*Supporting Information*

# A ring-shaped hemoprotein trimer thermodynamically controlled by the supramolecular heme–heme pocket interaction

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## Materials and Methods

Instruments: ESI-TOF MS analyses were performed with a Bruker Daltonics micrOTOF II mass spectrometer. UV-vis spectra were measured with a Shimadzu BioSpec-nano or Shimadzu UV-3600 plus double-beam spectrometer. Circular dichroism (CD) spectra were recorded with a JASCO J-820S spectrometer. Size exclusion chromatographic (SEC) analyses were performed with an ÄKTA Pure 25 system (GE Healthcare) at 4 ºC. Dynamic light scattering (DLS) and static light scattering (SLS) measurements were performed using a Zetasizer μV (Malvern Instruments). pH measurements were made with an F-72 Horiba pH meter.

Materials: Maleimide-tethered heme derivatives **1** and **2** were prepared according to procedures reported in the literature.[S1] Cyt $b_{562}$ and its mutants were prepared by our previously reported methods.[S2] Other reagents and chemicals were purchased and used as received. Unless mentioned otherwise, all protein solutions were dissolved in a 100 mM potassium phosphate buffer (pH 7.0). Deionized water was prepared using a Millipore Integral3 apparatus.

## Protein Sequence of Cytochrome $b_{562}$ Mutants

Cyt $b_{562}$

ADLEDNMETLNDNLKVIEKADNAAQVKDALTKMRAAALDAQKATPPKLEDKSPDSPEMK
DFRHGFDILVGQIDDALKLANEGKVKEAQAAAEQLKTTRNAYHQKYR

N80C

ADLEDNMETLNDNLKVIEKADNAAQVKDALTKMRAAALDAQKATPPKLEDKSPDSPEMK
DFRHGFDILVGQIDDALKLACEGKVKEAQAAAEQLKTTRNAYHQKYR

H63C

ADLEDNMETLNDNLKVIEKADNAAQVKDALTKMRAAALDAQKATPPKLEDKSPDSPEMK
DFRCGFDILVGQIDDALKLANEGKVKEAQAAAEQLKTTRNAYHQKYR

## Preparation of Surface Modified Cytochrome $b_{562}$ Mutants

The synthesized Fe porphyrin, **1** (0.1 mg) in a DMSO solution (0.1 mL) was added to 166 μM of reduced N80C mutant solution. The mixture was then gently stirred at room temperature for 75 min under an $N_2$ atmosphere. Then, reaction mixture was acidified using 1 M HCl for denaturation. The native heme and excess **1** were removed from aqueous phase by 2-butanone extraction performed five times. The solution was dialyzed against 1 L of 100 mM potassium phosphate buffer (pH 7.0). The obtained assembly, **1**-N80C assembly, was then concentrated using an Amicon Ultra concentrator (Merck Millipore) and stored at –80 °C. The obtained protein units were characterized by ESI-TOF MS, UV-vis spectroscopy and size exclusion chromatography using Superdex$^{TM}$ 75 Increase 10/300 GL and/or 200 Increase 10/300 GL (GE healthcare). Other assemblies, **2**-N80C assembly and **1**-H63C assembly, were prepared and characterized according to the same procedure for **1**-N80C assembly and characterized by ESI-TOF MS: **1**-N80C (*m/z* calcd: 1050.61 ($z$ = +12), found: 1050.30), **2**-N80C (*m/z* calcd: 1044.11 ($z$ = +12), found: 1043.88), **1**-H63C (*m/z* calcd: 1048.69 ($z$ = +12), found: 1048.43)

**SEC Analysis**

The analysis was performed using a Superdex™ 75 Increase 10/300 GL (GE Healthcare) column or Superdex™ 200 Increase 10/300 GL (GE Healthcare) column with a flow rate of 0.5 mL/min at 4 ºC with monitoring of the absorbance at 418 nm and 280 nm for detection. The column was validated using the following reagents (Gel Filtration Calibration kits HMW and LMW, GE Healthcare): Superdex™ 200 Increase: thymoglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), albumin (66.0 kDa), ribonuclease A (13.7 kDa); Superdex™ 75 Increase: blue dextran 2000 (2000 kDa), albumin (66.0 kDa), ovalbumin (44.0 kDa), chymotrypsin (25.7 kDa), ribonuclease A (13.7 kDa).

