Biomineralization

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Biomineralization of a Cadmium Chloride Nanocrystal by a Designed Symmetrical Protein**

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Abstract: *We have engineered a metal-binding site into the novel artificial β-propeller protein Pizza. This new Pizza variant carries two nearly identical domains per polypeptide chain, and forms a trimer with three-fold symmetry. The designed single metal ion binding site lies on the symmetry axis, bonding the trimer together. Two copies of the trimer associate in the presence of cadmium chloride in solution, and very high-resolution X-ray crystallographic analysis reveals a nanocrystal of cadmium chloride, sandwiched between two trimers of the protein. This nanocrystal, containing seven cadmium ions lying in a plane and twelve interspersed chloride ions, is the smallest reported to date. Our results indicate the feasibility of using rationally designed symmetrical proteins to biomineralize nanocrystals with useful properties.*

Monomeric proteins carrying a number of identical, symmetrically arranged domains do not occur naturally but are of great interest for the creation of novel materials with useful electrical or photoelectrical properties.[1] Symmetrical protein frameworks capable of directing the growth of metallic clusters or quantum dots, or placing them at selected locations, have been studied for years as possible means to achieve "bottom-up" routes to nanomaterials and nanocircuitry.[2] Pizza6 is a recently created artificial monomeric protein, consisting of six tandem copies of a 42-residue domain and having six-fold rotational symmetry.[3] The protein was computationally designed by reverse engineering the evolutionary pathway of a natural protein from a presumed symmetrical ancestor. It is a member of the β-propeller family, each domain having four β-strands in the shape of propeller blades.[4] Propeller proteins are very widely distributed in nature, and have a variety of functions; often they are involved in protein–protein interactions. The original Pizza design included a so-called "velcro strap", so that the N-terminal seven residues form a β-strand of the neighboring blade. Versions of Pizza with only two or three domains per polypeptide (called Pizza2 and Pizza3, respectively) are found to fold stably into the same shape by self-association to recreate the six-domain structure.

Pizza proteins are highly thermostable and have great promise as building blocks for numerous different purposes. By making such proteins associate only in the presence of metal ions it should be possible to control network assembly very simply.[5] Metal ions are cheap, soluble ligands, which also induce strength and stability in metalloproteins.[6] Any protein network dependent on such ions should therefore be highly stable, but easy to disassemble with chelating agents. We have engineered a version of Pizza2 with no velcro (nvPizza2), and observed that, in accordance with expectations, the protein has a tendency to remain monomeric. We also created a variant intended to trimerize in the presence of metal ions, and studied the structure and oligomeric form of both proteins under various conditions.

The central axis seemed to us an ideal location for creating a metal-binding site with three-fold rotational symmetry, which could be created by placing a histidine side-chain at an appropriate point in nvPizza2. This gives three equivalent sites in six domains. nvPizza2 is a circular permutant of Pizza2, with the N-terminus position shifted by seven residues, but for simplicity the same residue numbering is retained so that nvPizza2 begins at residue 8. Comparison with known metalloproteins suggested that residue Asn 58 would be a suitable position for introduction of a histidine side-chain. Residues Asn 16 and Asn 58 are found at the start of the β-strand running along the central channel of the propeller structure. Previous computational studies had shown serine to be energetically favorable in this position,[3] with Ser 16 and Ser 58 forming a hydrogen bond with the carbonyl oxygen of Pro 57 or Pro 15, stabilizing the structure. Serine also has the advantage of a small side-chain. Modeling showed that Asn 16 (of the original velcro-strapped Pizza protein) would interfere with metal binding by His 58, so two proteins, nvPizza2-S16S58 and nvPizza2-S16H58, were created by mutagenesis from the original sequence, and expressed using the same methods described previously.[3]

The oligomeric state of nvPizza2-S16S58 (at pH 8.0 with 100 mM salt present) was determined by analytical ultra-

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centrifugation, and found to be an equilibrium between monomer and trimer. Well-diffracting crystals of this protein were only obtained with one set of conditions, and revealed the trimeric structure (see Figure S2 in the Supporting Information). nvPizza2-S16H58 showed a somewhat greater proportion of the trimer in solution; crystals grew very readily in the presence of 0.2 M cadmium ions and diffracted to 1.55 Å resolution. The structure could be solved very simply by molecular replacement using known Pizza models, and refined to an R-factor of 16.6% and free R-factor of 19.0%. The asymmetric unit of the crystal contains three copies of nvPizza2-S16H58 forming a complete, six-bladed structure with a backbone structure essentially identical to the original Pizza6. The identity of the metal ions was confirmed by measuring anomalous diffraction at 2 Å wavelength. The cadmium and chloride ions could also be readily distinguished in the electron density map by height, the cadmium ions showing density peaks above 30 σ, and the chloride ions peaks above 10 σ (Figure S3).

