuZnSOD.[136] The fourth ligand in the copper site of CuZnSOD is replaced by an aspartate residue in hCCS. Although aspartate could be a ligand to copper, no copper binding is evident from the X-ray structure of hCCS[136] and hCCS exhibits no SOD activity.[137]

To elucidate the role of the fourth ligand, Schmidt et al. mutated the aspartate residue in hCCS to histidine as in CuZnSOD.[137] The resulting protein gained significant SOD activity, indicating that this mutation allows the formation of a new catalytically active copper site as in CuZnSOD and is critical in conferring the SOD activity. Furthermore, they demonstrated that this mutation did not inhibit the protein from functioning as a copper chaperone. This work strongly suggests that the aspartate residue is present in hCCS to preclude the deleterious SOD reaction as a result of copper binding, so that hCCS can function as a pure copper chaperone. Furthermore, it indicates that both the location and the exact nature of the amino acid residues are important for the formation of the metal-binding sites in proteins.

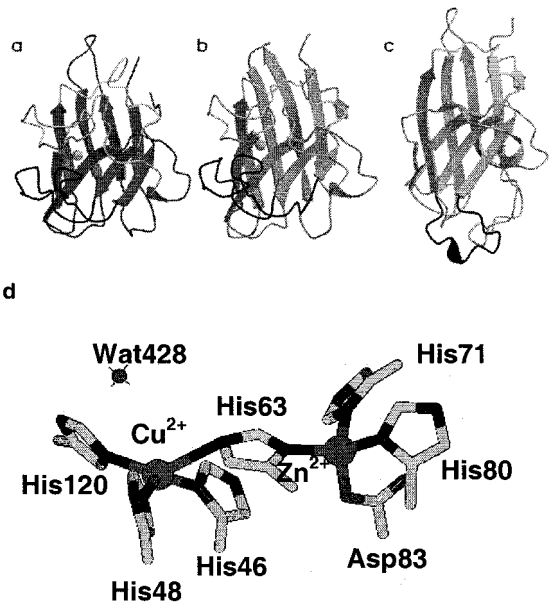

Figure 6. Structural comparison of (a) human CuZnSOD, (b) hCCS Domain II, and (c) yCCS Domain II. (d) Metal-binding site of human CuZnSOD. ((a−c) Reprinted with permission from ref 136. Copyright 2000 American Chemical Society.)

2. From Copper–Zinc Superoxide Dismutase to Type 1 Blue Copper Protein

Copper proteins perform a variety of functions, such as electron transfer (e.g., type 1 copper azurin), dioxygen binding (e.g., type 3 copper hemocyanin), or catalysis (e.g., type 2 copper CuZnSOD).[127,128] One may ask why a particular type of copper protein evolved to have the particular structural characteristics for the particular function. Although studies on the individual types of proteins are useful, redesigning one type of protein into another can address the above question in a much more powerful way. By changing the structure one residue at a time and looking for subsequent specific structural and functional changes, one can learn the key elements governing the characteristics of each type of protein and make future de novo design much more efficient and fruitful.

One of the most important questions in the field of copper protein chemistry is centered on the unique structural and spectroscopic properties of type 1 copper proteins.[138,139] The essential features of type 1 copper proteins appear to be two histidine imidazoles and a cysteine thiolate coordinated to Cu(II) in a trigonal planar (or distorted tetrahedral) geometry with one or two additional weak axial ligands such as methionine (Figure 7).[140−142] Most of the early discovered type 1 copper proteins are "blue copper" proteins due to the strong electronic absorption around 600 nm, which was assigned to a cysteine S π-to-Cu(II) charge transfer.[143] However, some type 1 copper proteins such as stellacyanin, pseudoazurin, and cucumber basic blue protein also have a moder-

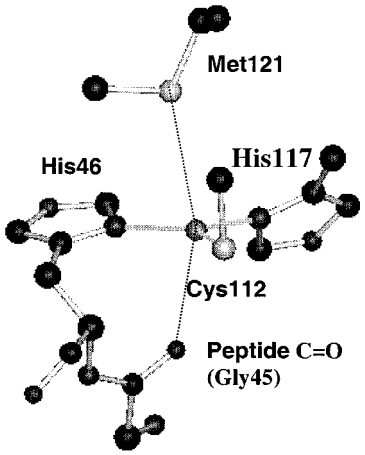

Figure 7. Blue type 1 copper center in azurin.

ately strong absorption around 450 nm in addition to the band at 600 nm. The most extreme example is nitrite reductase. Its 450 and 600 nm bands are of similar intensity, resulting in a "green copper" instead of a "blue copper" protein. Nonetheless, the type 1 Cu(II) in this "green copper" protein has all the other characteristics of type 1 copper proteins.

To elucidate the relationship between the type 1 and type 2 copper proteins and to take advantage of predefined scaffolds and metal-binding sites in the design of the unusual type 1 copper center, Lu et al. introduced cysteines into the metal-binding sites of yeast CuZnSOD, a type 2 copper protein.[144−148] When a cysteine was substituted for a histidine in the copper site, a site having a distorted square planar geometry (Figure 6d), a strong new absorption around 400 nm and a typical type 2 or "normal" EPR were observed, suggesting that the presence of cysteine was not sufficient in itself to produce the unusual type 1 or "blue" copper spectra.[144,148] On the other hand, substitution of a cysteine for a histidine in the zinc site, a site possessing a distorted tetrahedral geometry, resulted in a type 1 copper protein that displayed an electronic absorption spectrum that was similar to that of the type 1 copper center of nitrite reductase and an EPR spectrum that was similar to stellacyanin.[144−147] The magnetic circular dichroism[145] and RR spectra[146] of the copper derivative as well as the electronic absorption spectrum of the cobalt derivative[145] of this redesigned protein also resembled those of other type 1 or "blue" copper proteins. All three His-to-Cys mutant proteins reacted with ascorbate much faster than the wild-type protein, indicating that the presence of a thiolate greatly increased the redox reactivity of the metal-binding sites, consistent with the thiolate providing an efficient super exchange pathway for electron transfer.[147] The bridging histidine in CuZnSOD was also replaced with a cysteine by Banci et al. The resulting protein did not display any S-to-Cu(II) charge-transfer absorption, indicating the lack of a Cu−cysteine bond.[149] These studies demonstrated that both the cysteine ligand and the geometry defined the structural and functional properties of type 1 blue copper proteins. In addition, the work made a significant contribution to understanding the relationship between the geometric parameters and spectroscopic properties (such as the ratio of the electronic absorption at 460 nm over that at 600 nm) of the type 1 copper center.[145−147,150,151]

3. From Type 1 Blue Copper Azurin to Type 2 Copper Proteins

To provide direct evidence for the importance of the cysteine in maintaining the structure and function of the type 1 blue copper center, Mizoguchi et al. replaced the cysteine in *Pseudomonas aeruginosa* azurin (Figure 7) with an aspartate residue.[152] Electronic absorption studies of both Cu(II) and Co(II) derivatives of the protein as well as EPR spectral studies of the Cu(II) derivative all indicated that the type 1 copper center was redesigned into a type 2, five-coordinate copper site, with both oxygens of the aspartate residue serving as potential ligands to copper. This conclusion was confirmed by X-ray crystallographic and NMR studies.[153,154] More importantly, this mutation resulted in a dramatically reduced electron self-exchange rate and intramolecular electron-transfer rate compared to the native azurin.[155] A Cu K-edge X-ray absorption study of the redesigned protein indicated both a decrease in the covalency of the metal-binding site and an expansion of ∼0.2 Å in the copper coordination sphere upon reduction of the protein.[155]

The type 1 to type 2 copper conversion has also been accomplished by replacing one of the histidines in the type 1 blue copper azurin to glycine.[156−158] Interestingly the blue copper site can be restored by addition of external ligands such as *N*-methyl imidazole. Despite this restoration, the protein was not able to rapidly interconvert between the Cu(II) and Cu(I) forms, indicating that the covalent attachment of the ligand at the histidine position to the protein backbone is essential for the electron-transfer function of the protein.

In addition to the above studies, mutations of the methionine in azurin to other natural amino acids have also been carried out, many of which resulted in a copper site between that of type 1 and type 2 copper centers.[159−164]

C. Redesign of Non-Heme Iron Proteins

1. From Rubrerythrin to Ribonucleotide Reductase

Dinuclear iron proteins are an important class of non-heme iron proteins that include hemerythrin (Hr), rubrerythrin (Rr), ribonucleotide reductase (RnR), and methane monooxygenase (MMO).[165] They all share a similar di-iron oxo core and yet perform a variety of different functions, such as dioxygen binding (Hr), ribonucleotide reduction (RnR), and methane activation (MMO). Redesign of one dinuclear iron protein into another makes it possible to uncover fine structural differences that define the functional properties.

The di-iron oxo site of rubrerythrin (Rr), a protein whose physiological role remains to be determined, was redesigned in order to mimic that in RnR.[166] The majority of dinuclear iron proteins, including RnR and MMO, contain six ligands from two pairs of $EX_{30-32}EXXH$ motifs.[167−169] Only five of the six ligands

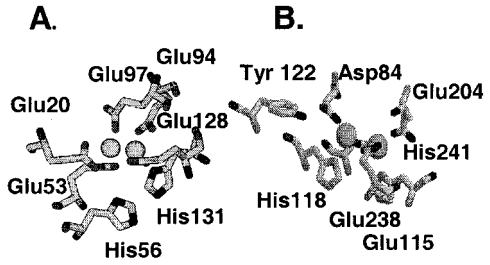

Figure 8. Di-iron oxo sites in rubrerythrin (A) and ribonucleotide reductase (B).

are found at the corresponding positions in Rr from *Desulfovibrio vulgaris* (Figure 8). The putative sixth ligand, His56, is too far away to serve as a ligand to the nearest iron in Rr. Instead, Glu97 provides the sixth ligand at the other end, and it is believed to be a unique ligand for Rr proteins.[170] To redesign Rr to a RnR, this Glu97 was mutated to an alanine. The X-ray crystal structure of the redesigned protein indicated that His56 was an iron ligand and this coordination was not a result of His56 moving closer to the iron. Instead, the iron moved ~1.6 Å relative to the same iron in the native Rr. The di-iron oxo site of the new variant is very similar to that of RnR.

Since Tyr122 played a critical role in RnR function, an effort was also made to introduce a Tyr at a similar position in Rr.[166] Even though a Tyr102 was present in Rr that seemed to correspond to Tyr122 in RnR, a sequence homology search indicated that a nonconserved residue (Leu60) in Rr corresponded to Tyr122 in RnR. Therefore, a Leu60Tyr mutation was made and the crystal structure of the variant was obtained. According to the structure, the side chain of the introduced Tyr60 was now in a similar position as the Tyr122 in RnR. While the introduction of the Tyr into Rr was successful, no Tyr radical was detected in the redesigned protein. This result was explained based on the difference that Tyr122 in RnR made direct hydrogen bonds to an iron-coordinating terminal carboxylate while the Tyr60 in the redesigned protein did not. Both the Glu97Ala and Leu60Tyr mutation studies suggested that significant local structural changes might occur. These changes may not be readily predicted without homology information.

2. From Ribonucleotide Reductase to Methane Mono-oxygenase

Redesign of the di-iron site of ribonucleotide reductase (RnR) introduced a methane mono-oxygenase (MMO) type function.[171] The metal site of RnR is surrounded by hydrophobic residues. A tyrosine (Tyr122) close to the dinuclear iron center is proposed to form a radical, initiating the catalytic cycle of RnR (Figure 8B). To examine the importance of the hydrophobic environment around the iron oxo site of RnR, Phe208 was mutated to a Tyr, placing another phenolic group near the site. UV−vis and RR spectral studies of the variant showed that it contains a ferric catecholate instead of a Tyr radical. The mechanism for generation of this protein-derived dihydroxyphenylalanine was proposed to be similar to that of MMO.

3. From One Type to Another Type of Fe–S Cluster

Iron−sulfur clusters, such as [2Fe-2S], [3Fe-4S], and [4Fe-4S], are found in proteins. Typically considered as electron-transfer centers, they have been shown recently to catalyze biological reactions such as hydration/dehydration of double bonds, stabilize protein structures, regulate metabolic pathways, and serve as biological sensors.[172−175] One of the most interesting aspects of Fe−S cluster proteins is their ready conversion from one type to another through chemical oxidation and reduction, pH changes, or site-directed mutagenesis.[176−199] More importantly, several studies indicated that some of the cluster conversions are physiologically relevant in that they play important roles in regulation of enzyme activity. A detailed account of the field is beyond the scope of this review, and the readers are referred to several excellent reviews.[172−175]

D. Redesign of Other Metal-Binding Sites

1. Redesign of Ca(II)/Mg(II) Specificity

Proteins in the calmodulin superfamily (including troponin C, parvalbumin (PV), and oncomodulin (OM)) have been used to redesign metal sites for selectivity of Mg(II) or Ca(II).[200−202] Most proteins in this superfamily contain multiple divalent cation sites, complicating studies at any one site. Troponin C contains four helix−turn−helix domains, two of which bind Ca(II) with low affinity and two that have a high affinity for Ca(II) but also bind Mg(II). PV and OM have two mixed Ca(II)/Mg(II) sites and two Ca(II)-specific sites.