**Light Scattering Analysis**

To analyze the protein solution, a 12 μL quartz cuvette was utilized. Before adding the sample solution into the cuvette, the sample was filtered with a Millex-GV 0.22-μm filter (Merck Millipore). The cuvette was inserted into the apparatus for 30 seconds of equilibration at the 25 °C before measurements were performed. The wavelength of the laser irradiation was 830 nm. The scattering measurement angle was 90°. For dynamic light scattering (DLS) measurements, the autocorrelation functions of the observed signals were analyzed in protein analysis mode. The hydrodynamic diameters of the proteins were obtained using the scattering intensity-based particle size distribution mode (Fig. S5a).

For static light scattering (SLS) measurements, the Rayleigh ratio ($R_\theta$) of the samples with different protein concentrations was analyzed from the time-averaged scattering intensity of the samples and toluene as a standard. The obtained $R_\theta$ values were analyzed with the Zimm plot equation[S3]

$$\frac{Kc}{R_\theta} = \frac{1}{M_w}\left(1 + \frac{q^2 R_g^2}{3}\right) + 2A_2 c \qquad \text{(S1)}$$

where $K$ is an optical constant, $c$ is the sample concentration, $M_w$ is the weight-average molecular weight, $q$ is the scattering vector, $R_g$ is the radius of gyration, $A_2$ is the second osmotic virial coefficient, and $K$ is given as

$$K = \frac{4\pi^2 n_0^2}{N_A \lambda^4}\left(\frac{dn}{dc}\right)^2 \qquad \text{(S2)}$$

where $\lambda$ is the wavelength of the light, $N_A$ is Avogadro's number, $n_0$ is the refractive index of the medium, and $dn/dc$ is the change in refractive index with sample concentration. In the case of a small protein ($R_g$ < 10 nm), the $qR_g$ factor is quite small ($qR_g \ll 1$) enough to regard as negligible and the Zimm plot equation can be expressed as:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w} + 2A_2 c \qquad \text{(S3)}$$

**Modeling of the Ring-shaped Trimer**

The modeling was performed using YASARA[S4] Structure Version 13.6.16 that employed force field AMBER03[S5] for protein residues and GAFF[S6] using AM1/BCC[S7] partial charges for the heme moiety covalently bound to Cys80. The calculation was carried out using YASARA Structure Version 13.6.16 employing force field AMBER03. To maintain the correct coordination geometry, the distances from the metal to all four pyrrole N atoms of the porphyrin ligand were constrained to 2.0 Å. The hexa-coordination by ligation with Met7 and His102 was represented by two force field arrows and the distances of the Fe–S and Fe–N dative bonds were constrained to 2.3 and 2.0 Å, respectively, according to the NMR structure (PDB ID: 1QPU) of Cyt $b_{562}$. The partial charge of the metal was set to +2 and the total charge of the heme was set to zero. Manually constructed ring-shaped trimer was solvated in a box of TIP3P water molecules using periodic boundaries at pH 7.0 and a density of 0.997 g/mL. The minimized structure was obtained by steepest descent minimization and simulated annealing at 298 K.

**High-Speed Atomic Force Microscopy (HS-AFM) Imaging**

HS-AFM imaging was carried out using a laboratory-designed HS-AFM apparatus operated with tapping mode.[S8] The dimensions of the cantilever (Olympus) were 6−7 μm length, 2 μm width, and 90 nm thickness. The nominal spring constant was 0.1−0.2 N/m, the resonant frequency was 0.7−1 MHz, and the quality factor in aqueous solution was ca. 2. For the HS-AFM imaging, the free oscillation amplitude was set to 1−2 nm, and the set-point amplitude was approximately 90% of the free oscillation amplitude. An amorphous carbon tip grown by electron-beam deposition under scanning electron microscopy was used.[S9] After the electron-beam deposition, the tip was sharpened by plasma etching under Ar gas (tip apex, d = ca. 4 nm).

In the HS-AFM imaging and processing, mica was used as a substrate. The solution (3 μL) of isolated ring-shaped trimer (1 μM as monomer) or **1**-N80C assembly (0.24 μM as monomer) soon after dilution of concentrated solution (1.2 mM as monomer) was deposited onto the freshly cleaved mica substrate. After the 3-min incubation, the substrate was thoroughly washed with a buffer (100 mM potassium phosphate buffer, pH 7.0) to remove excess molecules. The HS-AFM imaging was performed in the buffer at room temperature.

