Biosynthesis of Metal Sites

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1. Introduction

This contribution serves as a counterpoint to the other articles in this special issue on biomimetic inorganic chemistry by focusing on the diversity of biological approaches, rather than synthetic methods, to form desired metallocenters. Our hope is that biomimetic inorganic chemists may obtain useful synthetic insights by examining the ingenious ways by which nature carries out metallocenter biosynthesis. At the same time, careful analyses of synthetic strategies and fundamental metallocenter properties may help investigators to discern new biological pathways for metal incorporation. Metalloproteins have been characterized intensively for decades, yet only recently have investigators focused on the mechanisms of biological metallocenter assembly.[1,2] Here, we describe several general themes, summarized in Table 1, that arise from studies examining the biosynthesis of protein metallocenters. In addition, we highlight a few well-studied examples of metallocenter assembly that illustrate the complexity and diversity of approaches utilized in these biological systems. A particularly interesting example, the biosynthesis of the iron−molybdenum-cofactor (FeMoco) site of nitrogenase, is addressed in a separate contribution to this issue.[3]

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2. General Themes of Metallocenter Biosynthesis

2.1. Reversible Metal-Ion Binding

The interactions between some biological macromolecules (L) and their requisite metal ions (M^{n+}) are governed simply by thermodynamic control, such as that illustrated in eq 1.

$$L + M^{n+} \rightleftarrows L{\cdot}M^{n+} \quad (1)$$

In these situations, the ligand's specificity and affinity for the metal ion as well as the metal ion's coordination geometry depend both on the metal-ion properties and on factors associated with the macromolecular ligand.[4,5] Metal ions vary in their charge, radius, ligand exchange lability, ligand preference (e.g., hardness/softness), and position in the Irving−Williams series. Similarly, the fold of a protein or other biological macromolecule, its rigidity, and the properties of its side chains (e.g., pK_a, polarizability, and location in a particular secondary structure) dictate its interactions with the metal ions.

Intracellular Ca-regulated proteins provide a nice illustration of reversible metal-ion binding.[6] For example, calmodulin, a small acidic protein involved in the regulation of selected phosphatases, kinases, and other enzymes, reversibly binds four Ca^{2+} ions. Each of the hard Ca^{2+} ions is coordinated by seven hard oxygen ligands contributed by amino acid side chains that comprise the so-called "EF hand" helix−loop−helix motif (Figure 1). Analogous sites are found in troponin C, parvalbumin, calbindins, and additional Ca-binding proteins. For calmodulin, the reversible interaction shown in eq 1 is oversimplified because binding of the first two Ca^{2+} ions occurs in a cooperative manner with an average binding constant of approximately $2 \times 10^5\ M^{-1}$ whereas the third

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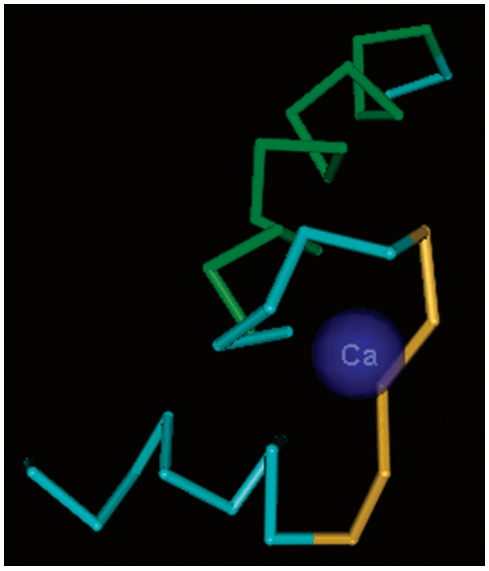


Figure 1. EF hand motif found in many Ca-binding proteins. The metal-binding site is located in a loop region connecting two α-helices (the E and F helices). The term "EF hand" relates the appearance that is similar to a right hand in which the blue helix is the extended thumb, the green helix is the extended pointer finger, and the loop represents the remaining fingers wrapped around the metal. The figure depicts the Cα positions of a portion of the structure of calmodulin in which Asp20, Asp22, Asp24, and Glu31 are ligands to the Ca (PDB accession code 1CLL).

and fourth ions are bound with binding constants of about 3×10^4 M^{-1}. The binding of calcium ions leads to exposure of hydrophobic sites on calmodulin, likely responsible for its interaction with the calmodulin-associated proteins listed above.

Thermodynamic control of metal-ion binding also accounts for many of the interactions between K^+, Na^+, or Mg^{2+} ions and macromolecules such as proteins, DNA, or RNA.[7,8] These ions are quite abundant in the cytoplasm of cells, with estimated concentrations of approximately 10^{-2}, 10^{-1}, and 10^{-3} M, respectively.[7] Extracellular interactions of metal ions with biological material (cell walls, polysaccharides, etc.) also are likely to be dominated by thermodynamically controlled processes.

Investigators studying metallocenter properties can exploit such thermodynamic control to substitute alternate metal ions into selected proteins.[9] In this process, the original metal ions are removed by chelation, often using extreme conditions such as low pH/denaturants, and the resulting apoproteins are incubated with high concentrations of the desired metal ions to obtain the metal-substituted species.

Table 1. General Mechanisms of Metallocenter Biosynthesis[a]

mechanism	equations representing the indicated mechanism[b]
1. reversible metal-ion binding	$L + M^{n+} \rightleftarrows L{\cdot}M^{n+}$
2. metallochaperone delivery of metal ion or cofactor	$L_1{\cdot}M^{n+} + L_2 \rightleftarrows L_1 + L_2{\cdot}M^{n+}$
3. posttranslational modification to create a metal-binding site	$L \rightarrow L^*$ and $L^* + M^{n+} \rightleftarrows L^*{\cdot}M^{n+}$
4. synergistic binding of metal with another component	$L + M^{n+} + X \rightleftarrows L{\cdot}M^{n+}{\cdot}X$
5. synthesis of metal-containing cofactors	$Y + M^{n+} \rightarrow Y{\cdot}M^{n+}$ and $Y{\cdot}M^{n+} + L \rightleftarrows L{\cdot}Y{\cdot}M^{n+}$
6. metal incorporation coupled with electron transfer	$L + M^{n+} \rightarrow L{\cdot}M^{n+(+1)} + e^-$ or $L + M^{n+} + X^{y+} + z\,e^- \rightarrow L{\cdot}M^{n+}{\cdot}X^{y+(-z)}$
7. requirement for an apoprotein-specific molecular chaperone	$C{\cdot}L + M^{n+}$ (+ NTP) $\rightarrow L{\cdot}M^{n+} + C$ (+NDP + P_i)

[a] These mechanisms are not mutually exclusive. Many examples of metallocenter synthesis utilize multiple mechanisms, as illustrated in section 3 of this review. In addition, it is important to note that metallocenter assembly can be driven by nucleotide hydrolysis reactions, typically involving mechanism 7. [b] L, biological macromolecular ligand; L*, covalently modified ligand; X, nonmetal component of a metallocenter; Y, component required for synthesis of a metal-containing cofactor; C, molecular chaperone.

For example, the metal sites of many Zn-containing enzymes have been replaced by Co to facilitate spectroscopic analyses. Five-coordinate Co^{2+} often exhibits intense LMCT transitions, and Co^{2+} is paramagnetic so that EPR spectroscopy can be used to monitor the metal site. While such an approach can help define the properties of selected metallocenters, this method of metal incorporation may have little relevance to the in vivo mechanism of metallocenter assembly.

Significantly, thermodynamic control cannot explain many metal-ion−protein interactions.[10] This point is highlighted by the Cu-containing proteins that form despite the reported absence of free copper ions in the cell.[11,12] This claim is based on the measured zeptomolar (10^{-21} M) sensitivity of the *Escherichia coli* copper regulatory protein CueR and on the estimation of less than 10^{-18} M Cu^{2+} in yeast, determined on the basis of superoxide dismutase activity measurements; for comparison, one atom of free Cu per yeast cell (10^{-14} L) would correspond to a concentration of 10^{-10} M. An analogous situation may well exist for Zn,[13] where the intracellular concentration is estimated at 10^{-11} M.[7] It is still unclear whether significant levels of "free" Fe, Mo, Ni, Co, and other biologically important metal ions (i.e., the free hydrated metal ions)[10] exist in the cell. Not unexpectedly, metal transporters and permeases that accumulate these ions are tightly regulated.[14] In this way, cells strike a balance in which metal concentrations are maintained at levels sufficient for growth while preventing deleterious effects. The low concentrations of free metal ions observed within cells imply that reversible metal-ion binding to ligands is unlikely to account for the synthesis of most metallocenters. Furthermore, the metallocenters of many proteins are buried within a rigid framework. This finding demands that the metal ions must bind to the ligand during the folding process, an unlikely scenario, or that an alternative mechanism exists for incorporating the metal (sections 2.2−2.7).

2.2. Metallochaperone Delivery of Metal Ion or Cofactor

Metallochaperones are metal-binding proteins (L_1) designed to deliver the appropriate metal ion or metal cofactor to a target ligand (L_2), as illustrated in eq 2.

$$L_1{\cdot}M^{n+} + L_2 \rightleftarrows L_1 + L_2{\cdot}M^{n+} \qquad (2)$$

It is important to note the distinction between metallochaperones and molecular chaperones that assist in protein folding. The best studied protein chaperones, such as GroEL, Hsp70, and Hsp40,[15] prevent the misfolding or stimulate the refolding of a wide range of proteins by processes that generally require nucleotide triphosphate hydrolysis.[16] These generalized or "housekeeping" protein chaperones are certain to play a role in the synthesis of many metalloproteins. In addition, section 2.7 describes a subset of metalloprotein-specific molecular chaperones that bind to a target apoprotein and confer an appropriate conformation needed to bind a metal ion or cofactor. Nucleotide triphosphate hydrolysis is carried out by a subset of these chaperones, presumably to provide a driving force for their reactions. In contrast to such molecular chaperones that bind proteins, metallochaperones bind metal ions or metal cofactors and deliver them to their highly specific targets.

