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Metal-Directed Protein Self-Assembly

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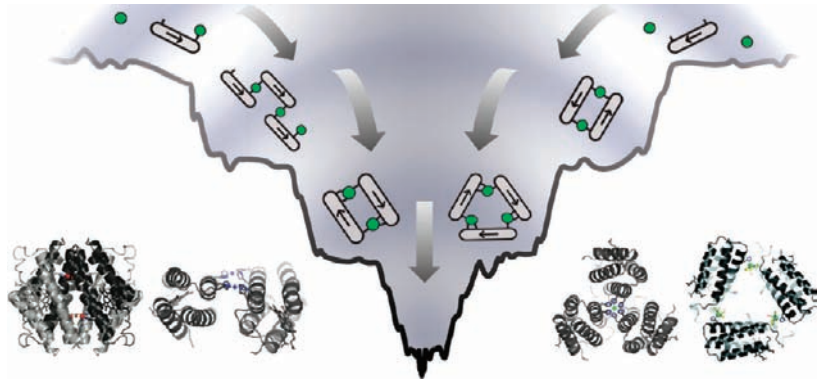

Proteins are nature's premier building blocks for constructing sophisticated nanoscale architectures that carry out complex tasks and chemical transformations. Some 70%−80% of all proteins are thought to be permanently oligomeric; that is, they are composed of multiple proteins that are held together in precise spatial organization through noncovalent interactions. Although it is of great fundamental interest to understand the physicochemical basis of protein self-assembly, the mastery of protein−protein interactions (PPIs) would also allow access to novel biomaterials with nature's favorite and most versatile building block. In this Account, we describe a new approach we have developed with this possibility in mind, metal-directed protein self-assembly (MDPSA), which utilizes the strength, directionality, and selectivity of metal−ligand interactions to control PPIs.

At its core, MDPSA is inspired by supramolecular coordination chemistry, which exploits metal coordination for the self-assembly of small molecules into discrete, more-or-less predictable higher order structures. Proteins, however, are not exactly small molecules or simple metal ligands: they feature extensive, heterogeneous surfaces that can interact with each other and with metal ions in unpredictable ways. We begin by first describing the challenges of using entire proteins as molecular building blocks. We follow with an examination of our work on a model protein (cytochrome cb_{562}), highlighting challenges toward establishing ground rules for MDPSA as well as progress in overcoming these challenges.

Proteins are also nature's metal ligands of choice. In MDPSA, once metal ions guide proteins into forming large assemblies, they are by definition embedded within extensive interfaces formed between protein surfaces. These complex surfaces make an inorganic chemist's life somewhat difficult, yet they also provide a wide platform to modulate the metal coordination environment through distant, noncovalent interactions, exactly as natural metalloproteins and enzymes do. We describe our computational and experimental efforts toward restructuring the noncovalent interaction network formed between proteins surrounding the interfacial metal centers. This approach, of metal templating followed by the redesign of protein interfaces (metal-templated interface redesign, MeTIR), not only provides a route to engineer de novo PPIs and novel metal coordination environments but also suggests possible parallels with the evolution of metalloproteins.

Introduction

Proteins are the most versatile class of biological molecules thanks to the wide array of chemical functionalities provided by their 20 amino acid constituents. These functionalities enable the construction of sophisticated 3D structures, aid in catalytic reactions, and importantly for bioinorganic chemists form an astonishing variety of coordination complexes.

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What makes proteins even more versatile is their ability to specifically recognize and bind other protein molecules. In fact, only a small fraction of proteins fulfill their physiological functions alone.

It is safe to say that protein−protein interactions (PPIs) are the chief contributors to cellular complexity. Transient PPIs are responsible for cellular dynamics, communication, and transfer of chemical currency (e.g., electrons, phosphate groups), whereas permanent PPIs are central to the construction of cellular machinery. These key roles of PPIs have motivated intense efforts to elucidate their physicochemical basis,[1] develop pharmaceutical agents that impede their formation,[2] and engineer them de novo.[3] Invariably, all these efforts are complicated by the fact that PPIs are composed of weak, noncovalent bonds distributed over large and sometimes flexible protein surfaces.[2]

To circumvent the challenge of ruling over numerous weak bonds, we envisioned that if protein surfaces are appropriately modified, PPIs could be controlled through metal coordination. Using metal coordination to guide PPIs offers several advantages. First, metal−ligand bonds are stronger than the noncovalent bonds that make up protein interfaces, obviating the need to engineer large surfaces to produce favorable protein−protein docking. Second, metal−ligand bonds are highly directional, and thus, the stereochemical preference and symmetry of metal coordination may be imposed onto PPIs. Third, metal−ligand bonds can be kinetically labile, allowing PPIs to proceed under thermodynamic control. Fourth, metal coordination can be formed or broken through pH changes or external ligands, rendering PPIs responsive to external stimuli. And fifth, metal ions bring along intrinsic reactivity (Lewis acidity, redox reactivity), which may be incorporated into protein interfaces.

All these advantages of metal coordination have been cleverly exploited in molecular self-assembly.[4,5] Under the umbrella of supramolecular coordination chemistry and the burgeoning field of metal−organic frameworks (MOFs), many groups have utilized metal coordination to organize small organic ligands into well-defined assemblies and multidimensional arrays that are finding applications in host−guest chemistry,[6] sensing,[7] storage/separation,[8] and catalysis.[9] So, we asked whether the same could be done using individual proteins, nature favorite and most versatile ligands, as building blocks.

Using metal coordination in biological self-assembly is of course nothing new; nature does it all the time. A major fraction of known proteins contain metal ions as integral components required for proper folding,[10] and more and more natural protein complexes with bridging metal ions are being discovered.[11,12] Such biological systems have inspired the design of a diverse array of artificial metalloproteins.[13] Impressively, some of such engineered metalloproteins manifest properties characteristic of highly evolved natural systems, such as selectivity,[14] redox tunability,[15] and open coordination sites that allow for reactivity.[16] Inspired by such advances in both protein design and supramolecular coordination chemistry, our ultimate goal is to meld the principles and strengths of these two approaches (1) to build novel biological assemblies with predictable shapes and dimensions, and (2) to construct functional and tunable metal coordination sites within these assemblies.

Metal-Directed Protein Self-Assembly

Challenges of MDPSA and Cytochrome cb_{562} as a Model Building Block. There are significant challenges in considering proteins as building blocks for metal-directed self-assembly. These challenges become immediately apparent upon comparing ligands typically used in supramolecular coordination chemistry with even a small protein such as cytochrome cb_{562} (cyt cb_{562}) employed in our studies (Figure 1). The first hurdle is the selective localization of metal coordination. Whereas with a small organic ligand the metal binding mode is essentially predetermined, surfaces of water-soluble proteins are inundated with metal-coordinating side chains. This can lead to the simultaneous binding of multiple metal ions on the protein surface and to the formation of heterogeneous mixtures of metal-cross-linked protein polymers. Not surprisingly, most soluble proteins precipitate in the presence of high concentrations of transition metals.

