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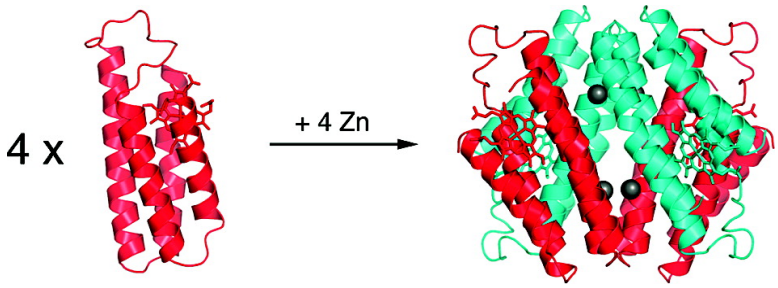

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Controlling Protein−Protein Interactions through Metal Coordination: Assembly of a 16-Helix Bundle Protein

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Protein−protein interactions (PPIs) are central to nearly all processes within cells, whether they are formed transiently in dynamic networks or permanently in macromolecular assemblies. There has been considerable progress toward our understanding of how proteins recognize their partners and how the energetics of their interactions are tuned.[1] Nevertheless, the ability to predict or interfere with natural PPIs or engineer new ones remains a great challenge, owing to the fact that protein−protein docking processes are guided by the superposition of many weak, noncovalent bonds that spread over large and often flexible surfaces. Our goal is to utilize the strength, directionality, and selectivity of metal−ligand interactions to control PPIs, thereby achieving specificity and affinity without requiring extensive binding surfaces. We describe here the Zn-mediated construction of a 16-helix architecture comprising four copies of cytochrome cb_{562} (cyt cb_{562}), a 4-helix bundle heme protein. Our results demonstrate that the self-organization of this macromolecule is controlled by metal coordination, with little or no thermodynamic bias from specific protein−protein contacts.

There are several factors that make cyt cb_{562} a good model system for investigating metal-mediated PPIs. It has a highly stable helical-bundle fold that is further strengthened through the engineering of covalent heme−polypeptide linkages into the parent protein, cyt b_{562}.[2,3] As a result, its structure is not perturbed by modifications on its surface. The all-α-helical makeup of cyt cb_{562} leads to uniform surface features that facilitate the introduction of metal-binding motifs. In addition, cyt cb_{562} has a rigid, roughly cylindrical shape, ideal for use as a building block for protein superstructures.

An earlier examination of the crystal structure of cyt cb_{562} revealed that the protein molecules are associated in pairs within the crystal lattice, despite being monomeric even at millimolar concentrations.[3] The pairing is mediated by a crystal packing contact formed by the antiparallel alignment of Helix 3 (residues 56−80) from individual molecules. On the basis of this nonfunctional packing arrangement, we envisioned that metal-mediated interactions between cyt cb_{562} monomers could be forged through the incorporation of metal-coordinating motifs onto the Helix 3 surface. To this end, we engineered a variant (His^4-cb_{562}) featuring two dihistidine motifs near the N- and C-termini of this helix at positions 59/63 and 73/77, which are located directly opposite each other in the crystal lattice and could potentially participate in interprotein metal coordination. Such di-His motifs placed at i and i+4 positions on an α-helix can coordinate metal ions with high affinity and have been widely used for the assembly of metalloproteins and peptides.[4−7] In addition, two Asp residues at positions 60 and 74 contained within each di-His motif were left intact in order to increase the likelihood of stable metal coordination.[8]

Our studies on metal-mediated PPIs have focused on late first-row transition metal ions. Zn(II), in particular, should have a high binding affinity for the di-His motifs on His^4-cb_{562} and exhibit rapid ligand exchange, which should prevent the formation of kinetically trapped protein−protein complexes. While dynamic light scattering measurements suggested the formation of multimeric His^4-cb_{562} structures upon Zn(II) addition, aggregates were formed at metal/protein ratios exceeding 2:1, even at protein concentrations below 100 μM. These protein aggregates were readily dissolved upon EDTA addition or lowering the solution pH below 6, indicating that Zn−His coordination is responsible for protein oligomerization. At high Zn loadings, both di-His clamps are likely charged with metal, which can lead to multiple modes of interprotein coordination and, thereby, aggregation. To capture the structures of any homogeneous protein assemblies, we screened for crystals of His^4-cb_{562} at low Zn loadings and obtained diffraction quality ones at less than 2:1 metal/protein ratios.

The 2.9 Å data reveal a unique quaternary structure (PDB ID: 2QLA) stabilized by four Zn(II) ions, wherein four His^4-cb_{562} monomers form two interlaced V-shaped dimers to yield a 16-helix bundle (Figure 1).[9] Each V-shape is formed by the parallel alignment of two cyt cb_{562} molecules related by a noncrystallographic two-fold symmetry axis, forming a ∼37° interprotein angle. The two V's, on the other hand, are wedged into one another in an antiparallel fashion. Both the formation of the V's and their interlacing are achieved entirely by interprotein Zn coordination. Each Zn shares an *identical* distorted tetrahedral coordination environment with ligands from three monomers (Figure 1c): the 73/77 di-His clamp from one molecule holds the Zn in a bidentate fashion, coordination of Asp74 from a second molecule stabilizes the V arrangement between the two, and coordination of His63 from a third molecule locks the two V's together. This striking arrangement suggests that the formation of the protein assembly and metal coordination should be highly cooperative. Interestingly, the 59/63 di-His clamp is not utilized for bidentate coordination despite the likelihood that it has a similar Zn affinity as the 73/77 couple. The His59 side chain is, in fact, H-bonded to Thr31 across the interface but is not involved in metal coordination (Figure 1b). The discrimination by Zn between the two di-His motifs is in accord with the dynamic self-assembly of the cyt cb_{562} superstructure: the lability of Zn likely permits the exploration of different coordination geometries, resulting in the formation of the thermodynamically most stable quaternary structure.