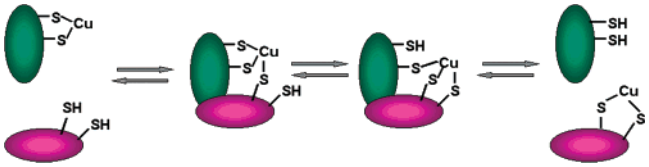


Figure 2. Mechanism of Cu transfer from a Cu metallochaperone to a target domain with a thiol-containing binding site. Cu(I) is transferred from a pair of Cys residues in one protein to a pair of Cys residues in the second protein via intermediates with three Cys ligands to the metal.

Perhaps the clearest examples of metallochaperones are small, cytoplasmic proteins (Atx1 in yeast, Hah1 in humans, or CopZ in bacteria) that function to deliver Cu^{1+} to secretory pathway ATPases.[17] Three-dimensional structures are known for Atx1,[18] Hah1,[19] CopZ,[20,21] and domain 4 of a target protein (the Menkes disease protein, Mnk4)[22] in various metal-bound states. Both the metallochaperones and the target proteins bind Cu^{1+} using two Cys residues found in a MT/HCXXC motif, and a model of the Hah1:Mnk4 complex identifies other critical residues likely to be needed for protein:protein interactions.[19] The mechanism of Cu^{1+} transfer between the metallochaperone and the target domain is proposed to involve ligand exchange reactions with two- and three-coordinate intermediates (Figure 2).[23] Significantly, reversible Cu^{1+} transfer between the two proteins occurs with an equilibrium constant near unity.[24] This equilibrium does not provide enough thermodynamic driving force for efficient metallocenter assembly; thus, these metallochaperones are hypothesized to function like enzymes that provide kinetic control. The mechanism by which Cu^{1+} initially becomes bound to the metallochaperones has not been elucidated. Also unclear is the affinity of the metal for the proteins and the rates of dissociation.

Other metallochaperones exhibit a range of more complex behaviors. For example, a protein often considered to be a mitochondrial copper metallochaperone, Cox17, contains a polynuclear copper site coordinated by thiolate ligands (section 3.4),[25] as opposed to a single metal-ion binding site. Another example, the three-domain copper chaperone for superoxide dismutase (CCS), has one domain that structurally resembles the target enzyme, another related to Atx1, and a third that contains a critical Cys-X-Cys motif.[26] The Cu^{1+} bound to the CCS cysteines is transferred to a site with four histidyl ligands in Cu,Zn−superoxide dismutase (SOD1) via a CCS:SOD1 complex that has been structurally characterized.[27] A more detailed discussion of CCS and SOD1 activation is found in section 3.1. In contrast to the Cu^{1+}:cysteine interactions observed in the copper delivery proteins, other proposed metallochaperones bind their metal ions less tightly, with

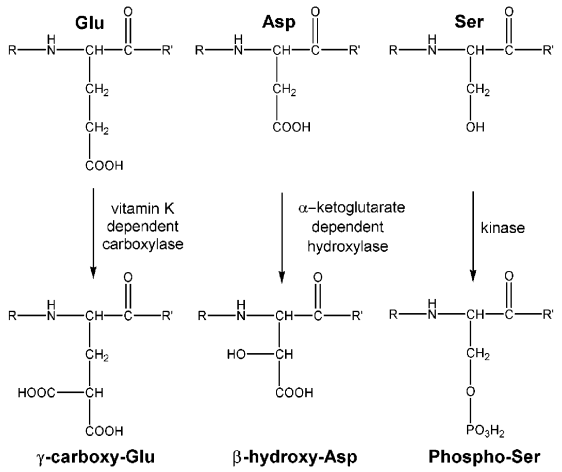


Figure 3. Posttranslational modifications to create a metal-binding site. Amino acid side chains in proteins may be specifically derivatized to create ligands with improved metal-binding affinities. In the reactions shown, vitamin-K-dependent carboxylation, α-ketoglutarate-dependent hydroxylation, and kinase-catalyzed phosphorylation reactions convert Glu, Asp, and Ser to *γ*-carboxy-Glu, *β*-hydroxy-Asp, and phospho-Ser.

binding constants on the order of 10^6 M^{-1}. Sections 3.5 and 3.6 describe two examples of putative Ni–metallochaperones that are proposed to function in Ni^{2+} delivery to urease and hydrogenase. Metallochaperones that bind metal cofactors rather than metal ions are described in sections 3.3 and 3.7, related to synthesis of *c*-type cytochromes and molybdenum cofactor (Moco) proteins.

2.3. Posttranslational Modification at the Metal-Binding Site

Some proteins tightly bind their cognate metal ion only after the nascent apoprotein (L) undergoes a covalent modification reaction (producing L^*), as shown in eqs 3 and 4.

$$L \rightarrow L^* \quad (3)$$

$$L^* + M^{n+} \rightleftarrows L^*{\cdot}M^{n+} \quad (4)$$

Figure 3 illustrates three examples of posttranslational modifications that are used to create metal binding sites. Several blood-clotting factors, other plasma proteins, and bone proteins are modified at selected glutamyl groups by the action of a vitamin-K-dependent carboxylase.[28,29] The resulting *γ*-carboxyglutamic acid residues chelate Ca^{2+} and aid in maintaining protein:protein and protein:membrane interactions. Similarly, a specific interaction with Ca^{2+} occurs in proteins containing enzymatically derivatized epidermal growth factor domains, such as those present in coagulation factors, complement proteins, and transforming growth factors.[29] Specific aspartyl and asparaginyl groups in these proteins are hydroxylated by an α-ketoglutarate-dependent dioxygenase to produce *β*-hydroxyaspartyl and *β*-hydroxyasparaginyl residues that bind the metal ions. Calcium ions also interact tightly with dentin or bone phosphoproteins that are very rich in phosphoserine residues.[30] These promote deposition of calcium

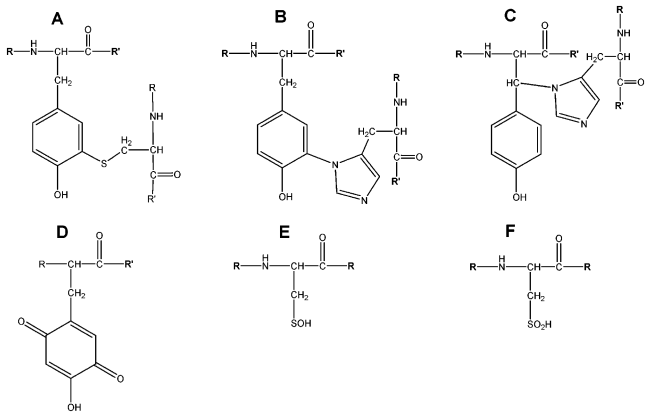


Figure 4. Posttranslational modifications that occur after metal binding. The modifications illustrated are observed in (A) galactose oxidase, (B) cytochrome *c* oxidase, (C) catalase, (D) amine oxidase, (E, F) nitrile hydratase.

phosphate or calcium hydroxylapatite during tooth and bone development. As a final example, the egg yolk protein phosvitin contains adjacent phosphoserine residues that form a tight complex (binding constant near 10^{18} M^{-1}) with Fe^{3+}, important for trace metal-ion storage during embryonic development.[31]

Posttranslational modifications to a protein alternatively can occur after a metal ion binds, in some cases involving a change in metal redox state or chemical transformations of another component. The resulting modifications may impart a specific enzymatic activity to the protein, tune the reactivity of the protein metallocenter, stabilize metal-ion binding, or inactivate the enzyme. Often, the modifications derive from autocatalytic reactions directly involving the bound metal; however, accessory enzymes may be needed to recognize the metal-bound form of the protein and catalyze the chemical changes. Figure 4 depicts several examples of metalloenzyme active sites with residues that are chemically altered after metal binding. Galactose oxidase contains a tyrosyl side chain linked by a thioether bond to a cysteine residue.[32] The modified tyrosinate binds Cu^{1+} and becomes oxidized to a tyrosyl radical as the metal is oxidized to Cu^{2+}.[33] Cytochrome *c* oxidases contain a cross-link between tyrosine and histidine side chains, where the histidine is a ligand to Cu.[34,35] The precise role of this modification is controversial, but its presence in enzymes from both bacteria and higher eukaryotes highlights its importance (section 3.4). A different type of Tyr-His cross-link is present in the heme-containing enzyme catalase.[36] Formation of this cross-link appears to be coupled to oxidative conversion of the bound tetrapyrrole to heme *d*. Many Cu-containing amine oxidases contain different versions of oxidized tyrosyl side chains such as the 2,4,5-trihydroxyphenylalanine quinone shown in the figure. The mechanism of formation of this altered side chain has been extensively investigated and shown to be autocatalyzed by a process requiring only Cu and oxygen.[37,38] Cysteinyl side chains can undergo oxidation reactions, as exemplified by the case of nitrile hydratase where crystal structures reveal both sulfinic and sulfenic acids bound to the Fe or Co ions.[39,40] While it is unclear whether these modifications are simply artifacts of crystallization, some

