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Journal:	*ChemComm*
Manuscript ID	CC-COM-11-2018-009314.R1
Article Type:	Communication

Journal Name

COMMUNICATION

A Ring-shaped Hemoprotein Trimer Thermodynamically Controlled by the Supramolecular Heme–Heme Pocket Interaction

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Engineered cytochrome b_{562}, a small hemoprotein, with an externally-attached heme moiety via a moderately long linker at a suitable position predominantly forms a thermodynamically stable ring-shaped trimer in dilute solution. In an equilibrium between supramolecular polymerization and depolymerization, the ring-shaped trimer is kinetically trapped even in a concentrated solution.

Supramolecular assemblies provide unique structures and thermodynamic behaviors due to reversible interactions between building blocks. These properties are attractive for development of functional materials.[1] Biomolecules have significant potential for use as building blocks and investigations of artificial supramolecular protein assemblies have been recognized as an emergent topic over the past decade.[2,3] The construction of the protein assemblies appears to be driven in a similar manner as formation of natural protein assemblies via electrostatic, hydrogen-bonding and hydrophobic interactions between amino acid residues. Chemical approaches employing coordination bonding, ligand–protein and cofactor–protein interactions are also used. These strategies have been used to generate artificial protein assemblies with various structures including rings,[4] 2D sheets,[5] tubes[6] and cages.[7] Our group has focused on a specific interaction between heme (iron protoporphyrin IX) and the heme pocket of a hemoprotein to drive construction of the hemoprotein assemblies.[8] The apoprotein, with a covalently attached heme molecule on its surface at a different location, spontaneously forms a supramolecular hemoprotein assembly triggered by the heme–heme pocket interaction. This approach has demonstrated one-,[9] two-[10] and three-dimensional[11] structures of the assembled hemoproteins. In our recent study using cytochrome b_{562} (Cyt b_{562}), a simple electron transfer hemoprotein, the reaction of a maleimide-tethered heme analogue with an N80C mutant of Cyt b_{562} was found to provide a rigid supramolecular assembly via a successive heme–heme pocket interaction with additional secondary hydrogen bonding interactions at the protein interfaces.[12] The secondary interactions in the rigid assemblies are effectively generated by a short linker connecting the heme and maleimide moieties, which is synthetically derived from ethylene diamine.[13] In this work, we found that the moderately long linker between the heme and the maleimide moiety in the N80C-based assembling system induces the predominant formation of the ring-shaped protein assembly in dilute solution (Fig. 1).

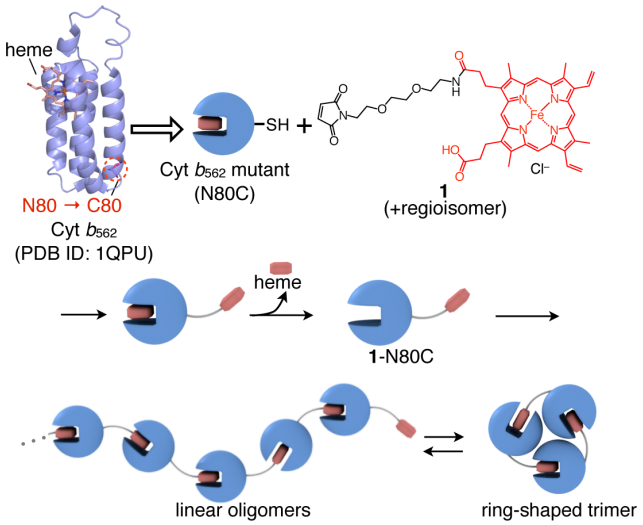

Fig. 1 Schematic representation of surface modification of an N80C mutant with maleimide-tethered heme **1** and two self-assembled states: linear oligomers and ring-shaped trimer.