The second hurdle stems from the large sizes of proteins. In most supramolecular coordination complexes and MOFs, the interactions between the ligands forming the backbone are so small as to be negligible. Only recently have there been examples where the effects of ligand−ligand interactions have been recognized and utilized in metal-directed self-assembly.[17] In contrast, proteins are considerably larger molecules whose surfaces are bound to come into extensive contact upon metal cross-linking. To compound the problem, the topography and composition of protein surfaces are highly irregular, making the outcome of protein self-assembly even harder to predict.

In order to circumvent some of these challenges, we decided to simplify our task a bit by choosing cyt cb_{562} as a model protein building block. This choice was made for several reasons. Cyt cb_{562} is a variant of the four-helix bundle hemeprotein cyt b_{562} that contains genetically engineered linkages between the heme and the protein backbone.[18] These

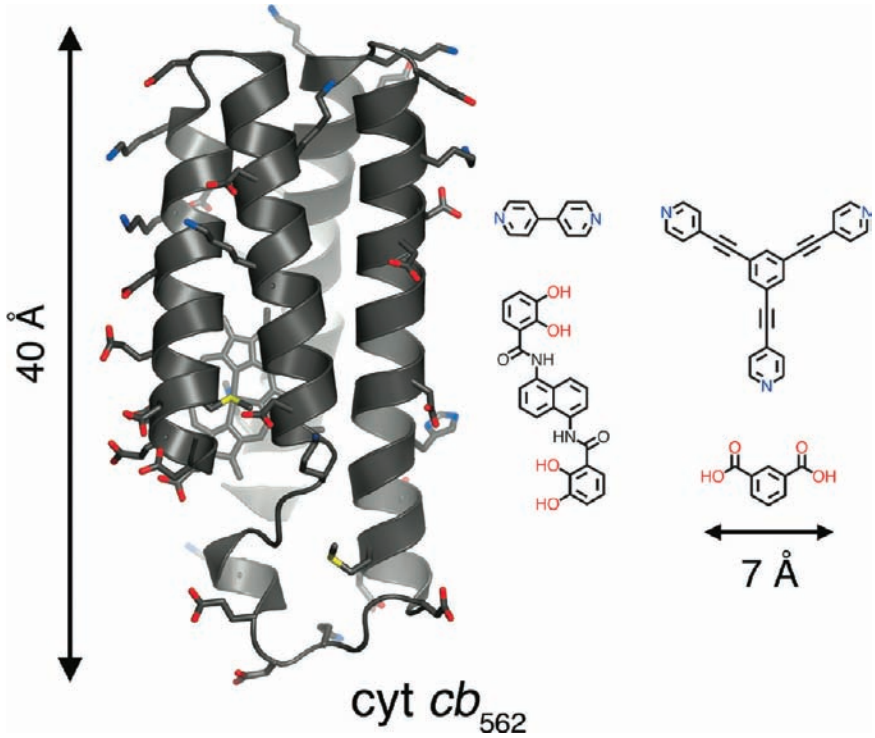

FIGURE 1. Cyt cb_{562} and representative organic building blocks used for constructing supramolecular coordination complexes and MOFs. Surface residues with coordinating ability are shown as sticks.

covalent bonds render cyt cb_{562} significantly more stable toward unfolding and thereby more resistant to structural perturbations as its surface is modified for metal binding. Much like an organic building block, cyt cb_{562} is readily synthesized, by bacteria, that is, and isolated in large quantities. It has a rigid, cylindrical shape, which not only is ideal as a building block for large assemblies but also makes such assemblies, in our experience, more amenable to crystallization and structural analysis. It has a uniform, all-α-helical topology, which, as discussed below, provides a nice handle for engineering metal coordination sites onto all facets of its surface. Finally, cyt cb_{562} is physiollogically monomeric and remains that way even at millimolar concentrations, meaning that its surface does not carry any bias toward oligomerization. This is crucial if metal coordination is intended to be the primary driving force for self-assembly.

Where to Start With MDPSA? Even when proteins possess a simple shape and a uniform topology like cyt cb_{562}, it can be challenging to visualize protein−protein docking geometries that would be amenable to metal cross-linking; surface composition and topography of proteins are still highly heterogeneous. For inspiration, we turned to crystal packing interactions (CPIs), which provide a source of feasible protein−protein docking geometries. A given CPI typically is not extensive (<1000 Å^2) and only stable in combination with other CPIs under crystallization conditions, yet it provides a metastable arrangement of proteins in which there are no steric clashes between them.[19] Importantly, CPIs may contain twofold or higher symmetry, which minimizes the number of metal coordination motifs that need to be engineered onto protein surfaces for MDPSA and provides a starting point to design high-order protein oligomers and superstructures.

An examination of CPIs in cyt cb_{562} crystals reveals that each monomer is paired in an antiparallel fashion with another monomer along their third helices (Figure 2a).[18] This relatively large (775 Å^2), C_2 symmetrical interface is clearly nonphysiological, since cyt cb_{562} is monomeric in solution at concentrations used for crystallization (2−5 mM). At the same time, this close-packed arrangement of monomers shows us

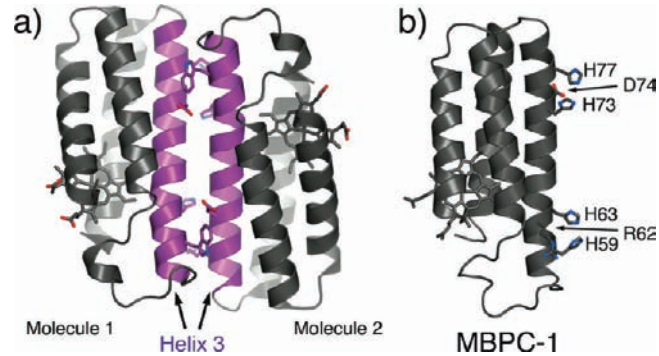

FIGURE 2. (a) Antiparallel arrangement of cyt cb_{562} molecules in the crystal lattice along their Helix3's (magenta). (b) Model of MBPC-1, where key residues involved in metal binding and secondary interactions are depicted as sticks.

a route for the self-assembly of cyt cb_{562}, whereby two metal binding motifs can be incorporated near each end of Helix3 for metal-mediated cross-linking.