This issue of metal-ion selectivity has been addressed in a number of ways by using a single helix−turn−helix domain. Chimeric Ca(II) (Mg(II)) sites have been made from OM and PV, showing that affinity in a paired domain can be dominated by one site.[202] Specific replacement of amino acids in the high-affinity Ca(II)/Mg(II) site of rat PV changed the mixed Ca(II)/Mg(II) site to a Ca(II)-specific site.[201]

2. Redesign of Mg(II)/Mn(II) Specificity

Many endonucleases require divalent metal cofactors (usually Mg(II)) for DNA cleavage. Alteration of metal-ion requirements is just one of many ways endonucleases are engineered.[203] EcoRV cleaves DNA in the presence of Mg(II) and to a lesser extent Mn(II).[204] Mutation of Ile91 to Leu reversed the cleavage efficiency for Mg(II) and Mn(II) (Figure 9). In the WT, ligands that coordinate Mg(II) are in the proper octahedral configuration at the cleavage recognition site. Coordination of Mn(II) at this site is less specific. Strangely, Ile91 does not interact with the metal or recognition site but points away from it into a hydrophobic core.[205] The Ile91Leu mutation thus alters the coordination sphere, providing better coordination of Mn(II). This single mutation shows that selectivity can be altered without directly replacing any of the metal ligands.

3. Redesign of Mg(II)/Zn(II) Specificity

Mammalian alkaline phosphatase (AP) contains a tetrahedral Zn(II) site, whereas *E. coli* has an octa-

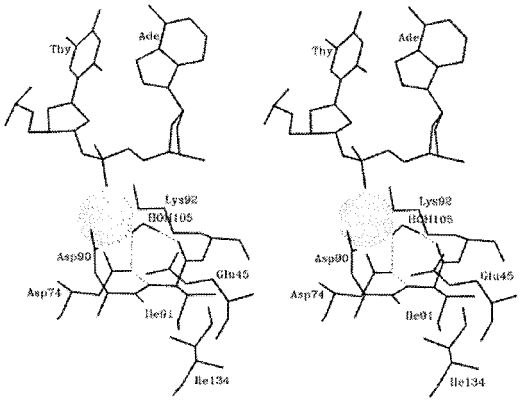

Figure 9. Stereodiagram showing the location of Ile191 and Mg^{2+} (dotted sphere) in EcoRV restriction endonuclease. (Reprinted with permission from ref 204. Copyright 1996 American Chemical Society.)

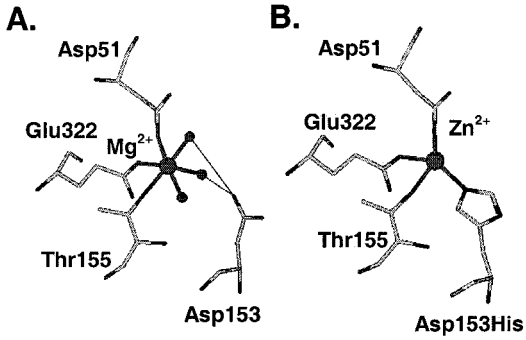

Figure 10. Structure of wild-type *E. coli* alkaline phosphatase (A) and Asp153His variant (B).

hedral Mg(II) at the corresponding location. Mutation of a single amino acid (Asp153His) in *E. coli* AP converted the specificity and geometry of the site to that of mammalian AP (Figure 10).[206]

4. Redesign of Fe(II)/Mn(II) Specificity

Among the many interesting cases of metal-ion specificity in proteins, the difference in specificity of Fe- and Mn-containing superoxide dismutase (SOD) is perhaps the most intriguing.[207,208] Both FeSOD and MnSOD share the same sequence and structure homologies. The overall structures and metal-binding sites of both proteins are virtually identical. However, even though FeSOD binds non-native Mn ions, it is active only with Fe ions. The same is true for MnSOD. The inactivity of Fe-substituted MnSOD has been attributed to the anomalously low reduction potential of the metal-binding site when the non-native metal ion is used.[209] Inspection of the X-ray crystal structure of the Fe-substituted MnSOD indicated that the metal-binding site is in an altered geometry.[210] The study also suggested possible coupling between remote active sites in the dimeric protein. Interestingly, a few "cambialistic" SOD enzymes have been isolated that are active with either Fe or Mn.[211,212] Therefore, redesign of the metal-ion specificity from one protein to another poses significant challenges and, if successful, can provide much insight into the principles of metal-ion selectivity.

Since the primary coordination spheres of FeSOD and MnSOD are quite similar, efforts in redesigning their metal-ion specificity have been focused on the secondary coordination sphere, including a conserved glutamate residue (Glu170) in the dimer interface of MnSOD and conserved glutamine residues believed to be central to the hydrogen-bonding network of FeSOD and MnSOD. Mutation of Glu170 to alanine destabilized the dimer structure.[213] Surprisingly, the purified Glu170Ala-MnSOD contains exclusively Fe and resembles native FeSOD in terms of spectroscopic properties, anion interactions, and pH titration behavior. Despite these similarities, the variant protein did not display any SOD activity.

It was recognized that one of the most striking conserved amino acid differences between FeSOD and MnSOD is the relative positioning of a glutamine residue near the metal-binding site. For example, while a glutamine residue (Gln69) is known to be important for the hydrogen-bonding network of FeSOD, other residues such as glycine are found at the corresponding position in MnSOD. Conversely, while a glutamine residue (Gln146) is known to be important in the hydrogen-bonding network of MnSOD, other residues such as histidine or glycine are found at the corresponding position in FeSOD. When the histidine in FeSOD from *Mycobacterium tuberculosis* is mutated to Gln to mimic that in MnSOD, the variant protein was still found to bind Fe preferentially.[214] On the other hand, similar mutations in *Porphyromonas gingivalis* Cambialistic SOD made to mimic MnSOD resulted in a variant whose electronic absorption spectrum resembled more closely that of MnSOD.[215] More importantly, the variant displayed increased SOD activity over the wild-type enzyme when Mn was in the metal-binding site. Finally, a mutation in MnSOD, to incorporate a Gln corresponding to that in FeSOD, did not result in a new, FeSOD-like active site. However, the MnSOD variant gained new SOD activity (~7% that of FeSOD) when Fe was in the metal-binding site.[216] These studies suggest that the presence and the position of the glutamine residue is important but not the sole determinant of the metal-ion specificity of FeSOD and MnSOD. Major differences in other conserved residues need to be elucidated before a successful redesign of metal-ion specificity can be accomplished.

From the work described in this section II on redesign of metalloproteins, we can see that most of the redesign efforts have been focused on heme proteins, with copper and non-heme iron proteins as a distant second. This condition may be a reflection of both the degree of understanding of the proteins and the ease of characterizing the redesigned proteins by spectroscopy. With a better understanding of native metalloproteins and advancement in spectroscopic and X-ray crystallographic techniques, we will see more successful examples of redesigning non-heme metalloproteins as well as more sophisticated designs of heme proteins that take into account the secondary coordination sphere and other long-range effects. The insight gained from the redesign approach lays a solid foundation for the design and engineering of new metal-binding sites that will be discussed in the next section.

III. Design and Engineering of New Metal-Binding Sites

A. Rational Design

1. Theoretical Approach Using Automated Computer Search Algorithms

A number of computer search algorithms have been written for general protein design and modification, and many of the algorithms can be applied to the design of metalloproteins. A particularly noteworthy approach uses retrostructural analysis to create so-called miniproteins with minimal sequence and structural features that mimic the general features of particular metalloproteins.[6,11,217] This approach is reviewed elsewhere in this issue. Here we will focus on computer search algorithms that design a new metal-binding site onto a native scaffold. Among several of these reported algorithms, two programs called Metal-Search[218] and Dezymer[219] have been the most successful in guiding metalloprotein design.

Both Metal-Search and Dezymer algorithms make predictions based on strict geometric principles.[220] They assume a fixed protein backbone and search for optimal locations of ligands using different amino acid side chain rotamers. In the Metal-Search program, the idealized metal positions at every residue, including its side chain and rotamer, are precalculated and those substitutions that result in idealized metal positions near each other are then grouped and evaluated. The Dezymer program, on the other hand, starts with an anchor point of an amino acid substitution with a selected rotamer and systematically searches for additional coordinating residues that can satisfy the desired geometry of the designed site. Once a site and its substitution are identified, other changes in the surrounding area may be needed to avoid steric conflicts and to optimize packing of the site. In its current form, the Metal-Search program is used mainly for design of tetrahedral metal-binding sites while the Dezymer program is more versatile in this regard. Discussed below are several successful examples.

i. Design of a Tetrahedral Zn(II)-Binding Site Containing Cys and His. Zn(II)-binding sites have been a favorite target for protein design, not only because zinc plays important roles in the structure and function of many proteins, but also because zinc-binding sites are well defined and can be readily studied by substitution of Zn(II) with spectroscopically rich high-spin Co(II).[221,222] Zinc sites were among the first to be successfully introduced into the de novo designed proteins such as α helical bundles[8,9,223,224] and native scaffolds using automated computer search algorithms.[225,226]

Choosing the B1 domain of IgG-binding protein G as the scaffold, Klemba et al. used the Metal-Search program to identify potential positions for the formation of a tetrahedral His_3Cys metal-binding site.[225] To avoid major perturbations to the overall structure of this small, 56-residue scaffold, the authors picked to mutate four residues that were mostly solvent exposed. Additional mutations of a residue that was in a predicted steric conflict with one of the introduced histidines were also carried out. The UV−vis spectra of the Co(II) derivative of the designed proteins were consistent with a His_3Cys tetrahedral site with a dissociation constant of 4 μM. A competition study with Zn(II) suggests that the designed protein binds Zn(II) about 1000 times more strongly, resulting in an estimated dissociation constant on the order of a few nanomolar.

Several tetrahedral Zn(II)-binding sites have also been designed into the hydrophobic core of thioredoxin using the Dezymer program,[226] giving us a rare opportunity to compare the designed proteins using two different programs. In this case, Wisz et al. tried to select amino acid positions that are buried inside the protein to improve the binding affinity. Despite the fact that no attempt was made to consider additional mutations around the primary coordination sphere to avoid steric clashes, UV−vis absorption studies of the Co(II) derivative of the designed proteins showed that one Co(II) binds in a tetrahedral site of the designed protein. The binding affinity (with a dissociation constant of 2−4 μM for Co(II) and 100−1000 tighter for Zn(II) derivatives) is very similar to that of the designed proteins by Klemba et al. using the Metal-Search program.[225] The strong binding affinity in both studies is remarkable for a designed protein. It would be interesting to find out whether the binding affinity of the designed protein by Wisz et al. will improve when the steric clashes are eliminated upon further mutation.

Another strategy to improve the metal-binding affinity of the designed proteins was demonstrated by Marino and Regan.[227] In the study the authors investigated the effect of altering the secondary coordination sphere around the primary $Zn(II)His_3Cys$ tetrahedral site designed into the B1 domain of IgG-binding protein G. Inspired by the work of Christianson and Firke's groups on the study of zinc protein carbonic anhydrase,[228] Marino and Regan placed Glu, Gln, Asp, or Asn close to each of the three histidine ligands in the designed $Zn(II)His_3Cys$ tetrahedral site of the B1 domain so that they could form hydrogen-bonding interactions with the imidazole nitrogens of the histidine ligands. Electronic absorption studies of Co(II) derivatives of the mutant proteins indicated that they maintained the same His_3Cys tetrahedral geometry and, more importantly, exhibited enhanced affinity from 1.5- to 6.4-fold over that of the designed protein without the secondary coordination sphere mutation. Double mutations in the secondary coordination sphere resulted in even further enhancement and the effects appeared additive. An intriguing find from the study is that the above mutant proteins with enhanced metal-binding affinity unexpectedly shared little secondary structure with the parent protein, probably because many of the mutations caused major perturbations of the protein scaffold.