Maleimide-tethered heme **1**,[8] which has a linker derived from 2,2'-(ethylenedioxy)bis(ethylamine), was reacted with the N80C mutant to yield the monomer unit, **1**-N80C (Fig. 1). The modified protein was characterized by LC-MS: found m/z = 1050.36, calcd m/z = 1050.61 (z = 12+) (Fig. S1). Analytical size exclusion chromatography (SEC) for **1**-N80C shows typical

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

profiles with a broad molecular weight distribution which was seen in our previous systems in concentrated solutions to form the **1**-N80C assembly (Fig. 2a: gray), whereas dilution of the protein solution increases the intensity of a peak which elutes at 10.9 mL (Fig. 2a: red). The addition of the apo-form of Cyt b_{562} to the solution of **1**-N80C assembly decreases the intensity of the 10.9 mL peak with concomitant increases of new adjacent peaks eluting at 10.5 and 11.7 mL (Fig. 2b).[14] Elution volumes of the monomer and linearly-assembled oligomers from dimer to hexamer were validated by the correlation between the estimated elution volumes against the logarithm values of the corresponding molecular weights (Fig. S2). It was then revealed that the component which increasingly forms upon dilution of the **1**-N80C solution elutes between the elution points of a linearly-assembled dimer (11.7 mL) and a trimer (10.5 mL). This behaviour is characteristic of **1**-N80C compared with a series of our other assembling systems.[15] To determine the molecular weight, we isolated the component eluted at 10.9 mL by SEC and performed ESI mass spectrometry measurements.[16] Interestingly, the mass number identified in the deconvoluted mass spectrum is consistent with the number calculated for the trimer of **1**-N80C (Fig. 3a,b), while the UV-vis spectrum of the isolated trimer as shown in Fig. 3b was almost consistent with that of wild type Cyt b_{562}, whereas slightly broad peaks were observed in UV-vis spectrum of the entire **1**-N80C assembly (Fig. 3c) due to the exposed heme moiety at the terminus.[8,9] This result suggests that the heme moiety is not exposed in the isolated trimer. Taken together, **1**-N80C is estimated to predominantly form a ring-shaped structure in a dilute protein solution. The dynamic and static light scattering measurements indicate that the hydrodynamic diameter and molecular weight of this structure are 6.47 nm and 38.0 kDa, respectively (Fig. S5). These values are generally consistent with expected values for the ring-shaped trimer.

The shape of the isolated characteristic trimer on the mica substrate was directly observed by high-speed atomic force microscopy (AFM). Images of ring-shaped objects with ca. 15-nm diameter were observed in the solution (see Fig. 4a and Movies S1 and S2). This structure is slightly larger than the hydrodynamic diameter determined from dynamic light scattering experiments. Considering the artifact caused by the interaction between the proteins and the flat substrate, we estimated a model for a flatly assembled ring-shaped trimer (Fig. S6). The simulated AFM image obtained by this model quite