A striking feature of the protein crystal structure is the presence of a 19-atom nanocrystal sandwiched between two nvPizza2-S16H58 trimers. This nanocrystal is coordinated by a set of symmetrically positioned histidines, inducing the dimerization of the trimers (Figure 1). One face of the

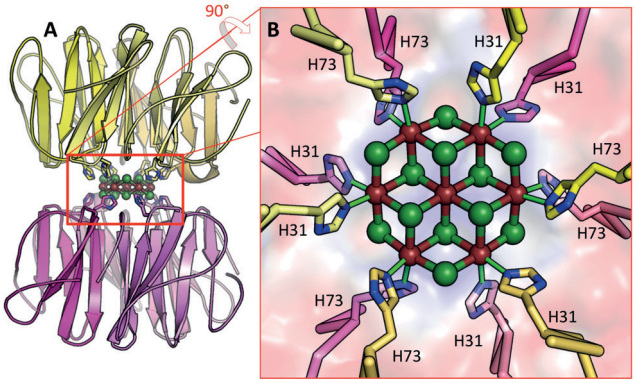

Figure 1. A) The overall structure of nvPizza2-S16H58, the protein being represented as a Cα trace. One molecule is colored yellow and one pink, the β strands being shown as arrows. The cadmium and chloride ions are shown as brown and green spheres, respectively. B) Closer view of the nanocrystal.

nvPizza2-S16H58 protein has a ring of six histidine residues, His31 and His73 from each subunit, which are pseudo-equivalent. Each copy of His31 and His73 coordinates a cadmium ion, one of seven lying in a plane perpendicular to the protein symmetry axis. The side-chain of His31 adopts a highly strained rotamer with an occluded conformation about the Cα-Cβ bond in order to coordinate a cadmium ion with its Nε atom, and also form a hydrogen bond with Thr14 of the same domain using its Nδ atom. The histidine introduced to create a metal binding site, His58, leaves no room for His73 to take up the same rotamer as His31. His73 therefore makes no hydrogen bond with Thr56, which is the only outlier in the Ramachandran plot.

A neighboring copy of the nvPizza2-S16H58 trimer sits over the ring of cadmium ions so that three cadmium ions are bonded by two copies of His31 (in adjacent trimers), and three ions are bonded by two copies of His73. These cadmium ions are therefore key crystal contacts holding two trimers face-to-face (Figure S4). Within this group lies a central cadmium ion on the protein symmetry axis; it is not directly coordinated by the protein, but is held in place by a group of 12 chloride ions distributed around the cadmium ions to form a lattice nearly identical to that of crystalline cadmium chloride.[7] Detailed comparison of the two lattices is shown in Figure 2 and Table 1. His31 and His73 each contact a cadmium ion with the same expected coordination bond length of 2.4 Å.

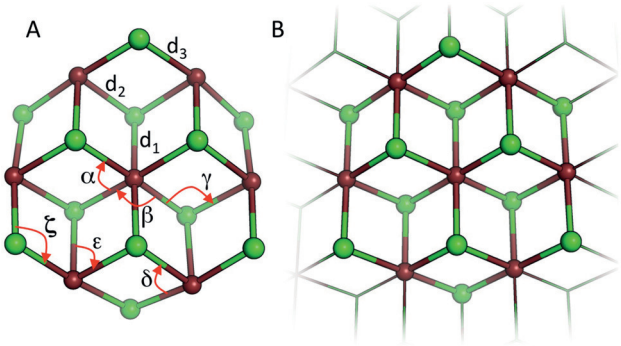

Figure 2. A) The cadmium chloride crystal biomineralized by nvPizza2-16S58H. The cadmium and chloride ions are shown as brown and green spheres, respectively. The distances and angles indicated are given in Table 1. B) The cadmium chloride lattice determined using a simple mineral crystal.