To address the challenge of selective metal localization, we exploit the simple principle of the chelate effect. All metal-coordinating groups on a protein's surface are either formally or effectively monodentate and are considered as weak ligands at neutral pH except His and Cys. Therefore, if a bi- or tridentate metal chelating motif can be installed on a protein surface, it should theoretically outcompete other functionalities for metal binding while leaving coordination sites free to accommodate other protein monomers. The *i*, *i*+4 bis-His motif on α-helices, in particular, is a high-affinity bidentate motif frequently utilized for the assembly of natural and engineered metalloproteins.[20] We thus constructed a model system (MBPC-1), which is a cyt cb_{562} variant containing two such bis-His motifs (His59/His63 and His73/His77) near the ends of Helix3 (Figure 2b).[21] We describe below our initial studies with MBPC-1, and its derivatives, that laid the foundation for MDPSA, revealed the challenges as well as unforeseen research avenues involved in this approach, and provided a springboard for future studies.

Zn(II)-Directed Self-Assembly of MBPC-1. A tenet of self-assembly is reversibility, which allows molecular components to avoid forming kinetically trapped complexes on their way to the thermodynamically favored product(s). With this in mind, we first examined the self-assembly properties of MBPC-1 in the presence of Ni(II), Cu(II), and Zn(II), which are all exchange labile and form stable complexes with the *i*, *i*+4 bis-His motif.[22] Importantly, each of these metal ions has a distinct stereochemical preference, providing an opportunity to investigate the effect of metal coordination geometry on supramolecular assembly.

Our first revealing observations come from experiments with Zn. Upon addition of an excess of Zn, MBPC-1 promptly precipitates out of solution. The precipitation is reversible upon addition of chelators such as EDTA or acidifying the solution (pH ≤ 5), suggesting that His−Zn interactions are partaking in the formation of heterogeneous aggregates. It is likely that, in the presence of excess Zn, both bis-His clamps as well as other sites on the protein surface are charged with metal, resulting in a mixture of insoluble metal-cross-linked protein polymers.

To probe the formation of any discrete supramolecular assemblies at equimolar or lower Zn/MBPC-1 ratios, we studied the hydrodynamic properties of the resulting solutions via sedimentation velocity (SV). SV measurements show that MBPC-1 is entirely monomeric at low protein and Zn concentrations, but gives way first to a dimeric and then to a larger

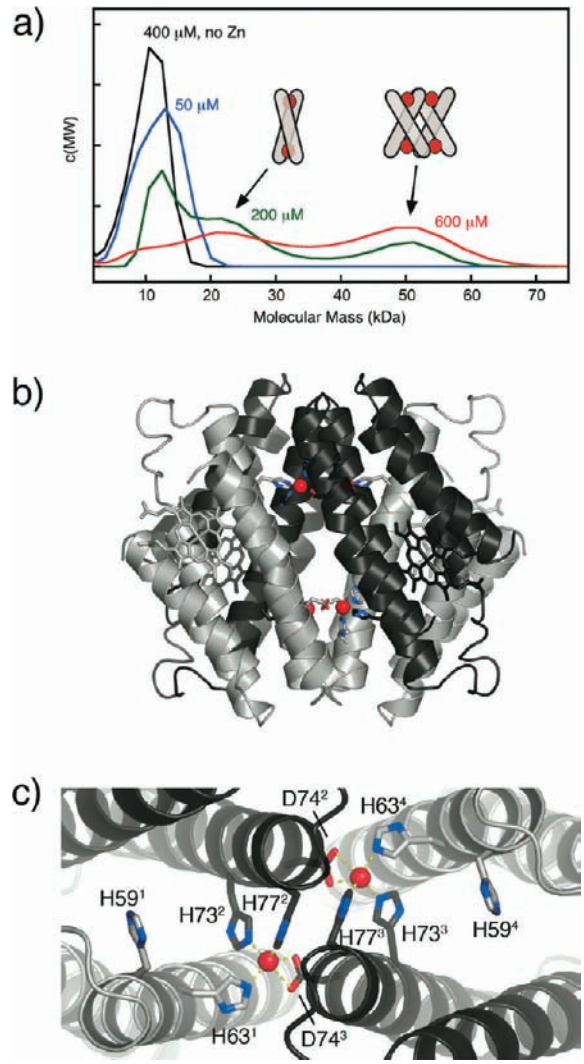

FIGURE 3. (a) Molecular mass distributions for MBPC-1 and equimolar Zn(II) (except where noted) determined by SV measurements, and proposed Zn-induced supramolecular geometries corresponding to dimeric and tetrameric species. (b) Side view of Zn_4:MBPC-1_4. (c) Top view of Zn_4:MBPC-1_4, highlighting the coordination environment of interfacial Zn ions.

species at increasing concentrations (Figure 3a).[21] We were able to structurally characterize the latter species which emerged as a tetrameric assembly (Zn_4:MBPC-1_4) with a unique D_2-symmetrical supramolecular topology stabilized by four Zn(II) ions (Figure 3b).[21] Each of these Zn ions are found in an identical, tetrahedral coordination environment completed by a 73/77 bis-His clamp from one protein monomer, His63 from a second, and unexpectedly Asp74 from a third instead of His59 (Figure 3c). This unforeseen cross-linking of three monomers by each Zn gives rise to the dihedral symmetry of the assembly, which is best described as two V-shaped MBPC-1 pairs wedged into one another.

This structure, along with the SV results, implies the following: (1) Zn-mediated assembly of MBPC-1 indeed proceeds

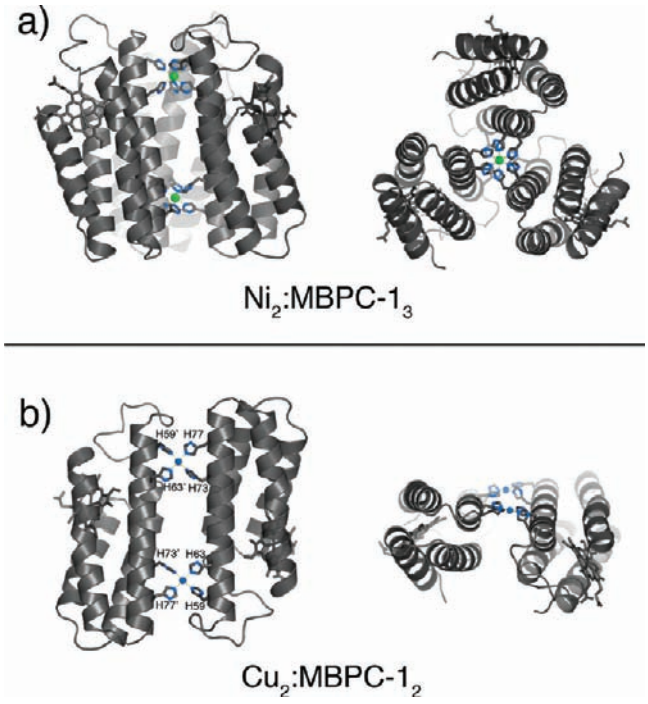

FIGURE 4. Crystal structures of (a) Ni_2:MBPC-1_3 and (b) Cu_2:MBPC-1_2 viewed from the side and the top.