**Simulation of AFM images**

Simulation of AFM images was performed using custom software based on IgorPro 6 (Version 6.3.6.0, Wavemetrics, Lake Oswego, Ore., USA) software. The pseudo AFM image was generated using a cone-shaped AFM tip with a radius of 0.5 nm and half cone angle of 10°, and the energy minimized structure of the ring-shaped trimer. During the simulation, the orientation of the ring-shaped trimer was rotated along the longitudinal axis of the shape and adjusted so that the appearance of the simulated image was close to the experimental AFM image. After construction of the simulated image, the image was filtered by a low-pass filter with a cut off spatial frequency of 2 nm because the spatial resolution of the HS-AFM is generally limited to 2 nm.

**Fitting Analysis of SEC Chromatogram**

The peak fitting was performed using IgorPro (Version 6.1.2.1). The elution peak of each oligomer in SEC was fitted using the Gaussian distribution function. The initial fitting values for lower molecular weight oligomers (monomer to pentamer) were determined from the exact peak top values in SEC and FWHW. The initial fitting values for peaks of oligomers longer than hexamer were assigned using peak top values calculated from the relationship between the molecular weight of monomer to pentamer and elution peak top values (Fig. S2).

**Supplementary Figures**

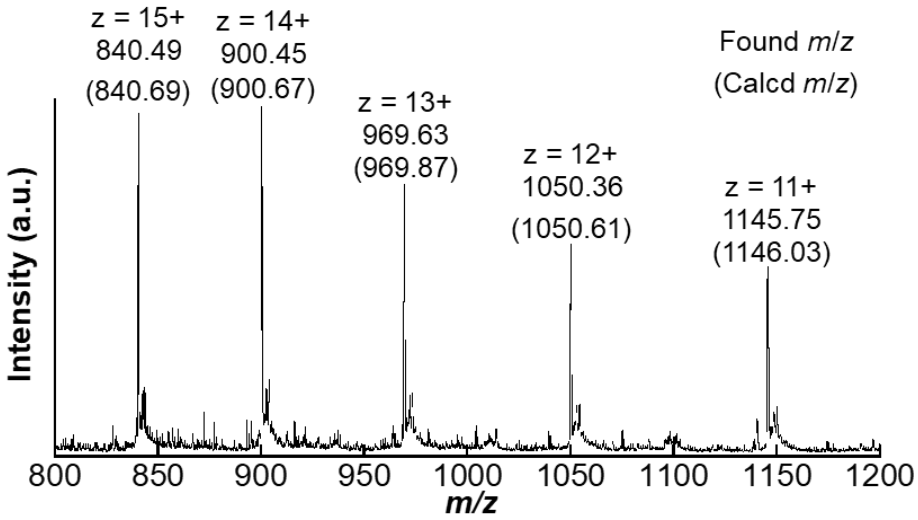


**Fig. S1** ESI-mass (positive mode) spectrum for **1**-N80C.

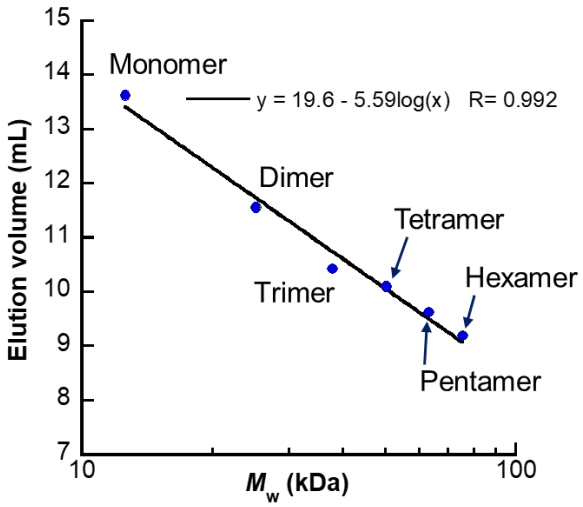


**Fig. S2** Plots of the estimated elution volumes of monomer and linear oligomers of **1**-N80C against the logarithm values of the corresponding molecular weight. The linear correlation in the plots indicates that the estimation is plausible.