Table 1: Comparison of geometry of various cadmium chloride crystals.

Length/angle[a]	nvPizza2-S16H58	30255[b]	62202	86440
d_1	2.7	2.65	2.72	2.64
d_2	2.8	2.65	2.72	2.64
d_3	2.6	2.65	2.72	2.64
α	85	87.1	89.9	86.4
β	95	92.9	90.1	93.6
γ	95	87.1	90.1	93.6
δ	82	87.1	89.9	86.4
ε	84	87.1	89.9	86.4
ζ	100	92.9	90.1	93.6

[a] Distances and angles are indicated in Figure 2. Distances are given in Ångstroms, angles in degrees. [b] ICSD (Inorganic Crystal Structure Database) entry.

Analytical ultracentrifugation and electrospray mass spectrometry showed that, in the presence of cadmium chloride, nvPizza2-S16H58 forms a dimer of trimers, with a total of 12 blades (Figures S5 and S7). Nickel, cobalt, and zinc were also tested, but did not give similar behavior (data not shown). The cadmium-selective dimerization of the nvPizza2-S16H58 trimer in solution indicates that the formation of the nanocrystal is not a crystallization artifact.

Apart from the cadmium ions within the planar nanocrystal, another cadmium ion was observed at the designed

position, forming the expected 2.4 Å coordination bonds with the three copies of His58. This cadmium ion is also coordinated by three water molecules with bonds of the same length, creating a nearly perfect octahedral coordination shell. These water molecules also form hydrogen bonds with a bound sulfate ion, lying on the symmetry axis of the protein within the central channel (Figure 3). The increased

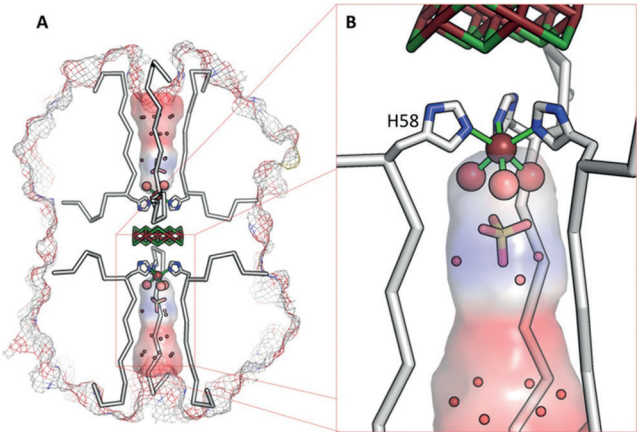

Figure 3. A) A cross-section through the nvPizza2-S16H58 structure showing the planar cadmium chloride crystal holding two protein oligomers together. His58 is shown as a stick model, and the Cα trace from residue Thr56 to Ala65 is shown as white sticks. The outer surface of the protein is shown as a mesh, and the inner surface of the cavity is shown as a translucent film. Both surfaces are colored by electrostatic potential (red negative, blue positive). B) A closer view of the cadmium ion (brown) coordinated by His58 and water (large spheres), with a sulfate ion and crystallographic waters in the channel shown as a stick model and small spheres.

tendency of nvPizza2-S16H58 to trimerize even in the absence of cadmium may arise from a central water molecule substituting for the metal ion, and forming hydrogen bonds with the three His58 residues. Such bonds must be much weaker and longer however than the coordination bonds formed by cadmium. By providing a rigid protein platform, the cadmium-His58 bonds favor the formation of the nanocrystal by His31 and His73. nvPizza2-S16S58 gives only small, disordered crystals unsuitable for X-ray analysis under the conditions used to grow nvPizza2-S16H58 crystals, possibly because the strain that would arise in His31 on nanocrystal formation is insufficiently compensated.

The cadmium ion closes off the central channel of the protein at one end, leaving a deep narrow tunnel, roughly 20 Å in length, filled with water molecules but which may only be accessed from one face of the protein, where it widens to almost 10 Å across (Figure 3a). The tunnel is reminiscent of buried enzyme active sites such as catalase[8] or superoxide dismutase,[9] which also rely on metal ions for reactivity. It may prove possible to create active sites from Pizza proteins in this way, using Pizza6 if needed to create a less symmetrical cavity.