Many metalloprotein design studies have focused on the secondary coordination sphere in terms of negative design (e.g., to eliminate steric clash or ligation from unexpected amino acid residues nearby). The work by Mario and Regan is a nice example of designing the secondary coordination sphere using

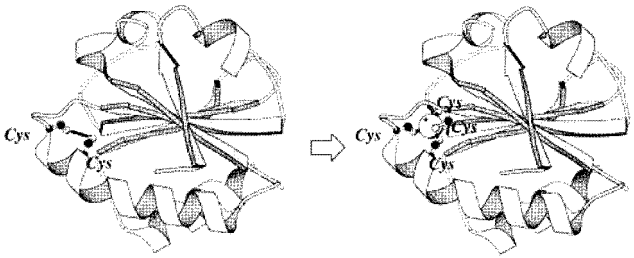

Figure 11. Conversion of a disulfide bridge in thioredoxin into a mononuclear [Fe(Cys)$_4$] rubredoxin center. (Reprinted with permission from ref 230. Copyright 1998 American Chemical Society.)

positive design approaches to enhance metal-binding affinity. The importance of the secondary coordination sphere in metalloprotein design was also demonstrated by several groups, who showed that, without an important feature in the secondary coordination sphere, the primary coordination sphere cannot even form (see section II.A.1.iv).[81,91,93]

ii. Design of Iron−Sulfur Cluster. Iron−sulfur clusters are a class of metal-binding centers that are the subject of recent intense research due to the discovery of new roles for these "old" clusters.[172−175] Fe−S clusters, typically considered as electron-transfer centers, can catalyze biological reactions such as hydration/dehydration of double bonds, stabilize protein structures, regulate metabolic pathways, and serve as biological sensors.[172−175]

The mononuclear [Fe(Cys)$_4$] rubredoxin center has been designed into native protein scaffolds using both the Metal-Search[229] and Dezymer[230] programs. The Dezymer program was used to convert a disulfide bond in thioredoxin to a mononuclear [Fe(Cys)$_4$] rubredoxin center (Figure 11).[230] In the other study, the Metal-Search program was used to design the same mononuclear [Fe(Cys)$_4$] center into the B1 domain of the IgG-binding protein G.[229] In both cases, visual inspection of the computer models of the selected variant indicated no additional mutations were necessary to alleviate adverse steric interactions. The UV−vis and EPR spectra of the Fe(III) complex of the designed proteins in both studies were very similar to those of oxidized rubredoxin. The UV−vis absorption studies of their Co(II) derivatives also confirmed that a tetrahedral site was created in both proteins. Furthermore, one of the designed proteins was capable of undergoing several successive cycles of air oxidation and reduction by β-mercaptoethanol.[230]

From the above study, Farinas and Regan recognized a limitation of Dezymer and Metal-Search programs. Both programs used the fixed backbone of protein coordinates from either X-ray or average NMR structures. Small backbone movements can occur in solution and are sometimes necessary to accommodate slight changes associated with introducing new amino acid ligands. Therefore, the authors decided to select at random 6 out of 60 calculated NMR structures to apply the Metal-Search program.[229] Indeed, they found that while certain sites can be identified in both the average and the individual structures, some sites could only be found in the individual structures. Since this strategy does not change the program algorithms, it can be applied to protein design using other programs or computer modeling packages. More study using this strategy is needed to find out how useful it is in metalloprotein design.

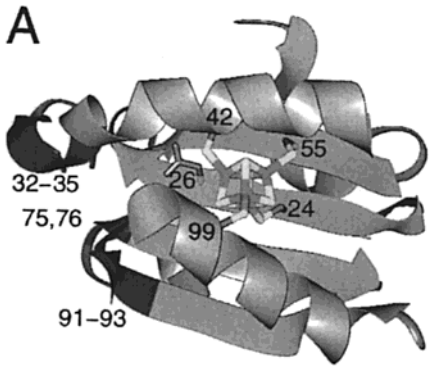

Figure 12. Creation of an Fe_4S_4 cluster in thioredoxin using the Dezymer program. (Reprinted with permission from ref 231. Copyright 1997 National Academy of Sciences.)

Most of the design of metal-binding sites has been limited to mononuclear centers. The design of tetranuclear [Fe_4S_4] clusters into proteins represents a new challenge. In this case the Dezymer program was used to search for backbone positions of thioredoxin to place Cys side chains to match the [Fe_4S_4] structures of a HiPIP protein (Figure 12).[231] In addition, two cysteines in the native thioredoxin were removed to avoid their competing chelation. A final isosteric Asp-to-Leu mutation was also included to improve the stability of the designed protein. The successful design of the center in thioredoxin was supported by the optical and EPR studies that showed similar spectral signatures as that in HiPIP proteins. Furthermore, the [Fe_4S_4] cluster was much more stable in the designed protein than when it was free in solution.

iii. Design of a Blue Copper Center. Design of a blue copper center in proteins represents a still further challenge in metalloprotein design. As pointed out by Hellinga,[232] for most successful examples of metalloprotein design, such as the tetrahedral Zn(II) centers, iron sulfur clusters, and mononuclear non-heme iron centers, construction of a geometrically correct, sterically compatible primary coordination was sufficient to reproduce the dominant features of the structure and function of the desired centers. However, certain metal-binding sites in proteins are unique in that metal ions are not necessarily in their preferred state, and to design such sites correctly, special caution has to be taken to prevent many alternative reactivities and coordination geometries. Blue copper centers are one of these centers.

Blue copper proteins, also called type 1 copper proteins, are classical electron-transfer proteins containing a mononuclear copper with $CysHis_2$ in a trigonal plane and another weak axial ligand, usually Met (Figure 7).[140−142] Since this geometry is not preferred by either Cu(I) or Cu(II), blue copper proteins display many unusually spectroscopic properties, such as an intense blue color and very small

$A_{||}$ in electron spin resonance spectroscopy.[138,139] In addition to the challenge of designing a site that allows incorporation of copper ions into a geometry that is not preferred, the assembly of the designed protein has to overcome the alternative reactivity of disulfide bond formation between proteins. This competing reaction is especially efficient in the presence of redox-active metal ions such as Cu(II). Despite these complications, Hellinga took the challenge and used the Dezymer program to carry out a series of four primary designs and 32 variants.[219,232,233] The most successful variant of the designed thioredoxin was able to mimic the type 1.5 blue copper center after a strong, exogenous ligand, azide, was introduced axially into the designed center. By using an iterative strategy of design, characterization, and evaluation, the author provided many valuable insights into the metalloprotein design process. These included the need to surround the designed primary coordination sphere with a hydrophobic shell to ensure the absence of potential alternative coordinating residues or even solvent (water) and the requirement of forming a strong Cu(II)−thiolate bond in order to destabilize disulfide bond formation. It demonstrated that negative design (i.e., destabilization of competing reactions and coordination) is a crucial aspect of the protein design process.

iv. Design of Mononuclear Non-heme Iron Centers with Catalytic Activities. Perhaps the biggest challenge in protein design is the introduction of catalytic activity into proteins so that they can activate small molecules, such as O_2 or N_2, and transfer the atoms to desired substrates. Unlike the design of ligand-saturated metal-binding sites such as [Zn(II)His_3Cys], [Zn(II)His_2Cys_2], [Fe$(Cys)_4$], or [Fe_4S_4], the design of catalytically active metalloproteins requires a careful design of open coordination sites for binding small molecules such as O_2 as well as substrates. The design is not trivial because other amino acids around the designed metal-binding sites may "fill in" the open binding sites. Thus, a successful design requires a careful consideration of positive features to introduce the open binding site and negative features to prevent unwanted binding. These principles were demonstrated in two studies.

Using the X-ray structure of the trigonal bipyramidal iron site in iron superoxide dismutase (SOD) as a guide, Pinto et al.[234] used the Dezymer program to search thioredoxin and identify positions to place one Asp and two His in a plane. Another His at the axial position was also chosen so that an open coordination site was created on the opposite end. Additional mutations were introduced to prevent competing chelation by two internal cysteines and one histidine and to improve packing for the designed site. The electronic absorption spectra of the designed protein as well as its N_3^- and F^- adducts are similar to those of native iron SOD, and the iron has a dissociation constant of less than 1 μM. More importantly, comparative studies with the unrelated *E. coli* iron SOD indicated that the designed protein can catalyze hydrogen peroxide dismutation with a rate on the order of 10^5 M^{-1} s^{-1}. Even though the catalytic rate is still about 10^4 fold slower than the native SOD, the rate is quite impressive considering that many of the long-range structural features in the native SOD have not been introduced into the designed protein.

In a recent study Benson et al.[235] extended the above work by designing six mononuclear iron-binding sites with three histidines and at least one open site at three different locations of thioredoxin. Unfavorable steric clashes were eliminated through further mutations. In almost all cases, the designed proteins were found to bind one Fe(III) with a K_d less than 5−10 μM. Two of the designs exhibit SOD activities appreciably above the background, with one of them approaching 1% of native *E. coli* SOD activity. An apparent correlation between the SOD activity and the number of positive charges in the vicinity of the designed site was also noted. This observation confirms the importance of electrostatic interactions between the superoxide anion and protein side chains in the SOD activity.

2. Empirical Approach

Before the automated computer search algorithms were available, empirical approaches were the only way for the design of novel metalloproteins. They include design by inspection, by homology, and by replacement of a modular unit such as a loop between α helices or β strands. Design by inspection is considered the simplest case of protein design; designers placed one or a few metal-coordinating amino acids in specific locations of a protein to form metal-binding sites. Knowledge of protein structure, minimally the secondary structure and preferably the three-dimensional structure, was needed to locate the best positions for these sites. A basic knowledge of the metals ligand and geometry preferences[236−238] was also required. Often, many variants with different combinations of metal-coordinating amino acids were created in the same scaffold. Metal-binding affinity assays were used to evaluate the sites. On the other hand, design by homology and by replacement of a modular unit utilized the sequence and crystal structures of both the scaffold protein and the target metal-binding site. A careful analysis of both structures was performed to locate regions of common secondary and tertiary structure. Although appearing less rigorous than the theoretical approach described in the section above, these empirical approaches have successfully created novel functional metalloproteins, some of which have been used in practical applications (such as in protein purification using immobilized metal affinity chromatography, enzyme activity regulation, and metal-ion sensing. See sections below for a detailed discussion). These empirical design approaches complement the theoretical approaches well by allowing investigators to gain experience and knowledge of metalloprotein design, which may in turn enhance protein design using automated computer search algorithms.

i. Metal Sites Designed by Inspection. The first generation of designed metal-binding sites, although considered simplistic by protein design standards, demonstrated several useful properties and functions. First, the metal-binding sites created by only a single

mutation or by the incorporation of a series of His residues have found many applications. Second, more design was involved in sites where an established metal-ion-binding motif was inserted into a protein. Finally, an even higher level of metal site design is apparent from work where two or more residues where mutated to create the site. The motivation for creating these sites was quite diverse, and the success of many of these examples sheds light on the principles of metal site design.

a. Single Amino Acid Substitutions. The first level of metal-binding site design involved the substitution of a single residue to either cysteine or histidine, creating a high-affinity site for metal ions or metal complexes. A common example is the use of cysteine residues to coordinate thiophilic metal ions to aid in X-ray structural determination.[239] Introducing a single histidine or cysteine residue on a protein surface allowed for selective binding of ruthenium complexes to study long-range electron transfer or redox-triggered protein folding.[240,241]

Replacing a residue in the hydrophobic substrate-binding pocket of staphylococcal nuclease with a cysteine resulted in a metal-dependent regulatory switch of the enzyme activity.[242] The crystal structure of the nuclease revealed Leu89 as a key residue in the substrate-binding pocket. The Leu89Cys mutation did not significantly change the catalytic activity or specificity of the enzyme. However, addition of various metal ions, including $HgCl_2$, resulted in inactivation of the variant's enzymatic activity. Importantly, removal of the metal ion through addition of chelating agents fully regenerated activity.

Similarly, activity of ion channel proteins was inhibited through mutations of channel-lining residues to cysteine and subsequent addition of metal ions.[243−246] A Thr449Cys mutation in the extracellular mouth of the *Shaker*-Δ K^+ channel created a "close" switch when Zn(II) or Cd(II) ions were present in micromolar concentrations.[243] In addition, the pore-exposed residues of the Na^+ [245] and K^+ [246] channel proteins were identified by random mutations of protein residues to cysteine. Those mutations that caused inhibition of channel function in the presence of Cd(II)[245] or Ag(I)[246] were thus determined to line the channel.

The metal-dependent regulation of protein activity through introducing the metal-binding site has been used to sense metal ions in the pore-forming protein, α-hemolysin (Figure 13).[247−249] The α-hemolysin protein is composed of a hexamer with 293 amino acids in each subunit. The residues important for pore formation (i.e., those between positions 126 and 134) were individually changed to histidine.[247] The activities of several of these variant proteins were inhibited by the addition of metal ions, as reflected by decreases in channel current. The metal ions (Cu(II), Zn(II), Ni(II), or Co(II)) could be differentiated based on differing current signals.

b. Incorporation of Histidine Repeats. The incorporation of a series of histidine residues on surface loops or at the N- or C-termini of proteins formed functional metal-binding sites. It is an efficient way of creating nonspecific metal sites of relatively high

Figure 13. Cross section of the pore-forming protein α-hemolysin with the designed metal-binding site extending into the pore. (Reprinted with permission from ref 249. Copyright 2000 American Chemical Society.)

binding affinity. The histidine repeats, including histidine tags, are widely used in protein purification with the technique called immobilized metal affinity chromatography (IMAC).[250−254] Also, the histidine repeats serve to reinforce metal-binding sites created by introducing single metal-binding residues as discussed in section III.A.2.i.a. For example, five consecutive amino acids, residues 130−134, were mutated to histidines in the pore-forming protein, α-hemolysin. This and other similar variants were shown to sense metal ions.[248,249,255−258] In addition, a segment of six adjacent histidine residues was inserted into lactose permease (LP).[259] Cysteine residues substituted at other positions in the LP proteins and then labeled with nitroxide spin labels. Copper(II) ions were added and presumed to bind at the histidine repeats' site of the different spin-labeled variants. The residue separation and thus protein topology was determined by measuring the EPR relaxation time of the nitroxide label in the proximity of Cu(II).

