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Metalloproteins and Metallopeptides –
Natural Metallofoldamers

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1.1 Introduction

By a combination of amino acids with various properties, it is possible to obtain the natural polymers, peptides and proteins, capable of catalyzing key transformations, sustaining energy flow, and maintaining life in a narrow range of allowable conditions and starting materials. The folding, flexibility, and structural changes of peptides and proteins are integral to their functions within living systems. Several factors affect their folding and conformation, including primary sequence, subunit composition, nature and type of subunit interactions, presence of cofactors, and compartment and sequence during folding in a well controlled environment. Emphasis must also be placed on the conformational flexibility of peptides and proteins necessary in the context of their folding and function. In the case of enzymes, such conformational flexibility renders the induced fit process possible during enzymatic catalysis, as found in the large domain movements in hexokinase, HIV protease, and many others upon substrate or inhibitor binding.

Protein conformation, structure, and function are often determined or modulated by metal ions. Therefore, it is instructive to discuss the effect of metal ions or cofactors on the conformational changes of proteins prior to and during their actions. Furthermore, by comparing the folding of metallopeptides and metalloproteins to those whose conformational changes take place in the absence of metal ions can provide further structural and functional information on the metal ions in the former molecules. Such discussion is
important in this context, as many proteins require metal ion(s) for their optimal structure and function.

1.2 Metalloproteins

1.2.1 Metalloproteins are Nature’s “Metallofoldamers!”

The term “foldamer” was defined as “polymers with a strong tendency to adopt a specific compact conformation” (Gellman) or “oligomers that fold into a conformationally ordered state in solution, the structures of which are stabilized by a collection of noncovalent interactions between nonadjacent monomer units” (Moore) [1]. In this respect foldamers share common characteristics with proteins and thus the term is adopted to differentiate synthetic oligomers and polymers from “nature’s foldamers” such as peptides, proteins, and nucleic acids. The term “metallofoldamers” thus is used to describe foldamers that adopt conformationally ordered states in the presence of metal ions or complexes [2]. Therein, the metal center(s) plays a key structural role in the formation of the specific conformation of the corresponding foldamer.

Metal ions may play a key role in the conformational changes of proteins or peptides crucial in their functions. Given the high occurrence of metal ions as cofactors and their integral role in the function of proteins in carrying out catalytic transformations, it is important to review how they are incorporated in the structure of proteins. The mechanism of metal incorporation ranges from a controlled manner through the use of metallochaperones to the direct incorporation from the cellular pool. In the former case this specific class of proteins binds metal ions and mediates the delivery into target enzymes through protein–protein interactions. In the case of iron transport and heme incorporation, transferrin transports iron into cells and hemopexin delivers apo-heme to the same compartment. For cytochrome-c, the heme must be attached before proper folding occurs [3], whereas the assembly of Fe–S clusters and incorporation into proteins and the folding of the F–S proteins require machinery encoded in the iron–sulfur cluster operon [4].

Nevertheless, metal ion incorporation is not always well controlled in such a manner. There are still many metalloproteins without a known chaperone counterpart for metal delivery and folding. Therefore, metal incorporation has also been suggested to be controlled by the choice of compartment in which the metal incorporation takes place. From studies in cyanobacteria, it becomes apparent that many copper and manganese containing proteins fold in different compartments where metal insertion can be controlled in a way that the Irwin–Williams series of stability can be circumvented [5].

A classic example of the structural role of metal ions that affects the function is the zinc finger domains, wherein the metal ions crosslink α–β domains and thus play a central role in the formation of the defined structures. The metal binding as well as the packing of a hydrophobic core drive the folding process. The zinc coordinates to the Cys$_2$His$_2$ motif and drives the folding process, while its removal causes the disruption of the proper folding. These zinc finger domains were first found in the transcription factor TFIIIA, which represents the most common nucleic acid binding motif in transcription factors [6]. Upon binding to DNA, the Zn finger domains undergo further conformational change in order to fit
into the major groove of DNA (Figure 1.1; PDB 1ZAA) [7], representing one typical example of metal- and ligand-mediated conformational change of a natural metallofoldamer.

1.2.2 Metal-Triggered Conformational Change of Proteins

1.2.2.1 Cytochrome c and Heme Binding

The cytochrome c family of electron-transfer proteins has a high α-helix content and a heme cofactor that is posttranslationally modified to covalently attach to the protein by two thioether bonds between the vinyl group of heme and two cysteine residues within the motif Cys-Xaa-Xaa-Cys-His [8]. The folding of cytochrome c has been studied by proton to deuterium exchange equilibrium monitored with NMR [9]. The folding sequences involve the interaction of the two terminal helices (N- and C-, respectively) first, followed by joining of the 60s helix. The heme center locks in the designated pocket and this is followed by final coordination of the Met80 in the sixth position. These three helices containing the axial methionine appear to be the minimal structural requirement in cytochrome c folding (Figure 1.2) [10]. The axial heme ligand is not conserved among all the proteins and can be found to be histidine, asparagine, or it can be absent [11]. The cytochrome c family has also been implicated in the apoptotic mechanism of cell death.

![Figure 1.1](image1.png)

*Figure 1.1*  Crystal structure of zinc finger–DNA complex (of Zif268). The Zn(II) ions (black spheres) in the three “fingers” (cyan) are bound to the protein through two Cys and two His residues and hold the α-helical/β-sheet structural motifs together.

![Figure 1.2](image2.png)

*Figure 1.2*  Comparison of the three-dimensional structures of two different cytochromes c. Left: crystal structure of tuna cytochrome c (PDB ID 3CYT). Right: solution structure of oxidized cytochrome c from *Bacillus pasteurii* determined by NMR. The three helices are in cyan, the extra loop in the tuna enzyme is shown in lavender, the heme is shown in red.
via a reactive oxygen species mechanism, scavenging of hydrogen peroxide, and in the assembly of cytochrome c oxidase [11].

The absence of the heme from cytochrome c causes complete destabilization of the protein due to a decrease of hydrophobic contacts as the heme resides at the hydrophobic core essential for the folding of the protein, an effect similar to guanidinium chloride denaturation [12]. The denaturant action is related to its competition with water molecules in the protein binding, resulting in an unfolding of the protein structure. Simulations of protein folding have shown that correct folding requires full heme contacts at the folding transition state in addition to the hydrophobic interactions as a critical “folding nucleus” [13]. The computational results are in agreement with H–D exchange NMR results, suggesting the initiation of the folding by the terminal helices followed by the 60s helix for the Met80 loop and β-sheets to build onto. The last helix to form is the 40s loop, concluding the significant folding role of the heme for providing a hydrophobic core to stabilize the protein and coordinating to the His and Met residues which results in further lowering the entropy en route to native folding. The heme center can also complicate the folding process as other residues or small molecules can compete for heme binding, which causes what is termed “chemical frustration” [14]. Folding of cytochrome c can therefore be modulated by choosing solvent conditions that favor one set of heme ligands over others. No crystal structures of the apo protein could be obtained for this reason [10], indicating the significance of the heme cofactor in forming and maintaining the folding this natural metallofoldamer cytochrome c.

1.2.2.2 α-Lactalbumin and its Ca$^{2+}$ Binding and Molten Globule

Calcium binding can cause significant conformational changes, which in turn may mediate a signaling cascade. The “EF-hand” folding is a major Ca-binding motif composed of a helix-loop-helix sequence as found in the multifunctional messenger calmodulin and S100. The assembly of the Ca binding can then be propagated to a protein partner with which the Ca-binding protein is interacting with. In the S100-type of proteins (which regulate cell cycles, cell growth, and differentiation), Ca binding influences protein folding to aid in their dimerization and further interaction with other partner proteins. Likewise, Ca binds α-lactalbumin at a domain containing a helix-loop-helix bend – close to the EF-hand domain – dubbed a Ca-binding “elbow” [8].

α-Lactalbumin is a main protein component of milk, which has been the target for investigation of calcium binding to proteins besides the EF-hand group of proteins and is used as a model for the study of protein stability. It is the regulatory subunit of lactose synthase for the synthesis of lactose from UDP-galactose and glucose in the lactating mammary gland. The protein possesses a single strong Ca$^{2+}$-binding site, which can also bind Mg$^{2+}$, Mn$^{2+}$, Na$^+$, and K$^+$, and a few distinct Zn$^{2+}$-binding sites. In bovine α-lactalbumin, Ca$^{2+}$ binds to the “elbow” region (Figure 1.3, lavender) via three carboxylates of Asp82, Asp87, and Asp88, two carbonyl groups of Lys79 and Asp84, and two water molecules in a distorted pentagonal bipyramid coordination sphere with the two carbonyl groups at the axial positions. The binding of cations to the Ca$^{2+}$ site increases the stability of α-lactalbumin against heat and various denaturing agents and proteases, while the binding of Zn$^{2+}$ to Ca$^{2+}$-saturated protein decreases the stability and causes aggregation.
There are four disulfide bonds in α-lactalbumin (Figure 1.3, red), but none in the EF-hand proteins, which dramatically stabilize the protein conformation [8]. The correct folding of the protein, which relies on the correct formation of the proper disulfide bonds, is promoted by the high Ca\(^{2+}\)-binding affinity to the protein. Ca\(^{2+}\) binding however does not change the secondary structure based on circular dichroism (CD) and fluorescence studies [15,16] which can effectively afford the molten globular state of the protein in the absence of Ca\(^{2+}\) possessing a native-like secondary structure but a flexible tertiary structure [16]. The molten globular state of the protein can be obtained by removal of calcium, in the presence of denaturants, or in an acid denaturant state [17].