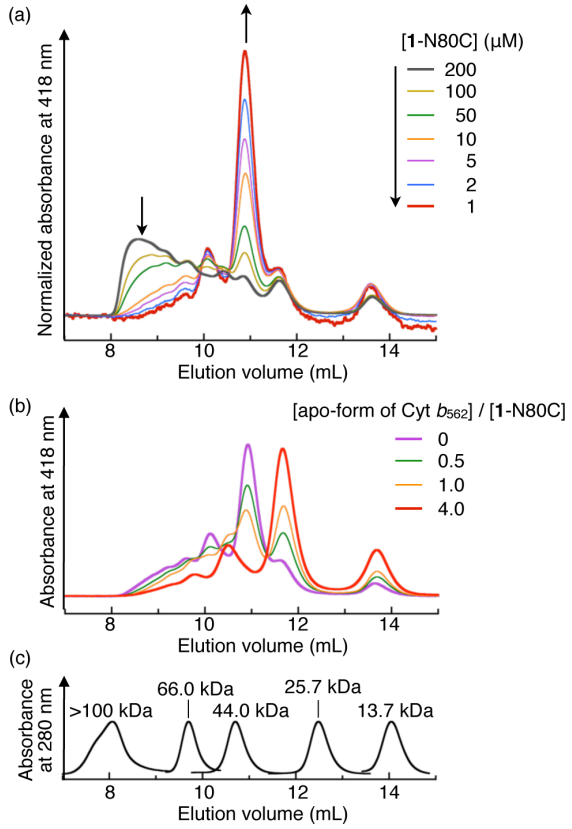

Fig. 2 (a) SEC traces of the **1**-N80C assembly at various concentrations. (b) SEC traces of the **1**-N80C assembly ([**1**-N80C] = 5 μM) formed upon capping by the apo-form of Cyt b_{562}. (c) authentic samples: blue dextran (>100 kDa), albumin (66.0 kDa), ovalbumin (44.0 kDa), chymotrypsin (25.7 kDa), ribonuclease (13.7 kDa). Conditions: eluent = 100 mM potassium phosphate buffer pH 7.0, temperature = 4 °C, column = Superdex™ 75 Increase, flow rate = 0.5 mL/min.

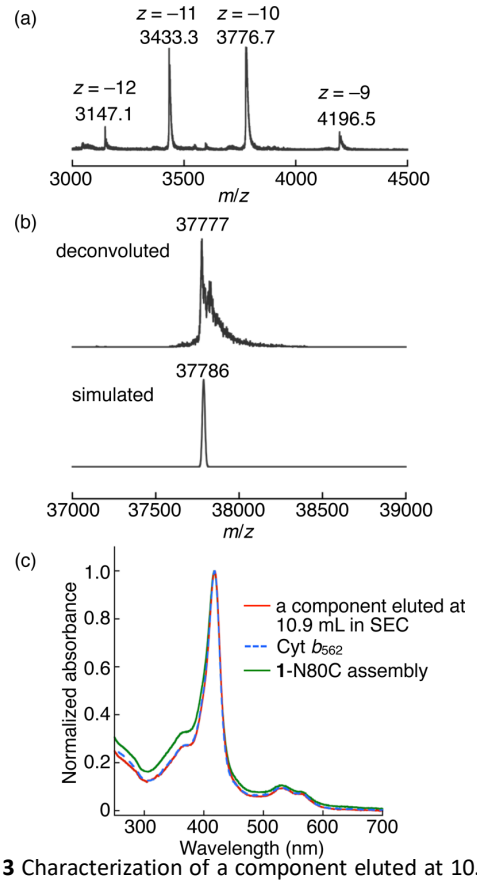

Fig. 3 Characterization of a component eluted at 10.9 mL isolated by SEC. (a) ESI mass spectrum in negative mode with the expected charge states. (b) Deconvoluted mass spectrum (top) and mass spectrum simulated as a trimer of **1**-N80C (bottom). (c) UV-vis spectra of the isolated component, **1**-N80C assembly and Cyt b_{562}.

matches the experimental results supporting the direct observation of the ring-shaped trimer by AFM (Fig. 4b,c).[17] In contrast to the isolated ring-shaped trimer, a sample observed immediately after dilution of concentrated **1**-N80C solution reveals the fibrous structures with submicrometer lengths (Movie S3). This behaviour is similar to the behaviour exhibited by previous systems using the short linker.[12] Structural changes occurring upon interactions with substrate require that the ring-shaped trimer is flexible. Evidence for such flexibility can be inferred from the circular dichroism (CD) spectrum of the Soret band region (wavelengths between 390-480 nm), which is similar to that of wild type of Cyt b_{562}. This result suggests that there is no heme–heme exciton coupling as seen in the flexible Cyt b_{562} assembly (Fig. S7).[12]

Thermodynamic stability of the isolated ring-shaped trimer, **1**-N80C assembly, and holo- and apo-forms of Cyt b_{562} was evaluated by observing CD signal changes at 222 nm during the denaturation which occurs upon addition of guanidinium chloride (GdmCl) as shown in Fig. S8. Table 1 summarizes the values of $[GdmCl]_m$, the concentration of GdmCl providing half denaturation. It is obvious that the ring-shaped trimer is more thermodynamically stable than the **1**-N80C assembly and the apo-form of Cyt b_{562}. Compared with holo-form of Cyt b_{562}, the ring-shaped trimer has a lower $[GdmCl]_m$ value, possibly due to the lack of the hydrogen bonding interaction of one of two propionate side chains with the protein matrix, the structural distortion and/or steric repulsion between proteins. The denaturation of the proteins which occurs upon increasing the temperature was also evaluated (Fig. S9). The values of T_m, the temperature in which half of the protein is unfolded, are