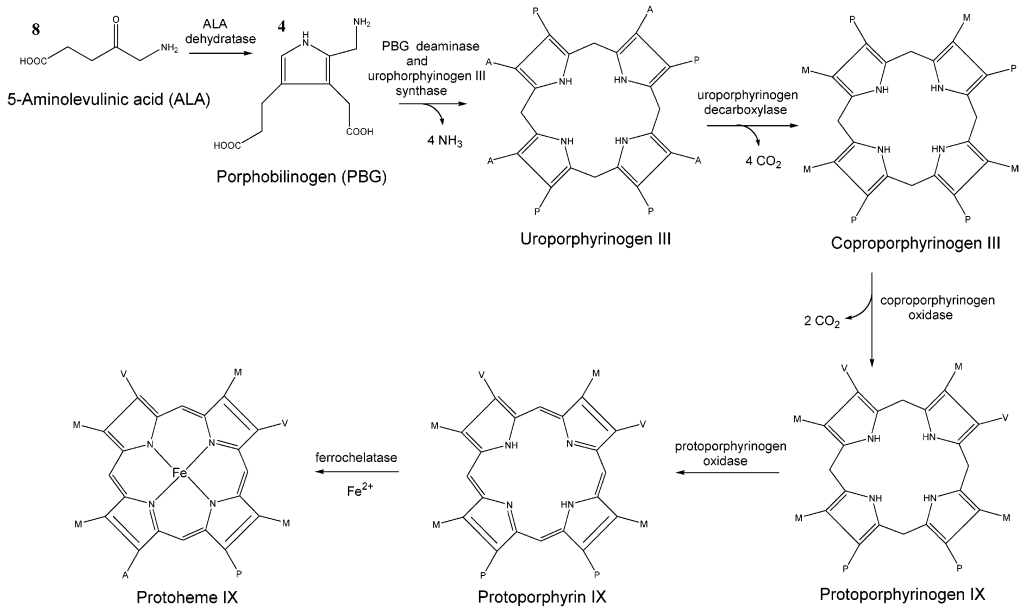


Figure 5. Heme synthesis. Depending on the organism, enzymes convert either succinyl-CoA or glutamate to 5-aminolevulinic acid (ALA). Two ALA combine to form porphobilinogen (PBG), and four PBG are condensed to uroporphyrinogen III (with side chains labeled as P = propionate and A = acetate). A series of decarboxylations and oxidations yield protoporphyrin IX (M = methyl and V = vinyl). Ferrochelatase catalyzes the final step in heme synthesis, the insertion of Fe to generate protoheme IX.

investigators have argued that cysteine oxidation is required for generation of in vivo activity[41,42] and the modifications could be used to modulate the metal-ion reactivity. Self-hydroxylations of aromatic amino acids near the active sites of many non-heme iron oxygenases are known to occur,[43,44] often accompanied by enzyme inactivation.

A related type of posttranslational modification accompanying metal binding involves proteolysis. An excellent example of such a situation is associated with hydrogenase activation,[45−48] as described in section 3.6. In this case, a protease recognizes the Ni-containing large subunit precursor and proteolysis leads to a conformational change that buries the metallocenter.[46] A similar proteolysis-based process may function in the stable incorporation of manganese into the oxygen-evolving complex.[49]

2.4. Synergistic Binding of Metal with Another Component

The interaction of metal ions with some proteins is accompanied by the binding of another nonmetal component (X), as illustrated in eq 5.

$$L + M^{n+} + X \rightleftarrows L{\cdot}M^{n+}{\cdot}X \qquad (5)$$

The identity of the accessory compound can range widely, as shown by the following examples. The metallocenter of ribulosebisphosphate carboxylase/oxygenase is comprised of both Mg^{2+} and an activating CO_2 molecule.[50] Assembly of this site proceeds in a stepwise fashion with CO_2 reacting with the ϵ-amino group of a specific lysyl residue to form a carbamate that serves as one of the metal ligands. Analogous CO_2-dependent metal-binding reactions occur during activation of a number of dinuclear hydrolases including phosphotriesterase[51] and urease (see section 3.5).[52] In these enzymes, the carbamate bridges the two metal ions bound to the active site. The metallocenter of transferrin consists of both ferric ion and the carbonate dianion, with the carbonate acting as a bridge between the protein and the metal.[53] Kinases and phosphotransferases often bind their required metal ions concurrently with their substrate (e.g., the MgATP complex). As a final example, Fe-only hydrogenases[54,55] and NiFe−hydrogenases[56] (described further in section 3.6) possess Fe sites with bound cyanide and carbon monoxide molecules. The activation of these enzymes requires that both the metal ions and the diatomic molecules be incorporated.

2.5. Synthesis of Metal-Containing Cofactors and Use of Scaffold Proteins

Activation of selected metalloproteins requires the prior synthesis of a cofactor that contains both metal and an additional organic or inorganic component (Y), as shown in eqs 6 and 7.

$$Y + M^{n+} \rightarrow Y{\cdot}M^{n+} \qquad (6)$$

$$Y{\cdot}M^{n+} + L \rightleftarrows L{\cdot}Y{\cdot}M^{n+} \qquad (7)$$

Perhaps the best-characterized example of this mechanism involves the synthesis of heme cofactors and their subsequent incorporation into various hemoproteins (the complexities of cytochrome *c* biosynthesis are described in section 3.3). Succinctly, enzyme-catalyzed reactions convert either succinyl-CoA or glutamate into δ-aminolevulinic acid. This molecule is further converted through a series of intermediates to form protoporphyrin IX, the metal-free cofactor, into which Fe is inserted by ferrochelatase (Figure 5).[57] Analogous reactions are required for synthesis of other tetrapyrrole macrocycles such as the cobalamins, various types of chlorophylls, and the metha-

nogen coenzyme F_{430} (containing Co, Mg, or Ni, respectively). Co- and Mg-chelatases have been described for insertion of these metals into the appropriate tetrapyrrolic ring structures.[58,59]

In a variation of this approach, some metallocenters are first synthesized on a scaffold protein and subsequently incorporated into the target enzyme. For example, FeMoco of nitrogenase[3] is first assembled on NifEN (i.e., the products of the *nifE* and *nifN* genes) and then transferred into the NifDK enzyme apoprotein.[60] Notably, the two scaffold proteins are structurally related to the nitrogenase subunits. A similar situation was proposed for HupK,[61] a protein resembling in sequence the hydrogenase large subunit with two Cys ligands absent and participating in activation of some NiFe hydrogenases (see section 3.6), but no direct evidence for such a role has been reported and the function of HupK remains unclear. Scaffold proteins need not always be related in sequence to the final protein targets, as demonstrated by accessory protein components involved in the synthesis of Moco-containing enzymes (section 3.7) and Fe−S cluster proteins (described in section 3.2).

2.6. Metal Incorporation Coupled to Electron Transfer

The synthesis of some metallocenters is coupled to one or more electron-transfer reactions. Depending on the example, the redox step may involve the metal, another component, or a side chain of the protein. Direct metal oxidation during metallocenter assembly (eq 8) is nicely illustrated by synthesis of the ferrihydrite core of ferritin or the formation of the tetramanganese cluster in the oxygen-evolving photoreaction center.

$$L + M^{n+} \rightarrow L{\cdot}M^{n++1} + e^- \qquad (8)$$

Ferritin is an iron storage protein comprised of 24 subunits (a mixture of H subunits with M_r 22−24 kDa and L subunits with M_r 20−22 kDa) that associate to form a hollow sphere capable of binding up to 4500 iron atoms.[62,63] Nucleation of the iron core is initiated at a ferroxidase site that binds two Fe^{2+} ions, reacts with oxygen to form a transient peroxodiferric species,[64] and releases H_2O_2 while yielding a μ-hydroxo (or oxo) diferric precursor. After its nucleation, the core grows primarily by oxidative processes on the surface of the mineral particle. As another example, the water-oxidizing complex of photosystem II is formed by stepwise binding and oxidation of four Mn^{2+},[65] with the electrons transferred to other components of the photosynthetic system. More generally, synthesis of Cu^{2+} and Fe^{3+} sites in proteins often involves initial binding of Cu^{1+} and Fe^{2+} followed by oxidation to stabilize the metal ions. Such a situation occurs in Cu,Zn−SOD1, as described in section 3.1.

Other examples illustrate how reduction of an accessory component may be required for metallocenter synthesis (eq 9).

$$L + M^{n+} + X^{y+} + z\,e^- \rightarrow L{\cdot}M^{n+}{\cdot}X^{y+-z} \qquad (9)$$

For example, incorporation of nickel into carbon monoxide dehydrogenase of *Rhodospirillum rubrum* requires the prior reduction of an Fe−S cluster.[66] Structural studies of this protein reveal that the added Ni completes a unique [1Ni−4Fe−4S] center that is required for activity.[67] Another example of a reductive activation step occurs during NiFe−hydrogenase biosynthesis (see section 3.6). These enzymes possess a $Ni-Fe(CN)_2(CO)$ site with the Fe-bound diatomic molecules derived from carbamoyl phosphate.[68,69] The overall reaction requires a reductive step that may involve participation of the Fe−S cluster in HypD.[70] Yet a third example from the Ni−enzyme literature involves the synthesis of methyl-*S*-coenzyme M reductase, a methanogen enzyme that contains the Ni−tetrapyrrole cofactor F_{430}.[71] Formation of active enzyme requires both the reduction of Ni^{2+} to Ni^{1+} and reduction of a C=N bond in the organic macrocycle.[72]

The third redox-dependent process involved in metallocenter assembly requires changes to the protein, typically involving cysteine residues. For example, disulfide bonds that are spontaneously generated or enzymatically formed in periplasmic proteins must be reduced to create metal-binding thiolates (see section 3.3 describing cytochrome *c* biosynthesis). The opposite reaction, oxidation of cysteines during metallocenter assembly, is exemplified by disulfide formation during SOD1 activation (section 3.1).