under thermodynamic control as evidenced by the formation of discrete species (monomer, dimer, tetramer) that appear to be exchanging. (2) In the absence of any specific PPIs, protein self-assembly is governed by metal coordination: the proteins adopt a supramolecular arrangement so as to saturate the metal coordination sphere and fulfill the stereochemical preference of the metal ion. Based on this argument, we propose that the dimeric species favored at intermediate Zn and MBPC-1 concentrations features a crisscrossed alignment of MBPC-1 monomers, in which two Zn ions are coordinated by both bis-His clamps in a tetrahedral arrangement (Figure 3a inset). At higher concentrations, the monomers rearrange into the crystallographically characterized Zn_4:MBPC-1_4, whereby the entropic cost of forming this eight-component assembly is overcome by the enthalpic gain due to the coordination of four Zn ions, likely with positive cooperativity.

Inner Sphere Coordination Geometry Governs Oligomerization Symmetry. If the extent and symmetry of MBPC-1 self-assembly is governed by metal coordination as suggested above, then metal ions with *non-tetrahedral* preferences should lead to the formation of alternate supramolecular architectures. Crystal structures of Cu(II) and Ni(II) complexes of MBPC-1 show that this is indeed the case (Figure 4).[23] Cu(II) induces an antiparallel C_2-symmetric dimer (Cu_2:MBPC-1_2), which features two Cu centers with a square planar coordination sphere formed by two equatorial bis-His clamps (59/63 and 73/77). Ni(II) coordination, on the other hand, accommodates three bis-His clamps in an octahedral geometry, yielding a parallel C_3-symmetric trimer (Ni_2:MBPC-1_3) cross-linked by two Ni(II) ions.

The distinct oligomerization geometries obtained with Cu, Ni, and Zn firmly indicate that the supramolecular arrangement of MPBC-1 can be controlled by metal coordination geometry. This finding demonstrates how a basic inorganic principle can be applied to a yet-to-be-solved biochemical problem, namely, the engineering of a protein surface that can assume multiple oligomeric states under the control of a simple external stimulus. Moreover, the facile access to different symmetries through metal coordination without the need to engineer large surfaces may open up the path for constructing multidimensional protein architectures, which require building blocks that simultaneously utilize a combination of these symmetry elements.

Bringing Non-Natural Ligands into the Mix. Following the footsteps of others,[13] we imagined that the structural and functional scope of MDPSA could be significantly broadened if synthetic metal ligands are interfaced with proteins. From a structural viewpoint, a multidentate ligand incorporated into a protein's surface via a single point attachment can offer more structural flexibility than, for instance, a bis-His motif. At the same time, the large number of ligands available in the synthetic toolbox would offer great diversity in terms of tuning metal reactivity.

In a recent proof-of-principle study, we employed the Cys-specific iodoacetamide derivative of phenanthroline (IA-Phen) to construct MBP-Phen1, which contains a single Phen group covalently bound to Cys59 near the N-terminus of Helix3, and His77 incorporated at the opposite end (Figure 5a). The crystal structure of the Ni(II) adduct of MBP-Phen1 reveals a unique triangular assembly, Ni_3:MBP-Phen1_3, whose vertices are formed by a Ni ion coordinated to PhenC59 from one protein monomer and His77 from another (Figure 5b).[24] The flat shape and dimensions of Ni_3:MBP-Phen1_3 are reminiscent of the threefold symmetrical components of biological cages such as the 432-octahedral ferritin shell and the 532-icosahedral viral capsids.

The phenanthroline group, instead of extending into the solvent, is located in a small hydrophobic crevice underneath the 50s loop (Figure 5c). This placement of Phen is the key to the open Ni_3:MBP-Phen1_3 architecture, as it protects the Ni ion from coordination by a second Phen group, resulting in an unsaturated, roughly square-pyramidal Ni coordination geometry (Figure 5d). This demonstrates that protein surface features can be used as steric encumbrance to yield coordinatively unsaturated metal binding sites. IR measure-

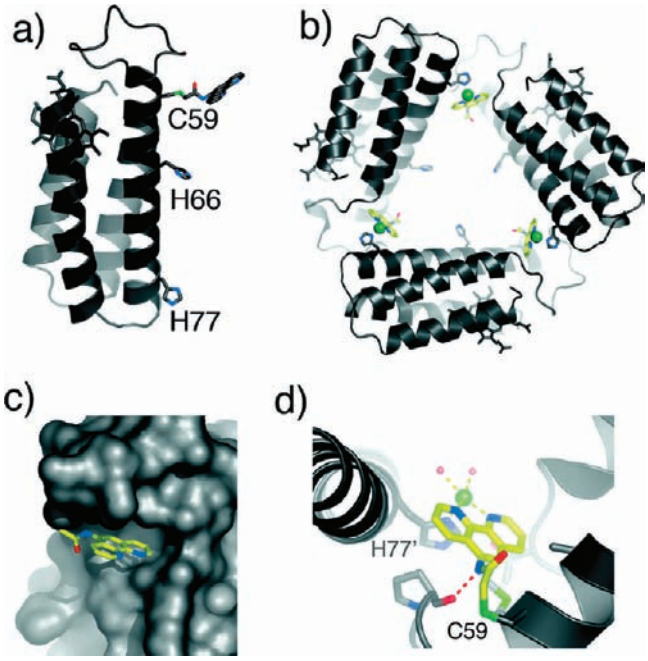

FIGURE 5. (a) Model for MBP-Phen1, highlighting potential metal binding sites on Helix3. (b) Crystal structure of Ni_3:MBP-Phen1$_3$. (c) Surface representation of Ni_3:MBP-Phen1$_3$, showing the burial of the Phen group under the 50s loop. (d) Ni coordination environment in Ni_3:MBP-Phen1$_3$. The H-bond between the P53 carbonyl and the PhenC59 amide nitrogen is indicated with a red dashed line.

ments on Ni_3:MBP-Phen1$_3$ crystals indicate that the interfacial Ni centers can indeed accommodate extrinsic ligands such as cyanate.[24]

The Second Challenge of MDPSA: The Influence of Noncovalent PPIs. With the dominant role of metal coordination established, we next asked if the noncovalent interactions formed between protein monomers had any effect on protein self-assembly. Zn_4:MBPC-1$_4$, in particular, features a very extensive set of close protein−protein contacts (~5000 Å^2). To quickly probe if these contacts have a sizable collective influence on the thermodynamics of self-assembly without introducing extensive surface mutations, we made a small perturbation in the metal coordination instead.

