Supporting Information

Construction of ligand-binding controlled hemoprotein assemblies utilizing 3D domain swapping

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Methods

Amino acid sequences of designed proteins

AVCP: MKKIWLALAGLVLAFSASAAGLSPEEQIETRQAGYEFMGWNMGKIKANLEGEYNAA
QVEAAANVIAAIANSGMGALYGPGTDKNVGDVKTRVKPEFFQNMEDVGKIAREFVGAANT
LAEVAATGEAEAVKTAFGDVGAACKSCHEKYRAK

CPC: MRKSLLAILAVSSLVFSSASFAVDPAKEAIMKPQLTMLKGLSDAELKALADFILRIAKQA
QEKQQQDVAKAIFQQKGCGSCHQANVDTVGPSLAKIAQAYAGKEDQLIKFLKG EAPAI

FP: MKKIWLALAGLVLAFSASAAGLSPEEQIETRQAGYEFMGWNMGKIKANLEGEYNAAQ
VEAAANVIAAIANSGMGALYGPGTDKNVGDVKTRVKPEFFQNMVDPAKEAIMKPQLTMLK
GLSDAELKALADFILRIAKQAQEKQQQDVAKAIFQQKGCGSCHQANVDTVGPSLAKIAQAY
AGKEDQLIKFLKGEAPAIEDVGKIAREFVGAANTLAEVAATGEAEAVKTAFGDVGAACKSC
HEKYRAK

The periplasmic targeting signal amino acid sequences are underlined. Blue: N-terminal region of AVCP; red: C-terminal region of AVCP; green: N-terminal region of CPC; yellow: C-terminal region of CPC; magenta: α-helical linker.

Preparation of FP

The pKK223-3 plasmid containing the gene of FP was enhanced with *E. coli* DH5α cells, and transformed into *E. coli* JCB387 cells containing the pEC86 plasmid. The pEC86 plasmid contained the cytochrome *c* maturation (CCM) genes. The *E. coli* JCB387 cells were cultured with the reported condition[1] and harvested by centrifugation. The cells were suspended with 100 mM Tris-HCl buffer, pH 7.0, containing 10 mM EDTA and 20% (w/v) sucrose, subsequently incubated on ice for 15 min, and centrifuged. The obtained cells were suspended with pure water, subsequently incubated on ice for 15 min, and centrifuged. The supernatant was dialyzed overnight with 25 mM sodium acetate buffer, pH 5.0, at 4 °C. The oxidised FP was prepared by adding excess potassium ferricyanide to the protein solution. FP was purified with a cation exchange column (SP Sepharose Fast Flow, Cytiva) with 25 mM sodium acetate buffer, pH 5.0, containing 25 mM and 300 mM NaCl for removing unnecessary proteins and eluting FP, respectively. After 5-times dilution of the sample solution with 25 mM sodium acetate buffer, pH 5.0, FP was further purified with a cation exchange column (HiTrap SP HP, Cytiva) using a fast protein liquid chromatography (FPLC) system (AKTA go, Cytiva) with a NaCl concentration gradient, 0−300 mM, and a flow rate of 1.0 mL/min at 4 °C. The monitoring wavelength was 280 nm. The eluent was 25 mM sodium acetate buffer, pH 5.0. After purification with cation exchange chromatography, FP was purified with a size exclusion column (HiLoad 26/60 Superdex 75

pg, Cytiva) using the FPLC system (AKTA go, Cytiva) with a flow rate of 1.5 mL/min at 4 °C. The monitoring wavelength was 280 nm. The eluent was 50 mM potassium phosphate buffer, pH 7.0.

Preparation of the FP 3D-DS dimer

Ethanol was added to the FP interface dimer solution to a final concentration of 40% (v/v) at room temperature. Concentration of FP after addition of ethanol was 250 μM. The solution was lyophilised, and the resulting lyophilised precipitate was dissolved in 500 μL of 50 mM potassium phosphate buffer, pH 7.0, at room temperature. After removal of precipitate by filtration, the FP 3D-DS dimer was separated from the monomer and high order oligomers with a SEC column (HiLoad 26/600 Superdex 200 pg, Cytiva) using the FPLC system (AKTA go, Cytiva) with a flow rate of 1.5 mL/min at 4 °C. The eluent was CO-bubbled 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM dithionite. The monitoring wavelength was 280 nm. The fraction of the FP 3D-DS dimer was further purified with the same SEC column. The concentrations of the proteins were calculated from the absorbances of the Soret band using the coefficients of oxidised FP (8.6×10^4 $M^{-1}cm^{-1}$) and the FP 3D-DS dimer (8.6×10^4 $M^{-1}cm^{-1}$) obtained by the pyridine hemochrome method.[2]

UV-vis absorption measurement

UV-vis spectra of oxidised AVCP, CPC, FP, and FP 3D-DS dimer (3 μM in heme unit) in 50 mM potassium phosphate buffer, pH 7.0, were obtained with a UV-2450 spectrophotometer (Shimadzu, Japan) at 25 °C. Reduced FP and FP 3D-DS dimer solutions were prepared by adding excess sodium dithionite to protein solutions under a N_2 atmosphere. Binding of CO to reduced FP and FP 3D-DS dimer was performed by incubation of the reduced samples at 25 °C under a CO atmosphere.