Ca\(^{2+}\) does not have a significant effect on the metal-binding site, but it does affect the cleft at the opposite face (underneath the β-sheets shown in Figure 1.3) of the molecule at the joining of the α-helical (Figure 1.3, light brown) and the β-sheet (Figure 1.3, cyan) lobes by disturbing the H-bonding pattern in this region, which results in a more open conformation in apo α-lactalbumin and demonstrates the significance of Ca\(^{2+}\) in the folding of α-lactalbumin [18].

Bovine α-lactalbumin (BLA) shares 38% sequence homology with hen egg white lysozyme (HEWL) with most of the differences at charged residues in BLA. However, their tertiary structures are nearly superimposable with all the four conserved disulfide bonds (Figure 1.3, yellow ribbon). Electrostatic interactions can stabilize partially unfolded conformations, which can aid in the formation of molten globule state in BLA, but not in HEWL. The thermodynamic folding barriers in the two proteins are different with a marginal barrier possible for BLA due to stabilization of partially unfolded conformations in the presence of Ca\(^{2+}\), which stabilizes the fully folded state of the protein [19]. Such electrostatic interactions can be potentially engineered in artificial systems as a means to aid in structure stabilization. These studies again reveal the significance of metal ions in the proper folding and stabilization of proteins in order to afford functional natural metallofoldamers.

Figure 1.3  Stereo view of crystal structure (PDB 1F6S) of bovine holo α-lactalbumin, showing the “Ca\(^{2+}\) elbow” in lavender. The structure of hen egg white lysozyme (3LZT; yellow) is superimposed onto the α-lactalbumin structure, showing the significant similarity in folding.
1.2.2.3 Metallothionein and Heavy Metal Regulation

The concentration of zinc must be well controlled in the cell, as high concentrations can be toxic and cause mitochondrial dysfunction. Metallothionein plays a central role in zinc homeostasis since it is the major protein important in regulation of the zinc level in the cell and its translocation [20] and it has been shown to induce an effect on brain neurons by binding to neuronal receptors and initiating pathways which cause neurite survival [21,22]. Metallothionein regulates the flow of zinc and copper in the cell and can further prevent poisoning from exposure to toxic cadmium and mercury. Its overall role is suggested to be the control of the distribution of zinc as a function of the cellular energy state and it has been implicated in the following functions [23,24]: (a) intracellular zinc transport, (b) zinc binding and exchange (e.g., with the zinc cluster protein Gal4, zinc finger transcription factors such as TFIIIA, and aconitase) as well as a zinc-specific chaperone, (c) oxidoreductive properties of cysteine bound zinc as cysteine ligands are redox-sensitive regulatory switches [25], (d) controlling cellular zinc distribution as a function of the energy state of the cell as shown by the interaction with ATP, GSH, and ROS and zinc distribution to enzymes in metabolic networks of gene expression and respiration [26], and (e) a possible role in neural activity, storing and distributing zinc for the neuronal network and protecting it against cellular damage as well as neuronal recovery through binding to neuronal receptors initiating signal transduction pathways [22].

One function of metallothionein may resemble that of the iron-storage protein ferritin in terms of its zinc-storage capability. It is a small protein rich in cysteine (20 cysteines in a total of 62 amino acids in human metallothioneine), but without aromatic amino acids such as tyrosine or histidine. The apo protein can bind a total of seven equivalents of divalent metal ions with $d^{10}$ configuration such as Zn$^{2+}$ or Cd$^{2+}$ in two noninteracting domains (Figure 1.4) [27] and up to six Cu$^{+}$ ions in each domain [28]. The protein can bind up to seven Zn$^{2+}$ or Cd$^{2+}$ ions in tetrahedral coordination spheres, or 12 three-coordinate Cu$^{+}$ ions with only cysteine residues. The binding of metal converts the random coil conformation of apo metallothionein into a folded two-domain structure (Figure 1.4). Cd$^{2+}$ binding has been extensively studied with C-13 NMR [29], wherein structural flexibility was observed. The absence of hydrophobic residues for stabilizing a folded form is compensated by the presence of the metal–thiolate core in the folding of this protein.

Apo metallothionein retains the backbone conformation imposed by the formation of the metal–thiolate clusters. REF computational studies indicate a potential H-bonding

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**Figure 1.4** Stereo view of the structure (PDB 4MT2) of Cd$_5$Zn$_2$, showing the N- (cyan) and C- (pink) domains with the coordinated Cys residue.
network present in apo metallothionein which plays a role in the formation of a constant but flexible backbone needed to adjust to the incoming metal to ensure specific metal incorporation [30]. The metal–thiolate clusters then direct the “wrapping” of the protein into its three-dimensional structure. There are two distinct metal binding sites in the C-terminal (a-domain) and N-terminal domains (b-domain). The C-domain contains four Zn$^{2+}$ ions bound to 11 Cys residues with five serving as bridging ligands, while the N-domain binds three Zn$^{2+}$ ions via nine Cys residues and the metal binding follows a non-cooperative model up to four equivalents, as shown by Co$^{2+}$ binding [31,32]. Computational studies also support this metal-binding mechanism. MM3/MD calculations of metallothionein structure after sequential removal of metals show that the last two metals bind to independent tetrathiolate sites from terminal thiolate ligands, which further supports independent Co$^{2+}$ binding to isolated sites prior to metal cluster formation and also confirms that the C-terminal site is the first to bind metal ions [24]. Taken together, metallothionein represents an excellent example for describing the role of metal ions in the proper folding of a natural metallofoldamer.

1.2.3 Conformational Change of Metalloproteins Caused by Ligand Binding

1.2.3.1 Calmodulin and Ca$^{2+}$/Ligand Binding

The role of metal ions in stabilizing the folded states of small proteins is well established, as illustrated in zinc finger proteins [33] (Figure 1.1). Reversible binding of metal ions, where both the metal-free disordered form and the metal-bound ordered form are functional, is very widely observed among calcium-binding proteins. The coupling of the N- and C-terminal lobes in the EF-hand Ca$^{2+}$-binding calmodulin is a good example [34]. Since calcium signaling is such an important process in many metabolic systems, it is likely that this kind of reversible order–disorder equilibrium is quite common. The binding of up to four Ca$^{2+}$ ions in the two different globular ends of apo calmodulin [35] causes significant conformational changes to the molecule (Figure 1.5), including straightening of the long interdomain helix (Figure 1.5, red). Calcium binds to the sites with different affinities (i.e., a higher preference for the C-terminal binding site than the N-terminus),

Figure 1.5  Right: structure of Ca$^{2+}$-free calmodulin determined with NMR in solution, showing a kink in the middle of the interdomain helix (PDB ID 1CFD). Left: crystal structure of Ca$^{2+}$-bound calmodulin, showing dramatic conformational change upon Ca$^{2+}$ binding (PDB 1EXR).
resulting in conformational changes and interaction with other proteins and enzymes to perform its regulatory role (Figure 1.5) [36]. Ca$^{2+}$ binding to the C-terminal sites stabilizes the long interdomain helix via a Tyr138–Glu82 interaction, which in turn disrupts two interaction helices by breaking an Asp/Glu2–Lys77 interaction, which is followed by Ca$^{2+}$ binding to the N-terminal sites to form a binding cleft for target proteins [37].

Calmodulin can bind various molecules, including drugs, peptides, and their regulating target proteins. Calmodulin-modulated Ca$^{2+}$ signaling is thus attributed to the different responses of these target molecules to the conformational change of calmodulin upon Ca$^{2+}$ binding. The difference in Ca$^{2+}$ binding and target interactions of the two lobes also enable calmodulin to work out local and global Ca$^{2+}$ sensing and signaling through conformational change [38]. Further, the binding of target molecule to calmodulin can also influence the Ca$^{2+}$ sensitivity of calmodulin [39]. The calmodulin-binding regions in the target proteins are comprised of short helical segments of ~14–26 amino acids with a high occurrence of hydrophobic and basic residues for high affinity and specificity without the need for sequence specificity [40]. Such dramatic conformational change is illustrated in Figure 1.6 for Ca$_4$-calmodulin binding binding to the “IQ” “motif” in the $\alpha_1$ subunit of the L-type voltage-dependent Ca$^{2+}$ channel Cav1.1, which undergoes Ca$^{2+}$- and CaM-dependent channel facilitation and inactivation [41]. The C-terminal conformation of the $\alpha_1$ subunit is critical for channel function and has been proposed to regulate the gating machinery of the channel [42]. The binding causes a significant conformational change in calmodulin, especially the kink at positions 79–81 of the interdomain helix, which results in wrapping around the peptide (Figure 1.6). Taken together, calmodulin represents one of the best examples showing significant metal- and ligand-induced conformational changes.