2.7. Requirement for an Apoprotein-Specific Molecular Chaperone

Several metalloproteins incorporate their metal centers by processes that appear to involve apoprotein-specific molecular chaperones (C). These proteins stabilize a required conformation of the target apoprotein, often catalyze nucleotide triphosphate hydrolysis, and dissociate after activation (eq 10); thus, they bind the apoprotein but are not part of the final holoprotein.

$$C{\cdot}L + M^{n+} (+ NTP) \rightarrow L{\cdot}M^{n+} + C\,(+NDP + P_i) \qquad (10)$$

These apoprotein-specific molecular chaperones target only their particular apoprotein partners but otherwise resemble in function the well-studied molecular chaperones such as GroEL, Hsp70, and Hsp40 that bind to and prevent misfolding or stimulate refolding of a wide range of proteins.[15] Putative GTP-dependent apoprotein-specific molecular chaperones for urease and hydrogenase activation are described in sections 3.5 and 3.6, respectively. Similarly, apoprotein-specific chaperones implicated in the biosynthesis of SOD1, Fe−S cluster proteins, and Moco-containing enzymes are mentioned in sections 3.1, 3.2, and 3.7. As described elsewhere in this issue,[3] at least two molecular chaperone-like activities are associated with nitrogenase biosynthesis. In one case, the γ or NifY proteins form complexes with nitrogenase lacking its FeMoco.[73−75] These proteins prop open and allow access to the enzyme active site, bind and insert FeMoco, and then dissociate from the

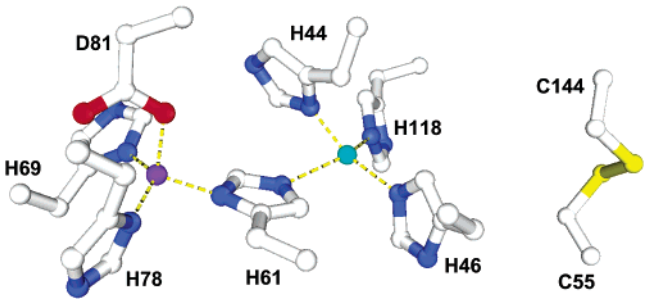


Figure 6. Structure of the dinuclear active site of yeast Cu,Zn−superoxide dismutase (SOD1; PDB code 1SDY). His 61 bridges the Zn (purple) and Cu (cyan) sites. Also shown is a nearby disulfide that appears to be important for enzyme activation.

nitrogenase holoprotein. Formation of the complex between γ or NifY proteins and FeMoco-free nitrogenase is achieved by action of the second nitrogenase-related chaperone, dinitrogenase reductase, in an ATP-dependent manner.[76,77] Of interest, the [4Fe−4S] center of this protein is required for its function in electron delivery to nitrogenase but not for its action as a chaperone.[78] A final example of an apoprotein-specific molecular chaperone involved in metallocenter activation is associated with *Streptomyces* tyrosinase. Insertion of Cu into the tyrosinase apoprotein (encoded by *melC2*) requires complex formation with the product of *melC1* which stabilizes an appropriate apoprotein conformation, inserts the metal, and assists in secretion of the active enzyme.[79−81]

3. Illustrations of Biological Metallocenter Assembly Pathways

The following sections describe several complex metallocenter assembly pathways that combine some of the multiple themes listed above and summarized in Table 1. Another notable enzyme with an elaborate activation process is nitrogenase, described elsewhere in this issue.[3]

3.1. Cu,Zn−Superoxide Dismutase

Cu,Zn−superoxide dismutase (SOD1) uses its unique histidyl-bridged metal ions (Figure 6) to catalyze the dismutation of two molecules of superoxide, producing oxygen and hydrogen peroxide. Little is known about how Zn^{2+} is placed into this enzyme, but Cu^{2+} insertion occurs by a fascinating process that incorporates the themes summarized in sections 2.1, 2.2, 2.6, and 2.7. Such a complex mechanism of Cu delivery is demanded by the finding that there is no detectable free Cu ion in the cell.[11,12] The key to SOD1 maturation resides in a protein termed Cu chaperone for superoxide dismutase (CCS).[82] Our discussion of SOD1 maturation focuses on studies involving the enzyme and CCS from yeast where protein structural investigations are most advanced.

As depicted in Figure 7, three-dimensional structures are known for homodimeric CCS,[83] heterodimeric CCS:SOD1,[27] and homodimeric SOD1.[84] CCS has three domains: an Atx1-like domain (see section 2.2) containing an MXCXXC metal-binding motif with Cys17 and Cys20, a SOD1-like domain that has the same β-barrel structure as the target enzyme but lacks residues needed for catalytic activity, and a flexible loop containing a conserved CXC motif with Cys229 and Cys231. Domain I is required for SOD1 activation only under copper-limiting conditions, whereas both domains II and III are essential for CCS function under all conditions.[26] The CCS:SOD1 structure was obtained by using the H48F variant of SOD1 (lacking a Cu ligand) to prevent transfer of Cu to the enzyme active site.[27] The structure shows that domain II of CCS replaces one of the SOD1 subunits of the homodimer to create a nearly identical interface in the heterodimer. Compared to the CCS homodimer, domain I shifts its position in the heterodimer and domain III becomes more ordered. A particularly interesting aspect of the latter domain is the disulfide bond between Cys229 of CCS and Cys55 of SOD1. The SOD1 structure reveals, in addition to features of the metallocenter, the presence of a disulfide bond between Cys55 and Cys144 (Figure 6) that is thought to be essential for its function.

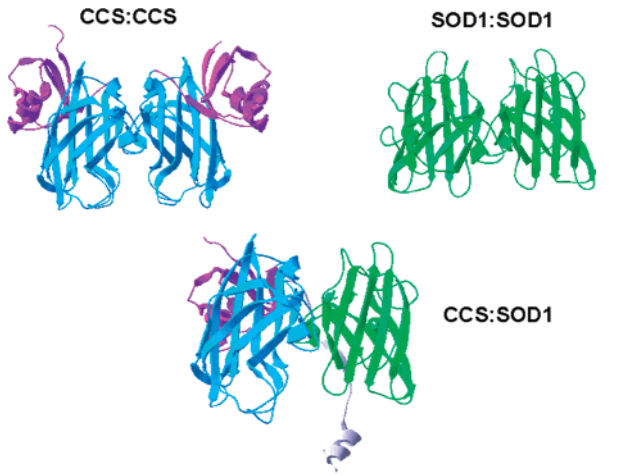


Figure 7. Summary of structural investigations of SOD1 activation. Ribbon diagrams are shown of the CCS homodimer (PDB code 1QUP), SOD1 homodimer (1SDY), and CCS:SOD1 heterodimer (1JK9). CCS contains three domains, one (purple) resembling Atx1, a second (blue) resembling SOD1 (shown in green), and a third (grey) that is disordered in the absence of SOD1.

SOD1 activation likely initiates by the swapping of domain II in dimeric CCS with one subunit of dimeric Zn-containing SOD1, affording two molecules of the heterodimer. Domain I of CCS obtains Cu^{1+} from an unknown source[26] and transfers the metal ion to domain III by the process illustrated in Figure 2. Domain III then rotates its position to deliver Cu to the SOD1 active site. Of note, this transfer involves a change in Cu coordination from Cys ligation to binding by His ligands and appears to require an oxygen-dependent step.[27] A reasonable scenario is that this step involves both oxidation of the metal and oxidation of Cys229 in CCS and Cys55 of SOD1 to form a disulfide bond, thus propping open the

Figure 8. *E. coli isc* operon involved in Fe−S cluster synthesis. The product of *iscR* functions in autoregulation of the operon. The *iscS* gene encodes a cysteine desulfurase that provides inorganic sulfur to the products of *iscA* and *iscU*. These scaffold proteins accept iron to form Fe−S clusters, perhaps using electrons donated by a ferredoxin (encoded by *fdx*). The products of *hscA* and *hscB* appear to serve as a molecular chaperone that transfers the clusters to target proteins in an ATP-dependent process.

active site, followed by disulfide exchange to yield the Cys55-Cys144 disulfide in SOD1.

3.2. Fe–S Cluster Proteins

Proteins containing Fe−S clusters are found throughout nature, where they play key roles in electron-transfer reactions, enzyme catalysis, and gene regulation.[85] Starting with purified apoprotein, it is sometimes possible to form a functional Fe−S cluster by direct addition of high concentrations of Fe^{2+} and S^{2-}.[86] Such in vitro cluster formation can be considered to involve reversible metal-ion binding and synergistic binding of metal with another compound (themes described in sections 2.1 and 2.4, above). In the cell, however, free metal and sulfide ions both are toxic; thus, more sophisticated methods are required to synthesize the required clusters. Although many details of Fe−S cluster assembly have yet to be elucidated, biochemical and genetic studies have been effectively combined to identify key components of this process and to partially define their roles.[87,88] As described below, Fe−S cluster synthesis uses a combination of the themes described in sections 2.1, 2.4, 2.5, 2.6, and 2.7.