Isothermal titration calorimetry showed several processes occurring as cadmium ions were added to nvPizza2-S16H58 (Figure 4). This is consistent with the different classes of

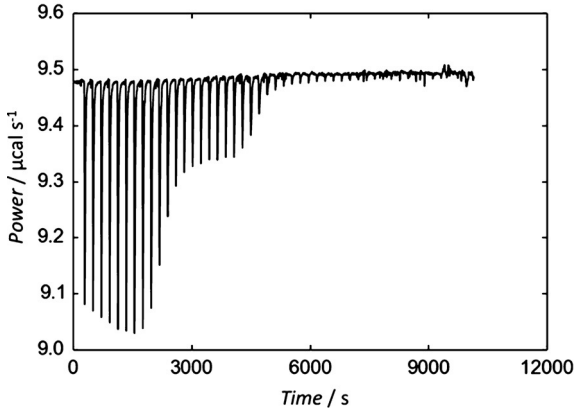

Figure 4. The heat released upon injection of cadmium chloride into nvPizza2-S16H58. The initial increase in heat release per mole of cadmium ions injected is indicative of a cooperative process among multiple sites. The marked plateau in the center of the raw thermogram also indicates saturation of higher affinity binding sites before lower affinity sites become appreciably occupied.

cadmium ions and metal-driven dimer formation seen in the crystal structure, but cooperative effects among the multiple binding sites complicate any quantitative analysis.

The two-dimensional cadmium chloride nanocrystal described here is the smallest nanocrystal reported to date, to our knowledge. A 1D crystal structure of potassium iodide has been grown within a carbon nanotube 1.6 nm in diameter, but these crystals may be tens of micrometers in length.[10] The lattice appeared distorted from that of macrosized KI crystals but the resolution of the electron microscope used in the analysis was much lower than the X-ray data described here.

Biomineralization is a dynamic field of study that attempts to answer the question of how natural proteins create materials such as sea-shells,[11] magnetic cores in bacteria,[12] and bones and teeth in animals.[13] Partly based on such natural examples, many groups are investigating the potential optical, magnetic, and electrical properties of novel protein-based materials for applications including memory devices,[14] biosensors,[15] and light-driven switches.[16] Other applications may include the creation of synthetic enzymes with inorganic or prosthetic catalytic groups,[17] and environmental bioremediation by adsorption of toxic elements.[18] The structure described here shows that metal ions are highly suitable ligands for controlling the oligomeric form of proteins with appropriately designed, symmetrical metal-binding sites, and that symmetrical proteins may likewise act as highly specific agents to order metal ions into crystal lattices.

Keywords: biomineralization · 2D crystals · metalloproteins · nanomaterials · oligomers

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[1] a) J. G. Heddle, *Nanotechnol. Sci. Appl.* **2008**, *1*, 67–78; b) J. G. Millán, M. Brasch, E. Anaya-Plaza, A. de La Escosura, A. H.

Velders, D. N. Reinhoudt, T. Torres, M. S. Koay, J. J. Cornelissen, *J. Inorg. Biochem.* **2014**, *136*, 140 – 146; c) J. G. Heddle, I. Fujiwara, H. Yamadaki, S. Yoshii, K. Nishio, C. Addy, I. Yamashita, J. R. H. Tame, *Small* **2007**, *3*, 1950 – 1956; d) A. D. Malay, J. G. Heddle, S. Tomita, K. Iwasaki, N. Miyazaki, K. Sumitomo, H. Yanagi, I. Yamashita, Y. Uraoka, *Nano Lett.* **2012**, *12*, 2056 – 2059.

[2] a) Y. Tamura, T. Kaizu, T. Kiba, M. Igarashi, R. Tsukamoto, A. Higo, W. Hu, C. Thomas, M. E. Fauzi, T. Hoshii, I. Yamashita, Y. Okada, A. Murayama, S. Samukawa, *Nanotechnology* **2013**, *24*, 285301; b) Y. Takezawa, P. Bockmann, N. Sugi, Z. Wang, S. Abe, T. Murakami, T. Hikage, G. Erker, Y. Watanabe, S. Kitagawa, T. Ueno, *Dalton Trans.* **2011**, *40*, 2190 – 2195.

[3] A. R. D. Voet, H. Noguchi, C. Addy, D. Simoncini, D. Terada, S. Unzai, S. Y. Park, K. Y. J. Zhang, J. R. H. Tame, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 15102 – 15107.