1.2.3.2 Carboxypeptidase A Catalytic Mechanism

Another example of dramatic conformational changes in a metalloenzyme is well represented in the action of carboxypeptidase A, a pancreatic proteolytic enzyme. It belongs to a family of exopeptidases responsible for catalyzing the hydrolysis of peptide bonds at the C-terminus of peptides and proteins. It plays a regulatory role or complements the action
of other proteolytic enzymes such as trypsin, chymotrypsin, and pepsin to aid in the production of essential amino acids [43]. Carboxypeptidase A is specific to hydrophobic C-terminal amino acid residues (such as phenylalanine, tyrosine, or tryptophan), while the B-type is specific to the charged residues Lys and Arg. Carboxypeptidase A is a monomer of 307 amino acids with a globular shape consisting of both $\alpha$-helices and $\beta$-sheets (Figure 1.7). The active-site zinc ion plays a key role in the catalysis as it participates in the stabilization of the intermediate, the deprotonation of the nucleophilic coordinated water, and the electrostatic interactions critical for recognition of the terminal amino acid in the substrate peptide chain. This enzyme shares a common active-site motif of H$_{69}$xxE$_{72}^{72}$ for zinc binding (with a bidentate carboxylate of E72), along with a second histidine (H196) located far downstream and a water molecule to complete the coordination sphere of the metal (Figure 1.7) [44].

As in the case of hexokinase, this protein also undergoes conformational changes upon substrate binding to the active site “pocket,” which closes upon substrate binding. More specifically, the negatively charged residues can interact with Arg145 residues in the active site while the hydrophobic interactions between the substrate and the hydrophobic pocket can help orient the substrate. Upon binding of potato inhibitor via its C-terminal carboxylate (Figure 1.7) [45], the bidentate Glu72 residue becomes monodentate which is accompanied by a $>10$ Å movement of Tyr248 to form a H-bond with the bound inhibitor along with a $\sim2$ Å movement of the backbone of the region around Tyr248 toward the metal. Once again, conformational flexibility in metalloproteins is illustrated herein with carboxypeptidase A during its catalytic action.

1.2.3.3 Aminopeptidase and Alternative Catalysis

Aminopeptidases are widely distributed hydrolytic enzymes catalyzing various processes, such as peptide digestion and hormone production, some requiring metal ion(s) in the active site for full activity. The nuclearity of the active site of metallopeptidases varies from mononuclear in the case of carboxypeptidase, to dinuclear in aminopeptidases, and trinuclear in phospholipase C. Even among the dinuclear aminopeptidases, such as those isolated from bovine lens (bAP) [46], *Escherichia coli* (eAP) [47], *Aeromonas proteolytica* (aAP) [48], and *Streptomyces griseus* (sAP) [49], there is a variation in the structural and mechanistic roles of the metal ions [50]. For example, the dinuclear aAP shows selective metal binding, but mononuclear catalytic activity with the second metal playing a regulatory role [51]. In contrast, the dinuclear sAP (as well as bAP) exhibits dinuclear...
catalysis, despite its very similar folding and active-site coordination (Asp/His on one metal, Glu/His on the other metal, and a bridging Asp) to those of aAP (Figure 1.8), which indicates that the microenvironment such as the proximal amino acid residues around the active-site coordination sphere must be more significant than the folding of the peptide backbone for proper functions and specific activities.

*Streptomyces* AP is a di-Zn\(^{2+}\)-containing 30-kDa enzyme which consists of a central \(\beta\)-sheet core surrounded by helices with the active site found within the \(\beta\)-sheet region (Figure 1.8), wherein the dinuclear site can selectively bind metal ions such as Co\(^{2+}\) and Mn\(^{2+}\) in the two metal-binding sites of sAP [52]. The various metal derivatives exhibit significant alternative catalysis toward a phosphodiester substrate, despite the fact the latter frequently serves as a transition-state inhibitor, which is not the case for aAP [53]. Moreover, the di-Cu\(^{2+}\) derivative of the enzyme shows significant activity toward catechol oxidation [54], despite its protein folding and active-site coordination environment being completely different from those of catechol oxidase with three His residues bound to each Cu (Figure 1.8). The observations presented herein indicate that protein folding in not the only control for showing specific enzyme catalysis (i.e., sAP vs aAP toward alternative catalysis) and the folding and/or active-site coordination sphere does not need to be restricted to a certain pattern to exhibit a specific catalysis (i.e., di-Cu-sAP vs catechol oxidase).

### 1.2.4 Protein Misfolding: Causes and Implications – Cu, Zn-Superoxide Dismutase

The superoxide dismutase (SOD) family has four distinct groups, that is, Cu-, Zn-, Fe-, Mn-, and Ni-containing, which are responsible for catalyzing the conversion of superoxide anionic radical to \(\text{O}_2\) and \(\text{H}_2\text{O}_2\) to protect the cellular environment from damage by superoxides generated during respiration or through the oxidative activity of immune cells [55]. The Cu,Zn-containing SOD (SOD1) is a dimeric protein, with each monomer consisting of an eight-stranded \(\beta\)-barrel and electrostatic and metal-binding loops (Figure 1.9) [56]. The electrostatic loop features Arg143 for hydrogen bonding to superoxide and Thr137 in conjunction with Arg143 to limit the anions coming into the copper-active site. The catalytic site features a unique bridging His63 residue between the two metal ions at

*Figure 1.8* Left: stereo view of superimposed sAP (lavender; PDB ID 1CP7) and aAP (cyan; PDB 1AMP), with the active-site di-Zn in red sphere and Ca (in sAP) in yellow. Right: stereo view of di-Cu catechol oxidase from sweet potato, showing the very different folding from that of sAP. The two Cu ions are shown in red.
6 Å apart. The coordination site of Cu$^{2+}$ is completed by three more His residues and a water molecule in a square pyramidal geometry, whereas the Zn$^{2+}$ tetrahedral site is comprised of two more His residues and an Asp residue. The catalytic cycle starts with the binding of superoxide to Cu$^{2+}$ by displacing the coordinated water followed by electron transfer to the copper and diffusion of oxygen, which results in a trigonal planar Cu$^{+}$ site. A second electron transferred by another superoxide results in the regeneration of the Cu$^{2+}$ center and the release of peroxide [57].

SOD1 is properly folded through posttranslational modifications which proceed via two distinct pathways, depending on whether or not the copper chaperone CCS is required for the insertion of Cu and the formation of an intramolecular disulfide bond [58]. The formation of disulfide bridges is crucial in the oligomerization of the protein as the reduced metal free protein favors the monomeric state. Proper folding of SOD1 is important since many mutant forms of this protein have been shown to cause amyotrophic lateral sclerosis (ALS), suggested to be due to destabilized or completely unfolded structures and aggregation at room temperature [59]. More specifically, the immature reduced forms of the mutant protein without the formation of disulfide bonds [60] have been implicated in the aggregation process as they can form incorrect intermolecular disulfide crosslinks. The spinal cords of ALS transgenic mice have been found to contain significant amounts of insoluble aggregates composed of such crosslinked multimers, which however are not observed in other tissues such as the brain cortex and liver [60]. Transgenic mice expressing the human mutant G85R SOD1 protein develop paralytic ALS symptoms along with the appearance of SOD1-enriched inclusions in their neural tissues. The crystal structure of this mutant supports that metal-deficient and/or disulfide-reduced SOD1 mutants may contribute to toxicity in SOD1-linked ALS [61].

The unfolding process of SOD1 has been shown to include more than two states, involving other intermediates in the unfolding process. The irreversible inactivation process due to thermal denaturation has distinct features between apo- and holoenzymes, with the rates of inactivation showing a biphasic response as a function of temperature for the apoenzyme but a monophasic function in the case of the apoenzyme [62,66]. The role of the metal ions has been also implicated in the stabilization of the β-barrel structure of the protein fold [63]. Moreover, the unfolded state of the protein is also stabilized by metal ions [64]. The derivative Cu, E-SOD1 without a bound Zn$^{2+}$, has a lower thermal stability, supporting the primary structural role of Zn$^{2+}$ in this protein. In addition, Co(II)
or Hg(II) can replace Zn(II) in order to maintain the thermal stability of the Cu(II)-free apoenzyme [65]. Overall, the apoenzyme is more sensitive to inactivating processes compared to the holoenzyme. Stability is also affected by the oxidation state of the bound copper. DSC measurements of dithionite reduced native SOD1 containing Cu\(^{+}\) and Zn\(^{2+}\) reveal one peak at 96 °C while native SOD1 containing Cu\(^{2+}\) and Zn\(^{2+}\) exhibits two melting transitions at 89 and 96 °C wherein the transition at 89 °C is affected by oxygen in the solution [66]. The effect of metal binding in protein stabilization is not unique to SOD1. For example the Cu-binding in \textit{P. aeruginosa} azurin stabilizes the protein, whereas in beta-2-macroglobulin it causes native-state destabilization [67].

Taken together, the presence and identity of the metal ion bound to SOD1 and the status of the disulfide bonds in SOD1 have significant effects on the folding, stability, and catalytic efficiency of the enzyme. Destabilization and misfolding of this enzyme may result in the formation of aggregations in neural tissues and cause neurodegeneration and ALS. The above sections have briefly described the folding, structure, and function of several natural metallofoldamers—metalloproteins, which serve as a foundation for the further design and investigation of synthetic metallofoldamers.

