Design of Novel Protein Cages

In nature, cage-like protein assemblies serve important roles in encapsulation, delivery of molecular cargo and in performing catalytic transformations. Inspection of natural protein cages reveals asymmetric protein monomers forming tightly packed symmetric assemblies via multiple self-associative patches on each monomer. Achieving the necessary selectivity and specificity to form a symmetric structure from scratch is a formidable challenge in protein design. Thus far, the construction of artificial cages has relied on connecting natural protein building blocks via designed protein-protein interactions or interfaces. Scientists in the Tezcan lab at UC San Diego used concepts from inorganic chemistry to selectively guide the formation of interfaces with two-fold (C_2) and three-fold (C_3) rotational symmetry. Asymmetric protein monomers are equipped with orthogonal metal coordination motifs to self-assemble into polyhedral cages. The resulting cages can assemble and disassemble in a stimuli responsive manner while maintaining a tightly packed protein shell, akin to naturally occurring cage-like protein architectures.

Previous efforts in the Tezcan Lab have used the monomeric cytochrome *cb*$_{562}$ to direct protein oligomerization via judicious placement of native metal coordination residues like histidine, aspartate and glutamate on the protein surface. Native coordination motifs ("soft" ligands) preferentially coordinate "soft" late-first-row transition metals (*e.g.* Ni^{2+}, Cu^{2+}, and Zn^{2+}) with minimal chemical discrimination. This complicates the design of selective protein-protein interfaces with different metal ions. In contrast, the biologically inspired chelating motif, hydroxamate (HA) prefers "hard" metals like Fe^{3+}. As an additional design advantage, HA groups preferentially form octahedral complexes with C_3 symmetry in contrast to the C_2-symmetric coordination of Zn^{2+} ions by native chelating motifs (Fig. 1a & 1b). Making use of these features, a heterometallic protein cage with distinct metal ions at C_2- and C_3-symmetric interfaces was generated by incorporating HA binding motifs, in conjunction with native chelating residues. As a model system, a cytochrome *cb*$_{562}$ variant (CFMC1), structurally characterized using SSRL BL9-2, was chosen. While only Zn-mediated dimers of CFMC1 were observed in solution, the protein was found to crystallize into rhombohedral dodecameric cage-like units via Zn-mediated interactions, thus providing a structural template to generate a solution-stable bimetallic protein cage with selective coordination of Zn^{2+} and Fe^{3+}. A bidentate Histidine-Aspartate motif was positioned along the protein's Helix 1 to mediate C_2 symmetrization via tetrahedral Zn^{2+} coordination. Positions 63 and 82 were identified at C_3 symmetric pores of the dodecameric units as suitable locations for installing HA functionalities by introducing cysteine residues and reacting the residues with iodo-hydroxamic acid groups *in vitro* after protein purification (Fig. 1c). Two initial variants designated BMC1 and BMC2 were prepared, both bearing a His-Asp Zn-binding motif and Cys-HA groups at position 63 (BMC1) or positions 63 and 82 (BMC2).

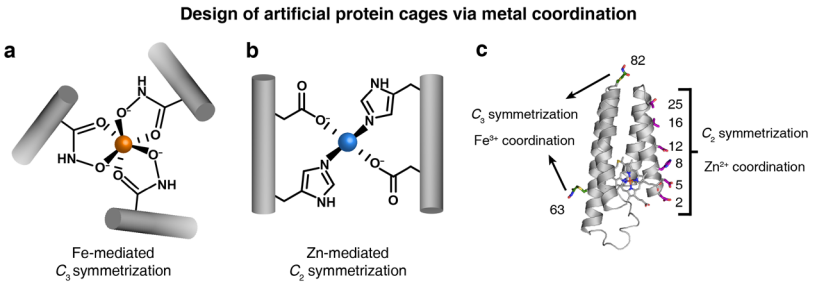

Figure 1. (a) C_2-symmetric protein dimerization induced by tetrahedral Zn^{2+} coordination of native amino acid sidechains. **(b)** C_3-symmetric protein trimerization induced by octahedral Fe^{3+}-tris-hydroxamate coordination. **(c)** Structural overview of the cytochrome *cb*$_{562}$ scaffold. Salient structural elements are shown as sticks.

Crystals of BMC1 and BMC2 were obtained upon concurrent binding of both Zn^{2+} and Fe^{3+} and using data collected at SSRL BLs 12-2 and BL9-2, both BMC1 and BMC2 formed the same dodecameric units as CFMC1 in the crystal lattice. A close inspection of the crystal structure revealed Zn ions exclusively located at C_2 interfaces and the desired Fe^{3+}:$(HA)_3$ coordination at C_3 interfaces (Fig. 2a), as was confirmed by anomalous x-ray diffraction data collected above and below Fe and Zn K-edges with no evidence of cross-metalation between the two sites. Whereas neither variant could form cages upon solution self-assembly, BMC2 crystals could be dissolved to reveal solution-stable uniform cage particles, as observed by transmission electron microscopy (TEM).