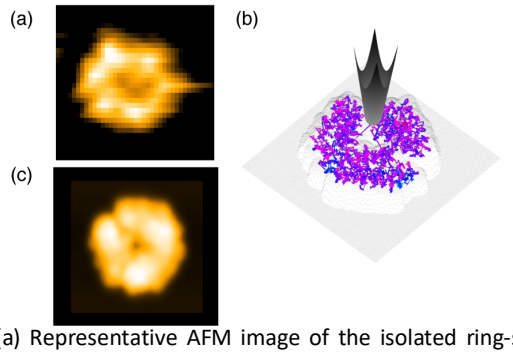

Fig. 4 (a) Representative AFM image of the isolated ring-shaped trimer. (b) AFM simulation of an energy-minimized model of the ring-shaped trimer (purple) on the substrate using a conical tip (gray). (c) Simulated AFM image. AFM images are depicted in the 20 nm x 20 nm squares.

Table 1 Denaturation parameters of ring-shaped trimer, **1**-N80C assembly, and apo- and holo-forms of Cyt b_{562}.[a]

	ring-shaped trimer	**1**-N80C assembly	apo-form of Cyt b_{562}	holo-form of Cyt b_{562}
$[GdmCl]_m$/M	1.8	1.4	1.0	2.1
T_m/°C	56.5	54.5	52.0	66.5

[a]Denaturation behaviors were evaluated by CD spectroscopic measurements (Fig. S7 and S8). $[GdmCl]_m$ and T_m are concertation of added GdmCl and temperature providing the half denaturation, respectively. Conditions: [protein] = 10 μM, solvent = 100 mM potassium phosphate buffer, pH7.0.

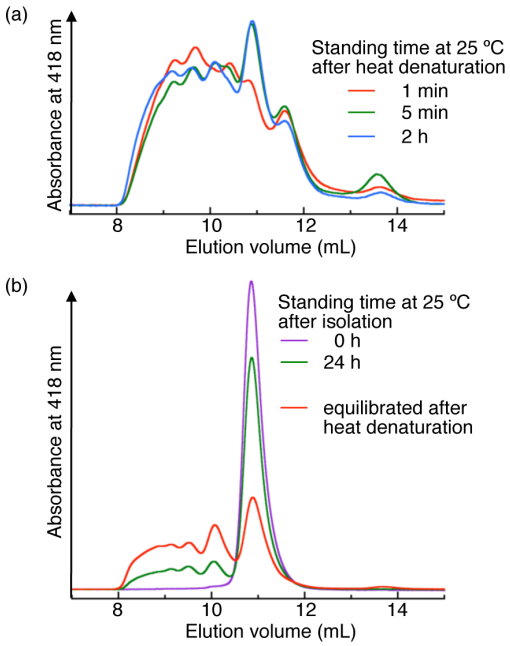

Fig. 5 (a) SEC traces of **1**-N80C cooled after heat denaturation. (b) SEC traces of the isolated ring-shaped trimer after concentration. Purple and green lines show the samples standing at 25 °C for 0 h and 24 h, respectively, after concentration. The red line shows the sample equilibrated after heat denaturation. Conditions: [**1**-N80C] = 20 μM in (a) and 100 μM in (b), other conditions are the same as described in Fig. 2.

summarized in Table 1 and the results are generally consistent with those obtained by denaturation using GdmCl. Both of the denaturation experiments indicate that the thermodynamic stability of the ring-shaped trimer is higher than that of **1**-N80C assembly. This stability could be caused by having the heme moieties fully incorporated within the protein matrix without exposure of the terminal structures in a linear oligomer.[8,9]

Heating of a solution of **1**-N80C appears to cause denaturation of the protein. The assembling behaviours of **1**-N80C cooled after the heat denaturation at 80 °C for 20 min were monitored by SEC measurements (Fig. 5). The SEC trace after a 1-min cooling period indicates the formation of oligomers including the transiently formed linear trimer eluted at 10.5 mL.[18] After a 5-min cooling period, the transiently formed linear trimer decreases and the ring-shaped trimer is predominantly formed. After 2 h, the size distribution is close to reaching equilibrium. These changes in the SEC profiles indicate that the ring-shaped trimer has a larger barrier to formation relative to the linear trimer,[19] but this barrier to formation is relatively low because the equilibration is almost complete within a 5-min cooling period.