The sulfur source for bacterial Fe−S cluster assembly was identified during pioneering studies of nitrogenase biosynthesis in *Azotobacter vinelandii*. NifS was shown to be a pyridoxal phosphate-dependent L-cysteine desulfurase that creates an enzyme-bound persulfide, an inorganic sulfur donor that is used for production of Fe−S clusters in nitrogenase proteins.[89] *A. vinelandii* later was shown to possess a second copy of this gene, named *iscS*, that is located in an *i*ron−sulfur−*c*luster operon (comprised of *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, and *fdx*), which also is found in *E. coli* (Figure 8).[90] Deletion analysis verified that *E. coli* IscS has a critical role in Fe−S cluster formation in this microorganism,[91] but this species also possesses SufS and CsdB homologues with related activities (the latter of which is a selenocysteine lyase).[92,93] *E. coli* CsdB and *Thermatoga maritima* NifS crystal structures confirm key features of their proposed persulfide-generating mechanisms.[94,95]

The sulfur donors indicated above transfer their sulfane sulfur atoms to a series of bacterial proteins (NifU, IscU, IscA, SufA) that appear to function as scaffolds for Fe−S cluster synthesis.[96−103] The sulfur donor and cognate sulfur acceptor proteins form complexes according to gel filtration chromatography, surface plasmon resonance, and isothermal titration calorimetry approaches, and sulfur transfer occurs as monitored by ^{35}S-radiolabeling and electrospray ionization quadrupole mass spectrometric methods. Questions remain about the precise types of interactions occurring between the donor/acceptor proteins, the source of Fe, and the possible types of clusters that can be generated in the scaffold proteins. Nevertheless, the overall pattern is conserved, with these proteins being acceptors of the Fe and S for cluster synthesis. As an informative complication, NifU possesses a permanent [2Fe−2S] cluster housed in its central domain while the newly formed [2Fe−2S] cluster is localized to its amino terminal domain. Of interest, a permanent cluster does not exist in IscU or IscA; however, a separate protein containing a [2Fe−2S] cluster is encoded within the *isc* operon (*fdx*, Figure 8) and may substitute for the permanent cluster domain. The role of the permanent cluster/Fdx is unknown but may involve an electron-transfer step required for Fe−S cluster synthesis. Consistent with such a function, the crystal structure of Fdx has been elucidated and resembles that of adrenodoxin-type ferredoxins.[104] Whereas the Fdx holoprotein is likely to function in cluster synthesis, the Fdx apoprotein has been reported to serve as a [2Fe−2S] acceptor using IscA holoprotein.[99]

Transfer of Fe−S clusters from the scaffold proteins to the target proteins is a poorly understood process but generally appears to require specific molecular chaperones. Hsc20 and Hsc66, encoded by genes within the *isc* operon, are related to the known molecular chaperones DnaK and DnaJ. Surface plasmon resonance and titration calorimetry approaches reveal that IscU holoprotein forms a complex with Hsc20, which directs it to Hsc66.[105] This interaction stimulates Hsc66 ATPase activity by 480-fold by IscU binding to the Hsc66 substrate binding site.[106] Using phage display approaches, the precise sites of IscU interaction with Hsc66 and Hsc20 have been identified and shown to be distinct.[107] Of possibly related interest, two other sets of bacterial components utilize energy-dependent steps for synthesis or repair of Fe−S clusters. SufC and SufB proteins, encoded by a wide range of bacteria (as well being found in plants and some other eukaryotes), are thought to function in iron homeostasis and the oxidative stress response. SufC exhibits ATPase activity and interacts with SufB at the membrane—perhaps indicating an Fe−S cluster exporter role.[108,109] Finally, Fe−S cluster metabolism in *Salmonella enterica* requires ApbC and ApbE, an ATPase and a periplasmic lipoprotein, respectively.[110] Of special interest, ApbC is related in sequence to dinitrogenase reductase and CooC that are required for biosynthesis of metallocenters in nitrogenase and carbon monoxide dehydrogenase. Similarly, ApbE is related to NosX that may play a redox role during incorporation of the Cu_A site into nitrous oxide reductase (NosR).

In summary, synthesis of Fe−S clusters in bacterial proteins is thought to initiate by the generation of sulfane sulfur in NifS, IscS, or related proteins; the sulfur is donated to NifU, IscU, IscA, or other acceptor scaffold proteins; Fe also is incorporated into the scaffold proteins (perhaps requiring Fdx or an Fdx-like domain for an electron-transfer step); and finally, the intact cluster is incorporated into target apoproteins by using the Hsc20/Hsc66 molecular

chaperones, the SufC/SufB system, or other cellular components.

An additional noteworthy feature of bacterial Fe−S cluster synthesis concerns the exquisite IscR-dependent autoregulatory mechanism for control of cluster formation, as described in *E. coli*.[111] This protein acquires a [2Fe−2S] cluster but only after maturation of other cluster proteins that are better able to sequester the cofactor. When Fe−S cluster production becomes saturating so that IscR holoprotein is formed, IscR[2Fe−2S] acts a repressor of the *isc* operon by binding immediately upstream of *iscR* which is adjacent to *iscSUA*.

Eukaryotes possess a related, but more complex, pathway for Fe−S cluster assembly.[88] Most significantly, the reducing environment of the mitochondrion may be necessary for formation of clusters required in both the mitochondria and cytoplasm.[112] Genetic screens of mutant *Saccharomyces cerevisiae* identified *NFS1*, *JAC1*, and SSQ1 (analogues of *iscS*, *hscA*, and *hscB*) as genes that encode mitochondrial proteins participating in Fe−S cluster assembly.[113] Studies involving Nfs1p were the first to show a role in synthesis of both mitochondrial and cytoplasmic Fe−S proteins.[112] Analogous findings later were obtained with Yah1, a yeast mitochondrial ferredoxin.[114] Also located in the *S. cerevisiae* mitochondria are two homologues of IscA (termed Isa1p and Isa2p) with nonredundant roles;[115] these proteins were originally thought to function in metal delivery, but subsequent investigation indicates they are likely to function as scaffold proteins for Fe−S cluster assembly. For example, the Isa1 protein in *Schizosaccharomyces pombe* can transfer its [2Fe−2S] to the ferredoxin apoprotein.[116] Similar results were obtained with *S. pombe* Isu1, an analogue of bacterial IscU, and both human proteins.[117−120] In addition to these proteins that have close bacterial counterparts, the mitochondrial protein frataxin has been identified as being essential for cluster biosynthesis in eukaryotes.[121,122] Mutations in the gene encoding this protein lead to Freidreich's ataxia, an autosomal recessive neurodegenerative disease associated with defective Fe metabolism.[123] In its Fe-containing form, frataxin binds and donates its metal ions to the apoprotein form of a scaffold protein [124] or to ferrochelatase.[125] In addition, frataxin exhibits ferroxidase activity and stores Fe much like ferritin.[126] In summary, the mitochondria possess Fe−S cluster synthetic machinery much like that found in bacteria with at least one additional component. An in vitro system using these protein components along with ATP, NADH, and Fe^{2+} successfully reconstitutes the yeast mitochondrial Fe−S cluster assembly process.[127] Notably, however, cluster precursors also must be transported to the cytoplasm for incorporation into Fe−S proteins localized there. Evidence has been presented that Atm1p is a yeast mitochondrial membrane transporter that functions in this role.[112] Maturation of cytoplasmic Fe−S proteins also requires glutathione, perhaps functioning to deliver clusters to the apoproteins or assisting in the transfer or insertion of the cofactor.[128]

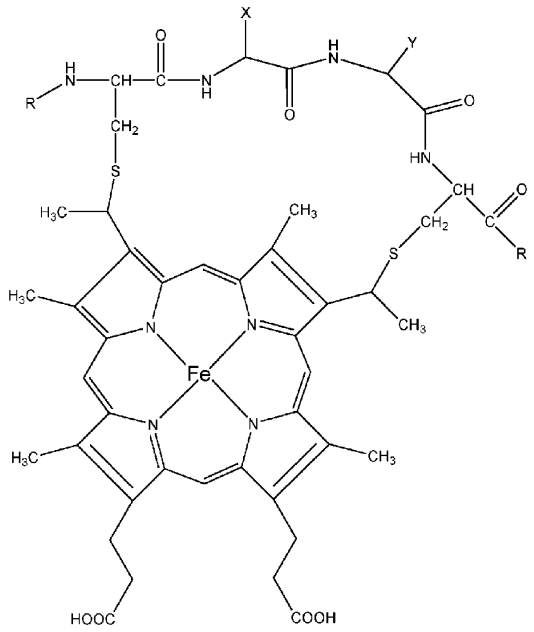


Figure 9. Structure of the cytochrome *c* metallocenter. Two Cys side chains in the protein covalently attach to the two vinyl groups found in protoheme IX (Figure 5) to yield the structure shown.

3.3. Cytochrome *c* Synthesis

The *c*-type cytochromes participate in critical energy-yielding electron-transfer pathways of aerobic respiration, anaerobic respiration, and photosynthesis. These nearly ubiquitous proteins possess redox-active Fe that is present in a covalently attached heme group, where two heme vinyl substituents are linked to cysteinyl residues via thioether bonds (Figure 9). At least three distinct pathways have been described for the translocation of cytochrome *c* apoprotein to its site of activity (outside the cytoplasmic membrane of prokaryotes, the intermembrane space of mitochondria, or the lumen of chloroplasts) followed by incorporation of heme.[129,130] One system utilizing a heme lyase for attachment of heme to protein is found in fungal, vertebrate, and invertebrate mitochondria.[131] A completely independent system that does not require a heme lyase is present in chloroplasts, cyanobacteria, Gram-positive bacteria, and some Gram-negative bacteria. Finally, most Gram-negative bacteria, including *E. coli*, as well as plant and protozoal mitochondria, and Archaea use the more complex system that we describe below for cytochrome *c* activation. Significantly, themes described in sections 2.2, 2.5, 2.6, and 2.7 are used for this system of metalloprotein assembly.

