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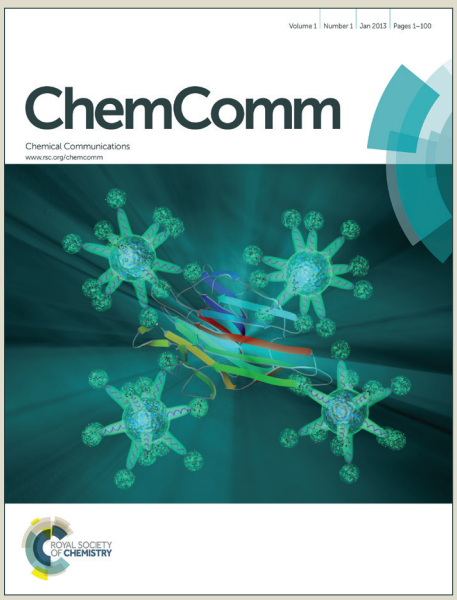

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Construction of an enterobactin analogue with symmetrically arranged monomer subunits of ferritin

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Hiroshi Nakajima,*[a] Mio Kondo,[b] Taiki Nakane,[c] Satoshi Abe,[c] Takahiro Nakao,[b] Yoshihito Watanabe[d] and Takafumi Ueno*[c]

A set of three catecholamide ligands mimicking the structure of enterobactin was constructed on ferritin, where the 3-fold symmetric arrangement of the monomer subunits served as a foundation to form a coordination space. Similar to enterobactin, the ligands showed strong affinity for the ferric ion and formed a tris-catechoyl complex. Crystallography revealed that the complex was embedded in the entrance of the 3-fold axis channel.

Ferritin is the ubiquitous Fe storage protein that ensures constancy of the cellular Fe level.[1, 2] Ferritin consisting of 24 monomer subunits forms a hollow dodecahedron assembled with octahedral (432) symmetry. There are two types of channels penetrating the shell along the C_3 and C_4 symmetry axes (Fig. 1a). The interior surface of ferritin contains abundant amino acid residues with carboxyl and imidazoyl side chains, which guide captured Fe ions from the C_3 axis channel to the ferroxidase centre located at the middle of the monomer subunit.[3-5] Recent studies have proposed that these residues may also fix metal complexes and other types of metal ions on the interior surface.[6-12] The fixed species retain their original properties, including their structures and functions, and work as active sites in the enclosed space. Regarding the exterior surface of ferritin, genetic engineering has allowed the regulation of the inter-subunit interactions by copper ions,[13] for which the C_2 symmetric arrangement is utilized to introduce coordination motifs at the interface between two subunits (Fig. 1a). These achievements demonstrate that ferritin is a versatile scaffold for fundamental experiments and applications of protein-based coordination chemistry.[14, 15] In order to further develop this chemistry, a possible strategy is to exploit the C_3 or C_4 symmetry of ferritin. As has been demonstrated via the structural transformation[16, 17] or functional modification of the spherical surface with organic molecules or proteins,[9, 18-20] the monomer subunits arranged in the C_3 as well as C_4 symmetry would serve as an useful foundation to create a highly symmetric coordination space on ferritin. Multiple symmetrically located reaction sites can be prepared on ferritin by the mutagenesis of a single amino acid residue near the target symmetry axis. Because the ferritin subunit contains limited numbers of cysteine residues, simultaneous mutagenesis to remove the original and introduce external cysteine residues would provide unique reactive thiol groups on the ferritin surface, which could then be utilized to conjugate a designed ligand. In light of these considerations, we decided to construct a structure on ferritin that mimics the form and function of a natural symmetrical molecule. In this study, we focused on enterobactin, a siderophore of *Escherichia coli*.[21, 22] Enterobactin is the strongest chelator of ferric ions reported to date. A circular tris-serinyl backbone contains three symmetrically located catecholamide ligands (Fig. 1b), which preferably form an octahedral Fe(III)-tris-catecholato complex with an extremely high absolute stability constant of $\sim 10^{49}$ M^{-1}.[22] The C_3 symmetry of ferritin appeared to be suitable to support a set of catecholamide ligands to mimic enterobactin, although the arm length of the ligand should be designed so that the catechol moiety can reach the centre of symmetry to form metal complexes without distortion.