1.3 Metallopeptides

Analogous to the metalloproteins discussed above which inspired the design and synthesis of metallofoldamers, a number of simple natural products such as oligopeptides and oligoketides and some antibiotics also adopt secondary or specific structures upon binding with metal ion(s) and can serve as templates for functional metallofoldamers. Metal ions play a key role in the actions of synthetic and natural metallopeptides [68,69] and are involved in specific interactions with proteins, membranes, nucleic acids, and other biomolecules. For example, Fe/Co-bleomycin binds DNA, which impairs DNA function and may also result in DNA cleavage; metallobacitracin binds the sugar-carrying undecaisoprenyl pyrophosphate to inhibit cell wall synthesis; and the specific binding of metal ions to ionophores or siderophores results in their transport through the cell membrane either causing disruption of the potential across the membrane or enabling microorganisms to acquire Fe from the environment.

In addition to the \(\alpha\)-helical and \(\beta\)-sheet secondary structures, the \(\beta\)-turn is another important secondary structure in peptides and proteins, in which Pro frequently found at the “break point” [70] and the \(\beta\)-turns [71] to afford an anti-parallel \(\beta\)-sheet structure. In addition to Pro, Gly is also a “structure breaker” and frequently associated with Pro to form a turn [72] as observed at the G12–P13 \(\beta\)-turn in Cu,Zn-superoxide dismutase (Figure 1.10). Combining with His, a metal-binding site can form near the turn in metalloproteins, such as the Pro86 turn in the copper site of plastocyanin (Figure 1.10, blue). Peptides are prototypical molecules which can adopt secondary structures to exhibit broad biological activities by interacting with specific receptors or target proteins, including a large number of G protein-coupled receptors, wherein a general “turn motif” is associated with the binding [73]. Peptides are involved in many physiological regulations and bioactivities, such as the opioid peptides dynorphin, endorphin, and enkephalin, galanin (which may regulate nociception), ghrelin (which may stimulate hunger), \(\text{Cu}^{2+}\)-regulating calcitonin, adrenocorticotropic hormone, and some neurotoxins. Such peptide-associated activities have triggered the design
and synthesis of a β-turn mimetic library targeting the major recognition motifs in protein–protein and peptide–receptor interactions [74].

1.3.1 Antibiotic Metallopeptides

There are many antibiotic peptides of diverse structures isolated from various sources which interact with a variety of biomolecules, resulting in the inhibition of the associated biochemical or biophysical processes which frequently are associated with conformational changes of the peptides and/or the targets. A number of antibiotics need metal ions to function properly, thus dubbed “metalloantibiotics” [75], such as the peptide bacitracin (Bc) from *Bacillus* species [76] and the peptides/ketide bleomycin (Blm) from the culture medium of *Streptomyces verticullus* [77]. Metal binding to these antibiotics results in significant conformational changes, such as the ~180° twisting of the simple antibiotic streptonigrin upon metal binding [78,79]. A few prototypical antibiotic metallopeptides are discussed in this section to show conformational changes of these peptides associated with metal binding and interaction with targets.

1.3.1.1 Metallo-Blm and DNA Binding

Blm is a Cu$^{2+}$-containing glycopeptidyl antibiotic excreted by *Streptomyces verticullus* [80,81] which also exhibits antiviral [82] and anticancer [83] activities and is widely used in chemotherapy. Blm contains a few uncommon amino acids, that is, β-amino-Ala, β-hydroxy-His, and methylvalerate and a peptidyl bithiazole chain for DNA binding (1, with potential metal-binding sites marked in red). It is the most extensively studied metalloantibiotic from various view points [75], including structures, oxidative DNA cleavage, and use as a model system to gain further insights into O$_2$ activation by nonheme Fe enzyme [84]. DNA cleavage by Fe-Blm is carried out by the active O = Fe$^{V/IV}$-Blm species via oxidation at C4’ and C2’–H proton abstraction immediately after the 5’GC and 5’GT sequences [84–87]. Fe$^{2+}$-Blm can also bind and cleave RNA molecules [88], including tRNA and its precursors and rRNA [89] mainly at the junctions between double- and single-stranded regions [90], and also DNA–RNA hybrids [8b,[91]].
Blm can bind various metal ions [92], including Mn$^{2+}$ [93], Fe$^{2+/3+}$ [94], Co$^{2+/3+}$ [95], Ni$^{2+/3+}$ [96,97], Cu$^{+/2+}$ [98,99], Zn$^{2+}$ [98], Cd$^{2+}$ [100], Ga$^{3+}$ [101], and Ru$^{2+}$ [102] as well as the radioactive $^{105}$Rh for radiotherapy [103], changing the conformation of the molecule from a supposedly more extended form (1) to a more compact form (Figure 1.11, green structure). Metallo-Blm has a distorted octahedral geometry with coordinated imidazole, pyrimidine, amines of β-aminoalamine, the amide nitrogen of β-hydroxyhistidine, and possibly the amide group of α-D-mannose, as shown by optical, NMR, EPR, and electron spin-echo envelope modulation (ESEEM) spectroscopic methods, crystallography, and chemical modeling [104–107]. This leaves an open or exchangeable site for O$_2$ or peroxo binding. Similar coordination chemistry was also suggested for Zn$^{2+}$-Blm from 2-D NMR [108], however excluding carbamoyl binding. The binding of Co$^{2+}$-Blm to DNA via the bithiazole rings does not influence the metal coordination, whereas the binding of O$_2$–Co-Blm affects the bound O$_2$ where the unpaired electron resides [109].

Low-spin diamagnetic Co$^{3+}$ complexes of Blm and analogues have structures similar to Zn-Blm based on 2-D NMR spectroscopy, except the axial ligands [110,111]. The structures of (DNA)$_2$-Co$^{3+}$-Blm and (DNA)$_2$-(HOO)Co$^{3+}$-Blm ternary complexes show further structural changes upon DNA binding (Figure 1.11) [110,112]. The metal-binding moiety is located in the minor groove of (DNA)$_2$ with the bithiazole rings intercalated into the DNA double helix, rendering the bound peroxide close to the 4'$\text{H}$ of the scissile ribose ($\leq$3 Å). Conversely, the bithiazole is in the minor groove in Zn$^{2+}$-Blm-DNA [113]. Nevertheless, intercalation of bithiazole may not be necessary for DNA cleavage since DNA cleavage by Fe-Blm is similarly effective compared to its derivative with the bithiazole tethered to a porous glass bead [114]. Fe$^{3+}$-Blm becomes low-spin ($g = 2.41, 2.18, 1.89$) with a bound hydroxide at slightly alkaline conditions [115,116]. O$_2$ binds to Fe$^{2+}$-Blm to afford a superoxide O$_2^-$–Fe$^{3+}$-Blm complex based on its $^{57}$Fe Mössbauer spectrum [117] which can afford an active HOO$^-$–Fe-Blm complex toward DNA cleavage [11b]. The paramagnetic Fe$^{2+}$-Blm ($S = 2$) with distance-dependent fast relaxing
isotropically shifted $^{1}H$ NMR signals has been studied by means of NMR [119,120]. The structural model of Fe$^{2+}$-Blm built by the use of NMR relaxation times as constraints is similar to those of Co$^{3+}$-Blm complexes. Upon binding to the Shble protein from Streptoalloteichus hindustanus, Cu-Blm adopts an extended conformation [121] (Figure 1.11), as opposed to that upon binding to DNA wherein bending of the bithioazole rings occurs in order to intercalate into the base pairs. Taken together, metallo-Blm represents a prototypical natural metallofoldamer which undergoes conformational change upon metal binding and binding with nucleic acid targets.

1.3.1.2 Bc and Cell Wall Biosynthesis

Bc is a metal-dependent peptidyl antibiotic produced by Bacillus subtilis and B. licheniformis, primarily against Gram-positive bacteria via inhibition of cell wall synthesis.
It is widely used as a feed additive for livestock [123] and in “triple antibiotics” (along with polymyxin and neomycin) for human external use [124]. Bc contains four D-amino acids, a thiazoline ring, and a cyclic heptapeptide structure formed via a linkage between the sidechain amine of Lys6 and the C-terminal carboxylate of Asn12 (2). It can inhibit metalloproteases presumably due to its metal-binding capability [125], can inhibit a membrane-bound protein disulfide isomerase (PDI) [126], and may serve as a selective inhibitor of β1 and β7 integrin following a not yet known mechanism [127]. However, it was recently shown to have only relatively minor effect on PDI activity in vitro [128] which requires re-evaluation of Bc as a specific inhibitor of PDI in cellular systems.