CD measurements

CD spectra of oxidised AVCP, CPC and FP (14 μM, 18 μM, and 9 μM, respectively) were obtained with a JASCO J-715 spectrometer (JASCO, Japan) using a 0.1 cm path length quartz cell in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C. CD ellipticity changes at 222 nm of AVCP, CPC, and FP (heme unit, 14 μM, 18 μM, and 9 μM, respectively) were measured in 50 mM potassium phosphate buffer, pH 7.0, from 20 to 80 °C with a JASCO J-715 spectrometer (JASCO, Japan) using the 0.1-cm path length quartz cell. Temperature increase speed was 1 °C/min. T_m values of AVCP and FP were determined using the CDpal program.[3]

SEC analysis

Purified AVCP, FP, and FP 3D-DS dimer were analyzed with a SEC column (Superdex 75 Increase 10/300, Superdex 200 Increase 10/300 or Superdex 200 Increase 5/150, Cytiva) using a FPLC system (DuoFlow, Biorad, or AKTApureμ, Cytiva) for measurements at 4 °C and a prominence HPLC system (Shimadzu), for measurements at room temperature with a flow rate of 0.35 mL/min at 4 °C or a flow rate of 0.5 mL/min at room temperature, respectively. The monitoring wavelength was 410 nm,

corresponding to the absorption of heme. Eluents were 50 mM potassium phosphate buffer, pH 7.0, the same buffer containing 5 mM sodium dithionite and bubbled with CO, or the same buffer containing 10 mM imidazole.

Titration of imidazole

Imidazole solution (5 mM) were added to oxidised FP and FP 3D-DS dimer (4.5 μM and 3.4 μM, respectively) in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C. UV-vis spectra were measured with the UV-2450 spectrophotometer (Shimadzu) at 25 °C.

Size exclusion chromatography with multi angle light scattering measurement (SEC-MALS)

The FP 3D-DS dimer was analyzed by SEC-MALS with a Superdex 200 Increase 10/300 column in line with a miniDAWN detector (Wyatt Technology Corporation) and RID-20A refractive index detector (Shimazu). The column was equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM imidazole at room temperature and operated at a flow rate of 0.5 mL/min. A total volume of 100 μL of the FP 3D-DS dimer solution at 25 μM in monomer unit was employed for each sample. Data processing and analysis were proceeded with the Astra X software (Wyatt Technologies), for which a typical dn/dc value for proteins (0.185 mL/g) was assumed.

High-speed atomic force microscopy (AFM) imaging

High-speed AFM imaging was conducted using a laboratory-built apparatus. The Olympus AC7 cantilever was employed as the AFM probe. The AFM probe was sharpened through carbon pillar formation by electron beam deposition under an organic gas atmosphere using a field emission electron microscope, followed by Ar plasma etching. A chemically modified mica surface was used as the substrate for immobilizing the molecules. Initially, 3-(Trimethoxysilyl)propylamine (0.01%) was drop-cast onto cleaved mica and incubated for 3 min, followed by rinsing with ultrapure water. Subsequently, glutaraldehyde (0.2%) was drop-cast, and after 3 min of incubation, it was rinsed with an observation buffer (100 mM potassium phosphate buffer, pH 7.0). A solution of the FP 3D-DS dimer (25 μM in monomer unit) was added in 3 μL drops. After 3 min of incubation, high-speed AFM imaging was conducted at room temperature in the observation buffer.

Structure prediction of the FP 3D-DS dimer with AlphaFold2

Ring-shaped and linear structures of $(\text{FP 3D-DS dimer})_2$ were obtained with ColabFold using a set of four manually constructed FP molecules as a template.[4,5] LocalColabFold (https://github.com/YoshitakaMo/localcolabfold) was used to operate ColabFold with following specified options: “--msa_mode mmseqs2_uniref_env”“--model_type alphafold2_multimer_v3""--amber””--use-gpu-relax””--custum-template-path”.

Simulation of AFM image

Simulated AFM images were generated utilizing collision simulations between the atomic coordinates of protein structures and modeled AFM probes. The probe employed in the simulations was designed as a cone shape with a 15-degree cone angle and a tip radius of 2.0 nm. To imitate the spatial resolution characteristic of experimental high-speed AFM images, we applied a low-pass filter with a 2 nm cutoff spatial frequency to the simulated images. These simulated AFM images were produced using a customized high-speed AFM data analysis tool, developed in Igor Pro 9 (WaveMetrics Inc.).