A catecholamide derivative, *N*-(2-(2,5-dioxo-1H-pyrrol-1-yl)ethyl)-2,3-dihydroxybenzamide (Cat, Fig. 1c), was prepared. This derivative contained a maleimide group to connect the catecholamide moiety to a cysteinyl thiol. The cysteine residue was introduced to the light-chain subunit of horse spleen ferritin by the genetic replacement of an alanine residue at the 119th position (A119C, Fig. 1a). The Cys119 was expected to create the smallest possible triangle (a side length of 15 Å) surrounding the C_3 axis channel on the protein surface. The original cysteine residue at the 126th position was replaced with alanine (C126A) to avoid unfavourable conjugation with Cat, while another cysteine residue at the 48th position, which was located in the interior surface of ferritin, was left unaltered in the mutant to allow for further cavity functionalization in future studies. The resulting mutant, A119C/C126A, was obtained as a recombinant protein from *E. coli*

[a] *Division of Molecular Materials Science, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Osaka, 558-8585, Japan. E-mail: nakajima@sci.osaka-cu.ac.jp*
[b] *Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan.*
[c] *Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259-B55, Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan. E-mail: tueno@bio.titech.ac.jp*
[d] *Research Centre of Materials Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan. E-mail: yoshi@nucc.cc.nagoya-u.ac.jp*
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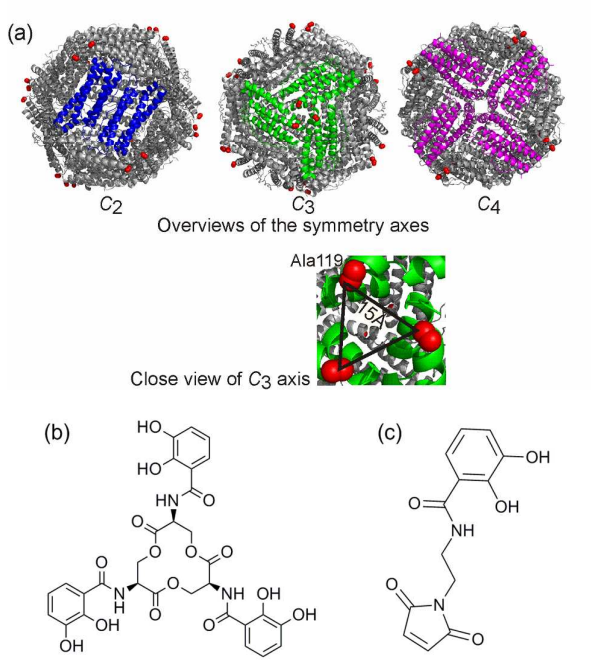

Fig. 1 (a) Symmetrical arrangements of the monomer subunits of ferritin (PDB: 1DAT). The locations of the Ala119 to be replaced with Cys and modified with Cat are illustrated with red spheres. (b-c) Molecular structures of enterobactin and Cat, respectively. For details regarding the preparation and characterization of Cat, see the ESI.

and was purified in the iron-free form using the conventional method.[10]

Modification of A119C/C126A with Cat was achieved after 3 h under the experimental conditions used here (See *Sup. Info.*). The MALDI-TOF mass spectrum of the modified protein variant showed a unique peak at m/z = 20120.9 (Fig. S1), which was in agreement with sum of the molecular masses of the A119C/C126A monomer subunit (19845.1) and Cat (276.1), confirming the modification of a single residue of the variant with Cat. The Cat-modified A119C/C126A mutant (Cat-Fn) showed no significant difference in its circular dichroism (CD) spectrum or its mobility in native PAGE compared with the unmodified mutant (Fig. S2). This observation indicates that the Cat modification did not affect the secondary or higher-order structures of ferritin.

UV-Vis titration of the ferric ion was performed to investigate the Fe-binding properties of Cat on Cat-Fn (Fig. 2a). In addition to a band attributable to Cat (λ_{max} = 310 nm, $\varepsilon_{310} = 2.9 \times 10^3$ M^{-1} cm^{-1}), the titration of Cat-Fn with $FeCl_3$ at pH 7.0 (20 mM MES-NaOH buffer) and 25°C showed the emergence of a new band at 498 nm. This signal was ascribed to an LMCT band of the Fe(III)tris-catecholato complex. The spectra collected during the titration also provided no evidence indicating the presence of mono- or bis-catecholato complexes, which show characteristic bands at 700 and 576 nm, respectively.[23, 24] This result supports the exclusive formation of the Fe(III)tris-catecholato complex (Fe-trisCat) at all metal:Cat ratios. The spectral signal appeared to be saturated when the total amount of Fe(III) was ca. 1 equiv. to the channel (i.e., 8 equiv. to Cat-Fn, Fig. 2b). Further addition of Fe(III) resulted in the precipitation of $Fe(OH)_3$, which caused gradual rise in absorbance of the spectra, finally followed by severe co-precipitation of the Fe(III)-bound Cat-Fn with $Fe(OH)_3$ (Fig. S3). This observation implies that Fe-trisCat is preferentially formed to suppress the precipitation of insoluble $Fe(OH)_3$. The absolute stability constant (K_{SC}) of Fe-trisCat was determined via EDTA titration (Fig. S4 and *Sup. Info.*)[25] to be approximately 1.2×10^{40} M^{-1}, which is extremely larger compared with that of EDTA with Fe(III) (6.3×10^{21} M^{-1})[26] and fairly similar to those of synthetic analogues, such as TRENCAM and MECAM (~10^{43} M^{-1}).[27, 28] The titration data indicated that a stoichiometric amount of Fe(III) is sufficient to obtain Fe-Cat-Fn in which the all sets of ligands attached to ferritin form Fe-trisCat. The UV-Vis spectroscopic and ICP-OES analyses of Fe-Cat-Fn revealed the complete retention of Fe(III)-trisCat, even after extensive dialysis against Fe-free MES-NaOH buffer (3 d at 25°C, Fig. S5). This result supports the negligible off-kinetics of Fe(III) from Fe-trisCat and its high K_{SC} value.

