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研究課題名（和文）Exploring the structure and mechanism of formation of an artificial protein capsid, toward the development of a novel redox-responsive nano-carrier system.

研究課題名（英文）Exploring the structure and mechanism of formation of an artificial protein capsid, toward the development of a novel redox-responsive nano-carrier system

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研究成果の概要（和文）：本研究では対称構造を有するタンパク質ケージを組み立てる新しい方法を提示する。変異導入したTRAPタンパク質は、外表面上にシステイン残基を有する11員環に折り畳まれる。金クラスターと共に反応させると非常に安定性の高い空洞のケージ（TRAP-ケージ）を形成し、還元条件下で脱集合する性質を有し、ドラッグデリバリーに応用できる可能性がある。TRAP-ケージ構造は予想外に金原子が協調的に作用して連結される結果を示した。このケージは金属配位による最初の新たなタンパク質ケージの構築を示し、特有対称性の変形立方体を有する。この結果は、別の対称性を探索することで大きなタンパク質ケージを構築する新戦略を提案する。

研究成果の概要（英文）：We present a new method for assembling protein cages with well-defined, symmetrical structures. The starting material, a mutated TRAP protein, folds into an 11-membered ring with cysteine residues on the outer surface. Reaction with the gold cluster Au55 leads to the formation of a hollow cage (TRAP-cage), which exhibits extreme stability, yet disassembles under reducing conditions. Potential applications in biomedicine (drug delivery) are suggested.
The TRAP-cage structure yielded unexpected results. Neighboring TRAP rings were linked by gold atoms coordinated via cysteines: TRAP-cage represents the first de novo assembled protein cage by metal coordination. Furthermore, with 264 protein subunits, TRAP-cage shows a unique symmetry. Instead of icosahedral symmetry, TRAP-cage has snub cube symmetry, with an 11mer ring occupying each of the 24 vertices with near-perfect regularity. Our findings suggest novel strategies for building large protein cages by exploring alternative symmetries.

研究分野：　生化学、バイオナノテクノロジー

キーワード：　protein cage　self-assembly　bionanotechnology　gold nanoparticle　metal coordination　artificial capsid　smart nanomaterial　supramolecular assembly

１．研究開始当初の背景

(1) The overall aim in bionanotechnology is the construction of novel or useful structures from simple biomolecular components, using the principles of molecular self-assembly.

(2) One very challenging area is in artificial or designed protein cages, which mimic symmetrical viral capsid structures. The controlled assembly of designed cages is expected to generate many significant applications in biomedicine and nanotechnology[1,2].

(3) One strategy for designed protein cages, metal-directed self-assembly, is especially promising due to the tunability of the assembly and disassembly processes. However, so far little progress has been made in this area[3,4].

(4) TRAP is a bacterial protein with a unique ring shape and very high stability, making it an ideal candidate for a building block in bionano applications[5,6]. We reported the efficient conversion of mutant $TRAP^{K35C}$ protein into capsid-like cage structures via the action of Schmid gold clusters (Au_{55})[7].

(5) Further experiments, show that TRAP-cage is a genuine stimulus-responsive nanomaterial with controllable self-assembly, extreme stability, and efficient disassembly in response to redox conditions. Most surprisingly, the structure of TRAP-cage demonstrates a unique supramolecular symmetry, and suggest novel directions in achieving large multicomponent assemblies.

２．研究の目的

(1) To solve the high-resolution structure of TRAP-cage; determine whether it adopts icosahedral symmetry (like virus capsids) or other types of symmetry.

(2) To elucidate the mechanism of TRAP-cage formation; identification of reaction intermediates and the role of Au_{55} gold clusters.

(3) To characterize the material properties of the TRAP-cage, especially in terms of overall stability.

(4) To determine conditions under which TRAP-cage undergoes disassembly.

(5) To encapsulate (and release) large molecular cargo within TRAP-cage, such as reporter proteins.

３．研究の方法

(1) Mutants of RNA-binding protein TRAP from *G. stearothermophilus* were generated standard *Pfu* polymerase techniques and purified using standard methods.

(2) Starting from purified $TRAP^{K35C}$ (8 nm rings), self-assembly of the 21-nm TRAP-cage was induced by addition of Au_{55} $[Au_{55}(Ph_2PC_6H_4SO_3Na)_{12}Cl_6]$ gold cluster or its variants under a variety of pH, temperature, concentration, and time conditions.

(3) Morphology of intermediates and products were monitored using 80 kV

transmission electron microscopy (TEM).

(4) Disassembly of the cage structures in the presence of reducing agents, etc., was monitored in real time using high-speed atomic force microscopy (in cooperation with Dr. Motonori Imamura, Kanazawa University).