Bc requires a divalent metal ion for its activity [129] and can bind several divalent transition metal ions, including Co²⁺, Ni²⁺, Cu²⁺, and Zn²⁺ [130], and it triggers a slight conformational change as discussed below [131]. Co²⁺-Bc binds C₅₅-isoprenyl (undecaisoprenyl or bactoprenyl) pyrophosphate with a formation constant of 1.05 × 10⁶ M⁻¹ [132], which can prevent dephosphorylation of this lipid pyrophosphate to bind UDP sugars for transport of the sugars during cell wall synthesis [133]. NMR study of Zn²⁺-Bc suggested that His-10 and the sulfur of thiazoline are coordinated to the metal [130]. EPR study of Cu²⁺-Bc indicated a tetragonally distorted Cu²⁺ center ($g_x = 2.058, g_y = 2.047, g_z = 2.261$, and $A_{z(Cu)} = 534$ MHz) with coordinated thiazoline ring nitrogen, imidazole of His10, and carboxylates of d-Glu4 and Asp11 [13b]. Extended X-ray absorption fine structure (EXAFS) study of Zn²⁺-Bc in solid form revealed three Ns and one O in the first coordination sphere with a tetrahedral-like geometry [134], suggested them to be thiazoline nitrogen, His10 imidazole, d-Glu4, and possibly the N-terminal amino group. The hyperfine-shifted ¹H NMR spectrum of Co²⁺-Bc revealed the Nₑ of His10, the carboxylate of d-Glu4, and the thiazoline nitrogen as the metal-binding ligands [131]. A structural model of Co²⁺-Bc built with relaxation times as distance constraints revealed a hydrophobic pocket formed by the side chains of Ile5 and D-Phe9 for possible binding with the hydrocarbon chain of the sugar-carrying undecaisoprenyl pyrophosphate (Figure 1.12), whereas the structure of apo-bacitracin from 2-D NMR showed that the side chains of D-Phe9 and Ile8 are close to Leu3 [135]. Investigation of the Co²⁺ complexes of Bc congeners, including the active Bc-B¹ and B₂ and the inactive stereo isomer A₂ and the oxidized form F, revealed that proper metal binding is essential for Bc activity. The crystal structure of a Bc-bound serine protease complex shows an extended structure [136] which would prevent metal binding. The conformational difference among Bc, its protease-bound complex, metallo-Bc, and farnasylpyrophosphate-metallo-Bc complexes reflects conformational flexibility of this peptide framework even for its seemingly rigid cyclic structure.
1.3.1.3 Antibiotic Salivary Peptide

The histatin (Htn) family is comprised of His-rich salivary peptides in higher primates [137–140], showing antibiotic activities against *Streptococcus mutans* [141] and *S. mitis* [142], *Saccharomyces cerevisiae* [143], *Cryptococcus neoformans* [144], and *Porphyromonas gengivalis*, as well as the opportunistic pathogenic yeast *Candida albicans* [145]. Htn5 has the highest concentration among the Htns in human saliva. It is the first 24 amino acids of Htn3 (DSHAK RHHGYKRKFHEKHHS HRGY) and exhibits its highest activity against *C. albicans* [138]. Htn5 does not form pores in the bacterial cell membranes [146] but internalizes by binding to the heat shock protein Ssa1/2 on the cell wall [147], followed by interaction with the K⁺ transporter TRK1 [148] which leads to apoptosis [149]. It may be internalized into mitochondria and interfere with the electron transfer processes to cause cell death [150].

Htn5 contains several potential metal-binding residues from His, Asp, Glu, and Tyr, has been shown to bind divalent metal ions in the order of Cu²⁺ > Ni²⁺ > Zn²⁺ ≫ Cu²⁺ ≈ Fe²⁺ [151], and is suggested to bind three equivalents of Zn²⁺ or Cu²⁺ from a calorimetric study. Cu²⁺ and Ni²⁺ bind Htn5 at the N-terminal DSH with a high affinity analogous to DAH in bovine serum albumin which folds the N-terminus [152] to afford a square planar coordination geometry on the basis of electronic, NMR, and EPR results [153], and potentially also at HEXXH and two His–His sites, as found in many metallopeptides and metalloproteins. However, Co²⁺ seems to bind to Htn5 first with three His residues and with two His residues in the second site [153]. Metal binding of Htn5 plays an important role in its bioactivities by fusing negatively charged vesicles (Zn²⁺ binding [151a]) and exhibiting oxidative nuclease activity (Cu²⁺ binding [154]). Similar to many Cu²⁺ complexes, the complex Cu²⁺–Htn5 also exhibits significant activity toward catechol oxidation [153]. Since Htn5 can effectively bind metal ions, conformation change upon each metal binding can be expected. However, it has not been revealed how all the metal centers are involved in the oxidative catalysis and in the antimicrobial activity of Htn5; and the correlation between structure, antimicrobial activities, and metallo-Htn5 is unknown.

**Figure 1.12** The structures of Bc-metal complex (pink) and a model of the ternary complex (blue) upon the binding of farnasylpyrophosphate (ball and stick) to metallo-Bc. Conformational changes due to formation of the metal complex and ligand binding are clearly seen including Phe9 and possible detachment of coordinated Glu4.
1.3.1.4 Ionophores and Siderophores

Ionophores [155] are small peptides or other kinds of molecules excreted by microorganisms which can selectively bind and transport alkali or alkaline earth metal ions across cell membranes and artificial lipid bilayers, whereas siderophores [156] selectively bind and transport Fe\(^{3+}\) [75]. These molecules can: (a) disturb the ionic balance across membrane, such as nactins, lasalocid, and valinomycin, (b) create pores on membranes, such as gramicidins, and (c) compete for iron in the environment, such as ferrichromes. The potential imbalance across the cell membrane may slow down cell growth or cause cell death. Consequently, the metal ions in metalloionophores serve as “magic bullets” to cause a potential imbalance and engender antibiotic activities. The mechanism of this type of antibiotic activity has been adopted in the design of channel-forming antibacterial agents [157].

Ionophores and siderophores exhibit significant conformational changes upon metal binding [155], from extended conformations to compact folded forms as in the case of nactins (e.g., nonactin, tetractin, and dinactin) and valinomycin [158,159]. The metalloforms then bind specific receptors on the cell surface and result in the transport of metal ions into the cell. Depending on the target metal ions, the structures of the metalloforms may vary dramatically. In the case of enniatin cyclic (L-N-methyl-valine-D-hydroxyisovalerate)\(_3\), the parent ionophore has a structure very similar to its K\(^+\) complex, yet quite distinct from its Rb\(^+\) complex [160]. This family of antibiotics contain an O-rich metal-binding environment, including ether groups, the carbonyl group of esters and amides, and carboxylates preferably for binding with alkali and alkaline earth metal ions in different metal:ligand ratios attributed to the structures, the size of their metal-binding site, the ionic radii of the metal ions, and/or the hydration energy of the cations [75].

The gramicidins family is a peptide ionophore family produced by Bacillus brevis [161], of which gramicidin A is the major component. Its primary structure contains six D-form amino acids, that is, formyl-Val-Gly-Ala-(D-Leu\(^4\))-Ala-(D-Val\(^6\))-Val-(D-Val\(^8\))-Trp-(D-Leu\(^10\))-Trp-(D-Leu\(^12\))-Trp-(D-Leu\(^14\))-Trp-ethanolamine (and Trp\(^{11}\) → Phe in gramicidin B and Trp\(^{11}\) → Tyr in gramicidin C). This family exhibits an antibiotic mechanism different from those cation-binding ionophores described above by inserting into the lipid bilayer as a dimer and folding into a unique β-double-stranded helix [162] to create a pore of \(\sim\)4 A (Figure 1.13) with selectivity in the order H\(^+\) > NH\(_4^+\) > Cs\(^+\) > Rb\(^+\) > K\(^+\) > Na\(^+\) > Li\(^+\) > N(CH\(_3\))\(_4^+\) in 0.1 M salt [163]. However, it does not show permeability to the divalent metal ions Mg\(^{2+}\), Ca\(^{2+}\), Ba\(^{2+}\), and Zn\(^{2+}\), which bind to the entrance of the channel and prevent the transport of monovalent cations [164]. Similar configurations are observed for the peptide backbone in both solution and solid state [165] (Figure 1.13) with hydrophobic amino acid side chains facing outward for better interaction with the lipid bilayer [165–170]. However, the structures in solution determined by NMR spectroscopy exhibit a higher degree of irregularity, such as the shape of the channel. The binding of monovalent metal ions does not seem to cause a significant conformational change in gramicidins. The insertion of gramicidins into membranes does not rely on the binding of metal ions as in the case of the ionophores described above, thus it does not create a further energy barrier for metal binding and transport.