The high stability of Fe-trisCat allowed crystallographic measurements of Fe-Cat-Fn with a resolution of 1.6 Å (Table S1 and Fig. 3). Overall, the spherical shape of the ferritin cage was conserved in Fe-Cat-Fn (Fig. 3a). In accordance with the mass analysis data, Cat was solely conjugated at Cys119 and Cys48 retained intact (Fig. 3b). The anomalous difference map indicated that a single Fe ion (Fe1) was located in the tris-catecholato complex (Fe1-trisCat, Fig. 3c). The Fe1-trisCat had an octahedral geometry of hexa-oxo-Fe(III) with bond distances of 2.1 Å for both Fe1-O1 and -O2 and a bond angle of 77.7° for O2-Fe1-O1. These values are in agreement with those found previously in Fe(III)-tris(catecholatoamide) complexes.[29-31] Thus, Fe1-trisCat has a normal coordination structure with no bond distortions. The planar geometry of the catecholamide group appeared suitable for facile interaction of the amide proton with the catechol *m*-hydroxyl group. An analogous interaction is observed in enterobactin, which is believed to reduce the pK_a of the catechol hydroxyl group and enhance the binding ability of the catechol moiety for ferric ions at neutral pH.[27, 28] This mechanism should also be adaptable to the Cat-Fn reported here. Data supporting this potential adaptation could be obtained from the crystal structure of intact Cat-Fn. However, attempts to obtain a single crystal of Cat-Fn failed because of the

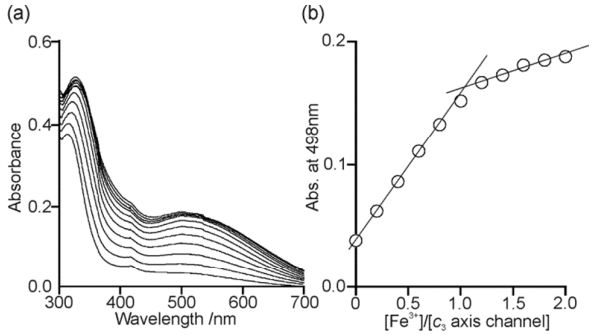

Fig. 2 $FeCl_3$ titration to Cat-Fn monitored via UV-Vis spectroscopy at pH 7.0 and 25°C. (a) Representative changes in the spectrum upon the addition of 0 to 2 eq. of Fe^{3+} to the C_3 axis channels were collected at 0.2-eq. intervals. The concentration of Cat-Fn was adjusted to 4.2×10^{-6} M. (b) Changes in the absorbance were observed at 498 nm.