Interest in removing toxic metal ions from industrial waste sites has spawned the design of proteins with engineered metal-binding sites. Generally, regions of His repeats are engineered into the outer loops of membrane-spanning proteins. The histidine repeat regions bind various metal ions in the environment and the organism via phagocytosis and take up large quantities of the sequestered metals.[260−262] Isolation and removal of the organism can result in removal of free metal ions from the environment. This principle was demonstrated with a few different proteins. For example, the *E. coli* outer membrane proteins, LamB[260] and OmpC,[262] were engineered with a His_6 motif. The strains of bacteria expressing these proteins accumulated 11 times more Cd(II)[260] and 3−6 times more Zn(II), Fe(III), or Ni(II),[262] respectively, than normal bacteria strains.

The His repeat sequence was also used to immobilize proteins to a surface for structure determination. First, the IgG_1 antibody heavy chain was modified at its C-terminus with the metal chelating

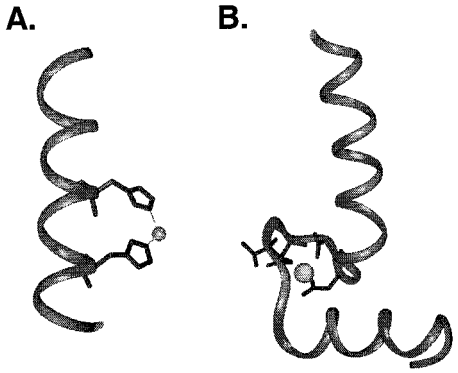

Figure 14. Hemocyanin HXXXH domain (A), and the calmodulin EF hand domain (B).

peptide, His-Trp-His-His-His-Pro.[263] The metal chelating site was utilized to affix the protein to a nickel-treated mica plate for the AFM imaging of the antibodies. Second, N-terminal labeling of the HupR protein[264] and streptavidin[265] with a His_6 tag allowed for two-dimensional crystallization of the proteins. Special Ni(II)[264] or Cu(II)[265] chelating lipids allowed for immobilization of the His-tagged protein on a lipid monolayer film. Electron microscopy of the layer resulted in an 18 Å resolution structure for the HupR protein. Third, the F_1-ATPase was immobilized on a Ni−NTA-coated glass slide for direct observation of its function by light microscopy.[266] Finally, the histidine repeats also function to affix proteins for facile study by surface plasmon resonance.[267−269]

c. Incorporation of a Metal-Binding Motif. Another level of metal-binding site design was to incorporate simple, metal-binding motifs into a protein. Motifs, such as the His-X_3−His motif (Figure 14A), common to zinc-finger proteins, hemocyanin, and thermolysin, and the EF-hand calcium-binding motif (Figure 14B), found in calcium proteins, can be incorporated into proteins with only a few residue substitutions.[250,252] This method has found similar applications as those incorporating either a single amino acid (see section III.A.2.i.a) or histidine repeats (see section III.A.2.i.b). For example, analogous to one of the histidine repeats applications,[259] the His-X_3−His motif was engineered into T4 lysozyme to aid the determination of distances between residues by measuring the EPR relaxation of a nitroxide spin label attached to a cysteine by a Cu(II) ion bound to the engineered His-X_3−His motif.[270] Because the metal-binding site in the His-X_3−His motif is better defined than that in the histidine repeats, the distances obtained can be more accurate.

The His-X_3−His arrangement was also engineered into iso-1-cytochrome *c* through Lys4His/Thr8His mutations to create a more stable variant and to aid in protein separation in the presence of metal ions. The addition of $[Cu(iminodiacetate)]^{2+}$ to the variant resulted in a protein with more resistance to thermal[271] and chemical denaturation.[271,272] This protein variant, together with several His-X_3−His variants of bovine somatotrophin,[273] was also used to test principles of protein separation using immobilized metal affinity chromatography.[274]

The EF-hand calcium-binding motif was engineered onto a particular loop in human lysozyme for increased stability.[275−278] The residues Gln86 and Ala92 were sequentially mutated to Asp residues.[279] Only the Gln86Asp/Ala92Asp lysozyme double variant had increased thermal stability and more protease resistance with Ca(II) present.[275,276,278] At 80 °C, the variant was 1.7 times more active than the wild type at 70 °C.[275] X-ray structures of both the wild-type and designed sites were determined.[277] This created a unique opportunity to carefully study the effects of the engineered metal site on the structure of the protein.[280]

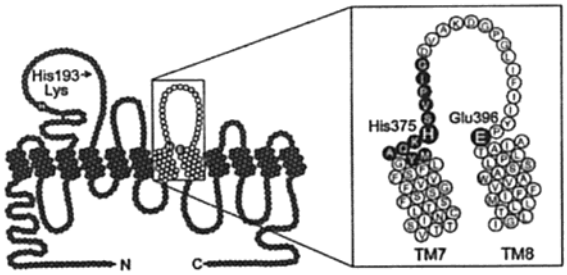

Figure 15. Two-dimensional representation of hDAT showing the location of His375 and the surrounding residues which were mutated in order to determine the topology of the segment. (Reprinted with permission from ref 285. Copyright 2000 American Chemical Society.)

The same motif was also engineered between two green fluorescent protein (GFP) variants to form an effective calcium sensor.[281−284] The binding of Ca(II) ions to these constructs caused the two GFP variants (such as cyan and yellow fluorescent proteins) to come in close contact, resulting in an increase in the efficiency of fluorescence resonance energy transfer.[281−284] The resulting sensor was capable of detecting $[Ca^{2+}]$ in the 10^{-2}−10^{-8} M range[282] and has been used in vivo to detect the cytoplasmic $[Ca^{2+}]$ in *Arabidopsis thaliana* guard cells.[284]

d. Incorporation of Two or More Chelating Residues. Many successful examples of creating metal-binding sites by incorporating two or more chelating residues have been reported. To create these metal-binding sites, more intricate design strategies were necessary. These sites were generally created either through a knowledge of secondary structure or by analyzing the three-dimensional structure of the target protein. Residues thought to be in close proximity and capable of binding were changed to histidine or cysteine, and the success of the site was tested by experiments. In many cases it remained to be shown if all of the designed ligands actually coordinated to the metal ion. Despite this concern, the designed metal-binding sites have been used in a variety of applications such as elucidation of protein topology, increase in protein stability, aid in protein purification, and regulation of enzymes activity and selectivity.

To Elucidate Protein Topology. The relationship of helices and location of helix residues has been determined in a number of proteins by designing bis-coordinated metal-binding sites. A transmembrane segment in the human dopamine transporter (hDAT) was probed with new metal-binding sites.[285] A bis-histidine site was created with a residue at position 375 plus neighboring residues (Figure 15). Inhibition

of the transporter with Zn(II) coordination was observed only with histidine residues located at position 375 and those located at residue 371, 377, 378, or 379. The results from Zn(II) binding to the mutants suggested that the transmembrane segment was helical, ending at position 375 and then changing to another loop structure beyond there. This study is one of numerous examples of using bis-His sites to identify helix position, orientation, or other types of protein topology.[286−297]

To Stabilize Proteins. Metal-binding sites were created between subunits of multimeric proteins to help stabilize their interactions. For example, Zn(II) sites were designed into the interface of two GFP variants, yellow (YFP) and cyan (CFP) fluorescent proteins, with the goal of stabilizing the heterodimer.[298] Four different Zn(II) sites were designed by examining the GFP crystal structure and mutating potential residues at the dimer interface to His and/or Cys. Binding was assayed by the observed FRET signal with increasing Zn(II) concentrations. An increase in FRET signaling of 8−10% was obtained in the presence of 10^{-4} M $ZnCl_2$.

A metal-binding site was also created to increase binding affinity between two different proteins: the human growth hormone (hGH) and the hGH receptor (hGHr).[299] The site was designed based on sequence alignments of the hGHr with the human prolactin receptor, known to also bind hGH through Zn(II) coordination. A mutation of Asn218His, located on the interface of the hGH-binding pocket of the hGHr, resulted in a 20-fold increase in hGH binding affinity with Zn(II) present.

The metal-dependent stabilization of the protein−protein interface may be the key for enzyme activation. For example, glycogen phosphorylase can be activated only through phosphorylation. However, examination of the X-ray structure indicated that substitution of opposing residues at the dimer interface of glycogen phosphorylase to histidines may allow the formation of new metal-binding sites. Interestingly, addition of Ni(II) to the engineered enzyme resulted in the activation of the enzyme without the need of phosphorylation.[300] The metal-binding site at the interface was believed to lock the enzyme in the dimeric and active form.

Finally, metal-binding sites were created between helices to stabilize the protein. The magnitude of stabilization that can be achieved has led researchers to introduce metal-binding sites into proteins for catalysis at higher temperatures. For example, Leu58His mutation in one of the helices of iso-1-cytochrome *c* was carried out (Figure 16).[271,301] This His58 together with the naturally occurring His39 on a neighboring helix was shown by crystal structural analysis to be able to chelate metal ions or metal complexes. Cross-linking of the helices with $[Ru(2,2'\text{-bipyridine})_2]^{2+}$ [301] or $[Cu(\text{iminodiacetate})]^{2+}$ [271] resulted in 16.5 and 5 °C increases, respectively, in melting temperature over the wild-type protein. In another study, a metal-binding site was incorporated between two domains of the serine protease trypsin.[302] Due to the difficulty of designing a rigid metal-binding site into proteins, a flexible region was chosen as the metal-binding site. It was thought that the amino acid ligands would be capable of enough movement to accommodate the coordination requirements of different metals. The mutations of Thr21His and Thr142His yielded a variant trypsin that displayed affinity for Zn(II), Co(II), and Ni(II). Metal binding increased the thermal stability of the protein by a few degrees over the wild type. Crystallographic analyses revealed that the preferential coordination geometry of each metal ion was obtained with the flexible coordination site: tetrahedral for zinc and octahedral for cobalt and nickel.

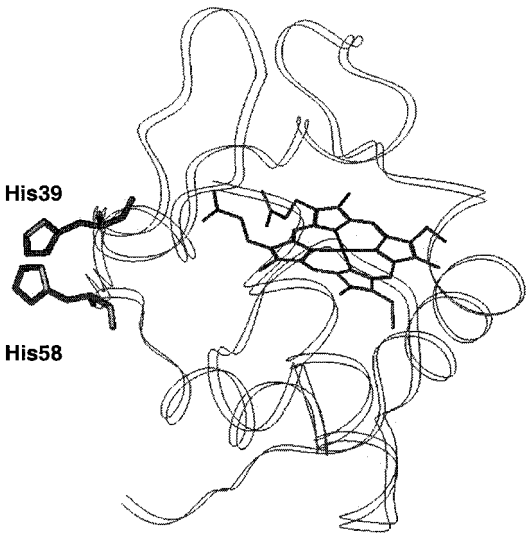

Figure 16. Structural model of Leu58His variant of yeast iso-1-cytochrome*c*. The His58 and the naturally occurring His39 form a bis-His site for a metal-binding site on the protein surface.

To Aid in Protein Purification. In addition to the increase of protein stability,[271,301] the His58/His39 chelating site of iso-1-cytochrome *c* later became an important part of a dual gene/protein system for the homologous expression and purification of nonfunctional cyt *c*.[303] Homologous expression of nonfunctional mutant proteins of iso-1-cytochrome *c* in yeast *Saccharomyces cerevisiae* lacking the wild-type cytochrome *c* gene was not possible because the yeast cells lacking this essential electron-transfer protein could not grow. A strategy was designed to overcome this problem by coexpressing both the nonfunctional cytochrome *c* variant and a functional cytochrome *c*.[303] To separate the two proteins of similar properties, a Leu58His mutation was made on the functional cytochrome *c* so that a bis-His site was formed with the His39 originally in the protein. The functional cytochrome *c* was then easily separated from the nonfunctional cytochrome *c* by a metal affinity column.