The extremely small solubility product \(K_{sp}\) of \(\sim10^{-38}\) for Fe(OH)\(_3\) under aerobic physiological conditions makes soluble Fe\(^{3+}\) in aqueous solutions very scarce. To overcome
this obstacle, microorganisms excrete Fe\(^{3+}\)-specific siderophores which bind Fe\(^{3+}\) with extraordinarily high affinity constants in the range of \(\sim 10^{30} - 10^{52} \text{ M}^{-1}\) and transport Fe\(^{3+}\) into cells via specific receptors [156, 171]. There are three families of siderophores, differing from each other by their iron-binding sites: hydroximate- (such as ferrichrome from *Penicillium* and the edible *Ustilago*), catechol-containing (e.g., enterobactin from *E. coli*), carboxylate-containing (like the simple citrate), and their combinations (such as aerobactin) [172]. Upon Fe\(^{3+}\) binding, the ferrichromes fold to afford an octahedral metal coordination sphere with a more compact conformation than their metal-free apo-forms (Figure 1.14, top). The complexes are recognized and transported into the cells by species-specific receptors. For example, although ferrichrome A serves as an iron carrier for fungi, it does not in bacteria [173]. The folding is also stereo-specific. While the iron complexes of ferrioxamines B [174], D\(_1\) [175], and E [176] and desferrioxamine E [177] fold into a mixture of \(\Delta\)- and \(\Lambda\)-*cis* isomers, ferrichrome complexes [178] are exclusively \(\Lambda\)-*cis* isomers. A few structures of the transporter protein FhuA (ferric hydroxamate

\[\text{Figure 1.13} \quad \text{Stereo relaxed eye view of the crystal structures of one channel of gramicidin A dimer (top, viewed through the channel; bottom, side view; PDB ID 1AV2). Cs}^+ \text{ ions located in the channel are shown in green spheres, water molecules are shown in small red spheres.}\]
uptake A protein) with and without a bound Fe\(^{3+}\)-siderophore have been determined (Figure 1.14, bottom) [179]. Some Fe\(^{3+}\)-siderophore complexes have quite similar folding and coordination chemistry upon their binding to FhuA. The binding of Fe\(^{3+}\)-ferrichrome (comprised of Gly and ornithine hydroximate; Figure 1.14, top) to FhuA induces a significant conformational change at the N-terminal domain of the receptor. However, the studies could not identify how the conformational change results in the uptake and transport of the complexes through the cell membrane.

### 1.3.2 Metallopeptides in Neurodegenerative Diseases

Protein misfolding, self-assembling, and/or aggregation can be associated with neurodegenerative diseases, such as the Cu,Zn-superoxide dismutase and amyotrophic lateral sclerosis discussed above, the abnormal aggregation and accumulation of \(\alpha\)-synuclein in Parkinson’s disease, aggregation-prone mutant huntingtin in Huntington’s disease, and the aggregation of prion protein in a few types of transmissible spongiform encephalopathies. In this section, the conformation and metal binding of two prototypical metallopeptides/proteins associated with neurodegenerative diseases are discussed: \(\beta\)-amyloid and prion, as well as their fragments.
1.3.2.1 β-Amyloid Peptides

Alzheimer's disease (AD) is a progressive brain-degenerative disease among elderly people and is the most common cause of dementia, which eventually leads to the loss of ability to perform daily routines. An estimate of about 2.4–4.5 million Americans have this disease, showing first symptom after age 60 in most cases [180]. Although the etiology of this disease has not been fully established, evidence for possible causes has been hypothesized and demonstrated. The aggregation of β-amyloid peptide (Aβ) in the brain as plaques and fibrils have been hypothesized to be associated with the pathogenesis of AD [181], which has gained further support from the fact that Aβ plaques are toxic to neurons and some rat models [181–183] and the overexpression of Aβ in familial AD and Down's syndrome results in early onset of the disease [184]. Nevertheless, the role of Aβ coagulation in AD has still been challenged [185]. The linkage between the structure and reactivity of Aβ and AD must be further clarified to provide possible prevention and treatment of this disease.

The Aβ peptides are generated by cleaving the ubiquitous amyloid precursor protein (APP) by α, β, and γ secretases, wherein Aβ(1–40/42) fragments (with sequence DAEFR HDSGY10 EVHHQ KLVFF20 AEDVG SNKGA30 IIGLM VGGVV40 IA) are generated by secretases β and γ while Aβ(1–16) is released by secretases α and β [186]. Aβs have several potential metal-binding residues, including Asp, His, Tyr, and Glu frequently found as ligands in metalloproteins [187]. Growing evidence has pointed out the involvement of metal ions, including Fe2+/3+, Cu2+, and Zn2+, in the conformational changes of Aβ into fibrils and plaques and the role of metallo-Aβ in causing oxidative stress in the brain of AD patients [188]. The variant His13 → Gln revealed the importance of this His for metal binding and the formation of Aβ plaques [188g]. Likewise, mouse Aβ with His13 → Arg is much less apt to form aggregates in the presence of metal ions [188a]. Redox-active Cu- and Fe-Aβ complexes may exhibit neurotoxicity via the generation of reactive oxygen species (ROS), which can cause damage to cell membranes, proteins, nucleic acids, and other biomolecules and lead to cell death [189]. Moreover, the significance of the soluble forms of Aβ in the pathogenesis of AD has also been proposed and verified [190].

The structure and metal binding of Aβ and the chemistry associated with metallo-Aβ have emerged from studies using different physical methods [188,191]. For example: the morphology of Aβ fibrils was revealed by electron microscope; [192] Aβ fibers and the hydrophobic C-terminal fragments of the peptide were shown by X-ray diffraction [193] and solid-state NMR techniques [194] to adopt a β-sheet structure (Figure 1.15a), whereas Aβ in micelles [195] and an intermediate during Aβ fibrillogenesis [196] have α-helical structures (Figure 1.15b); an extended structure of Aβ upon binding to insulin-degrading enzyme was shown by X-ray crystallography (Figure 1.15c) [197]; the structure of the Zn-Aβ complex was determined by NMR to show a coordinated Asp11 residue along with three His residues (two Nδ- and one Nε-coordinated; Figure 1.15d) [198]; the structure of Co2+-Aβ was determined on the basis of hyperfine-shifted His ring protons and molecular mechanics calculations to show three Nε-coordinated His residues as well as the presence of an extended H-bonding framework (Figure 1.15e) [199]; and damage to cellular components such as membranes by metallo-Aβ-mediated ROS was determined on the basis of the reaction products [200]. From these studies, the conformational change of Aβ peptides upon
metal binding and/or the formation of aggregates and fibrils under different conditions can be established, providing a good example of metallopeptides as natural metallofoldamers.

1.3.2.2 Prion Proteins and Fragments

The cellular form of prion protein PrP(C) (209 amino acids for the mammalian one) is a Cu$^{2+}$-binding glycoprotein which is attached to the cell surface via a glycosylphosphatidylinositol anchor [201] and contains Cu$^{2+}$ when isolated from a diseased brain [202]. The C-terminal domain of PrP(C) is mainly α-helical [203, 204], whereas the N-terminal domain is unstructured in the absence of Cu$^{2+}$ (Figure 1.16a, b) [205, 206]. PrP(C) is converted into an infectious form PrP$^{Sc}$ (scrapie isoform) when misfolded and aggregated (Figure 1.16c). This is responsible for transmissible spongiform encephalopathies such as bovine spongiform encephalopathy, ovine scrapie, and human Creutzfeldt–Jakob disease. Misfolding of PrP(C) with three α-helices and a short anti-parallel β-sheet to afford the self-assembled oligomeric β-sheet-rich PrP$^{Sc}$ is essential to the transmissible spongiform encephalopathies. Unlike the disordered protein moieties which are integrated parts of protein structure and function commonly found in signaling and regulatory proteins that can induce/adopt certain conformations for specific interactions with targets [207], the misfolded PrP$^{Sc}$ can “infect” normal PrP(C) and convert it into the disease-causing misfolded form. Recent studies on PrP(C)-knockout cells and on truncated PrP(C) devoid of the Cu$^{2+}$-binding repeats pointed to a possible role of this protein serving as a metal transporter [208]. Moreover, the Cu$^{2+}$ centers can generate reactive oxygen species to cause oxidative stress in the brain [209] and can result in

![Figure 1.15](a) Structure of extended β-sheet Aβ(17–42) from solid state NMR (PDB ID 2BEG). (b) Solution structure of helical Aβ(1–28) in micelles by NMR (1AMB). (c) Crystal structure of human insulin-degrading enzyme with a bound Aβ(1–42) of an extended structure, showing one monomer in cyan (2WK3). (d, e) Stereo views of (d) Zn$^{2+}$-bound Aβ (1ZE9) and (e) Co$^{2+}$-bound Aβ.
oxidative modification of PrP\textsuperscript{C} which is also proposed to link to prion diseases \cite{210}. However, PrP knockout mice are sensitive to Cu\textsuperscript{2+}-induced oxidative stress \cite{211}, which suggests a possible anti-oxidation role of PrP\textsuperscript{C}. Further research will be needed to fully establish the biological function of this structurally two-faced Janus protein.