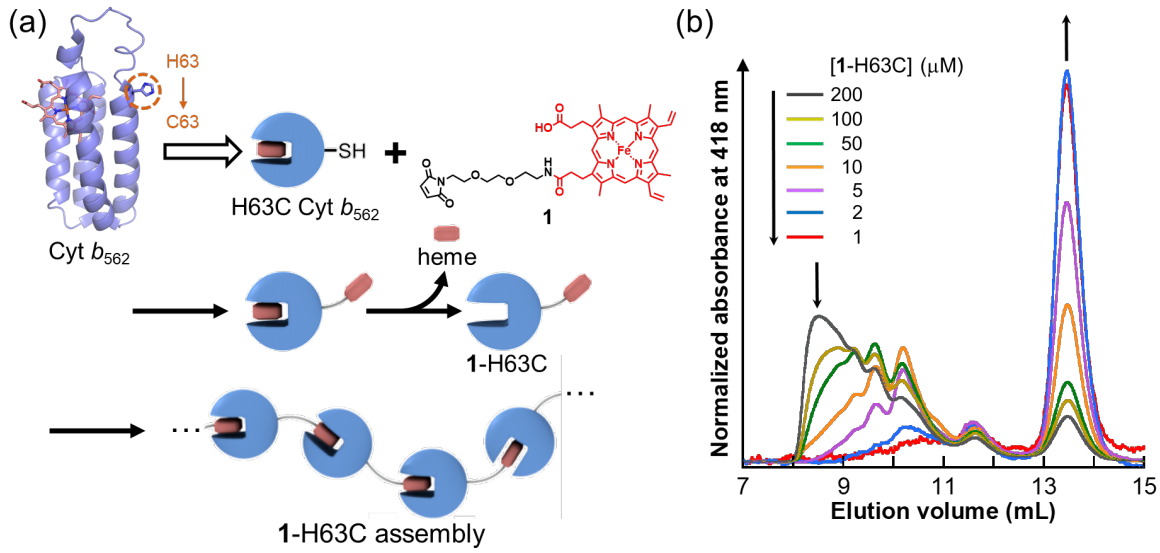


**Fig. S3** (a) Schematic representation for preparation of **1**-H63C assembly. (b) SEC traces at various concentrations.