As discussed above, each Zn in Zn_4:MBPC-1$_4$ is coordinated by an Asp74 located within the 73/77 bis-His clamp in the *i*+1 position. Ligation by Asp74's, instead of the originally expected His59's, is key to the supramolecular architecture of Zn_4:MBPC-1$_4$, in that they cross-link the MBPC-1 monomers at the Helix3 C-termini to yield the V-shaped dimers. We reasoned that if noncovalent interactions between protein monomers had negligible effect and metal coordination was the sole determinant of protein self-assembly, then the whole oligomeric assembly could be "inverted" simply by moving the coordinating Asp residue from within the C-terminal 73/77 bis-His clamp into the N-terminal 59/63 bis-His clamp motif at

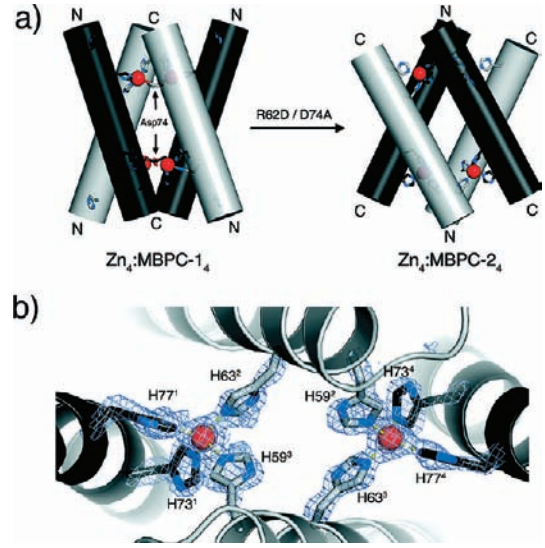

FIGURE 6. (a) Cylindrical representations of Zn_4:MBPC-1$_4$ and Zn_4:MBPC-2$_4$ Helix3's and side chains involved in Zn coordination viewed from the side. N- and C-termini of Helix3's in each assembly are labeled accordingly. (b) Closeup of the interfacial Zn coordination environment.

the *i*+3 position. To this end, we engineered MBPC-2, the D74A/R62D variant of MBPC-1 (Figure 2b), and determined its Zn-induced self-assembly properties.[25]

As planned, MBPC-2 forms tetramers upon binding one equivalent of Zn according to SV measurements, which indicate that the stability of Zn_4:MBPC-2$_4$ is significantly elevated compared to that of Zn_4:MBPC-1$_4$. The crystal structure of Zn_4:MBPC-2$_4$ reveals a D_2-symmetrical architecture, which indeed is the "inverse" of Zn_4:MBPC-1$_4$ (Figure 6a).[25] Whereas the V shapes are joined at the Helix3 C-termini in the latter, they are cross-linked at the N-termini in the former. Unexpectedly, however, the newly engineered Asp62 is not involved in Zn binding. Instead, each Zn ion in the assembly is ligated by the 73/77 bis-His motif from one monomer, His59 from a second, and His63 from a third, again yielding a tetrahedral Zn coordination geometry. In this arrangement, the V's are stabilized by His59 and His63 coordination, instead of the planned Asp62 and His63 coordination, from two monomers, which spread apart to bind two Zn ions, joining the Helix3 N-termini (Figure 6b).

Similarities between Zn_4:MBPC-1$_4$ and Zn_4:MBPC-2$_4$ structures suggest convergence in their mechanisms of self-assembly, that is, tetrahedral Zn coordination enforces D_2 supramolecular symmetry. Yet, there are also some important distinctions between the two. Why does MBPC-2 not oligomerize through the available His_3-Asp_1 Zn-coordination motif? Conversely, why does MBPC-1 not self-assemble through the same His_4 motif as MBPC-2? And why is Zn_4:MBPC-2$_4$ more stable than Zn_4:MBPC-1$_4$ despite the fact that

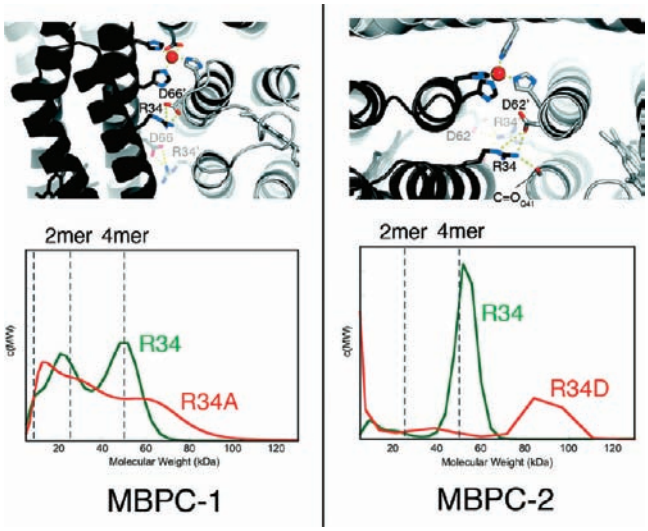

FIGURE 7. (top) Interfacial H-bonding interactions in Zn_4:MBPC-1_4 and Zn_4:MBPC-2_4. (bottom) Molecular weight distributions of MBPC-1 and MBPC-2 species as determined by SV measurements. All samples contain 600 μM protein and 600 μM Zn, with the exception of R34A-MBPC-1, which contains 300 μM Zn.

both assemblies are held together by four Zn ions in unstrained, tetrahedral coordination geometries? The answer clearly lies in the noncovalent interactions found in protein interfaces.

Zn_4:MBPC-1_4 and Zn_4:MBPC-2_4 both possess interfaces that possess only about one-third of the average H-bond density of a natural protein−protein interface[26] and no obvious hydrophobic interactions that would be expected to drive protein oligomerization. The majority of the H-bonding interactions involve two pairs of residues that form salt bridges: Arg34-Asp62 in Zn_4:MBPC-2_4 and Arg34-Asp66 in Zn_4:MBPC-1_4 (Figure 7). Significantly, when these interactions are abolished through the Arg34Asp (MBPC-2) and Arg34Ala (MBPC-1) mutations, the tetrameric assemblies are replaced by heterogeneous ensembles that contain higher order aggregates (Figure 7).[25] Apparently, secondary interactions can have a major influence on the outcome of MDPSA.