Peak fitting of the SEC trace of the **1**-N80C assembly before heat denaturation was performed to estimate the population of each oligomer (Fig. S10). The population of the linear trimer was found to be ca. 10% of the ring-shaped trimer, indicating that the ring-shaped trimer is more thermodynamically stable relative to the linear trimer. The isolated ring-shaped trimer is kinetically stable because the isolated solution can be concentrated up to 100 μM and maintain the ring-shaped

trimer at 25 °C (Fig. 5). After allowing the solution to stand for 24 h at 25 °C, ca. 70% of the ring-shaped trimer remains. After the heat denaturation and subsequent cooling of the isolated ring-shaped trimer, the SEC trace shows a mixture of the ring-shaped trimer and higher oligomers, which is similar to that of **1**-N80C assembly. These findings indicate three thermodynamic and kinetic characteristics of the **1**-N80C assembly: (i) the ring-shaped trimer is thermodynamically more stable than the linear oligomers in dilute solution; (ii) Especially, the linear trimer is much more unstable than the ring-shaped trimer; and (iii) the kinetic barrier from the linear trimer to the ring-shaped trimer is low, whereas the kinetic barrier of the reverse reaction is relatively high and provides kinetic stability to the ring-shaped trimer even in a concentrated solution.

In summary, the present hemoprotein assembling system prepared via the heme–heme pocket interaction is found to form a thermodynamically stable ring-shaped structure. The chain-ring equilibrium generally occurs in a broad distribution in supramolecular polymerization of the simple molecules/proteins.[1,4a] In contrast, the specific and unique stabilization of the ring-shaped trimer is generated in this system due to the structural factors derived from the structure of Cyt b_{562}, the heme attachment position and the length of the linker between the heme moiety and the protein. Further detailed investigations of the mechanism of formation of the unique ring-shaped structure are expected to contribute to the construction of supramolecular protein assemblies.

This work was supported by Grants-in-Aid for Scientific Research provided by JSPS KAKENHI Grant Numbers JP15H05804, JP15H00873, JP16K14036, JP16H06045, JP16H00837, JP18KK0156, JP16H00758 and JP18H04512. We appreciate support from JST PRESTO (JPMJPR15S2) and SICORP.

Conflicts of interest

There are no conflicts to declare.

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13 The detailed molecular structure of the rigid assembly using a short linker is displayed in Fig. S4.

14 Our previous work shows that the addition of 20 equiv of apo-form of Cyt b_{562} mostly dissociates the assembling system into the dimer and denatured monomer.[9c]

15 In the assembling system obtained by combining heme **1** with the H63C mutant, which has a different heme-attached position, the dilution increases the monomeric component (Fig. S3). A rigid assembly formed by the N80C mutant reacted with maleimide-tethered heme via a short linker indicates a simple shift of molecular weight distribution toward a smaller distribution upon dilution (Fig. S4).

16 The isolated component is kinetically stable against concentration in the further evaluations (vide infra).

17 Similar triangular assembly of an engineered cytochrome cb_{562} mediated by Ni coordination has been reported, indicating that Cyt b_{562} intrinsically possesses a tendency to form the trimer.[4d]

18 Although the SEC measurement takes over 20 min, the obvious and definite peaks observed in SEC traces indicate that the oligomeric components have already formed before the injection. Therefore, it rules out the possibility of the drastic changes of the molecular weight distribution during the SEC measurement. If the oligomerization could occur during the measurement, a broad and undefined trace would be obtained.[9c]

19 This kinetic barrier appears to be generated by the structural changes because the CD signal of the isolated ring structure in the far-UV region is slightly weaker relative to the CD signals of the **1**-N80C assembly and Cyt b_{562} (Fig. S7).