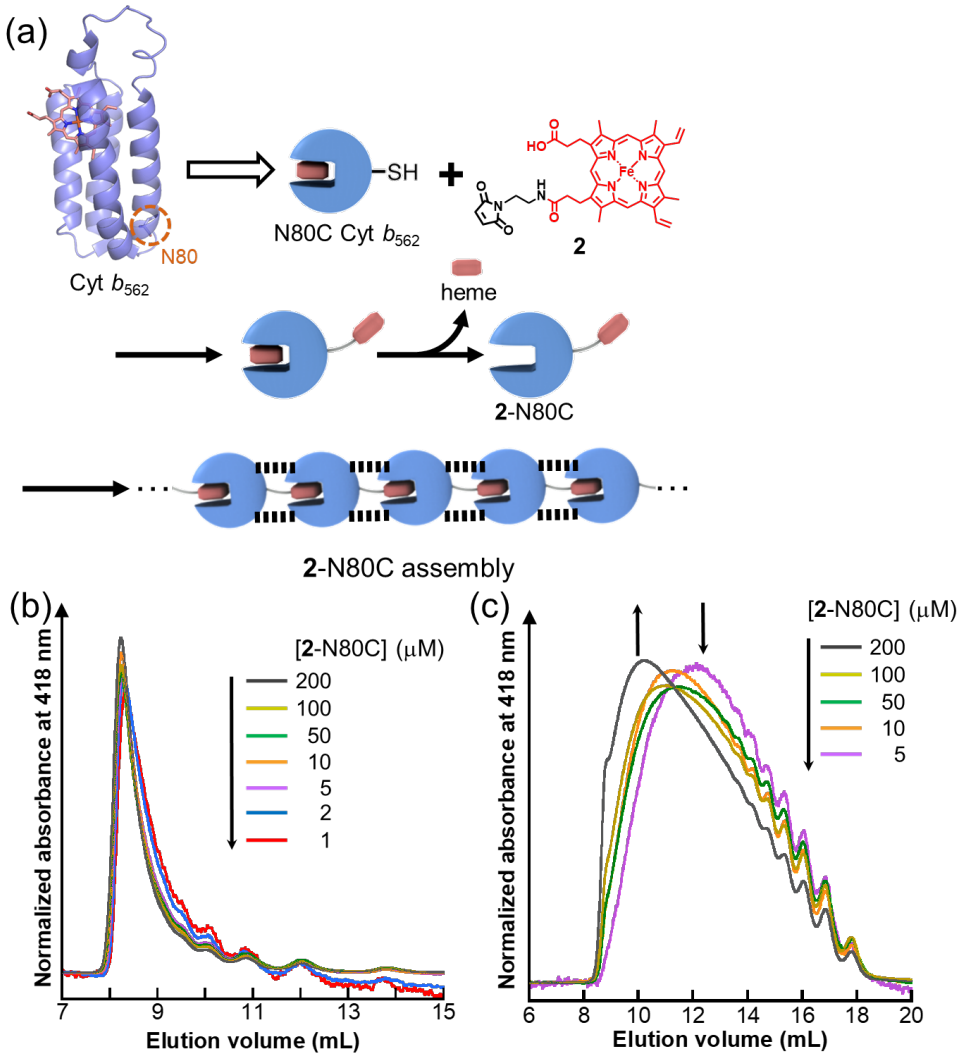


**Fig. S4** (a) Schematic representation for preparation of **2**-N80C assembly. (b) SEC traces at various concentrations using Superdex$^{TM}$ 75 Increase column. (c) SEC traces at various concentrations using Superdex$^{TM}$ 200 Increase column.

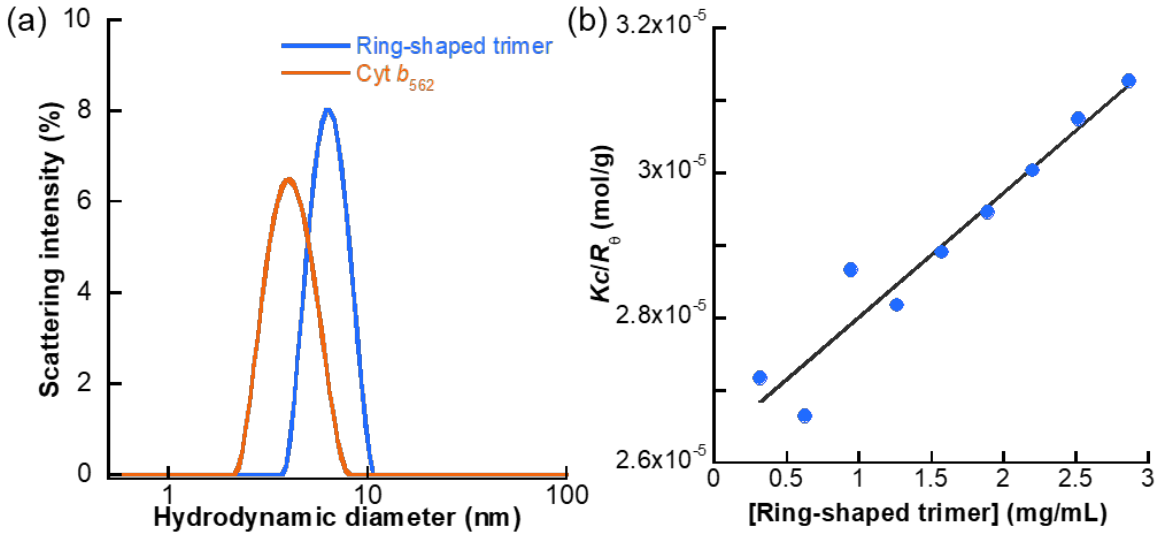


**Fig. S5** (a) Diameter distribution of Cyt $b_{562}$ and the isolated ring-shaped trimer by dynamic scattering measurements. Conditions: [Monomer] = 100 mM, in 100 mM potassium phosphate buffer (pH 7.0) (b) Zimm plots obtained from static light scattering results of the isolated ring-shaped trimer. Protein concentration was calculated using the monomer concentration determined from the UV-vis spectrum and the molecular weight of the monomer unit. The optical constant, $K$, was calculated using $dn/dc$ = 0.2 mL/g.