(5) The high-resolution structure of TRAP-cage was determined using cryo-electron microscopy (in collaboration with Dr. Kenji Iwasaki, Osaka University). Molecular models were built using an array of different software (Coot, CPP4, Phenix, Chimera, etc.). Analysis of the resulting symmetry was also analysed in cooperation with Prof. Craig Kaplan, Waterloo University, Canada.

(6) Differential scanning calorimetry (nano-DSC) was used to measure the thermal stability of TRAP ring and the final TRAP-cage structure.

(7) To test the hypothesis regarding the mechanism of TRAP-cage formation, the gold-based drug auranofin was used in place of Au_{55} and the results monitored.

(8) Attempts to encapsulate cargo within TRAP-cage was carried out by mixing purified enhanced GFP (eGFP) with the TRAP-cage reactants under various conditions, and results monitored using electrophoresis and fluorescence scanning.

４．研究成果

(1) Conditions for TRAP-cage formation: Cages do not form spontaneously even under oxidizing conditions. Upon addition of Au_{55} gold clusters, monodisperse cages (21 nm diameter, with 15 nm inner cavity) form at above pH 7, suggesting involvement of thiolate anions. Cages will form under sub-stoichiometric Au_{55} concentrations, with rates dependent on relative concentrations (Malay *et al.*, *Nano Letters*, 2012).

(2) Intermediates are clearly visible at high TRAP:Au_{55} ratios (**Fig. 1**). Addition of Au_{55} leads to rapid aggregation of protein, followed by formation of "rings-on-a-string" structures and eventually the complete transformation into monodisperse, hollow cage structures (unpublished results).

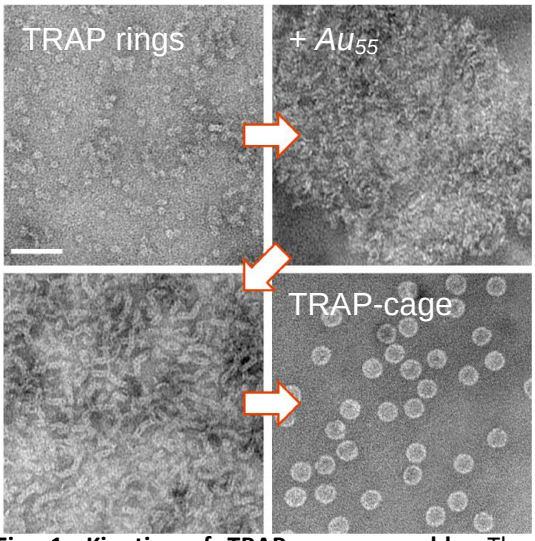

Fig. 1. Kinetics of TRAP-cage assembly. The reaction was initiated by mixing purified $TRAP^{K35C}$ protein with Au_{55} at pH 8, monitored using 80 kV TEM (scale bar, 50 nm).

(3) *In vitro* reactions revealed conditions promoting complete disassembly of TRAP-cage reactions. These include thiol-based reducing agents (e.g., DTT), gold chelators (dimercaprol), TCEP, etc. High-speed AFM experiments captured the catastrophic disassembly of TRAP-cage into constituent rings in real time (Imamura *et al.*, *Nano Letters*, 2015).

(4) Elucidation of the TRAP-cage structure

was achieved using cryo-EM techniques, with highly surprising results (**Fig. 2a-b**). It was expected that the native 11mer rings would rearrange into rings with symmetries compatible with icosahedral assembly (*i.e.* 10mer, 12mer rings) prior to cage formation. Instead, stable 11mer rings were retained, with the TRAP-cage assembled from 24 identical rings (264 subunits in total), with an overall chiral snub-cube symmetry, a configuration never previously reported (unpublished results).