To Regulate Enzyme Activity and Selectivity. The ability to control the activity of enzymes has been desired for many industrial and pharmaceutical applications. Steps toward this goal were achieved in enzymes with engineered metal-ion-binding site switches.[304] A switch was created in rat trypsin by changing Arg96 to His.[305,306] From examining the X-ray structure, this residue was found in proximity to the active site residue, His57. The addition of Zn(II) to this variant switched off enzyme catalysis,

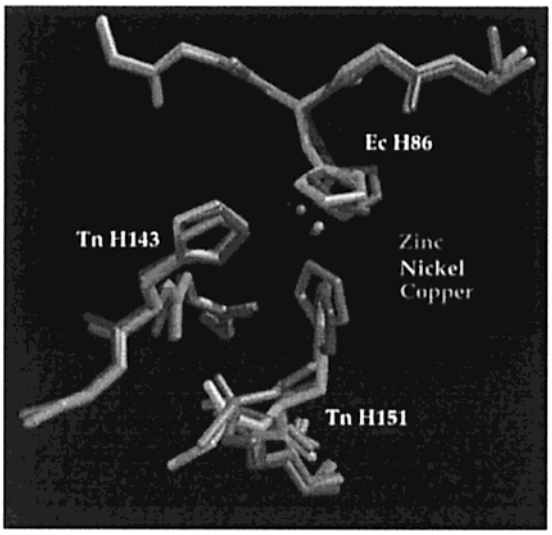

Figure 17. Engineered metal-binding pocket in trypsin. Overlayed are the crystal structures of the trypsin/substrate complex with bridging metal ion. (Reprinted with permission from ref 310. Copyright 1996 American Chemical Society.)

while EDTA restored full activity.[305] The crystal structure of this site reveals the mode of inhibition.[306] The Zn(II) binds to His96 and the naturally existing His57, reorienting its position away from the active site catalytic triad and inhibiting activity. In another study, Ser102His/Gly131His mutations in aqualysin I, a subtilisin-type bacterial serine protease, also resulted in a metal-dependent inhibition of the enzyme activity.[307]

Similarly to the design of a metal-dependent enzyme activity switch, the design of a metal-binding site that can fine-tune enzyme activity or substrate specificity in the metal-bound form is quite interesting. Bis-His variants of trypsin were made with the intention of modulating substrate specificity.[308−310] On the basis of the substrate-bound X-ray structure of trypsin the mutations Asn143His and Glu151His were chosen because of their potential to form a metal-ion bridge to a His residue on the substrate strand. This created a new, metal-ion-dependent, substrate specificity to trypsin. In the presence of metal ions, substrates with a histidine residue located two positions away from the scissile peptide bond were cleaved with a high level of efficiency.[308,309] X-ray structures of the Zn(II)-, Ni(II)-, and Cu(II)-bound mutants clearly show the bridging between His143, His151, and the substrate strand His (Figure 17).[310] In a different set of experiments, the K188 residue, located in the substrate-binding pocket, was mutated to His in an attempt to regulate trypsin activity.[311] Addition of Cu(II) to this protein resulted in a 30−100 fold decrease in K_m with no affect on K_{cat}. Full activity was returned with the addition of EDTA.

Many different Zn(II) sites were introduced into a family of rhodopsin-like G-protein-coupled receptors that have a common motif of seven transmembrane segments. "Off" and "on" switches were designed, and helix positions were elucidated. First, three mutations on two different helices in the κ-opioid receptor created a Zn(II) site that reduces agonist and antagonist binding with Zn(II) present.[290,291] The cytoplasmic ends of two transmembrane segments in both the β_2-adrenoreceptor and the parathyroid hormone receptor were substituted with His residues.[292] The addition of Zn(II) inhibited the enzyme. Work on the tachykinin NK_1 receptor created metal-binding sites that contributed to the determination of helix positions[293−296] and located agonist and antagonist binding sites.[293,294] The bis- and tris-His sites created between transmembrane segments of the tachykinin NK_1 receptor created a metal-binding site that inhibited the enzyme[293] or activated the enzyme.[296] A new metal-binding site at the antagonist binding site of this receptor resulted in antagonism with the addition of Zn(II).[293] Similarly, a new metal site created near the natural agonist binding site resulted in 25% agonism in the presence of Zn(II) (Figure 18).[296] Finally, a metal-binding site engineered into the β_2-adrenoreceptor protein by a His mutation on segment-III and a Cys mutation on segment-VII allowed for the activation of the enzyme with Zn(II).[297] Apparently the binding of metal ions moved these enzymes into the active conformation and allowed for the estimation of distances between these two helices in the active signaling conformation.

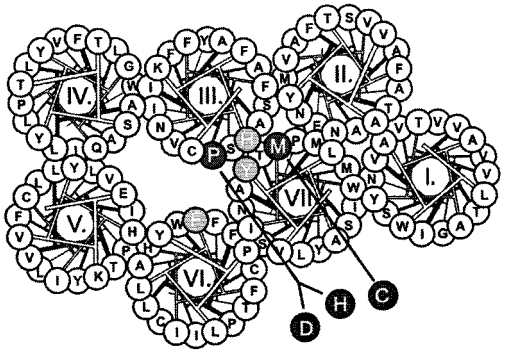

Figure 18. Helical wheel diagram of the NK_1 receptor viewed from the extracellular side. Highlighted residues are those that interact with the agonist, and those with lines indicate the corresponding mutations to create the agonist zinc site. (Reprinted with permission from ref 296. Copyright 2000 American Society for Pharmacology and Experimental Therapeutics.)

To Detect Metal Ions. Similar to the studies mentioned in section III.A.2.i.a, a new metal-binding site in α-hemolysin was utilized as a metal-ion sensor (Figure 13). By mixing wild-type monomers with the mutant α-hemolysin monomers, a different channel current through the pores was measured with the addition of different metal ions.[312] The concentrations of two and three divalent metal ions were simultaneously determined with one variant of these pores.[313]

ii. Metal Sites Designed through Homology. The second empirical approach to the design and creation of new metal-binding sites is based on either sequence or structural homology between a target protein lacking the metal-binding site and the template protein with the metal-binding site. This approach relies on guidance from homology and offers a better chance of success than design by inspection. It is an important exercise for protein designers, especially in the early stages of protein design when

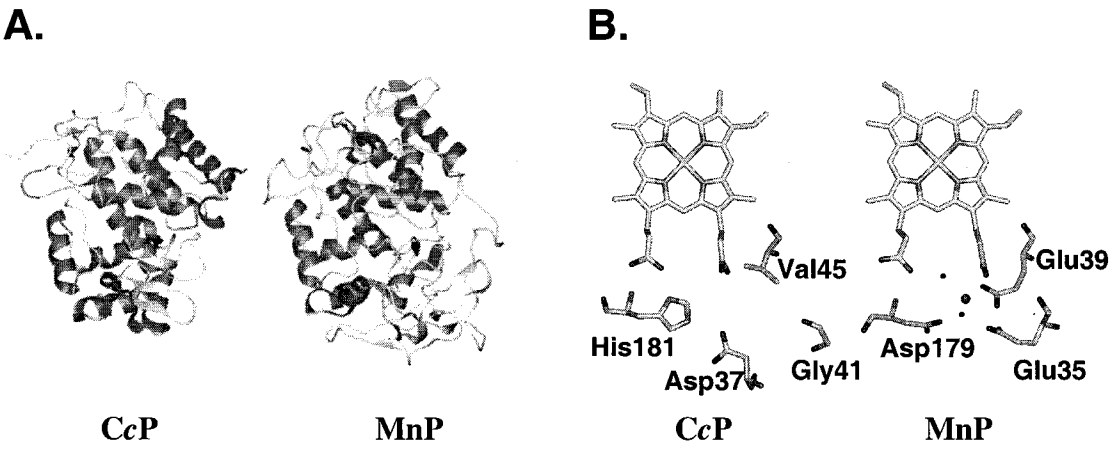

Figure 19. (A) Overall structure of C*c*P and MnP. (B) Active site structure of C*c*P and MnP.

our knowledge of creating metal-binding sites is limited.

a. Creation of a New Tetrahedral Zn(II)-Binding Site in Charybdotoxin. The X-ray structure of the 37 amino acid protein, charybdotoxin, was examined for a Zn(II)-binding site analogous to that in carbonic anhydrase.[314−316] A region containing two antiparallel strands of β-sheet was identified in charybdotoxin, and the appropriate residues were mutated to His, as in carbonic anhydrase. A few other sterically conflicting residues were also mutated. The engineered protein bound metal ions as demonstrated by Trp fluorescence quenching and UV−vis absorption spectroscopy. The same metal-ion specificity as carbonic anhydrase, with Cu(II) preferred over Zn(II), was obtained with the new metal-binding site. This study demonstrated the success obtained from careful modeling and structural homology searches.

b. Creation of a New Mn(II)-Binding Site in Cytochrome c Peroxidase: Toward a New Manganese Peroxidase. Cytochrome *c* peroxidase (C*c*P) and manganese peroxidase (MnP) are members of the plant peroxidase superfamily.[317] C*c*P catalyzes the oxidation of ferrocytochrome *c*, while MnP plays a key role in the oxidative biodegradation of lignin and many aromatic pollutants. Despite the limited sequence homology (less than 20%) between the two peroxidases, the overall structural folds of the two enzymes are quite similar (Figure 19A).[318] One major difference is that C*c*P lacks the Mn(II)-binding site found in MnP (Figure 19B). The Mn(II)-binding site was proposed based on the X-ray structure of MnP.[318] Kinetic studies of MnP and its variants containing mutations of the proposed Mn(II)-binding site ligands confirmed their role in binding Mn(II) and supporting MnP activity.[319−323] The oxidized Mn(III) then serves as a diffusible oxidant for biodegradation processes.[319,320] On the basis of a comparison of X-ray structures of the two peroxidases, the Lu group[324−326] and Goodin group[327] succeeded in engineering a Mn(II)-binding site in C*c*P by introducing amino acids found in MnP into the corresponding positions in C*c*P.[328] More importantly, they showed that the introduction of the new Mn(II)-binding site resulted in a significant increase in Mn(II) oxidation activity.

c. Creation of a New Cation-Binding Site in Cytochrome c Peroxidase: Toward a Cation-Controlled Molecular Switch. Heme peroxidases catalyze oxidation of a variety of substrates using H_2O_2.[28,329,330] Most peroxidases share common reaction intermediates called compounds I and II. Compound I is one oxidation state higher than compound II, which is a heme Fe(IV)=O species. While the extra oxidation state manifests itself as a porphyrin π cation radical in compound I of most other peroxidases, it is a tryptophan radical, located at position 191,[331] in C*c*P. Early structural based alignment of plant peroxidases indicated that most peroxidases contain a phenylalanine at the corresponding position of Trp191 in C*c*P. Therefore, a straightforward explanation for the difference in the location of the extra oxidation state is that since Trp is more easily oxidized than Phe, Trp191 can be further oxidized once a porphyrin π cation radical is formed in C*c*P. However, this explanation proved to be too simplified, especially after the observation that ascorbate peroxidase, a peroxidase with similar overall structural homology, contained a regular compound I with Fe(IV)=O plus porphyrin π cation radical, even if it possesses a Trp at exactly the same location as Trp191 in C*c*P.[332,333] Therefore, the protein environment around the Trp residue must play a role.

The first clue to the puzzle came from several mutagenesis studies that showed the Trp191Gly mutation resulted in a cavity that binds cations such as K^+ and imidazolium.[334,335] These results and electrostatic potential calculations[334,336,337] strongly suggested that the local environment created a negative potential that can stabilize the Trp191 radical in C*c*P. Further structural comparison showed that in APX and many plant peroxidases, a cation-binding site (a K^+ site for APX and a Ca(II) for most other peroxidases) was located ~8 Å away from the Trp/Phe residue while only a water molecule was present at the same location in C*c*P.[332,336] Therefore, Patterson et al. proposed that this cation-binding site may play a role in destabilizing the Trp radical in APX.[333]

To provide proof for the above hypothesis and to engineer a protein whose activity can be controlled by a designed cation site, Poulos' group introduced the APX K^+-binding site into C*c*P (Figure 20).[336,338] On the basis of a strong structural homology between APX and C*c*P, the investigators changed the amino

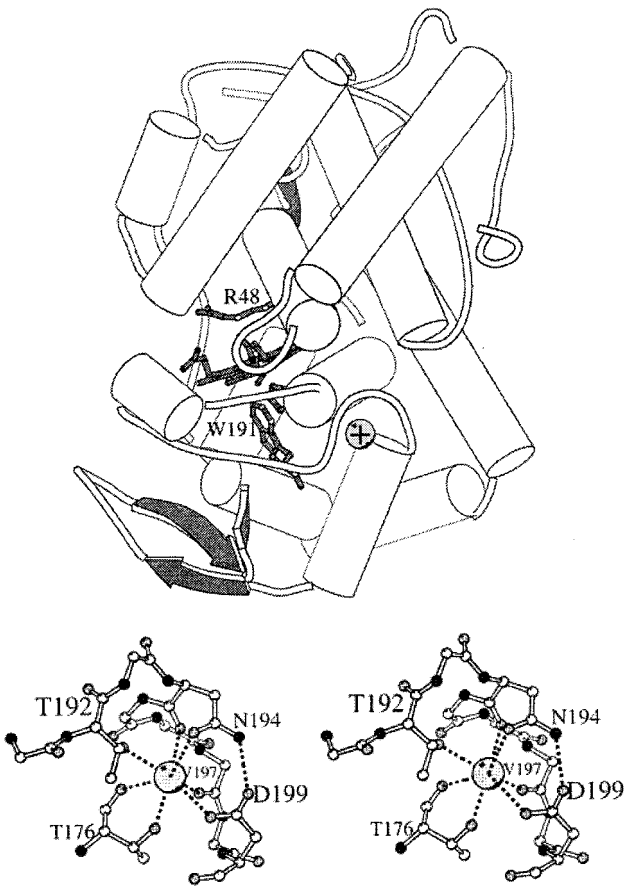

Figure 20. (A) Overall structure of C*c*P with the engineered cation-binding site. (B) Detailed structure of the cation-binding site. (Reprinted with permission from ref 338. Copyright 1999 American Chemical Society.)

acid residues in C*c*P to those responsible for the formation of K^+ site in APX. X-ray crystallography, EPR, and steady-state and stopped-flow absorption studies all showed that a K^+-binding site was created in C*c*P at a similar location as in APX. More importantly, both the enzyme activity and the characteristic EPR signal associated with the Trp191 radical decreased as the concentration of K^+ was increased. These results demonstrated that long-range electrostatic effects can control the reactivity of a redox-active amino acid chain.