Cu\textsuperscript{2+} binding to PrP\textsuperscript{C} triggers structural changes of the protein \cite{212} which enhances resistance against proteases \cite{213} and is linked to the prion diseases. The N-terminal domain of PrP\textsuperscript{C} can bind up to six Cu\textsuperscript{2+} ions at physiological pH, with the first two Cu\textsuperscript{2+} ions binding to the amyloidogenic region (residues 90–126) and the rest to the four highly conserved octapeptidyl repeats of PHGGGWGQ in residues 58–91 (Figure 1.16b) \cite{214}. The four Cu\textsuperscript{2+}-octarepeats are not interacting with each other as they are magnetically isolated \cite{214a}. Synthetic octapeptide repeats show higher preference toward Cu\textsuperscript{2+} binding than other metal ions \cite{215}. The crystal structure of the Cu\textsuperscript{2+} complex of the simple peptide HGGGW reveals a square pyramidal coordination sphere with the equatorial sites occupied by the His imidazole, two deprotonated Gly-amides, and a Gly-carbonyl and an axial water H-bonded to the Trp indole \cite{214a}. The similarity of the EPR spectra between this simple complex and the Cu\textsuperscript{2+}-bound octarepeats suggests their similar coordination sphere. Zn\textsuperscript{2+} also binds mammalian PrP with a weaker binding affinity than Cu\textsuperscript{2+} and can affect Cu\textsuperscript{2+} binding modes in PrP \cite{216}. However, there is no structure solved for the entire protein or the N-terminus domain of this protein in either the native or the unfolded

\textbf{Figure 1.16} The NMR structures of recombinant human prion protein (globular domain extending from residues 125–228) 1QM2 (a, left) and the crystal structure of the globular domain of sheep prion protein 1UW3 (a, right). (b) Solution NMR structure of the octapeptide repeats in mammalian prion protein (1OEI). (c) Solid-state NMR structure of amyloid fibrils from the prion-forming domain of the HET-s protein (2RNM). Each monomer is highlighted in a different color for clarity.
form. In addition to the disease-causing mammalian prions, many fungal prions are also known to undergo structural change to form amyloid filaments, which may be involved in normal or diseased states, such as the prion-forming domain at residues 218–289 of the HET-s protein from the filamentous fungus *Podospora anserine* [217] (Figure 1.16c). These fungal prions may be involved in fungal epigenetic processes [218] and also serve as good models systems for a better understanding of the structure and function of mammalian prions.

### 1.3.3 Other Metallopeptides

Peptide chains undergo dramatic conformational changes upon formation of secondary structures, which can be triggered by metal binding or interacting with target molecules, as discussed above. In addition to the natural metallopeptides discussed above, there are a number of synthetic peptides that can also undergo similar conformational changes. Such a structural property is applicable to the design of metallopeptides for further investigation of the structure and function of natural metallopeptides and metalloproteins and as therapeutic agents. A couple examples are given here.

#### 1.3.3.1 N-Terminal Binding Peptides and Ni-SOD

The N-terminal metal-binding site in serum albumin represents a typical coordination in metallopeptides and is comprised of a large number of natural and synthetic peptides, as in the case of histatin discussed above. Moreover, both the C- and N-termini of proteins are the “loose ends” in protein folding and thus can significantly contribute to protein stability when they are “tightened up” [219]. The binding of Cu$^{2+}$ to a deprotonated peptidyl amide results in the formation of a square planar metal center and a change in conformation of the peptide, as in the case of Cu$^{2+}$ binding to the octarepeats in prion discussed above [215]. A square planar geometry is also formed in several Cu$^{2+}$ complexes of Tyr-containing peptides at elevated pH through a proposed binding to the N-terminal amine and a deprotonated Gly-amine and binding to the phenolate of a Tyr side chain by showing a charge transfer transition at $\sim$400 nm, such as the monomeric [Cu$^{II}$L$_{1}$] (L = Phe-Gly-Pro-Tyr) complex and the dimeric [(CuL$_{1}$)$_{2}$] (L = Tyr-Gly-Pro-Phe) complexes at $\sim$pH 8–10 on the basis of the distinct (O$^{−}$)Tyr to Cu$^{2+}$ charge transfer transitions at $\sim$400 nm [220]. Once again, the coordination sphere was proposed based on spectroscopic features since the structures of these complexes were not solved. Cu$^{2+}$ binding to $\alpha$-synuclein may play a role in the fibrillogenesis of Parkinson’s disease [221]. Cu$^{2+}$ binds to the N-terminus Met-Asp of $\alpha$-synuclein and folds this moiety into a square planar geometry with the N-terminal amine, Asp-amido (H$^{−}$), and Asp-carboxylate as the ligands, along with His50 which may or may not be involved [222], and a dissociation constant of 0.10 nM [222,223].

Another kind of N-terminal metal-binding site is found in Ni-containing superoxide dismutase [224] (SOD; see Section 1.2.4 for a summary of this family of enzymes). The Ni center undergoes redox cycle between Ni$^{2+}$ and Ni$^{3+}$ during the catalytic cycle, accompanied by conformational change at the N-terminal Ni coordination sphere. The Ni ion is bound to the N-terminus of the sequence His$^{1}$-Cys$^{2}$-Asp-Leu-Pro-Cys$^{6}$ of the protein through the N-terminal amine of His1, the peptidyl amido-N of Cys2, and the thiolates of Cys2 and Cys6 in the reduced Ni$^{2+}$ state with a square planar geometry, and an
additional axial ligand from His1 residue in the oxidized Ni^{3+} state (Figure 1.17) [224]. Here, the axial His seems to serve as a redox and/or conformational switch of the N-terminal active-site Ni coordination of this enzyme, detaching from the metal to stabilize the Ni^{2+} state via a lowering of the d_{z^2} energy level and binding to the metal to donate further electron density to Ni^{3+}. A large conformational change during the redox cycle may not take place so that electron transfer is not slowed down by the conformational movement. This catalytic cycle is thus a good example for demonstrating the significance of the conformation and coordination of active-site metal in retaining the proper function of “natural metallofoldamers.”

The correlation between conformational change and redox activity of Ni-SOD has been modeled with the simple peptide H₂N-Gly-Cys-OMe, which folds to bind to Ni^{2+} via the N-terminus, deprotonated amide, and Cys side chain S, as well as a couple dipeptide mimics to form complexes with a coordination sphere of N₂S [225]. Various external thiols are introduced to the complexes to complex the square-planar coordination sphere of N₂S₂ as in reduced Ni-SOD. The coordination sphere and reactivity of Ni-SOD is also mimicked with the Ni^{2+} complex of the tripeptide Asn-Cys-Cys with a coordination sphere of N₂S₂ (including a deprotonated amide), as in Ni-SOD, consistent with a diamagnetic Ni^{2+} in square-planar geometry [226]. This complex undergoes chiral structural transformation that is associated with its SOD activity.

1.3.3.2 Metal-Triggered Conformational Change in Peptides

Helical coiled-coil structures can be designed on the basis of the tendency of amino acids to form helix structures [227] and inter-stranded interactions. However, a peptide designed to form a double-stranded parallel coiled-coil structure ended up showing triple-stranded “up up down” \( \alpha \)-helices [228], reflecting the great conformational flexibility of peptides and their high degree of freedom in assembling into higher-order structures. Synthetic peptides can afford a helical conformation upon metal binding to metal-binding sequences such as His-x-x-x-His and Cys-x-x-x-His [229], consistent with an \( \alpha \)-helical \( i-(i+4) \) conformation, or to moieties with unnatural metal-binding ligands [230]. Short peptides can also be triggered to form \( \alpha \)-helices with metal ions by the use of such a method [231], which can be as short as only one helical turn [232]. Despite their similar ligand-binding capabilities, Ni^{2+} and Cu^{2+} ions were found in one case to induce
conformational change differently: Ni$^{2+}$ induced the formation of a helical structure, but not Cu$^{2+}$ [233].

The role of metal ions has further been demonstrated in assisting peptides to adopt a specific conformation and then assemble into a certain tertiary or quaternary structure, such as the helical bundles [234] found in many metalloproteins, including cytochrome c (Figure 1.2). A designed helical bundle-forming metallopeptide demonstrates that hydrophobic interactions are sufficient to induce polypeptide folding, while the introduction of metal-binding sites can further “tighten up” a four-bundle helical structure [235]. Therein, the four-helix bundle is formed as a dimer of helix-turn-helix peptides and two Zn$^{2+}$-binding sites are built in to form a dinuclear center (Figure 1.18) [236]. An overall C$_2$-rotation symmetry of the dimer and the dinuclear metal sites is revealed from the crystal structure. The metal binding sites in this dimeric peptide and a few variants [237] can also accommodate various dinuclear metal centers with an overall folding and assembly analogous to the di-Zn peptide with some variations in the coordination sphere of the metal-binding site [238]. The variant L9G/L13G has a larger opening to the dimetal site than the original peptide, affording substrate accessibility and significant oxidation activities for the di-Fe complex, with $k_{cat}/K_m = 105 \text{ M}^{-1} \text{s}^{-1}$ for the oxidation of 3,5-dimethylcatechol and $23 \text{ M}^{-1} \text{s}^{-1}$ for 4-aminophenol oxidation [23b].

In addition to metal binding to the helical bundles, the prosthetic group metal–porphyrin can also be incorporated into peptides and assist the folding of the peptides and in some cases render catalysis possible. A designed peptide containing two His residues can be folded and assembled into a tetrameric helical bundle upon binding two Fe(III)-porphyrin molecules, with each one coordinated to two peptide chains via two His residues [239]. Moreover, a heterodimeric peptide with a protoporphyrin IV covalently linked to two peptide chains folds mainly to a helical structure. Here the heme-Fe is five-coordinate via binding to one His from one peptide chain, leaving an open site for possible substrate and/or peroxide binding, as in the case of heme-containing peroxidases. This heme–peptide complex indeed

Figure 1.18  A dimeric four-helical bundle peptide with a dinuclear metal center, represented herein by the di-Zn form solved with NMR (PDB 2KIK). The crystal structure of this helical bundle metallopeptide has a similar structure (PDB 1EC5). The peptide folding is shown in a stereo view with the image on the right also showing the molecular surface and Gly9 labeled in red.
shows a significant peroxidase activity toward 2,2'-azino-di(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS; $k_{\text{cat}}/K_m = 4417 \text{ M}^{-1} \text{ s}^{-1}$) and 2-methoxyphenol (870 mM $k_{\text{cat}}/K_m$)$^{-1} \text{ s}^{-1}$) [240].