Combining Metal Coordination and Noncovalent Interactions: A Terraced Energy Landscape for MDPSA. MDPSA, or protein self-assembly in general, is analogous to protein folding in terms of the physical processes involved: loss of conformational entropy due to the formation of ordered structures with a concomitant decrease in enthalpy due to bond formation. Like protein folding,[27] the energy landscape of MDPSA can also be described in terms of a funnel that qualitatively incorporates both the entropic and enthalpic contributions (Figure 8). One important distinction between protein folding and MDPSA is that the latter is dominated by the enthalpic term due to the formation of strong

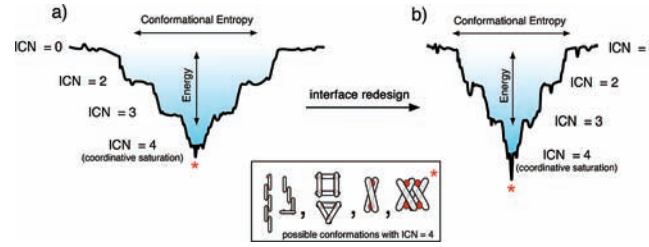

FIGURE 8. Terraced energy landscapes for MDPSA in the absence (a) or presence (b) of specific protein−protein interactions. The funnels shown apply to the Zn-driven oligomerization of MBPC-1 (a) and RIDC-1 (b). ICN denotes "interprotein coordination number" for interfacial metal ions. The asterisk denotes the preferred conformation, which is a D_2-symmetrical assembly shown in the Inset.

metal−protein bonds, especially in the absence of many specific noncovalent interactions between protein monomers (Figure 8a). This leads to a "terraced" landscape for MDPSA, where each terrace is defined by the number of bonds formed between protein monomers and interfacial metal ions (interprotein coordination number, ICN).

Given the large number of metal binding functionalities on the protein surface, there are many different protein alignments that can satisfy the same ICN. The ruggedness of the terraces, then, are defined by the noncovalent interactions formed between the protein monomers. As more interfacial protein−metal bonds are formed, the terraces become progressively narrower, ultimately leading to the most stable conformation(s) with saturated metal coordination. As our results indicate, even the lowest basin of the MDPSA funnel is somewhat wide and smooth. In the case of Zn and MBPC-1, one can imagine several different oligomeric conformations with similar energies that can satisfy tetrahedral Zn coordination (Figure 8 inset). As it turns out, even a single set of salt bridges can make the difference between the formation of a discrete oligomeric assembly (Zn_4:MBPC-1_4 or Zn_4:MBPC-2_4) and a heterogeneous mixture.[25]

Such sensitivity to secondary, noncovalent interactions makes our goal of predictably forming discrete superprotein architectures challenging. At the same time, access to these secondary interactions provides an additional handle to control MDPSA and, importantly, to influence the coordination environment of the metal ions embedded in protein−protein interfaces. These possibilities are discussed below.

Metal-Templated Interface Redesign

Despite having access to only a handful of metal coordinating groups, the ability of proteins to control and harness the reactivity of metal centers is unmatched in small molecule metal complexes. This is primarily due to the highly evolved

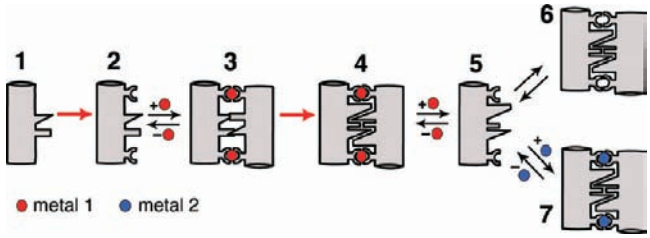

FIGURE 9. Cartoon outline for MeTIR. (1) Protein/peptide with a non-self-associating surface; (2) (1) modified with metal coordinating groups; (3) initial Metal1-templated protein complex with noncomplementary interfaces; (4) Metal1-templated protein complex with optimized, complementary interfaces; (5) protein with a self-associating surface; (6) metal-independent protein complex biased toward Metal1 binding; (7) protein complex with distorted Metal2 coordination.

noncovalent bonding networks of proteins, within which the coordination and solvation environments of metals can be exquisitely tuned.

Since one of the goals of MDPSA is to engineer functional metal coordination sites within protein interfaces, we found it useful to think about how functional metal centers and the surrounding protein environments may have evolved in natural systems. It is probable that some metals were incorporated into pre-existing protein scaffolds that presented the right coordination spheres, which subsequently were optimized for metal-based functions through cycles of natural selection. The feasibility of this evolutionary route is amply demonstrated by de novo designed metalloproteins which are based on pre-existing protein folds.[15,16] It is also quite likely that, early in the evolution of proteins, metals could initially have nucleated the assembly of random peptides or proteins around them, followed by rigidification and optimization of the surrounding peptide chain(s) for metal reactivities beneficial to a particular organism. With this hypothetical evolutionary time course in mind, we developed a rational engineering approach, metal-templated interface redesign (MeTIR) (Figure 9).[28]

Computationally Guided Redesign of Zn-Templated Interfaces. As illustrated in Figure 9 (species 3), the first critical step for MeTIR is the initial complexation of non-self-interacting proteins by metal coordination. The stage for MeTIR is therefore already set by the aforementioned Zn-, Cu-, and Ni-mediated superprotein architectures. We chose Zn_4:MBPC-1_4 as the initial focus of surface redesign efforts, because it features the most extensive set of contacts among its monomeric components. The majority of PPIs in Zn_4:MBPC-1_4 are found over two pairs of interfaces **i1** and **i2** (Figure 10). We envisioned that these nonspecific protein surfaces could be optimized through computational design for favorable packing, which should stabilize not only the tetrameric assembly but also its specificity for Zn binding.

Using Rosetta,[29] in collaboration with Xavier Ambroggio and Brian Kuhlman, we consecutively reengineered interfaces **i1** and **i2** in Zn_4:MBPC1_4 (Figure 11). Computational optimization of **i1** converged on six specific mutations (R34A/L38A/Q41W/K42S/D66W/V69I), which would yield a well-packed hydrophobic core. We termed the resulting protein construct RIDC-1. Optimization runs for **i2** also yielded six target mutations (I67L/Q71A/A89K/Q93L/T96A/T97I). Given the less-than-optimally packed core of redesigned **i2**, we predicted that it would not contribute significantly to the stability of the Zn-induced tetramer on its own. Hence, we constructed the second-generation variant, RIDC-2, which includes the six calculated mutations in **i2** in addition to the six incorporated into **i1**.[28]

In accord with our predictions, the redesign of **i1** leads to a significant stabilization of the Zn-induced tetramer as shown by SV measurements (Figure 12a). Again, as expected, the optimization of **i2** has a small incremental effect. Significantly, the overall structures of Zn_4:RIDC-1_4 and Zn_4:RIDC-2_4 assemblies are identical to the parent Zn_4:MBPC-1_4 complex as intended by "templating" (Figure 12b) despite 24 total interfacial mutations in Zn_4:RIDC-1_4 and 48 in Zn_4:RIDC-2_4.[28]