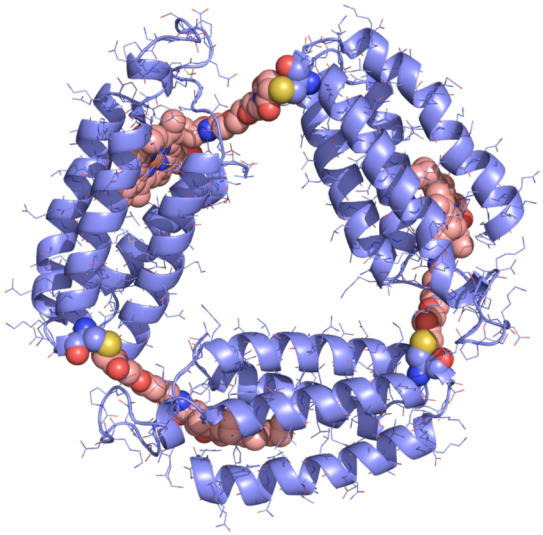

**Fig. S6** An energy-minimized model of the ring-shaped trimer. The locations of the C80 residue and **1** are highlighted with red spheres.

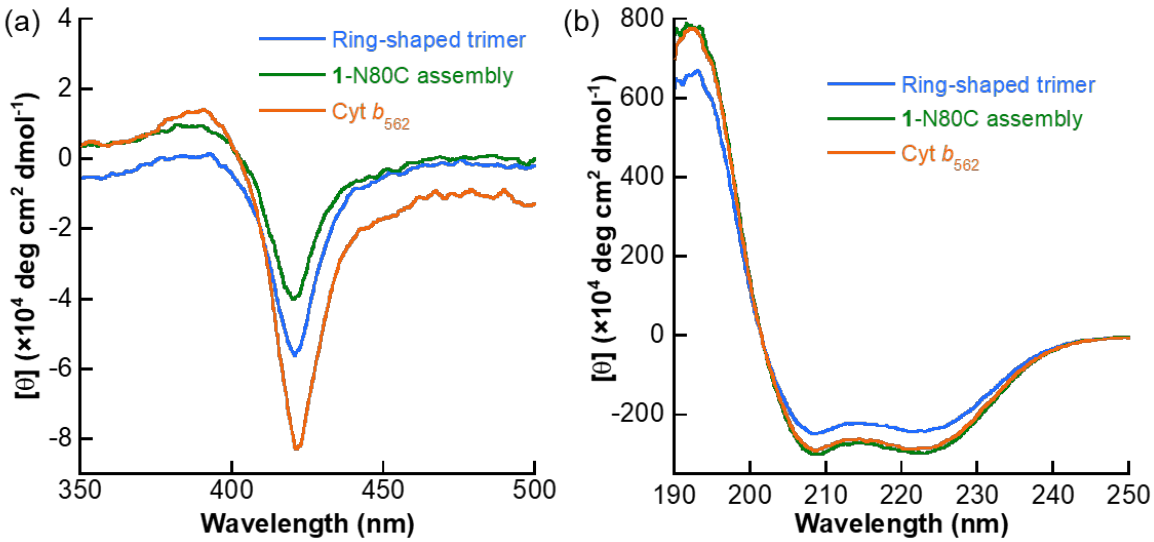


**Fig. S7** CD spectra of isolated ring-shaped trimer, **1**-N80C assembly and Cyt $b_{562}$. (a) Soret band region. (b) Far-UV region.