Building on the above success, Poulos' group further investigated how to convert the engineered K^+-binding site in C*c*P into a Ca^{2+}-binding site and how to control the metal-binding selectivity through structure-based protein design.[339] This work was based on the observation that while the K^+-binding site was found in APX, a Ca^{2+}-binding site was present in many other structurally homologous peroxidases such as lignin peroxidase and manganese peroxidase. Using the C*c*P variant with engineered K^+ binding as a template protein, together with structural based sequence alignment and modeling, Bonagura et al. replaced amino acid residues in the C*c*P variant with those responsible for binding Ca^{2+}.[339] The success of conversion was confirmed by X-ray crystallography. In contrast to what was observed with the C*c*P variant containing K^+-binding site, EPR signal and enzyme activity of the new variant are much more selective for Ca^{2+} than for K^+.

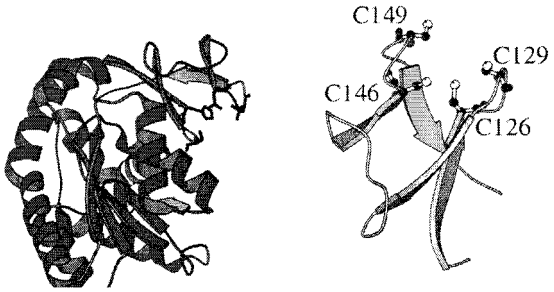

Figure 21. Representation of the adenylate kinase protein with the incorporated zinc-binding motif. (Reprinted with permission from ref 340. Copyright 1998 American Society for Biochemistry and Molecular Biology, Inc.)

d. Metal Binding Sites for Protein Stabilization. The stabilization of an adenylate kinase was achieved by placing the metal-binding site common to one protein family member into another member of the protein family. For the adenylate kinase family, those from Gram-positive bacteria contain a Zn(II) site while those from Gram-negative bacteria do not. The Cys-X_2-CysX_{16}-Cys-X_2-Cys zinc-binding motif of adenylate kinases from Gram-positive organisms was engineered into a Gram-negative protein (Figure 21).[340,341] The resulting protein displayed increased thermal stability.

A mammalian serum retinal-binding protein (RBP) variant was also stabilized with the introduction of a Zn(II) site.[342] The Zn(II) site was modeled after the carbonic anhydrase (CA) site. As in CA, three residues on adjacent antiparallel β-strands of the RBP were mutated to His. Monitoring Trp fluorescence while increasing guanidinium hydrochloride revealed the increased stability of the metal-binding site variant.

iii. Modular Approach: Loop-Directed Mutagenesis. The modular approach is commonly used by nature to create new structures and functions since exon shuffling and recombination is often observed in biological systems. Thus, similar techniques, such as domain shuffling and insertion, have been used for protein design and engineering.[343,344] A particularly noteworthy module in metalloprotein design is the loop connecting regular helix and sheet secondary structures.

On average, loops account for ~30% of globular protein structural elements.[345] Two important features make loops ideal for modular protein design and engineering. First, most loops lie on the surface of proteins and are highly tolerant of amino acid replacement, insertion, and deletion. Early studies have demonstrated that loop swapping[346] or even deletion[347,348] did not affect the core structure of the proteins. Second, loops often form the binding and recognition sites of proteins. Therefore, transplanting the active site in the loop from one protein into another can be a general approach for designing novel proteins. Analogous to site-directed mutagenesis, the replacement of one loop with another loop is termed loop-directed mutagenesis (LDM). Important progress in using LDM for designing nonmetalloproteins has been made[343,348−351] Here we focus on the use of LDM for the design and engineering of new metal-binding sites.

a. Grafting of Calcium-Binding Loop To Increase Stability. Calcium plays many important roles in biology, including the ability to confer stability to proteins. Two groups have taken advantage of this property and engineered new calcium-binding sites into homologous proteins lacking the calcium sites.

In the first case, Toma et al. reported a successful grafting of a calcium-binding loop from thermophilic thermolysin into the mesophilic neutral protease from *Bacillus subtilis*.[352] The work was based on the close sequence (~50%) and structural homology between the two proteins. From a comparison of a structural model of the neutral protease and a crystal structure of thermolysin, the author identified a ω loop that binds Ca-4 in thermolysin to test the feasibility of transferring the calcium-binding site from thermolysin to the neutral protease. Toward that end, the ω loop containing 10 amino acids in thermolysin was introduced into neutral protease to replace a seven-residue loop in the corresponding position. CD and fluorescence emission studies showed that the LDM did not lead to appreciable alteration of the scaffold of the protein. The resulting neutral protease also maintained similar activity as the native enzyme. More importantly, the engineered neutral protease was found to bind a new calcium ion with a $K_d \approx 0.1$ mM. When the calcium concentration was below 10^{-3} M, the engineered protein was found to be less stable than the native protein. However, in the presence of higher concentrations of calcium (0.1 M), it was 2-fold more stable than the native protein. This modulation of enzyme stability by calcium ions was similar to that observed in thermolysin.

In another case, Braxton and Wells used a similar strategy to confer autoproteolysis stability to subtilisin BPN′.[353] First, two loops of high mobility in subtilisin BPN′ were identified as the sites of autoproteolysis. Then, a structural comparison between the mesophilic subtilisin BPN′ and its thermophilic homologue, thermitase, allowed the author to locate two loops in thermitase that corresponded to the loops of autoproteolysis in subtilisin BPN′. One of the two loops in thermitase contained a calcium-binding site, and its sequence was introduced into subtilisin BPN′ at the corresponding location. The engineered protein had the same k_{cat} as the wild type and a K_M value 8-fold larger than the wild type. The protein was also found to bind a new calcium ion with $K_d \approx$ 0.1 mM. This new calcium site is apparently responsible for the observation that the engineered protein was 10-fold more stable to irreversible inactivation at 60 °C than the wild-type subtilisin BPN′.

b. Introduction of Metal-Binding Loop To Regulate Activity. In an extension of their work on metal-ion regulation of enzyme activity (see section III.A.2.i.d), Halfon and Craik succeeded in regulating trypsin activity by introducing a metal-binding loop from its homologous protein called tonin.[354] On the basis of computer modeling, two histidines in the loop, together with the catalytic histidine of trypsin, could complex metal ions. This metal binding would render the catalytic histidine unavailable for function and thus switch off the enzyme. Indeed, addition of Cu(II), Ni(II), or Co(II) ion to the engineered protein substantially inhibited the proteolytic activity of trypsin, and the inhibition can be fully reversed by removal of the metal ions using EDTA. Significantly, LDM made it possible to create a tridentate histidine ligand set, resulting in much higher metal-binding affinity (~100 nM for Cu(II)) and tighter regulation of enzyme activity than the previously reported bidentate histidine ligand sets based on site-directed mutagenesis.

c. Incorporation of Metal-Binding Loop To Create New Functionality. LDM can also be used to introduce new metal-binding sites and functionality into proteins. This principle has been demonstrated in several cases of protein design study based on the Greek key β-barrel scaffold. Like α helical bundles, the Greek key β-barrel is one of the most common scaffolds that form the framework of different families of proteins, such as immunoglobins, dismutases, blue type 1 copper proteins, and a Cu_A-containing subdomain of multicopper oxidases and reductases. Both blue copper and Cu_A centers are redox-active centers capable of transferring electrons efficiently over long distances. While the blue copper proteins are among the most well-characterized metalloproteins, the Cu_A center in multicopper oxidases, such as cytochrome *c* oxidase, remained a mystery for a long time because its spectral features were often dominated by those from other metal sites in the protein and because many of the features were not observed before.[129] Protein design using LDM played an important role in the elucidation of the structure and function of the Cu_A center.

It was recognized that subunit II of cytochrome *c* oxidase (C*c*OII) was structurally and functionally homologous to the CyoA subunit of cytochrome *o* quinol oxidase and shared the same Greek key β-barrel fold. However, the CyoA did not contain the Cu_A center as in C*c*OII. A careful sequence alignment revealed that the amino acids capable of forming the Cu_A center were replaced by those that could not coordinate copper, and those amino acids were clustered in a loop between two β strands F and G (called the FG loop) (Figure 22).[355] Because the loop sequences between the two proteins were different and, without structural information, it was difficult to pinpoint which amino acids were responsible for the Cu_A site formation, van der Oost et al. decided to replace the whole loop sequence of CyoA with the corresponding loop sequence of C*c*OII in order to restore the Cu_A center.[355] Spectroscopic[355] and X-ray crystallographic[356] studies confirmed that the Cu_A center, similar to that in C*c*O, was restored in CyoA. Since the CyoA was expressed in *E. coli* as a water-soluble protein, separate from other subunits of the cytochrome *o* quinol oxidase, CyoA with the restored Cu_A center became a model protein for the study of the Cu_A center. Subsequent studies using this system provided some of the strongest evidence that the Cu_A center contains a dinuclear center with two cysteines, two histidines, and one methionine.[357]

Similar sequence alignments also revealed that C*c*OII shared similar structural homology with the mononuclear blue copper proteins, and the main

```
                A         B      B'       C                    D
             ========    ===  =======  =========          ========
Azurin 1  AECSVDIQGNDQMQFNTNAITVDKSCKQFTVNLSHPGNLPKNVMGHNWVLSTAADMQGVVTDGMASGLDKDYLKPD
CcOII 155            ..LLATDNPVVVPVGKKVLVQVTA-----TDV-IHAWTIP-------------------------
CyoA 147             ..GIATVNEIAFPANTPVYFKVTS-----NSV-MNSFFIP-------------------------
                    E          E'              F              G
                  ======   =========       =======          ======
Azurin  77   DSRVIAHTKLIGSGEKDSVTFDVSKLKEGEQYMF-FCT-F--PGHSALMKGTLTLK
CcOII  187   --AFAVKQDAVPG-RIAQLWFSV----DQEGVYFGQCSELCGINHAY-MPIVVKAV..
CyoA   179   --RLGSQIYAMAGMQTRLHLIANE-----PGTYDGISASYSGPGFSG-MKFKAIAT..
```

Figure 22. Amino acid sequence alignment of blue copper azurin from *P. aeruginosa* (azurin), subunit II of cytochrome *c* oxidase from *P. denitrificans* (CcOII), and CyoA subunit of cytochrome *o* quinol oxidase from *E. coli* (CyoA).

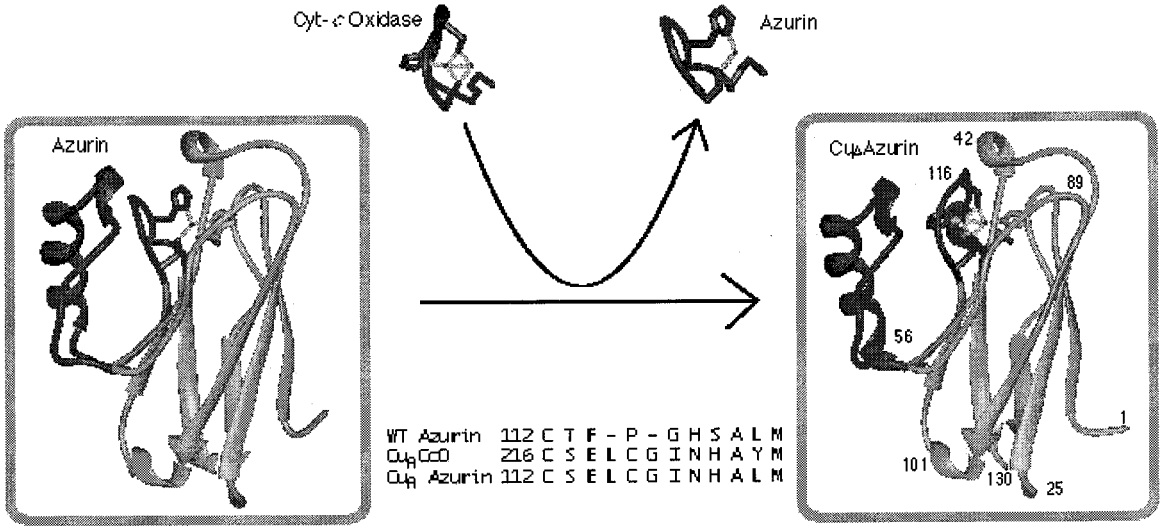

Figure 23. Schematic illustration of engineering the Cu_A center into azurin through loop-directed mutagenesis.

difference between the two families of proteins resided in the FG loop sequence (Figure 22).[355,358−360] The mononuclear blue copper center is coordinated by one cysteine, two histidines, and one methionine, and three of the four ligands appear in the FG loop, which is several amino acids shorter than the corresponding loop in C*c*OII. To allow the conversion of the blue copper center into a Cu_A center using LDM, the blue copper protein had to accommodate not only the longer C*c*OII FG loop, but also the extra copper ion. This was exactly what happened in two successful cases where the blue copper center in amicyanin[359] and azurin[360] was redesigned into the Cu_A center using LDM (Figure 23). In addition, LDM was used to convert one blue copper protein into another blue copper protein with different spectroscopic and redox properties[361] and from a Cu_A center in C*c*O to a blue copper protein.[362]

The X-ray structures of the engineered Cu_A center in CyoA[356] and in azurin[363] indicated that, with the exception of the FG loop region, the protein backbones of the engineered proteins were superimposable on that of the wild-type proteins (the RMSD's were less than 1 Å). While this result in the engineered CyoA may not be as surprising since LDM involved swapping loops of equal sizes, the close overall structural similarity between engineered Cu_A azurin and wild-type azurin is quite interesting when considering the LDM replaced a shorter, 10 amino acid loop with a longer, 12 amino acid loop. A careful comparison of the loop structures before and after the LDM revealed that azurin was able to accommodate the insertion of extra amino acids and the additional copper ion by a simple "breathing" motion of ~3 Å.[363] These findings are encouraging because they suggest that protein scaffolds can be rigid enough to withstand large changes in the loop region and at the same time are plastic enough to accommodate incorporation of new and very different metal-binding sites. Therefore, in addition to its value in protein design, LDM may be a general way to introduce metal-binding sites with known sequences but unknown structures into a well-characterized protein for facile spectroscopic and X-ray crystallographic study.