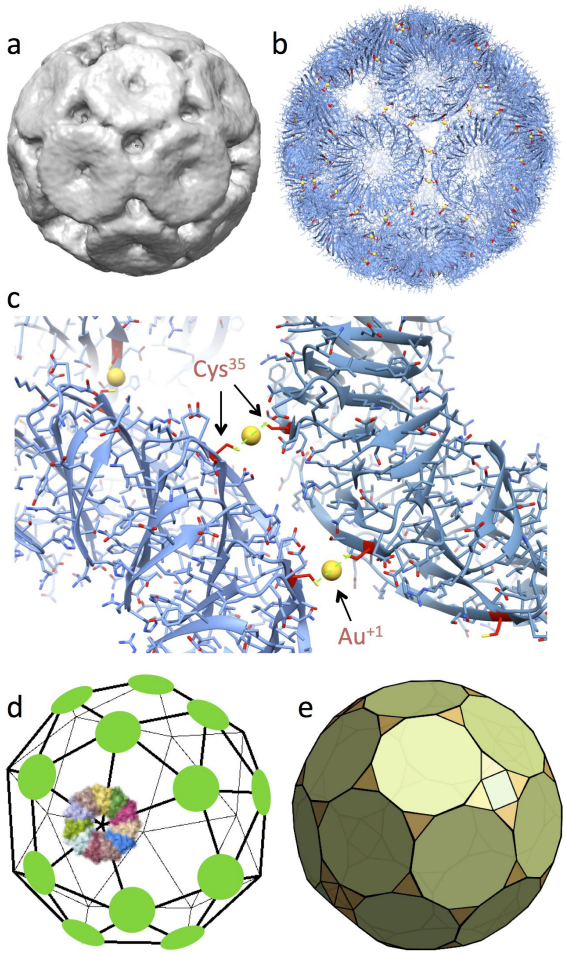

Fig. 2. Structure of TRAP-cage. (a) Cryo-EM density map, viewed down a 3-fold rotational axis. **(b)** Model of TRAP-cage, with 24 identical TRAP ring structures. **(c)** Close-up of the interactions between 2 adjacent rings in TRAP-cage; 120 such S-Au^{+1}-S linear coordination bonds hold the entire assembly together. **(d)** One C_{11}-symmetric TRAP ring is shown superimposed on a vertex of the Archimedean snub cube. **(e)** A geometric construction illustrating the near-perfect tiling of 24 11-sided polygons, resulting in a spherical shell.

(5) Evidence from the reaction stoichiometry, cryo-EM density maps, and structural modeling all suggest that the ring structures in the TRAP-cage are held together via the coordination of individual gold atoms between neighboring cysteine-thiolate groups (two linkages per pair of neighboring rings) via linear metal coordination complexes (S-Au^{+1}-S) (**Fig. 2c,** unpublished results).

(6) What is the explanation for the unusual symmetry of TRAP-cage? Theoretical studies typically state that, due to the rules of symmetry, only cages having exactly 12, 24, 60 (or multiples of 60) subunits arepermissible[8,9] but TRAP-cage has 264 subunits, and each "capsomer" has 11 subunits. The underlying mathematical concept has been previously explored in a paper on "symmetrohedra" by Kaplan and Hart[10]. It is shown that 11-sided polygons can be tiled almost perfectly into a sphere upon placement on the vertices of an Archimedean snub cube (**Fig. 2d-e**). In practical terms, this expands the possible strategies for self-assembling large, protein cage structures having regular, defined symmetry (unpublished results).

(7) Different experiments demonstrated the extreme stability of the TRAP-cage assembly. The cages retain their structural integrity between pH 4-13 (but denature at pH 3). DSC studies showed the cage structure to be stable up to 116 °C, much higher than the ring structures that unfold at 105-106 °C, and point to cooperative stabilization similar to viral capsids[11] (unpublished results).

(8) Attempts were made to encapsulate guest molecules, in the form of eGFP protein, within the interior of the TRAP-cage, but the final results were inconclusive. Further modifications of the TRAP / eGFP components, such as through the genetic incorporation of high-affinity interacting modules, would be expected to facilitate the efficient encapsulation of eGFP or other cargo of interest within the TRAP-cage cavity (**Fig. 3**).

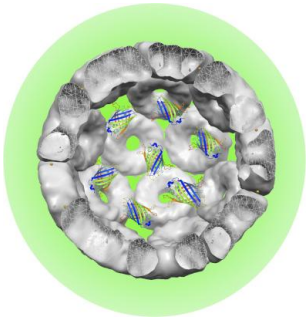

Fig. 3. Theoretical representation of eGFP guest proteins (ribbon structures) encapsulated within the cavity of TRAP-cage.

(9) To test the hypothesis regarding the mechanism of TRAP-cage assembly; *i.e.* interaction of Au_{55} clusters with the thiolate groups on $TRAP^{K35C}$ leads to the dissociation of Au^{+1} ions, that form linear coordination complexes with two flanking thiolates, we used the gold-based drug auranofin, which exert its inhibitory effects via related pathways[12,13]. Results are highly promising, with formation of TRAP-cage, although conditions require optimization (unpublished results).

(10) We expect to publish the research results in major journals (target: *Nature*).

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５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計 2 件）

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〔学会発表〕（計 4 件）

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(2) Malay AD. Development of a nano-delivery system based on an engineered protein cage structure. Asia-Pacific International Molecular Biology Network (A-IMBN) 17th Annual Conference, Manila, Philippines, December 1-2, 2014. (Invited Plenary Speaker)

(3) Imamura M, Malay AD, & Heddle JG. Live imaging of protein structural change by high-speed AFM. The 52nd Annual Meeting of the Biophysical Society of Japan, Sapporo Convention Center, Sapporo, Japan, September 25-27, 2014.

(4) Malay AD & Heddle JG. A new protein cage architecture assembled through gold cluster catalysis. The 28th Annual Symposium of the Protein Society, San Diego, CA, USA, July 27-30, 2014.

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