The interfaces between the four His^4-cb_{562} monomers feature a large number of polar interactions and bury a surface area exceeding 5000 Å^2. While the surface of cyt cb_{562} is not optimized for self-association and the interfacial contacts are likely to be nonspecific, they may collectively impart sufficient thermodynamic driving force to bias the formation of the observed assembly. To probe the existence of any metal-independent preorganization between the His^4-cb_{562} monomers and to examine their metal-dependent oligomerization behavior in solution, we carried out NMR and sedimentation velocity (SV) experiments. Indicative of a monomeric

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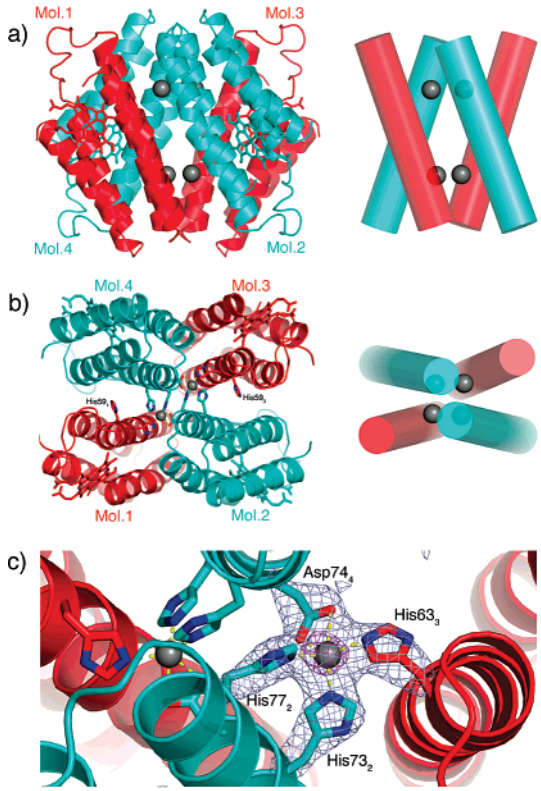

Figure 1. Crystal structure of the 4 Zn/4 His^4-*cb*$_{562}$ assembly. Pairs of protein molecules that form the V-shaped dimers are colored alike. Zn ions are shown as gray spheres. (a) View of the assembly parallel to the noncrystallographic two-fold axis and the corresponding cylindrical representation of Helix 3 involved in Zn coordination; (b) view down the noncrystallographic two-fold axis; (c) close-up view of the Zn coordination environment and simulated annealing $F_o - F_c$ omit electron density map (gray, 4σ; purple, 12σ). The parent molecules for metal ligands are shown in subscripts.

species, the 1D proton NMR spectra of His^4-*cb*$_{562}$ at high protein concentrations (>1 mM) feature significant chemical shift dispersion. Upon addition of 1 equiv of Zn, the peaks broaden considerably, as expected from the formation of a high-order oligomer. In order to quantitatively determine the hydrodynamic properties of this oligomer, we utilized pulsed field gradient (PFG) diffusion NMR spectroscopy.[10] These experiments yielded diffusion coefficients of 1.17×10^{-6} and 0.785×10^{-6} cm^2/s for the protein in the absence and presence of 1 equiv of Zn, respectively, with corresponding Stokes radii of 17.6 and 26 Å. The ~9 Å expansion is consistent with the formation of a tetramer.

The molecular mass distributions of the His^4-*cb*$_{562}$ species determined by SV measurements under different solution conditions are shown in Figure 2. In the absence of Zn, the protein is monomeric at all concentrations tested (up to 400 μM), with a single maximum at ~11 500 Da (12 328 Da actual). Upon addition of Zn, two new peaks centered at ~22 and ~50 kDa emerge at the expense of the monomeric species. The 22 kDa species presumably corresponds to either one of the two possible dimeric halves of the 16-helix bundle: (a) the V-shaped His^4-*cb*$_{562}$ dimer (e.g., Mol1 and Mol3 in Figure 1) held together by two Zn ions with His73/77 and Asp74 coordination, or (b) the antiparallel dimer (e.g., Mol1 and Mol4) with His73/77 and His63 coordination. These two species can further dimerize into the observed 16-helix bundle structure through His63−Zn or Asp74−Zn coordination, respectively, and become the predominant species at high protein concentrations.

Metal coordination chemistry has been used successfully for directing the formation of discrete nonbiological supramolecular

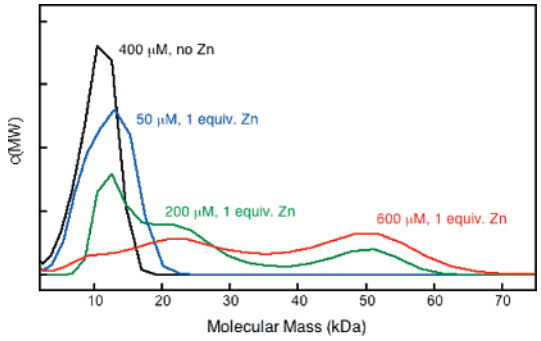

Figure 2. Molecular mass distributions of His^4-*cb*$_{562}$ species determined by sedimentation velocity experiments. The distributions are normalized with respect to the area covered under the curves.

complexes.[11] We have demonstrated here that protein building blocks with noninteracting surfaces can be assembled into self-healing superstructures through metal coordination. Such chemical control of protein−protein interactions paves the way for the generation of new biomaterials and manipulation of cellular processes.

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Supporting Information Available: Materials and methods for protein expression/purification/characterization, experimental details for DLS, NMR, and SV measurements, crystallographic data collection, and refinement statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

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