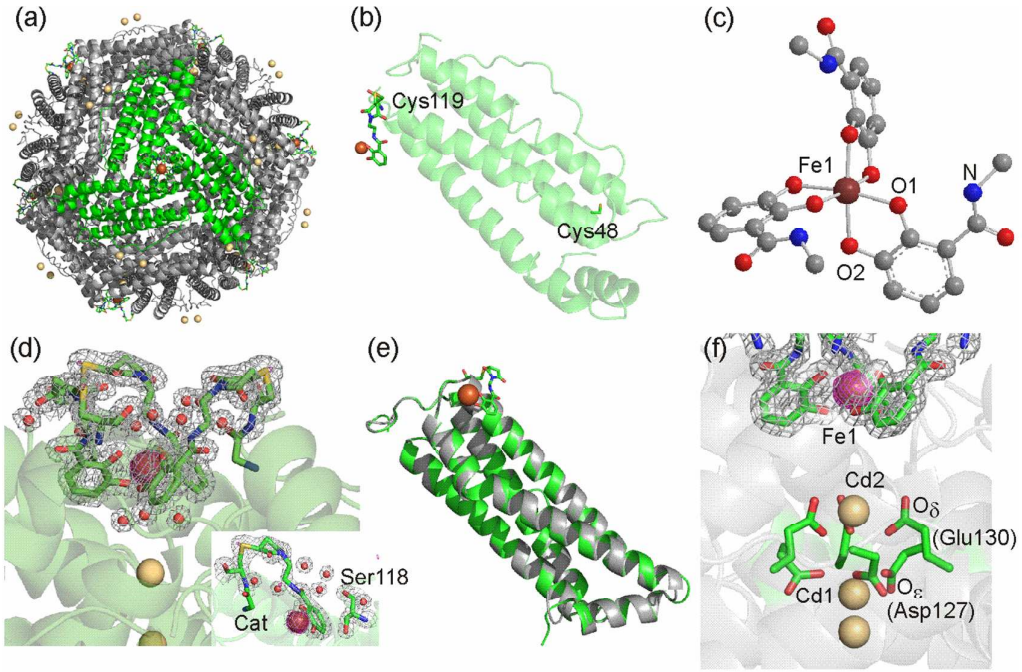

Fig. 3 Crystal structures of Fe-Cat-Fn (PDB: 5CZU). (a) Overall structure. The C_3 axis channel modified with three Cats is located at the centre of the green-coloured subunits. (b) Cys119 is uniquely modified with Cat. (c) Coordination structure of the Fe1-trisCat complex. (d) The Fe-trisCat complex is embedded in the exterior entrance of the channel. The inset shows the arrangements of Cat and Ser118 of a neighbouring subunit (e) Comparison of C_α positions in wild-type ferritin and Fe-Cat-Fn. (f) Interactions of Cd ions with amino acid residues in the channel. The Fe and Cd ions are represented by brown- and ivory-coloured spheres, respectively. The anomalous difference fourier maps at 4.0 σ indicate the positions of ferric ion which are shown in magenta. The selected 2|Fo| - |Fc| electron density maps at 1.0 σ are shown in light grey.

formation of a trisCat complex containing a Cd(II) ion, which was an essential additive to promote the formation of a single crystal; as a result, only the crystal structure of Cat-Fn in the Cd(II)-bound form could be obtained (Cd-Cat-Fn, PDB: 5AXS, Table S2 and Fig. S6).

Interestingly, Fe1-trisCat appeared to be smoothly embedded in the entrance of the C_3 axis channel, blocking the access of any ions and molecules to it (Fig. 3d). Despite this large construct at the channel entrance, negligible deviation was observed in the protein scaffold compared with the wild type (Fig. 3e). The obtained root mean square deviation (rmsd) of Cα atoms in Cat-Fn (Ser2 to Leu172) relative to those of the wild type was 0.35 Å. In addition, no particular interaction was found to stabilize the arrangement except for a possible weak hydrogen bond between the catechol O atom and the hydroxyl group of Ser118 from a neighbouring subunit separated by 3.5 Å. Thus, the observed arrangement of Fe1-trisCat might be due to the coincidental fit of Fe-trisCat with the channel entrance. This implies that although crystalline Fe1-trisCat existed only in the channel-blocking configuration, other configurations should exist in thermodynamic equilibrium in solution. This assumption is supported by the observation of Cd ions in the crystal structure (Fig. 3f). Two Cd(II) ions (Cd1 and Cd2) were located at the centre of the C_3 axis channel. The average distances between Cd1 or Cd2 and the carboxylate groups of Glu130 or Asp127, respectively, were 2.5 Å, suggesting weak interactions between the ions and carboxylate groups.[32, 33] It is important to note that Cd(II) ions, which were added to the crystallization solution, were able penetrate into the channel despite the apparent blockage of the channel entrance Fe1-trisCat before crystallization was attempted. Temporal dissociation of the Fe ion from Fe1-trisCat to open the entrance is unlikely because no observable formation of the Cd(II)-bound tris-catecholato complex (Cd-trisFn) was noted in the crystal structure. Indeed, the stable Cd-trisCat should be partially involved in the Fe(III)/Cd(II) exchange reaction upon the temporal disassembly of Fe-trisCat in the Cd(II)-rich crystallization solution. A tentative interpretation of the incorporated Cd ions is that Fe1-trisCat has multiple configurations in solution. Some of these configurations may protrude from the protein surface, like a trigonal pyramid, such that there are apertures around the base of pyramid that readily allow Cd(II) to permeate into the channel.

In summary, we successfully constructed enterobactin-like set of ligands by utilizing the C_3 symmetric arrangement of the ferritin subunits. The set of ligands formed an Fe-trisCat (Fe(III) tris-catecholato complex) with a high absolute stability constant ($\sim 10^{-40}$ M^{-1}). Crystallographic data revealed that Fe-trisCat took a configuration embedded in the entrance of the C_3 axis channel. The results obtained here demonstrate that the symmetric structure of ferritin is a useful basis for the design of a coordination sphere of high symmetry. Depending on the choice of the residue used to tether the ligand, we can prepare different coordination spaces on the exterior or interior surface of ferritin around the C_3 or C_4 axis. Such versatility will expand the potential applications of protein-based coordination chemistry.

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