In addition to providing a simple model protein for learning the principle of protein design and for elucidating the structure and function of the metal-binding sites in proteins, protein design using LDM offers a unique advantage over the study of native enzymes. It allows comparison of different metal-binding centers in the same protein framework. For example, it was difficult to provide the direct and conclusive evidence for which copper center, the blue copper and the Cu_A centers, is more efficient in ET. This is because the ET rates were measured through different protein frameworks and it has been difficult to quantify the medium effect such as the ET pathways through different proteins. The engineering of the Cu_A center into the same framework of the blue copper azurin allowed a direct comparison of the electron-transfer properties of the two centers because the distances and pathways between the donor (a disulfide anion radical) and acceptors (copper

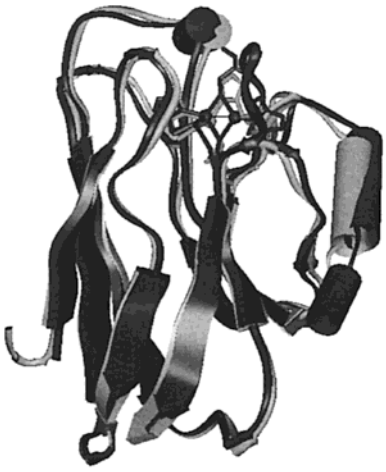

Figure 24. Overlay of X-ray structures of the wild-type blue copper azurin (light) and the redesigned Cu_A azurin through loop-directed mutagenesis (dark). With the exception of the loop region where the mutagenesis occurred, the backbones of the two proteins are superimposable. When all residues relevant to the ET study are overlaid, one of the dinuclear copper centers in Cu_A azurin matches closely with the mononuclear blue copper center, thus making it possible to compare the ET properties of the two centers in the same protein framework.

centers) are almost identical in the two proteins (Figure 24).[363,364] Therefore, a comparative study of ET in the two systems provided conclusive evidence that the dinuclear Cu_A is more efficient than the mononuclear blue copper site.[364]

3. Semitheoretical Approach

As discussed above, theoretical approaches, using automated computer algorithms, and empirical approaches, using visual inspection, have been used successfully to design and create new metal-binding sites in proteins with little homology to the target protein. In the former approach a thorough search of all locations in the protein and evaluation of the many solutions is required. In the latter approach, the inspection relies heavily on the knowledge of the designer. The semitheoretical approach falls between the above two approaches in its rigor by combining visual inspection of proteins to find proper locations for creating new metal-binding sites and computer program evaluation for the energetics of positioning appropriate amino acid residues. It is most suitable for creating new metal-binding sites where location of the site is fairly certain from previous knowledge or study. As in the design by inspection approach, this semitheoretical approach uses previous knowledge to locate the site and thus bypasses the exhaustive search process of theoretical approaches. At the same time, this approach uses similar rigorous methods for evaluation of energetics of positioning appropriate amino acid residues for the site formation.

i. Design and Creation of a New Tetrahedral Zn(II)-Binding Site in Antibodies. Metalloantibodies offer much promise toward obtaining new catalytic metal-binding sites. One method is to create a catalytic metal center near the antigen-binding region. The antigen specificity can then be separately selected to vary the enzyme substrate specificity. Another method uses both a metal ion and the substrate as the antigens. The dimeric antibody binds to the metal ion with one monomer and the substrate with the second monomer. Metal-ion and substrate specificity could be separately selected.

For the former method, much work at creating metal-binding sites in antibodies, primarily for hydrolysis, has been performed. By examining the morphology of Zn(II) sites in PDB structures of carbonic anhydrase and carboxypeptidase and then the structure of the anti-fluorescein antibody 4−4−20, multiple potential Zn(II) sites were identified.[365−370] Many of the designed proteins successfully bound Zn(II) in addition to other divalent metal ions. Metal binding was analyzed by tryptophan fluorescence quenching,[365−368,370] fluorescein quenching,[365−367] and spectroscopic studies of the metal ion.[370] In all of these variants, only metal binding was assayed. It remains to be seen if the sites can successfully catalyze a reaction. Interestingly, one particular protein was used as a metal-ion sensor.[368]

ii. Design and Creation of a Cu_B Center in Myoglobin and Cytochrome *c* Peroxidase: Toward a Novel Heme−Copper Oxidase. Heme−copper oxidases (HCO's) are a superfamily of terminal oxidases in the respiratory chains of both eukaryotic mitochondria and bacteria.[371,372] At the heart of these enzymes is the dinuclear center consisting of a heme with a proximal histidine and a Cu(II) with three distal histidines. Spectroscopic studies have shown that the heme−copper center contains a spin-coupled Cu(II)−Fe(III) site, probably bridged by an unknown ligand.[373−377] One major difference between heme−copper oxidases and other heme proteins is the presence of the Cu(II), called the Cu_B center. It would be quite interesting to find out how to design and engineer a Cu_B center into other heme proteins at a similar location with respect to the heme center and if the presence of the new Cu_B center can transform heme proteins such as globins and peroxidases into an oxidase. Furthermore, the designed proteins, being smaller, more homogeneous, and free from other metal-binding sites, can serve as a model system for investigating the structure and reaction mechanism of heme−copper oxidases.

The Cu_B site has been designed into both cytochrome *c* peroxidase[378] and myoglobin.[379] It was accomplished through a careful overlay of the heme−copper center in cytochrome *c* oxidase (C*c*O) with the heme site in either C*c*P or Mb (Figure 25). Different orientations of the C*c*P/Mb active sites were sampled until an appropriate match of residues in C*c*P/Mb to the histidine ligands of the Cu_B site in C*c*O was found. The matched residues in C*c*P/Mb were then mutated to histidines, and the energetic parameters after energy minimization of the resulting proteins were evaluated and compared with the parameters of wild-type proteins. After satisfactory mutations were validated, the mutations were carried out in the laboratory and the resulting proteins (called Cu_BC*c*P and Cu_BMb, respectively) were purified and studied by a variety of spectroscopy.

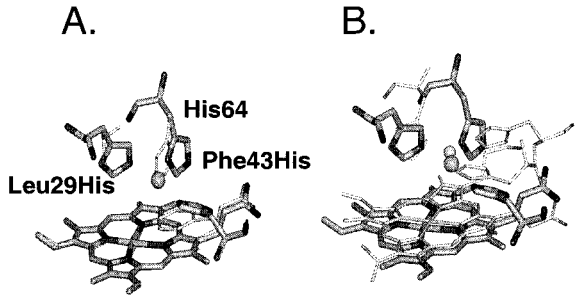

Figure 25. (A) Overlay of the crystal structure of WTMb (thin) and a structural model of Cu_BMb based on computer modeling and energy minimization (thick). (B) Overlay of the crystal structure of the heme−copper center in C*c*O (thin) and the same structural model of Cu_BMb (thick) as in part A.

When isolated, the heme-incorporated, Cu(II)-free Cu_BCcP (C*c*P(Arg48His, Trp51His, His52Ala, Ser81-His)) displays a low spin heme UV−vis and EPR spectra, typical of heme with either bis-His or His/hydroxide ligands. However, upon addition of increasing amounts of Cu(II), the UV−vis spectrum of the low-spin heme is converted to that of a high-spin heme, with clear isosbestic points at 396, 513, and 565 nm.[378] The Cu_B−heme center in C*c*O is known to be in a high-spin state. More interestingly, EPR analysis of the sample shows that the addition of increasing amounts of Cu(II) results in a decrease of the heme signals, demonstrating spin coupling of heme Fe(III) and the Cu(II), much the same as in C*c*O. Further evidence for spin coupling comes from the observation of broad integer spin signals at $g' = 3$ and $g' = 12$, much the same as in C*c*O. When Zn-(II) is added to the Cu(II)-free proteins, the low-spin heme to high-spin heme conversion is also observed by UV−vis but no spin coupling is detected by EPR. These results show that a Cu(II)-binding site has been created in C*c*P that mimics both the high-spin state and the spin coupling of the Cu_B−heme center in respiratory oxidases. This Cu_BCcP represents the first protein model of a Cu_B−heme center and is the only model that shows spin coupling between heme Fe(III) and Cu(II) without added exogenous ligands. Similar results were also obtained on the Cu_BMb.[379] In addition, the study also revealed that the presence of Cu(II) and Ag(I) (as a Cu(I) mimic) increased the affinity of heme for diatomic ligands such as CN^- and O_2. More importantly, the presence of Cu(I) in the designed Cu_B center transformed sperm whale Mb from an O_2 carrier into an O_2 activator.

iii. Other Examples. Work toward using the green fluorescent protein (GFP) as a metal-ion sensor was accomplished by creating a metal-binding site on its surface.[380] The coordination of metal ions in these designed proteins resulted in quenching of the proteins fluorescence. The first round of design was a bis-histidine site that loosely bound Ni(II). The second round of design involved the addition of a third metal-coordinating residue, aspartate. This variant yielded an effective metal site for Cu(II), Ni-(II), or Co(II) with binding constants in the low micromolar range.

In this section reviewing the rational design of new metal-binding sites in protein, we have described many successful examples of both the theoretical and empirical approach to the design process. On one hand, the theoretical approach uses automated programs and provides guidance to and evaluation of the designed metal-binding site. On the other hand, insights gained from the empirical approach may help design better programs. Before a comprehensive understanding of the distinct structural features of different metal-binding sites and their selectivity is achieved and programs incorporating these features are available, both approaches will make important contributions to the field of metalloprotein design. The semitheoretical approach described in this section is one way of combining the benefits of both theoretical and empirical approaches. Design by combinatorial/evolution methods, described below, is another effective empirical approach that will complement the theoretical approach.

B. Design by Combinatorial/Evolution Methods

So far this review has focused on rational design and redesign of metalloproteins. While rational design or redesign is an effective tool for optimizing and altering protein function, one drawback of rational design is that the structure and mechanism of the proteins to be designed must be understood. Unfortunately, the number of structures in the protein databases is increasing dramatically and our knowledge about the protein structure and function is quite limited. Furthermore, it is extremely difficult to predict long-range effects of residues far from the active site on the structure and function. More studies show that those long-range effects are important in protein design.

In contrast to the rational design, "irrational" design or design through combinatorial and evolution methods requires little prior knowledge of the protein structure.[381−383] Random mutations can be generated in the whole gene by error-prone-PCR or at specific residues by introducing oligonucleotides with degenerated sequences. The effect of these mutations can be enhanced with DNA shuffling.[384] However, this method does require a means to screen large libraries for the desired phenotype.[385,386] Several methods such as phage, yeast, or ribosome display have been reported to help the selection and decoding process.[387−394] Preparing monoclonal antibodies against metal cofactors or their analogues is an important branch in the field of catalytic antibodies.[395,396] It has also proven to be an effective way to obtain new metalloproteins. The rational design and "irrational" design methods can complement one another.[397]

1. Selection of Metalloproteins through Phage Display

One efficient means of screening for the desired phenotype is phage display, where proteins of interest are presented on the surface of phage.[387−389] This method has been applied to select zinc-finger proteins, an important class of DNA-binding proteins, so that they can recognize a chosen target DNA.[398−405] The studies focused on one of the three fingers in the transcription factor Zif268, and random mutations were made on residues known to be important for DNA-binding specificity. The protein variants were

then displayed and selected for binding of specific DNA sequences. This method has been extended to multiple fingers, where one finger was optimized at a time, with consideration of the interaction of neighboring fingers.[406] The selected protein bound target DNA with high affinity (nanomolar dissociation constants) and specificity (greater than 20 000-fold discrimination against nonspecific DNA). A combination of selection by phage display, refinement by site-directed mutagenesis, and characterization of the zinc-finger domains allowed a better understanding of the interaction between zinc-finger proteins and target DNA molecules. It also made possible the construction of tailor-made zinc-finger proteins for specific DNA sequences.[407] Interestingly, some of the selected zinc-finger proteins have been tested in cell cultures and were shown to block DNA transcription.[408]

In another study, variants of carbonic anhydrase containing random mutations in the hydrophobic residues surrounding the metal-binding site have been displayed on phage.[409] Those with high metal-binding affinity have been selected and enriched.