Metal ions have characteristic coordination geometries, such as the presence of bridging carboxylate and hydroxide/oxide in di-Fe centers, as illustrated above. In the case of Hg$^{2+}$, both linear and trigonal geometries are present in Hg$^{2+}$ complexes and proteins such as the Hg$^{2+}$-detoxification regulatory factor MerR [241]. The peptide Ac-G(LKALEEK)$_2$G-CNH$_2$ forms triple-helical bundles [242] which can be further modified with Cys at position 12 or 16 to afford mutants L12C or L16C that bind Hg$^{2+}$ in a pH-dependent manner [243], that is, a linear geometry as Hg(L12C)$_2$ at low pH and a trigonal planar coordination sphere as Hg(L12C)$_3$ at high pH [244] and as Hg(L16C)$_2$ at low L16C concentrations and Hg(L16C)$_3$ at high peptide concentrations at pH 8.5 [245]. These Cys-containing peptides also bind Cd$^{2+}$ and As$^{3+}$ to fold into triple-helical bundles [244,246]. The crystal structure of the complex As(L9C)$_3$ reveals parallel helical bundles and Cys-coordinated As$^{3+}$ with a tripodal coordination sphere (Figure 1.19a). This As–peptide complex mimics possible As$^{3+}$ binding to the bacterial As-responsive repressor protein ArsR which dissociates from DNA upon As$^{3+}$ binding as the regulatory control in bacterial arsenic resistance [247].

Further metal-binding properties of the peptide were pursued by the use of a double mutant L9C/L19C which binds two Cd$^{2+}$ ions sequentially, the Cys-9 site first followed by the Cys-19 site, to form a triple-helical bundle structure [248]. An analogous sulfur- and His-containing peptide L9PenL23H (Pen = penicillamine) with the sequence Ac-E WEALEKK (Pen)AALESK LQALEKK HEALEHG-NH$_2$ binds both Hg$^{2+}$ (at the L9Pen site) and Zn$^{2+}$ (at the L23H site) to fold into a triple-helical bundle structure (Figure 1.19b) [249]. Therein the Zn$^{2+}$ site is four-coordinate with one coordination site occupied by a water molecule analogous to the catalytic Zn$^{2+}$ site in carbonic anhydrase, rendering hydrolytic/hydration catalysis possible by this mixed-metal metallopeptide. In a different case, a much longer designed peptide of 73 amino acids is folded by Zn$^{2+}$ ($2.5 \times 10^{-8} \text{ M}$) or Co$^{2+}$ ($1.6 \times 10^{-5} \text{ M}$) into a four-helical bundle structure with two metal-binding Cys-x-x-x-His sequences on adjacent helices, wherein a tetrahedral $N_2S_2$

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**Figure 1.19** Stereo views of the crystal structures of (a) As(L9C)$_3$ (PDB 2JGO) and (b) Zn,Hg (Leu9Pen/Leu23His)$_3$. The metal-binding sites are shown as spheres (As, red; Hg, gray; Zn, green).
metal-binding coordination is formed that mimics the Zn$^{2+}$ finger motif [250]. A shorter Zn-binding peptide of 27 amino acids was suspected to form a helical bundle structure in the absence of Zn$^{2+}$, whereas it forms a folded helix-$\beta$-sheet structure triggered by Zn$^{2+}$ binding [251] which represents a typical example of metal switching in peptide folding.

A number of peptides are synthesized with built-in metal-binding motif(s) which include nonamino acid ligands. An amphiphilic peptide with a 2,2′-bipyridine (bpy) group attached to the N-terminus via a 4-carboxyl group, for example, (4-carboxyl-bpy)-Gly-Glu-Leu-Ala-Gln-Lys-Leu-Glu-Gln-Ala-Leu-Gln-Lys-Leu-Ala-NH$_2$, is expected to have the four Leu side chains facing to the same side once a helical structure is formed [252]. In the presence of Ni$^{2+}$, Co$^{2+}$, or Ru$^{2+}$, the peptide assembles into a 45-residue three-bundled coiled-coil structure that is confirmed by mass spectrometry of the inert Ru$^{2+}$-bound three bundle with $m/z = 5563$ [253]. The CD spectra of the metallopeptide reveal 80% $\alpha$-helicity in 150 mM NaCl solution and the formation of one diastereomer with a $\Lambda$-isomeric tris-bipyridyl-M$^{2+}$ center and a left-handed coiled-coil structure. The introduction of an additional sequence of Ala-Ala-His-Tyr to the C-terminus of the above peptide affords another three-bundled assembly in the presence of Ru$^{2+}$ with an additional tri-His binding site from the three bundles for Cu$^{2+}$ binding [254]. The use of a N-terminal monodentate metal-binding site in a peptide affords nicotinyl-$\gamma$-aminobutylic-Gly-Leu-Ala-Gln-Lys-Leu-Leu-Glu-Ala-Leu-Gln-Lys-Ala-Leu-Ala which binds Ru$^{2+}$ in a 4 : 1 ratio to assemble into an inert four-bundled coiled-coil structure, demonstrated by means of atomic absorption spectroscopy and electrospray mass spectrometry [255]. A few 3,3′-peptidyl derivatives of 2,2′-bipyridine show an extended configuration which fold to render $\beta$-sheet structures upon Cu$^{2+}$ binding based on CD spectra [256], representing another example of metal-triggered peptide folding.

1.4 Conclusion and Perspectives

The biological activities of proteins and peptides rely on the proper folding of their peptide chains. In some diseased stages, misfolded natural peptide chains can form organized tertiary and higher-order structures which may be further triggered by metal binding, as found in amyotrophic lateral sclerosis due to Cu,Zn-superoxide dismutase, Alzheimer’s disease due to $\beta$-amyloid, and prion diseases due to the prion proteins discussed above, reflecting the great conformational flexibility of peptide chains. Metal ions can also mediate the assembly of synthetic/designated peptides to form nanoscale spheres and fibrils [257] and microflorettes [258]. In some other instances, the binding of different metal ions to a peptide chain may afford conformational changes to a certain extent and afford different bioactivities, as in the case of the various metal-dependent activities of the dinuclear aminopeptidase from Streptomyces discussed above. The Ni$^{2+}$- and Fe$^{2+}$-substituted forms of acireductone dioxygenase serve as another example of metalloprotein foldamers with different enzymatic reactions [259]. Future structural studies about substrate binding modes in the ES complexes and the transition state are expected to provide further insight into the mechanisms for the different catalyses by the different metal forms of each individual protein. Proteins and peptides have chiral-specific properties as their amino acids building blocks are chiral and only the $\text{l}$-form is incorporated into living systems. Consequently, a totally synthesized Desulfiwibrio iron–sulfur protein rubredoxin
with only d-form amino acids binds Fe$^{3+}$ and folds as the mirror image of the l-form protein and exhibits the opposite CD spectrum [260]. Moreover, a dramatic conformational change of unfolded rubredoxin in 5 M urea upon metal binding was observed, wherein the addition of a 100-fold molar excess of Fe$^{2+}$ refolded the protein to >90% recovery with a $t_{1/2}$ of <10 ms [261]. In addition to the structures of peptides and proteins which can be dramatically affected by metal ions to afford various foldameric conformations, the structures of nucleic acids are also known to be affected by the binding of a metal center, such as the dramatic conformational change of duplex DNA upon binding with cisplatin (cis-diamminodichloroplatinum), a cancer chemotherapeutic agent. Here the metal center binds a DNA duplex at two N$_7$ of adjacent guanidine bases or guanidine–adenine bases in the major groove or two proximal guanidine bases of different strands in the minor groove, bending the duplex structure by 40–60$^\circ$ along the helix and twisting the helix by 25–32$^\circ$ [262]. Nucleic acids can also be designed to contain metal-binding bases to form a metallofoldermers, analogous to the case of designed peptides with a metal-binding bipyridyl group (Section 1.3.3.2), such as the dramatic conformational change from a monomeric hairpin structure to a dimeric double-stranded structure for the sequence 5'-TTAATTT-Im-Im-Im-AAATTAA upon Ag$^+$ binding (Figure 1.20) [263]. More detailed discussions about “metallo-DNA” and “metallo-PNA” can be found in this volume (Chapters 7 and 8). These studies and the several examples described in the above sections demonstrate the significant flexibility of the backbones of peptides/proteins and nucleic acids which can adopt various foldameric conformations under different conditions and/or upon metal binding. Future exploration along this direction can be expected to produce novel metalloproteins, metallopeptides, and metallo-nucleic acids with unique structures and chemical, physical, and/or biological properties for various applications.

**Figure 1.20** Left: the hairpin structure of a designed nucleic acid with three imidazole-containing (Im; blue) units as shown on top. Right: the double-stranded helical structure of this nucleic acid upon Ag$^+$ (pink sphere) binding to bridge the Im pair.
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