Biosynthesis of cytochrome *c* in *E. coli* requires the products of eight cytochrome *c* maturation (*ccm*) genes as well as several multipurpose proteins.[132] The process initiates with ribosomal synthesis of a precursor form of the cytochrome *c* apoprotein. This extended protein is secreted across the cytoplasmic membrane into the periplasmic space by the Sec system, accompanied by cleavage of the amino-terminal signal sequence. As the apoprotein emerges from the membrane, DsbA oxidizes two Cys residues located in a CXXCH motif (reduced DsbA is then recycled by DsbB-catalyzed oxidation). The disulfide

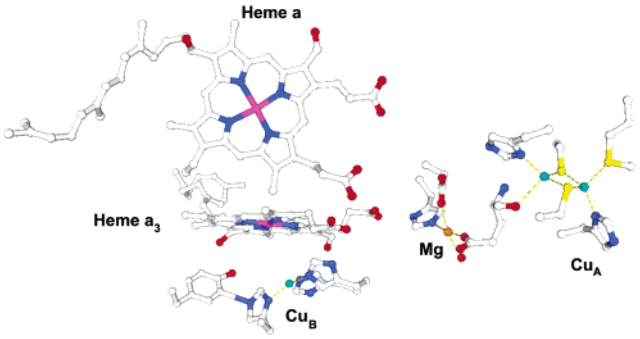


Figure 10. Cytochrome *c* oxidase metallocenters. Reduced cytochrome *c* donates electrons to the dinuclear Cu_A site. These electrons are relayed through heme a (possessing two axially coordinated His ligands, not shown) to the heme a_3/Cu_B dinuclear center. Heme a_3 possesses one axial His ligand (not shown) on the face opposite Cu_B. One His ligand of Cu_B is covalently attached to a Tyr side chain. The heme a_3/Cu_B center is the site of oxygen reactivity. Product water is released in a process that involves the bound Mg. Cu atoms are cyan, Fe atoms are pink, the Mg is orange, and the Mg-bound water is a red sphere.

within cytochrome *c* apoprotein must be reduced in order to react with the heme vinyl groups. Meanwhile, the heme is transported across the membrane by a still poorly understood process that might involve CcmA and CcmB. Within the periplasm heme is covalently bound to a His residue of CcmE, a heme chaperone.[133] CcmC is essential for heme attachment, but CcmD, CcmA, and CcmB facilitate this process.[134,135] An NMR structure of CcmE reveals further details of the heme binding site,[136] but the structure of the linkage to the His residue remains unclear. In the last step of biosynthesis, heme is transferred from CcmE to the reduced cytochrome *c* apoprotein. The results of in vitro studies suggest that this reaction can occur spontaneously, without the participation of other proteins.[137] In contrast, other studies are consistent with CcmF being a bacterial heme lyase,[138] perhaps in association with CcmG and/or CcmH. The thioether linkages of heme to the protein ensure that cytochrome *c* remains a stable redox cofactor.

3.4. Cytochrome Oxidase

Cytochrome *c* oxidase catalyzes the terminal step of respiration using electrons derived from cytochrome *c* to reduce molecular oxygen to water. Crystallographic studies have revealed the remarkable structure of this multisubunit, membrane-bound enzyme using proteins isolated from bovine heart mitochondria and the bacteria *Paracoccus denitrificans*, *Thermus thermophilus*, and *Rhodobacter sphaeroides*.[139−142] Although 13 different proteins are present in the enzyme of higher eukaryotes, the essential metallocenters (two hemes, three Cu atoms, and a Mg atom, depicted in Figure 10) are localized to two subunits in the core that are also present in the bacterial enzymes. Information related to cytochrome *c* oxidase biosynthesis is most advanced in yeast, where mutagenesis studies have shown that more than 30 gene complementation groups are needed for assembly of the complex.[143] The precise roles of each of these maturation factors are not defined, but evidence is available that themes discussed in sections 2.1, 2.2, 2.3, 2.5, and perhaps 2.7 are employed in cytochrome *c* oxidase activation.

One aspect of cytochrome *c* oxidase maturation involves synthesis of the unique heme A groups of the enzyme. First, Cox10, a farnesyl transferase, is used to modify heme B (see protoheme IX in Figure 5) to produce heme O.[144] In the next two steps, Cox15, ferredoxin, and ferredoxin reductase are used to sequentially oxidize the C-8 methyl group of heme O to form first the alcohol and then aldehyde, thus generating heme A.[145] One molecule of heme A, termed heme a in cytochrome *c* oxidase, is bound to the enzyme via two axial His ligands and serves as an electron shuttle. A second heme A molecule, termed heme a_3, binds a single axial His ligand and, on its opposite face, is closely juxtaposed (∼4.5 Å) to one of the Cu atoms, called Cu_B.

Synthesis of the Cu_B site requires a multicomponent Cu delivery system. Cytoplasmic transfer of Cu from the membrane transporter (Ctr1) to the mitochondria requires a metallochaperone, a function attributed to either Cox17[146] or Cox19.[147] These proteins are located both in the cytoplasm and the intermitochondrial membrane space. While little is known about Cu binding to Cox19 (other than it possesses four Cys residues that align with those in Cox17), a Cys-ligated polynuclear cluster was identified in Cox17 (6 Cu per dimer or 12 Cu per tetramer) on the basis of X-ray absorption and luminescence studies.[25] After Cu arrives at the mitochondria, the mitochondrial membrane-bound protein Cox11 transfers it into the Cu_B site of cytochrome *c* oxidase, probably mediated by an inner mitochondrial protein termed Sco1. Studies with a soluble portion of Cox11 indicate that the dimeric protein binds two Cu in a dinuclear cluster, with each metal coordinated by three His residues.[148] The Cu_B site in cytochrome *c* oxidase similarly contains three His ligands (Figure 10), where one of these residues is covalently linked to a nearby Tyr residue.[34,35] This Cu_B center, in combination with heme a_3, forms a unique dinuclear center that is the site of oxygen reactivity.

The remaining two Cu atoms in cytochrome *c* oxidase are found in a dinuclear site termed the Cu_A center. Biosynthesis of Cu_A again requires Cu transport through the cytoplasm by Cox17, Cox19, or other protein. Upon reaching the mitochondria, Cu is transferred to either Sco1 or Sco2, homologous mitochondrial membrane proteins that bind a single Cu per monomer via a His residue and a CXXXC motif.[149−153] These proteins subsequently insert the metal into the Cu_A site. Two Cys residues bridge the metal ions in the cytochrome *c* oxidase Cu_A center. One metal additionally has Cys and His ligands, and the other metal has a His and a backbone carbonyl oxygen ligand (Figure 10). The Cu_A site serves as the entry point for electrons donated by cytochrome *c*.

Mg is the remaining cytochrome *c* oxidase metal shown in Figure 10. No genes have been implicated in biosynthesis of this metallocenter, and the site can be substituted by growth of the organism in elevated concentrations of Mn. His, Asp, and Glu side chains bind the metal atom, where the carbonyl oxygen of this Glu residue is a ligand to Cu_A. The Mg site is

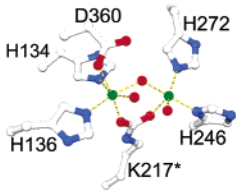
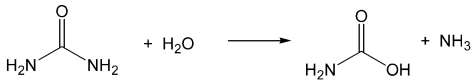


Figure 11. Dinuclear Ni active site of *Klebsiella aerogenes* urease (PDB code 1FWJ). The carbamylated lysine is shown as K217*, metal-bound waters are red spheres, and the Ni atoms are green.

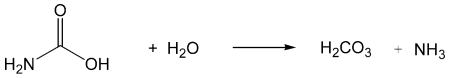
important for release of water, a product of the reaction catalyzed by cytochrome *c* oxidase.

3.5. Urease

Urease catalyzes the hydrolysis of urea to yield ammonia and carbamate, which spontaneously decomposes to yield carbonic acid and another molecule of ammonia (eqs 11 and 12). Structural studies of the enzymes from *Klebsiella aerogenes*,[154,155] *Bacillus pasteurii*,[156] and *H. pylori*[157] have revealed a dinuclear Ni active site (Figure 11) with a modified amino acid side chain—a carbamylated lysine residue—bridging the deeply buried metal atoms. Synthesis of the urease metallocenter is a complex process that illustrates themes described in sections 2.1, 2.2, 2.4, and 2.7 and requires Ni, carbon dioxide (used for carbamylation), several accessory proteins, and GTP hydrolysis.[158]



(11)



(12)

Our current understanding of the key events in cellular urease activation is derived primarily from studies involving the *ureDABCEFG* gene cluster of *K. aerogenes*. As illustrated in Figure 12, the process begins with the ribosomal synthesis of the $(UreABC)_3$ apoprotein. Crystallographic studies reveal the overall structure is identical to that of native enzyme but lacks Ni and carbamylation.[159] UreABC forms a series of complexes with the UreD, UreF, and UreG accessory proteins,[160−163] and these species can be activated by added Ni plus bicarbonate to varied extents: UreABC[52] < UreABC−UreD[164] < UreABC−UreDF[161] < UreABC−UreDFG.[165] In vitro immunoprecipitation studies and yeast two-hybrid analyses using components from other bacteria reveal the expected interactions among the gene products.[166−168] Significantly, UreABC−UreDFG exhibits GTP-dependent urease activation associated with a nucleotide-binding site located on the UreG component.[165] The precise roles of UreD, UreF, and UreG in urease activation remain unclear, but one reasonable possibility is that the proteins act together as a GTP-dependent molecular chaperone that alters the conformation of urease apoprotein to increase accessibility to the buried active site. Alternative possible roles include participation in CO_2 activation, establishing

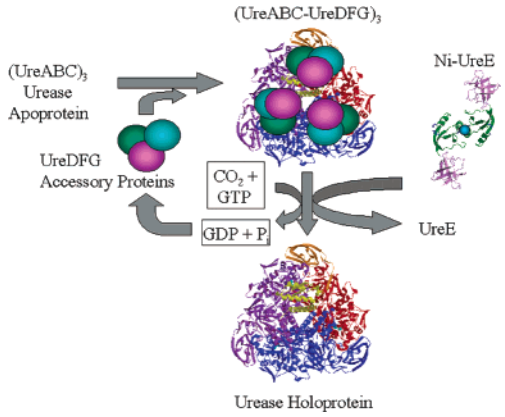


Figure 12. Model of urease activation. Synthesis of active urease requires the formation of a complex between urease apoprotein $(UreABC)_3$ and the UreDFG heterotrimer. This complex accepts Ni from the UreE metallochaperone (e.g., PDB code 1CFZ for the *K. aerogenes* protein) and inserts it into the carbamylated apoenzyme (thus the requirement for CO_2) in a GTP-dependent reaction. Dissociation of the accessory proteins results in active enzyme (e.g., PDB code 1GMU for the *K. aerogenes* enzyme).

proper docking of UreE to urease, and selecting the correct transition metal for incorporation.