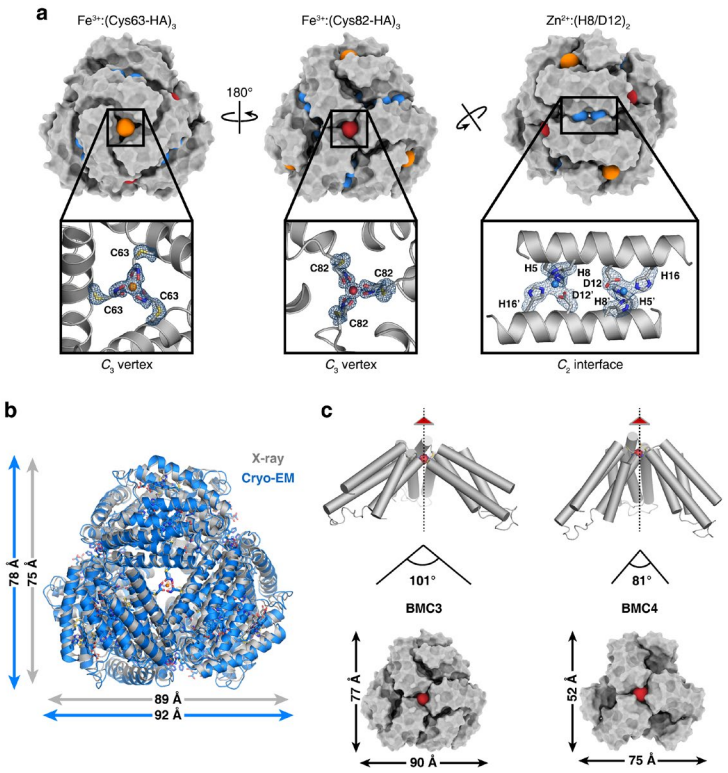

Figure 2. (a) Surface representations of the BMC2 cage, with metal ions shown as colored spheres. Atomic details of each metal coordination site are shown in the insets, with the mF_o-DF_c electron density omit map (blue mesh) contoured at 3 σ. **(b)** Overlay of the BMC3 x-ray and cryo-EM structures. **(c)** Comparison of the apical angle formed by the Fe:$(Cys82\text{-}HA)_3$–mediated vertices in BMC3 and BMC4 cages.

Therefore, two second-generation variants (BMC3 and BMC4) were produced in which the Zn-mediated interactions were bolstered to strengthen C_2-symmetric interfaces. BMC3 was altered to form two Zn-coordination sites whereas BMC4 was engineered to form a central histidine-asparagine site. The Cys63-HA group was removed and the BMC3 cages self-assemble into the desired dodecameric cages in solution forming two Zn coordination sites at each C_2 interface and maintaining the Fe centers.

Crystallographic data collected at the ALS and in-house single particle cryo-EM characterization revealed nearly identical cage architecture in solution to that observed in the crystal lattice. Importantly, proper self-assembly required the presence of both Fe and Zn ions and addition of either metal in isolation resulted in smaller oligomers. Surprisingly, addition of Fe and Zn to BMC4 resulted in the serendipitous formation of a D_3-symmetric hexamer. A comparison of BMC3 and BMC4 cages revealed a reduction in the apical angle formed by the Fe^{3+}:$(Cys82\text{-}HA)_3$-mediated vertices from 101° in BMC3 to 81° in BMC4. Furthermore, this structural shift at the C_3 interface was accommodated by an unexpected Zn-coordination motif resulting in a ~9 Å slip along the C_2 symmetric interfaces. These results highlight the conformational flexibility at the HA-mediated vertices to accommodate both sets of protein geometries and present a new avenue for the design of flexible and reversible protein architectures.

References

1. Ni, T.W. & Tezcan, F.A. Structural Characterization of a Microperoxidase Inside a Metal-Directed Protein Cage. *Angew. Chem. Int. Ed.* **49**, 7104-7018 (2010).

Primary Citation

E. Golub, R. H. Subramanian, J. Esselborn, R. G. Alberstein, J. B. Bailey, J. A. Chiong, X. Yan, T. Booth, T. S. Baker and F. A. Tezcan, "Constructing Protein Polyhedra via Orthogonal Chemical Interactions", *Nature* **578**, 172 (2020) doi: 10.1038/s41586-019-1928-2

Contact

Akif Tezcan, University of California, San Diego

SSRL is primarily supported by the DOE Offices of Basic Energy Sciences and Biological and Environmental Research, with additional support from the National Institutes of Health, National Institute of General Medical Sciences.