Programming the Memory of Metal Coordination onto Protein Surfaces. Because the redesign of **i1** introduces an extensive set of hydrophobic residues, we examined whether this interface could sustain stable monomer–monomer interactions also in the absence of metals. Hydrodynamic measurements show that RIDC-1 indeed forms a stable metal-free dimer (RIDC-1_2) with a dissociation constant of ~30 μM.[28] The crystal structure of RIDC-1_2 confirms that the dimer interface is predominantly formed along Helix2 and

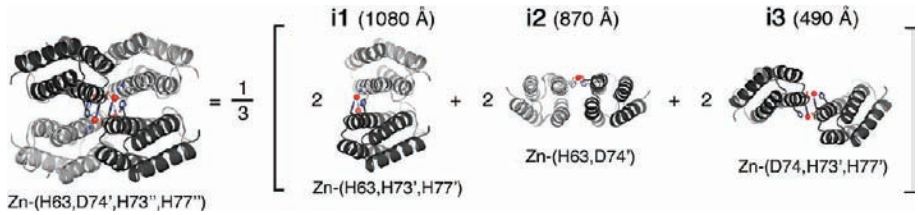

FIGURE 10. Three pairs of interfaces (**i1**, **i2**, **i3**) formed within the Zn_4:MBPC-1_4 tetramer; Zn-coordination environment in each interface is listed below.

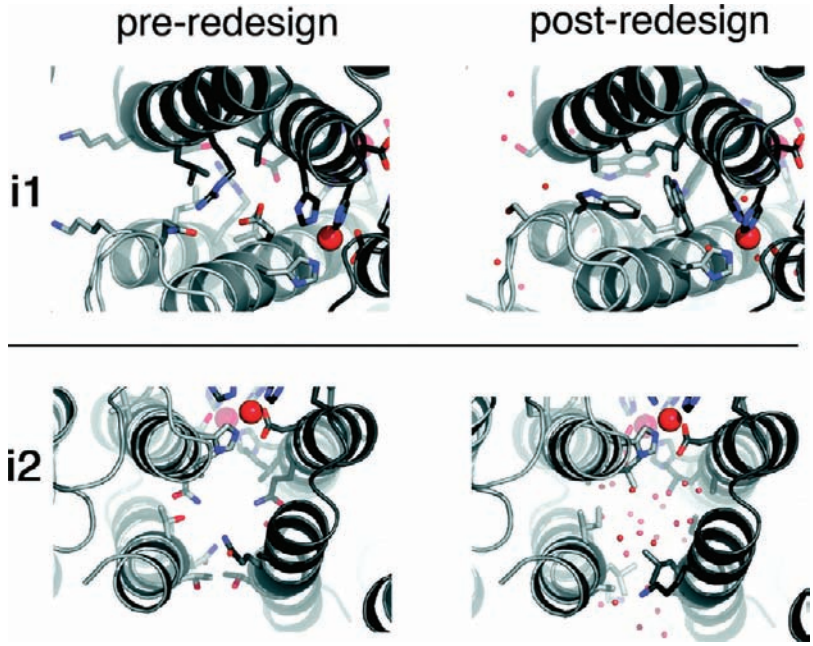

FIGURE 11. Detailed view of the crystalographically characterized side chain conformations in **i1** and **i2** before (left) and after (right) redesign (Zn, large red spheres; water molecules, small red spheres.)

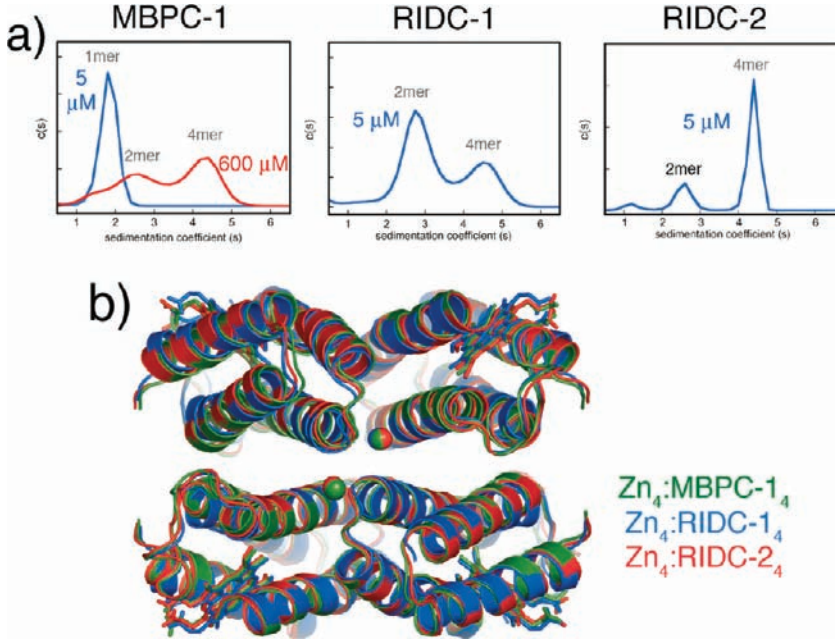

FIGURE 12. (a) From left to right: Sedimentation coefficient distributions for MBPC-1, RIDC-1, and RIDC-2 in the presence of equimolar Zn(II). (b) Backbone superposition of Zn_4:MBPC-1_4 (green), Zn_4:RIDC-1_4 (blue), and Zn_4:RIDC-2_4 (red).

3 where most of the hydrophobic mutations, especially the Trp's, were incorporated (Figure 13).[28] The antiparallel monomer−monomer alignment in RIDC-1_2 is somewhat distorted from the crisscrossed arrangement of monomers across **i1** in Zn_4:RIDC-1_4. This suggests that the engineered hydrophobic interactions do not impose strict geometric specificity, but they do orient the monomers to form a complex that closely approximates the conformation induced by Zn coordination.