Ni is delivered to UreABC−UreDFG by the metallochaperone, UreE. This urease accessory protein binds Ni, ~6 Ni per dimer for the *K. aerogenes* protein[169] versus a single Ni per dimer for *B. pasteurii* UreE,[170] with the function being independent of the number of metals bound. The metal content differences arise from a His-Asp-His sequence near the middle and a histidine-rich region at the carboxyl terminus of the former protein. Truncated *K. aerogenes* UreE protein, missing the His-rich region, and UreE variants lacking the His-Asp-His motif also function in urease activation.[171,172] Structures of the truncated *K. aerogenes* protein[173] and full-length *B. pasteurii* UreE[174] reveal nearly identical protein folds, with a conserved Ni binding site located at the dimer interface. Surprisingly, the Ni-binding domain of UreE closely resembles the structure of the Cu-binding Atx1 metallochaperone.[18] In addition, both UreE proteins contain a second module that structurally resembles a domain of an Hsp40-type molecular chaperone protein.[175] It may be that the chaperone-like module participates in UreE docking to its partner protein while the metal-binding domain transfers Ni to UreABC. In vitro studies demonstrate that full activation of urease within UreABC−UreDFG requires UreE.[176] The metallochaperone functions even in the presence of chelators, indicating that UreE is not simply a reversible carrier of Ni in this process.[176] Upon activation of urease, the accessory proteins dissociate and are likely recycled (Figure 12).

3.6. NiFe–Hydrogenase

Hydrogenases catalyze the reversible oxidation of hydrogen gas (eq 13), a central reaction in microbial energy metabolism.

$$H_2 \rightleftharpoons 2\,H^+ + 2\,e^- \qquad (13)$$

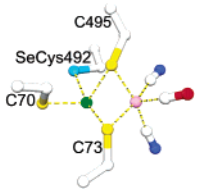


Figure 13. Dinuclear Ni−Fe active site of the reduced, active hydrogenase from *Desulfomicrobium norvegicum* (PDB code 1CC1). Ni is shown in green, Fe in pink, and Se (from a Se−Cys ligand) in light blue. The diatomic species bound to Fe are CO and two cyanide molecules. Alternative redox states contain a bridging ligand between the Fe and Ni. Enzymes from many other species have Se−Cys replaced by Cys.

Three phylogenetically distinct classes of hydrogenases are known: NiFe−hydrogenases, Fe−hydrogenases, and metal-free hydrogenases.[177] The former grouping contains the vast majority of these enzymes, with the name denoting the presence of Ni and Fe at the active site. Crystallographic studies of NiFe−hydrogenases isolated from *Desulfovibrio gigas*,[178,179] *Desulfovibrio vulgaris* Miyazaki,[180,181] *Desulfovibrio fructosovorans*,[182] *Desulfomicrobium norvegicum* (formerly *D. baculatum*),[183] and *Desulfovibrio desulfuricans*[184] reveal the unique structure of the dinuclear Ni−Fe active site illustrated in Figure 13. Four cysteine residues (or three Cys plus a selenocysteine in the case of the *D. norvegicum* enzyme illustrated) coordinate the Ni, with two of these Cys also binding the Fe atom. In addition, the Fe possesses three nonprotein diatomic ligands, identified by distinctive FTIR absorptions and chemical analyses as one carbon monoxide and two cyanide groups for enzymes from *D. gigas* and *Allochromatium vinosum*, formerly *Chromatium vinosum*.[179,185] Alternatively, one research group claims these diatomic molecules are SO, CO, and cyanide for the enzyme in *D. vulgaris*.[180,186] The structure shown represents the reduced, active form of the enzyme, whereas alternative states of these proteins additionally possess oxo or sulfido groups bridging the Ni and Fe atoms.[181,183] Elsewhere in the proteins are multiple Fe−S clusters that form a conduit for electron transfer to the appropriate electron carrier. An intricate pathway involving themes discussed in sections 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, and 2.7 is required for biosynthesis of these enzymes.

This discussion focuses on components and properties of the best-studied hydrogenase maturation system, that responsible for activation of the *E. coli* enzyme encoded by *hycG* and *hycE*. This is one of four two-subunit hydrogenases encoded by *E. coli* and requires an assortment of accessory genes for activation, with some acting pleiotropically and others being specific to a subset of the enzymes. The vast array of gene/protein designations used within this species and in different microorganisms can be confusing, but excellent reviews are available to assist interested readers.[177,187] In the following paragraphs we describe the reasonable working hypothesis illustrated in Figure 14 for NiFe−hydrogenase maturation.

Hydrogenase biosynthesis begins with ribosomal translation of the gene encoding the large subunit

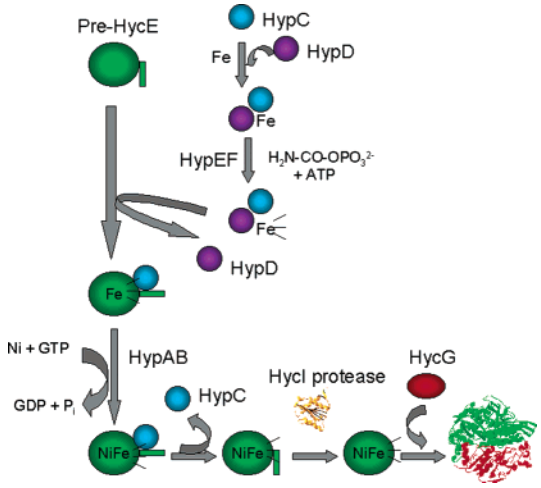


Figure 14. Model for activation of Ni−Fe hydrogenases. The large subunit of the enzyme is initially synthesized as a precursor containing a carboxyl-terminal extension (represented here by pre-HycE, one of four such gene products in *E. coli*). HypC delivers $Fe(CO)(CN)_2$ (the three diatomic molecules are shown by the three lines) and acts as a chaperone protein that forms a complex with pre-HycE and enhances access to the active site. In previous steps, HypC forms a complex with HypD and acquires Fe by an unknown mechanism. HypE and HypF generate the cyanide and CO ligands to HypC-bound Fe using carbamoyl phosphate in reactions that require ATP and reductant, perhaps using electrons shuttled through the Fe−S cluster of HypD. Addition of Ni to pre-HycE:HypC occurs in a GTP-dependent step that requires HypB and HypA. Dissociation of HypC allows action of a specific protease (HycI is used for pre-HycE, but we show the related and structurally characterized *E. coli* HybD, PDB code 1CFZ) that removes the carboxyl terminus. The mature length HycE binds to the small subunit (HycG), which also undergoes processing events including the incorporation of several Fe−S centers, to form the functional enzyme. Because no crystal structure is available for any *E. coli* hydrogenase, the functional enzyme is illustrated by the *D. gigas* hydrogenase (PDB code 1FRV).

to form a precursor protein (pre-HycE) with a ~15 residue extension at the carboxyl terminus. HypC provides the active site $Fe(CO)(CN)_2$ and acts as a molecular chaperone by forming a complex with pre-HycE[188] in a process that requires three structural features: the carboxyl terminal extension of the subunit precursor, one of the hydrogenase cysteines that eventually becomes a Ni ligand, and the cysteine at the amino terminus of the chaperone protein.[189,190] Truncated HycE lacking the carboxyl-terminal extension cannot be activated.[191] Prior to forming the pre-HycE:HypC species, Fe is incorporated into a HypC:HypD complex[192] and HypF/HypE add the diatomic ligands to the Fe.[70,193] Critical insight into this process was obtained by experiments with an *E. coli carAB* mutant, deficient in carbamoyl phosphate (CP) synthesis, that required CP for hydrogenase activation.[68] Purified HypF exhibits several activities: CP hydrolysis to release inorganic phosphate, CP-dependent pyrophosphate exchange into ATP, and (most importantly) carbamoyl transferase activity that transfers the carbamoyl group from carbamoyladenylate to the carboxyl terminus of HypE.[69,193] HypE dehydrates the protein-bound thiocarbamate to yield an enzyme thiocyanate which, after reduction, provides the cyanide ligand.[193] CO ligands may

derive from similar chemistry with an added hydrolysis step. HypD, an Fe−S cluster-containing protein, may serve as the source of reductant in the reactions forming the diatomic ligands.[70] Consistent with the proposed reactions, HypF contains sequence motifs characteristic of *O*-carbamoyl transferases[68] (as well as an acylphosphatase domain that has been crystallized and structurally characterized).[194]

The next step in hydrogenase biosynthesis is the GTP-dependent insertion of Ni into the $Fe(CO)(CN)_2$-containing HypC:pre-HycE complex, an event involving at least two accessory proteins. The nucleotide-binding accessory protein required for this process (HypB) is related in sequence to one of the urease accessory proteins (UreG) and exhibits low levels of GTPase activity.[195−198] In some microorganisms this protein possesses a histidine-rich motif and binds multiple Ni atoms reminiscent of the situation for some UreE proteins involved in urease activation,[197,198] but the *E. coli* and *H. pylori* proteins lack such a motif and do not bind Ni. Significantly, HypA binds stoichiometric levels of Ni and forms a complex with HypB.[199] The combined action of HypA and HypB forms a HypC:pre-HycE species containing the $Ni-Fe(CO)(CN)_2$ center.