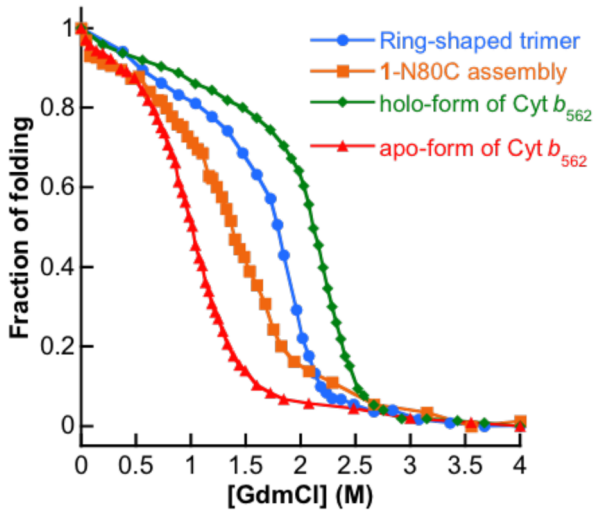


**Fig. S8** Fraction changes of folded proteins obtained by CD signal changes at 222 nm upon addition of GdmCl.

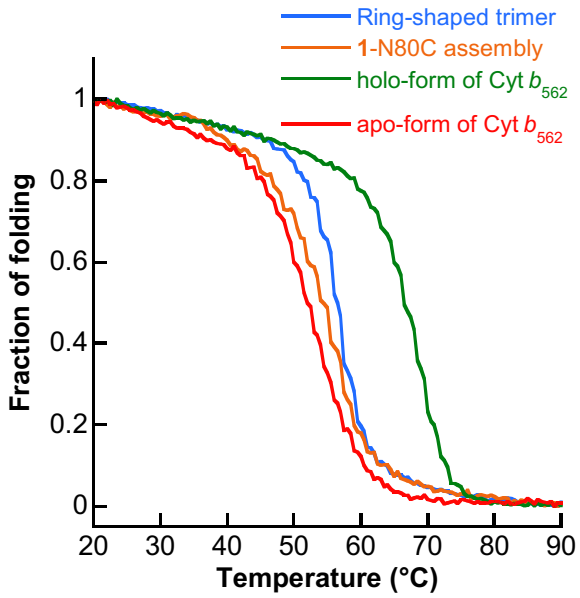


**Fig. S9** Fraction changes of folded proteins obtained by CD signal changes at 222 nm by increase of temperature.

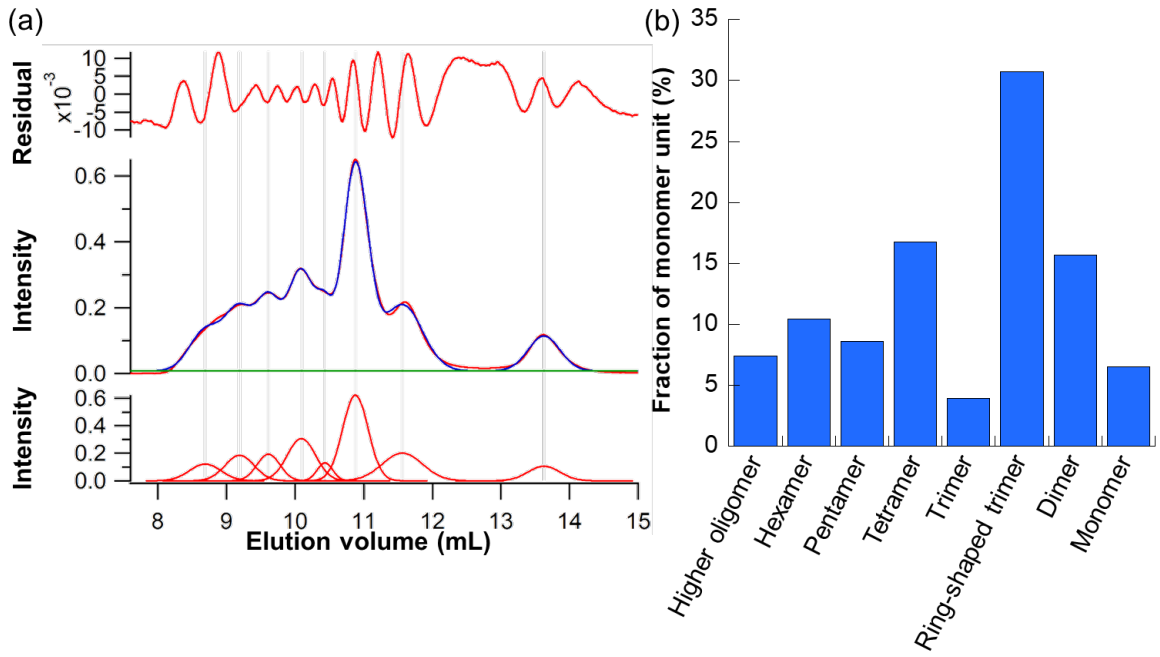


**Fig. S10** (a) Fitting results of the SEC trace of the **1**-N80C assembly. (b) Population of each oligomer estimated from the fitting results.

**Captions for Supporting Online Movies.**

**Movie S1.** Representative high-speed AFM images for isolated ring-shaped trimer. Scan area: 70 × 56 $nm^2$ 300 × 240 $pixels^2$). Frame rate: 10 fps.

**Movie S2.** Representative high-speed AFM images for isolated ring-shaped trimer. Scan area: 500 × 500 $nm^2$ 450 × 450 $pixels^2$). Frame rate: 2 fps.

**Movie S3.** Representative high-speed AFM images for **1**-N80C assembly. Scan area: 100 × 100 $nm^2$ 360 × 360 $pixels^2$). Frame rate: 6.7 fps.

**References**

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