[4] V. Fülöp, D. T. Jones, *Curr. Opin. Struct. Biol.* **1999**, *9*, 715 – 721.

[5] a) B. S. Der, M. Machius, M. J. Miley, J. L. Mills, T. Szyperski, B. Kuhlman, *J. Am. Chem. Soc.* **2012**, *134*, 375 – 385; b) W. J. Song, P. A. Sontz, X. I. Ambroggio, F. A. Tezcan, *Annu. Rev. Biophys.* **2014**, *43*, 409 – 431; c) P. A. Sontz, W. J. Song, F. A. Tezcan, *Curr. Opin. Chem. Biol.* **2014**, *19*, 42 – 49.

[6] a) A. F. Peacock, *Curr. Opin. Chem. Biol.* **2013**, *17*, 934 – 939; b) I. J. Minten, K. D. Wilke, L. J. Hendriks, J. C. van Hest, R. J. Nolte, J. J. Cornelissen, *Small* **2011**, *7*, 911 – 919.

[7] a) L. Pauling, J. L. Hoard, *Z. Kristallogr. Kristallgeom. Kristallphys. Krista* **1930**, *74*, 546 – 551; b) D. E. Partin, M. O'Keefe, *J. Solid State Chem.* **1991**, *95*, 176 – 183; c) W.-L. Yu, M.-G. Zhao, *J. Phys. C* **1986**, *19*, 6761 – 6766.

[8] G. N. Murshudov, W. R. Melik-Adamyan, A. I. Grebenko, V. V. Barynin, A. A. Vagin, B. K. Vainshtein, Z. Dauter, K. S. Wilson, *FEBS Lett.* **1992**, *312*, 127 – 131.

[9] K. Djinovic, G. Gatti, A. Coda, L. Antolini, G. Pelosi, A. Desideri, M. Falconi, F. Marmocchi, G. Rotilio, M. Bolognesi, *J. Mol. Biol.* **1992**, *225*, 791 – 809.

[10] R. R. Meyer, J. Sloan, R. E. Dunin-Borkowski, A. I. Kirkland, M. C. Novotny, S. R. Bailey, J. L. Hutchison, M. L. Green, *Science* **2000**, *289*, 1324 – 1327.

[11] N. K. Dhami, M. S. Reddy, A. Mukherjee, *Front. Microbiol.* **2013**, *4*, 314.

[12] D. Faivre, T. U. Godec, *Angew. Chem. Int. Ed.* **2015**, *54*, 4728 – 4747; *Angew. Chem.* **2015**, *127*, 4810 – 4829.

[13] a) H. C. W. Skinner, *Mineral. Mag.* **2005**, *69*, 621 – 641; b) A. Veis, J. R. Dorvee, *Calcif. Tissue Int.* **2013**, *93*, 307 – 315.

[14] a) A. K. Yagati, J. Min, J. W. Choi, *J. Nanosci. Nanotechnol.* **2014**, *14*, 433 – 446; b) K. Sano, A. Miura, S. Yoshii, M. Okuda, M. Fukuta, Y. Uraoka, T. Fuyuki, I. Yamashita, K. Shiba, *Langmuir* **2013**, *29*, 12483 – 12489.

[15] M. Naito, K. Iwahori, A. Miura, M. Yamane, I. Yamashita, *Angew. Chem. Int. Ed.* **2010**, *49*, 7006 – 7009; *Angew. Chem.* **2010**, *122*, 7160 – 7163.

[16] J. M. Reis, D. C. Burns, G. A. Woolley, *Biochemistry* **2014**, *53*, 5008 – 5016.

[17] a) M. L. Zastrow, A. F. Peacock, J. A. Stuckey, V. L. Pecoraro, *Nat. Chem.* **2012**, *4*, 118 – 123; b) W. J. Song, F. A. Tezcan, *Science* **2014**, *346*, 1525 – 1528; c) B. Maity, K. Fujita, T. Ueno, *Curr. Opin. Chem. Biol.* **2015**, *25*, 88 – 97.

[18] L. Zhou, M. Bosscher, C. Zhang, S. Ozcubukcu, L. Zhang, W. Zhang, C. J. Li, J. Liu, M. P. Jensen, L. Lai, C. He, *Nat. Chem.* **2014**, *6*, 236 – 241.

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