The final steps in hydrogenase biosynthesis include HypC dissociation, proteolytic removal of the carboxyl terminus, and binding of the processed HycE to HycG. Dissociation of HypC from the Ni-containing pre-HycE was shown to precede the proteolytic step.[47] HycI cleaves pre-HycE a few residues beyond the last Ni ligand in the sequence. The structure of a related protease (HybD) was elucidated and found to topologically resemble the metzincin protease superfamily.[48] Of particular interest, the protease possesses a metal-binding site that has been suggested to interact with Ni bound to its cognate large subunit, accounting for a key feature of its substrate recognition motif.[46] Only after proteolysis is HycE capable of binding to HycG.[47] The latter protein must also undergo maturation events, including insertion of Fe−S clusters and processing of its amino-terminal signal sequence.

In addition to the accessory proteins noted above, two other gene products have been suggested to participate in activation of some hydrogenases. HupK, first noted in *R. leguminosarum*,[61] is related in sequence to the large subunit of the enzyme but lacks two of the four cysteines that function as Ni ligands. This feature led to the suggestion that HupK could act as a scaffold to support synthesis of the enzyme Ni site,[61] but no supportive evidence has been published, many hydrogenase-containing microorganisms lack a comparable protein, and *R. eutropha hupK* mutants retain 30% of the hydrogenase activity.[200] HypX, first described in *R. leguminosarum*, is related in sequence to tetrahydrofolate-dependent enzymes and enoyl-CoA hydratase/isomerases.[201] A proposal that HypX is involved in delivery of the CO and cyanide ligands of the Fe, presumably as formimino and formyl groups bound to the one-carbon carrier, has not been substantiated. Furthermore, homologues to HypX are absent from many hydrogenase-containing species and *hypX* mutants of *B. japonicum* retain nearly one-half of hydrogenase activity compared to wild-type cells.[202]

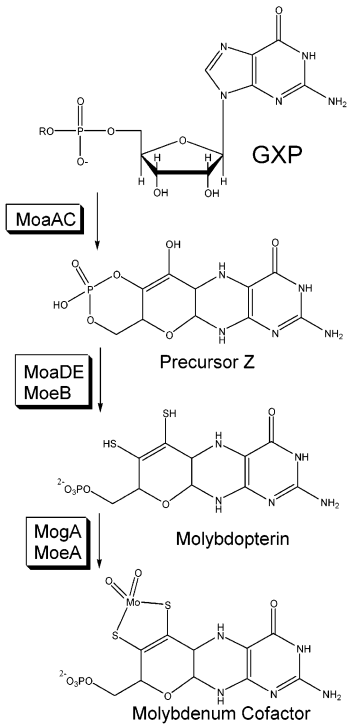


Figure 15. Moco biosynthesis. Starting with GTP or a closely related molecule, MoaA and MoaC insert the guanine C-8 atom into the ribose ring to form precursor Z. Thiol groups are added to precursor Z, generating MPT, in a series of reactions that require MoeB, MoaD, and MoaE. Addition of Mo by MogA and MoeA yields Moco. Conversion of Moco to a dinucleotide form is common, e.g., formation of MGD by MobA (not shown).

3.7. Moco Enzymes

Molybdenum-containing enzymes, with the exception of nitrogenase described elsewhere in this issue,[3] possess a complex with Mo coordinated to one or two molecules of a tricyclic pyranopterin (also known as molybdopterin, MPT) forming the molybdenum cofactor (Moco).[203] Moco biosynthesis has been extensively detailed in *E. coli* by using a combination of biochemical, genetic, and structural approaches, and additional insights are provided by studies in other microorganisms and eukaryotes. As summarized below, activation of Moco enzymes utilizes themes that were described in sections 2.1, 2.2, 2.5, and 2.7.

The overall pathway of Moco synthesis is illustrated in Figure 15. The first step involves the conversion of GTP or a closely related compound (labeled GXP) into precursor Z by MoaA and MoaC. In this remarkable reaction, the C-8 carbon of the guanine base is inserted between the 2′ and 3′ carbons of the ribose ring.[204,205] The detailed mechanism of this reaction is unknown, but the sequence of MoaA indicates similarity to members of the "radical SAM" family of proteins that catalyze radical-based reactions using *S*-adenosylmethionine and Fe−S clusters.[206] The presence of an Fe−S cluster was confirmed in recombinant *Arthrobacter nicotinovorans* protein.[207] The role of MoaC in the reaction is unclear, but its structure has been elucidated.[208]

The second phase of Moco synthesis involves conversion of precursor Z to MPT in reactions catalyzed by MoaD, MoaE, and MoeB.[209,210] Biochemical and structural studies involving complexes formed by MoeB plus MoaD[211] and MoaD plus MoaE[212] reveal critical insights into the roles of the individual proteins. MoeB activates the C-terminus of MoaD by adenylation, a reaction analogous to that utilized for ubiquitin activation.[213] The activated MoaD is modified by a sulfur transferase using cysteine-derived sulfur, resulting in a protein ending in a thiocarboxylate.[214] The sulfur atoms from two MoaD thiocarboxylates are transferred by MoaE onto precursor Z to form the dithiolene group of MPT.

MogA and MoeA carry out the next step in the biosynthetic pathway, the conversion of MPT to Moco. Because high concentrations of Mo can rescue *mogA* mutants, MogA was first thought to be a Mo chelatase.[215] Subsequent studies discount this notion and suggest that MogA acts prior to MoeA, perhaps as an MPT carrier.[216] The MogA structure identifies a likely active site region but does not clarify the role of the protein.[217] Similarly, the function of MoeA is uncertain and an initial suggestion that MoeA converts molybdate to thiomolybdate[218] has not found support during further biochemical studies. Rather, evidence suggests that MoeA facilitates incorporation of Mo into MPT at low concentrations of the metal ion.[216,219] The structure of MoeA identifies four domains including one that resembles MogA, consistent with a role in MPT or Moco binding.[220,221]

In most prokaryotic Mo-containing enzymes, Moco is further modified by conversion to the guanine, cytosine, or hypoxanthine dinucleotide forms. For example, MobA transforms Moco into molybdopterin guanine dinucleotide (MGD).[222] The structure of MobA has been determined[223,224] and the biochemical properties of this reaction unraveled.[225] These enzymes appear to be structurally and functionally similar to previously characterized nucleotide diphospho sugar transferases.

In some systems, the incorporation of Moco, MGD, or other Mo-containing cofactor into a molybdoenzyme requires an additional protein component. This situation was first noted for maturation of nitrate reductase where NarJ was shown to be needed after Moco synthesis was complete.[226] NarJ and related proteins (e.g., TorD involved in activation of trimethylamine *N*-oxide reductase)[227,228] are suggested to play molecular chaperone-type roles in the activation process.

4. Concluding Comments

So what lessons might biomimetic inorganic chemists learn from the metallocenter biosynthesis pathways used by Nature? Cellular synthesis of metal sites makes use of several recurring themes, often with multiple themes combined into a single pathway. In the simplest situation, metal binding to a biological ligand occurs by reversible thermodynamic control. The prevalence of buried metallocenters, the exceedingly low concentrations of free metal ions within cells, and the sophisticated structures of many metal-containing active sites provide evidence that alternative approaches also must exist. In some cases, metallochaperones are used to deliver the metal of interest to an apoprotein. In other cases the target protein undergoes posttranslational modification at the metal-binding site, either prior to or after the metal is bound. Many metallocenters contain additional components that are added along with the metal ion or used to form an independent metal-containing cofactor, perhaps with the assistance of a scaffolding protein. Electron-transfer reactions involving the metal, an added compound, or the protein ligand can participate in activation. Finally, molecular chaperones that bind and alter the conformation of the target apoprotein may be utilized. In many cases, the function of the molecular chaperone is coupled to nucleotide triphosphate hydrolysis. Additional complications of metallocenter biosynthesis exist within cells but were not highlighted here: uptake of metal ions, sensing and controlling of cellular metal levels, and intercompartmental trafficking of metals and metal-containing proteins. Although some of these complications will not apply to biomimetic inorganic synthesis, several approaches used by nature parallel strategies used by inorganic chemists. Thus, both kinetic and thermodynamic control must be considered during the chemical synthesis of a metallocenter. Optimal efficiency in a synthesis may require that the metal be delivered as a particular complex rather than as the free ion. A scaffold may be used to build a substructure of the metallocenter complex prior to transfer to the final ligand. Electron-transfer reactions involving the metal, the ligand, or other components can facilitate the synthesis. Finally, the ligand architecture must be designed to accommodate the correct metal in the proper geometry with appropriate affinity. As demonstrated by other contributions to this issue, synthetic strategies developed by biomimetic chemists often rival the complexity of biosynthetic approaches used by nature.

5. Abbreviations

CCS	copper chaperone for superoxide dismutase
CP	carbamoyl phosphate
FeMoco	iron−molybdenum cofactor
Moco	molybdenum cofactor
MGD	molybdopterin guanine dinucleotide
MPT	molybdopterin
SOD1	superoxide dismutase

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