2. Search for New Metalloantibodies

In an approach that is complementary to the rational design of metal-binding sites in antibodies (see section III.A.3.i), preparing antibodies against metal complexes such as metalloporphyrins or the transition-state analogues of the reactions they catalyze has resulted in new metalloproteins with catalytic functions. For example, monoclonal antibodies have been prepared using metalloporphyrin,[410,411] nonmetalloporphyrins,[412,413] or transition-state analogues of porphyrin metalation[414] as antigens. The antibodies isolated were found to bind the antigen metal complexes more tightly than other similar complexes.[410] More importantly, by choosing the porphyrin antigens carefully, several groups have been able to obtain metalloporphyrin-containing antibodies that can catalyze a variety of reactions such as porphyrin metalation,[414] electron transfer,[415,416] peroxidation,[417−423] mono-[411,424,425] and dioxygenation.[426] Spectroscopic and X-ray structural analysis suggests the metalloantibodies are similar in structural properties and catalytic mechanisms to native heme proteins.[427−434] In addition, antibodies containing non-porphyrin complexes, such as copper, zinc, iron, lead, and cerium complexes, have also been obtained.

3. Directed Evolution of Heme Enzymes

Myoglobin, peroxidases, and cyt P450 contain the same heme cofactor and yet perform different biological functions. Myoglobin, normally an oxygen carrier, displays some peroxidase activity. However, the specific activity is much lower than that of peroxidases (see section II.A.2.i). To improve the peroxidase activity of Mb, Wan et al. carried out directed evolution of horse heart Mb.[435] The Mb gene was first subjected to several cycles of PCR random mutagenesis and then expressed in *E. coli*. After spraying the cell plates with a low concentration of a common peroxidase substrate (2,2-azino-bis(3-ethyl)benzothiazoline-6-sulfonic acid (ABTS)), the investigators were able to select Mb variants with high peroxidase activity because cells containing those variants produced a green color due to the substrate oxidation. Four cycles of mutagenesis and screening resulted in several variants with enhanced peroxidase activity. One variant contains four substitutions in the heme pocket and exhibits ~25-fold higher peroxidase activity than WTMb. In another study, directed evolution of cyt P450 was carried out to improve its hydroxylation reactivity by more than 20-fold over that of the native enzyme.[436] The investigators were able to efficiently screen for the improved variant P450s by coexpressing them with horseradish peroxidase, which converts the products of the P450 reaction into fluorescent compounds amenable to digital imaging screening.[437] In both studies, careful design of the screening system was the key in high-throughput selection of desired variants. The mutations found in the selected variants with enhanced activities were not obvious and would have been difficult to uncover using a rational design approach.

IV. Summary and Outlook

In this review several approaches to the design and engineering of novel metalloproteins based on native protein scaffolds have been described. Whether it is for redesign of an existing metal-binding site or to create a new one, both rational and combinatorial methods can be used. To design rationally, theoretical, empirical, and modular approaches can all be effective. It is difficult to judge which method is best as the choice is highly dependent on the goal of the research and the protein systems involved.

Rational design using automated computer search algorithms such as Metal-Search and Dezymer (section III.A.1) has clear advantages for efficient crafting of metal centers and a judiciary choice of the best amino acid side chains for the formation of the metal-binding sites. It is best used for creation of new metal-binding sites in proteins where neither site location nor side chain choice can be determined based on the investigator's knowledge or protein homology. It is especially useful for designing metal-binding sites where construction of a geometrically correct, sterically compatible primary coordination sphere is sufficient to reproduce the dominant features of the structure and function of the desired center. Tetrahedral Zn(II) centers, iron−sulfur clusters, and mononuclear non-heme iron centers are primary examples of the successful designs.

For many other metal centers, such as blue copper and purple Cu_A centers, construction of a geometrically correct, sterically compatible primary coordination may not be sufficient to result in the desired metal-binding sites because their formation may be opposed by several alternative reactivities and geometries. Most metal-binding sites in proteins are not in idealized geometries as in their small metal complex counterparts.[438−440] For example, the geometry of the blue copper center is preferred by neither Cu(II) nor Cu(I). Without a careful design that includes a secondary coordination sphere or even longer range interactions, the amino acid side chains may be

flexible enough to accommodate the geometry of the free metal ions and ruin the design by computer programs. Therefore, it has been recognized that negative design (i.e., suppression of competing reactions and competing coordination) is a crucial aspect of the protein design process.[232] Furthermore, recent studies also showed that positive design in the secondary coordination sphere (such as hydrogen-bonding and hydrophobic interactions) is also quite important.[81,91,93,227] Until now, few effective programs have been known to overcome these problems and much iteration, mostly based on knowledge and inspection, has been needed for a successful design. Moreover, structural features responsible for protein selectivity of one metal ion over the other still remains to be fully understood, despite much progress in this area.[236−238] Thus, it is difficult to write programs capable of designing metal-binding sites selectively. In this regard, protein redesign by taking advantage of existing metal-binding sites with known geometry may offer a better chance of success by gaining insight into the structure and function of the centers one (or a few) residue(s) at a time (see section II). Another equally attractive empirical approach is the design of new metal-binding sites based on protein homology (see section III.A.2.ii). This approach depends highly on the degree of sequence or structural homology. Generally speaking, higher homology more often leads to a better chance of success. At the same time, factors that are common to both template protein and the target protein cannot be revealed from the design exercise. This problem also applies to the protein redesign approach. Despite these limitations, both approaches are extremely useful at elucidating the role of specific structural features in protein design and function and at revealing principles of protein design.

Design of metal-binding sites by inspection (see section III.A.2.i) is one of the early forms of protein design. Although it appears less elegant than other methods, design by inspection has produced proteins with many practical applications, such as proteins with a His-X_3-His chelating site on the surface and with poly-His tags for protein purification, enzyme activity regulation and metal sensing. The designed metal-binding sites also resulted in proteins with increased stability, altered activities, altered selectivity, new functions, and elucidated protein topologies. It can be an effective approach for creating metal-binding sites with more complexity if the investigators are armed with a thorough understanding of the structure and function of both the target and the template proteins and have a reasonably good idea where the metal site can be created. This approach can be enhanced further if it can utilize computer programs to help evaluate and modify the initial design through energy minimization. This is the basis of the semitheoretical approach (see section III.A.3). On one hand, the semitheoretical approach is similar to the empirical design by inspection in that it relies on investigator knowledge to pinpoint the location of the metal-binding site. On the other hand, the semitheoretical approach resembles the theoretical approach in that it uses rigorous computer programs to evaluate the initial design and to suggest better designs with better energetic terms.

All the rational methods described above rely heavily on our knowledge of principles governing the structure and function of metal-binding sites. However, despite much progress made so far, our knowledge is still quite limited. For example, the high affinity and selectivity of many metal-binding sites remains to be elucidated. Fascinating new metal centers, such as the Cu_Z center in nitrous oxide reductase,[130] a multinuclear copper center with Cu−Cu bonds, are being discovered. To fill the gap of our knowledge and to keep pace with the rapid development of the field, combinatorial engineering of metal-binding sites is of great value (see section III.B). However, this approach depends highly on the design of a clear and convenient selection scheme, which is often difficult to do. More importantly, the sampling size of a typical protein is vastly huge for selection. For example, given the typical transformation efficiency of *Escherichia coli*, a host commonly used for expressing combinatorially engineered proteins, the practical library size of the system is $\sim 10^9-10^{10}$, which corresponds in the best case to a fully randomized peptide of less than eight residues.[441] Combinatorial engineering using native protein scaffolds and focusing on a specific region of the protein can minimize this problem because structural space is much smaller than the sequence space.

Modular approaches (see section III.A.2.iii) may bridge the gap between rational design and combinatorial engineering. It allows easier rational generation of a site by swapping structural elements, such as a loop, that are important for the formation of the metal-binding site. At the same time, it is easier to search combinatorially for optimal sequences since the search is narrowly focused on the region of difference and its surrounding areas. Modular approaches, particularly loop-directed mutagenesis, may also play an important role in bioinformatics and structural genomics. Generally speaking, automated computer programs are reasonably good at predicting the overall scaffolds of unknown proteins in genomes; it is much harder to predict the structure of active sites including metal-binding sites. Using loop-directed mutagenesis to transfer the sequences containing amino acids for the formation of unknown metal-binding sites into easy-to-crystallize, well-characterized proteins may allow elucidation of these metal-binding sites without the elaborate cloning, expression, purification, and crystallization of the native protein containing the unknown structure.

Recognizing the advantages and disadvantages of each approach, one can combine the advantages of two or more approaches. For example, rational approaches can be used to create a new metal-binding site and combinatorial searches can then be used to fine-tune the metal-binding affinity, selectivity, or in the case of catalytic sites the substrate-binding pocket. Furthermore, programs such as Metal-Search and Dezymer can be used to design or redesign geometrically strained metal-binding sites, such as the blue copper center, in proteins with high homol-

ogy to these proteins. This strategy offers a higher chance of success. Study of the designed protein may help improve the programs.

Another focus of future research should be the role of the secondary coordination sphere in the design and formation of novel metalloproteins. This role has been well recognized in the study of metalloproteins.[228] Most of metalloprotein design study has so far been concerned with the negative design aspect, i.e., to avoid steric conflict between residues in the primary coordination sphere and those in the secondary coordination sphere. However, positive interactions such as hydrogen-bonding and electrostatic interactions are also very important, and consideration of these positive design aspects is evident only in a few limited cases.[81,91,93,227] Without these considerations, design and engineering certain metal-binding sites may not even be possible.[81,91,93]

Finally, more three-dimensional structural studies of the designed metalloproteins by crystallography or NMR are needed to bring this field to another level of sophistication and success. Most spectroscopic techniques routinely used in metalloprotein design can confirm the success (or failure) of only certain main features of the design. It is difficult to reveal subtle changes from the design work. Since metalloprotein design using native protein scaffolds makes it possible to choose a template protein of reasonable characteristics for NMR study or crystallography, a major commitment is required to use crystallography or NMR to learn not only if the design worked, but also how it worked and what improvement is necessary to make it better.

Protein structures are like many big puzzles that nature presents to us. For many years, scientists in different fields have been trying to solve the puzzle by studying each piece and its variants. It is now possible to design and put the pieces together like nature does, albeit quite awkwardly at this stage. With development of the many approaches described above, together with approaches discussed in other papers in this issue, it will not be too long before we can design and make the same puzzle or maybe even better puzzles than nature does.

V. Abbreviations

ABTS	2,2-azino-bis-(3-ethyl)-benzothiazoline-6-sulfonic acid
AFM	atomic force microscopy
AP	mammalian alkaline phosphatase
APX	ascorbate peroxidase
C*c*O	cytochrome *c* oxidase
C*c*OII	subunit II of cytochrome *c* oxidase
C*c*P	cytochrome *c* peroxidase from *Saccharomyces cerevisiae*
CCS	copper chaperone for SOD
CD	circular dichroism
CFP	cyan fluorescent protein
CO	carbon monoxide
compound I	ferryl and porphyrin π cation radical
compound I′	ferryl and Trp radical
compound II	ferryl
CooA	CO sensing heme protein
CPO	chloroperoxidase
Cu_A	dinuclear copper center of C*c*O and N_2OR
Cu_B	dinuclear Cu/heme center of C*c*O
Cu_BC*c*P	C*c*P with designed Cu_B site
Cu_BMb	Mb with designed Cu_B site
Cu_Z	tetranuclear copper cluster of N_2OR
CuZnSOD	copper−zinc superoxide dismutase
cyt	cytochrome
EPR	electron paramagnetic resonance
ET	electron transfer
ferryl	heme Fe(IV)═O
FeSOD	Fe-containing superoxide dismutase
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
hCCS	human CCS
hDAT	human dopamine transporter
hGH	human growth hormone
hGHr	hGH receptor
HiPIP	high potential iron protein
HO	heme oxygenase
HO-1	human heme oxygenase 1
Hr	hemerythrin
IR	infrared spectroscopy
LDM	loop-directed mutagenesis
LP	lactose permease
Mb	myoglobin
MCD	magnetic circular dichroism
MMO	methane monooxygenase
MnP	manganese peroxidase
MnSOD	Mn-containing superoxide dismutase
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
NMR	nuclear magnetic resonance
N_2OR	nitrous oxide reductase
NOS	nitric oxide synthase
NTA	nitrilotriacetic acid
OM	oncomodulin
P450	cytochrome P450
P450cam	camphor-hydroxylating cytochrome P450 from *Pseudomonas putida*
PDB	protein database
PV	parvalbumin
RBP	retinal-binding protein
RMSD	root mean standard deviation
RnR	ribonucleotide reductase
Rr	rubrerythrin
RR	resonance Raman spectroscopy
SHE	standard hydrogen electrode
SOD	superoxide dismutase
TIM	triosephosphate isomerase
UV−vis	electronic absorption in the ultraviolet and visible region
yCCS	yeast CCS
YFP	yellow fluorescent protein

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