The fact that RIDC-1 forms a metal-independent dimer suggests that the contribution of **i1** mutations to Zn_4:RIDC-1_4 stability is not only enthalpic but also entropic. Dimerization of RIDC-1 halves the number of protein components toward tetramerization while preorganizing the Zn-coordinating residues (H63 from one monomer, and H73′/H77′ from a second monomer) into close proximity. In the energy landscape model, the Zn-templated redesign of the MBPC-1 surface (to make RIDC-1) thus constrains the conformational search space

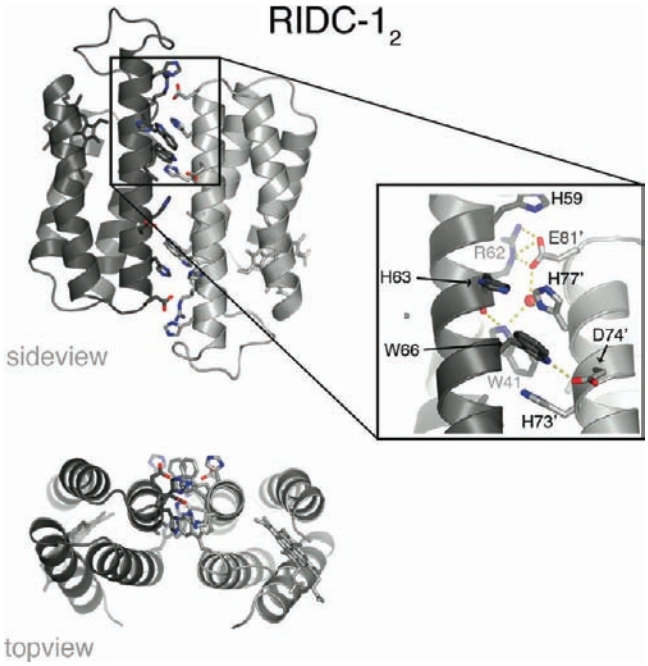

FIGURE 13. Side and top views of the RIDC-1_2 crystal structure, along with the closeup of one of the two symmetrical interaction zones in the dimer interface detailing the interfacial contacts. An ordered water molecule is highlighted as a red sphere.

toward the formation of the desired tetramer and restricts the number of possible protein complexes with a given ICN, ultimately giving rise to a steeper funnel with a deeper well (Figure 8b).

An implication of the increased stability of the Zn-induced tetramer is the rigidification of the tetrahedral Zn-coordination environment, which should raise Zn affinity and specificity. Conversely, this rigidification should lead a decreased preference for other metals with non-tetrahedral coordination geometries. This is indeed borne out by the crystal structure of the Cu(II) complex of RIDC-1, Cu_2:RIDC-1_2 (Figure 14a).[28] Whereas Cu(II) originally dictated a flat, antiparallel arrangement of two parent MBPC-1 molecules through a square planar His_4 coordination in Cu_2:RIDC-1_2 (Figure 4), it is now found in an open coordination environment with a His_3-$(H_2O)_{1\ or\ 2}$ ligand set (Figure 14b). This unfavored, "high-energy" configuration of Cu(II) is produced by the crisscrossed arrangement of the RIDC-1 monomers, which, just like in the Zn-induced tetramer, brings H63 and H73'/H77' in close proximity while pushing H59 out of the coordination sphere (Figure 14c). These results suggest that the memory of Zn coordination has indeed been programmed onto the RIDC-1 surface, which significantly alters the energy landscape for Cu-mediated self-assembly, such that the lowest energy conformation is no longer defined solely by coordinative saturation.

What We Have Learned and What Lies Ahead

In traditional inorganic chemistry circles, proteins are sometimes referred to as "spaghetti" or "chicken fat" that surround the precious metal ions. In the bioinorganic community, proteins are treated with a little more awe; this has prompted the

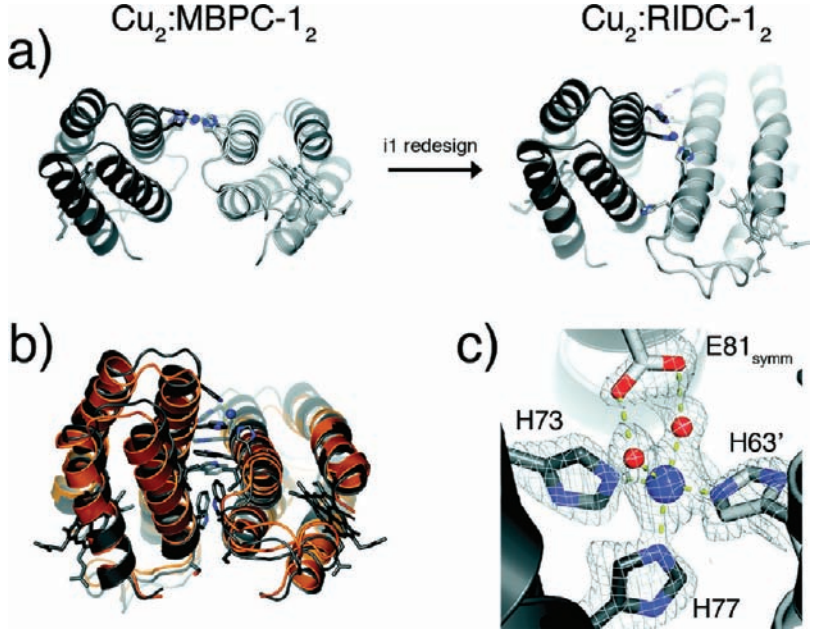

FIGURE 14. (a) Influence of Zn-templated interfacial mutations in **i1** on the conformations of Cu-mediated dimeric assemblies. (b) Backbone superposition of Cu_2:RIDC-1_2 (gray) and a dimeric half of Zn_4:RIDC-1_4 (orange) that contains **i1**. (c) Cu coordination environment in Cu_2:RIDC-1_2, highlighting the open coordination sites occupied by two water molecules. The Glu81 side chain from a crystallographic symmetry-related dimer is also shown.

entire field of biomimetic model chemistry, which aims to elucidate the inner workings of metal centers without the intricacies of the surrounding protein framework. No matter what the perspective, there is no denying that proteins are sophisticated metal ligands that guide metals to impressive chemical feats.

When we embarked on our endeavor four years ago, we took the notion of "proteins are ligands" somewhat literally. Could we use individual, folded proteins as ligands and control their self-assembly through metal coordination? We were, and still are, interested in this question from both biological and inorganic perspectives. Can we build complex multiprotein assemblies like nature does every time it needs to do something more involved than what is achievable with simpler, single protein systems (e.g., photosynthesis, respiration, nitrogen fixation)? At the same time, can we construct novel inorganic sites in biological interfaces, exploiting the functionality, and functionalizability, of protein surfaces to rule over the reactivities of these sites? The work that we have described in this Account not only showed us the feasibility of these goals but also opened our eyes to new research prospects such as the possible roles of metal ions in the evolution of protein folds/complexes and de novo design of protein interfaces.

One thing that we have certainly learned along the way is that proteins indeed make complicated ligands, which, more often than not, interact with metals and assemble in ways that are hard to predict. This is the major hurdle that still remains before we can construct made-to-order metalloprotein assemblies with tailored functions. Another hurdle is the technical difficulty of monitoring the dynamics of protein self-assembly and obtaining detailed chemical information about the protein complexes formed in solution, especially at low protein concentrations (which are readily accomplished through NMR in the case of small molecules). Yet, with perfect hindsight afforded by structural studies and some inorganic intuition, mixed in with a bit of computational protein design and advances in NMR and perhaps mass spectroscopy, we are certain that these hurdles will be overcome. We hope that others join in the fun.

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