Reference

1. S. Fujii, M. Masanari, H. Inoue, M. Yamanaka, S. Wakai, H. Nishihara and Y. Sambongi, *Biosci. Biotechnol. Biochem.*, 2013, **77**, 1677-1681.
2. R. G. Bartsch, *Methods Enzymol.*, 1971, **23**, 344-363.
3. M. Niklasson, C. Andresen, S. Helander, M. G. Roth, A. Zimdahl Kahlin, M. Lindqvist Appell, L. G. Martensson and P. Lundstrom, *Protein Sci.*, 2015, **24**, 2055-2062.
4. J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Zidek, A. Potapenko, A. Bridgland, C. Meyer, S. A. A. Kohl, A. J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A. W. Senior, K. Kavukcuoglu, P. Kohli and D. Hassabis, *Nature*, 2021, **596**, 583-589.
5. R. Evans, M. O'Neill, A. Pritzel, N. Antropova, A. Senior, T. Green, A. Žídek, R. Bates, S. Blackwell, J. Yim, O. Ronneberger, S. Bodenstein, M. Zielinski, A. Bridgland, A. Potapenko, A. Cowie, K. Tunyasuvunakool, R. Jain, E. Clancy, P. Kohli, J. Jumper and D. Hassabis, *bioRxiv*, 2022, DOI: 10.1101/2021.10.04.463034.

Supplementally Figures

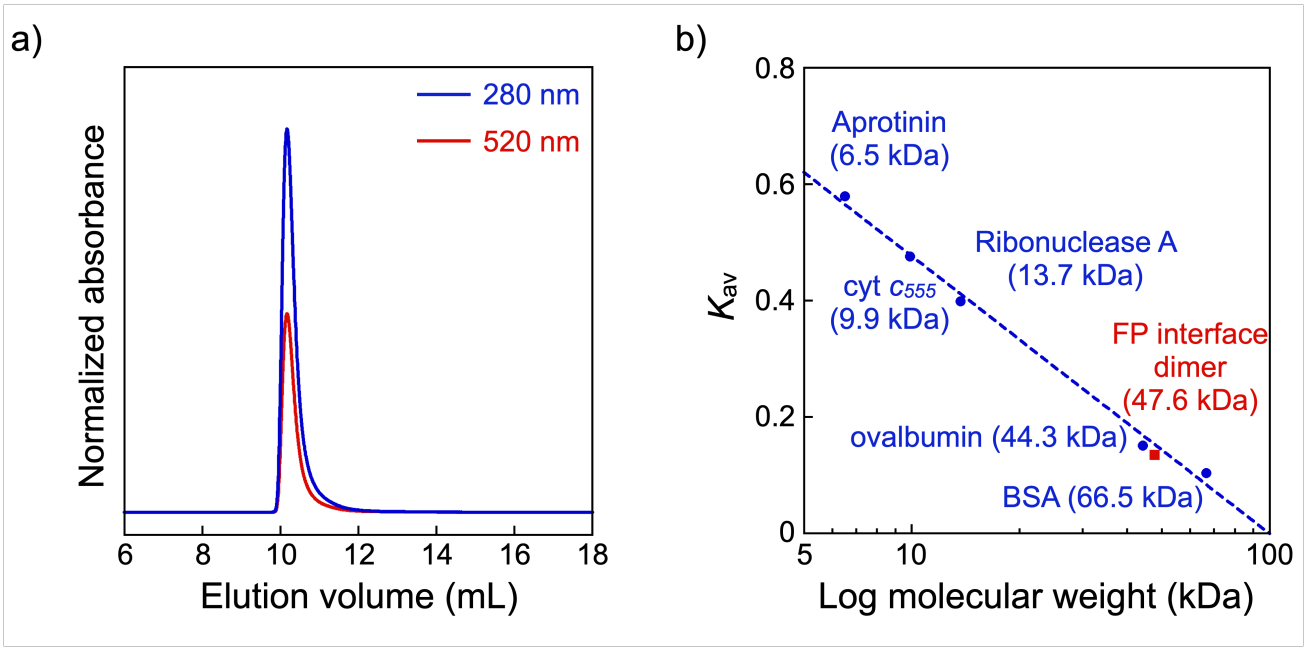

Figure S1. a) SEC traces of the FP interface dimer using a Superdex 200 Increase 10/300 column eluted with 50 mM potassium phosphate buffer, pH 7.0, at 4 °C. The monitoring wavelengths were 280 nm (blue) and 520 nm (red). The FP concentration was 50 μM. b) Calibration curve of the protein molecular weight. The calibration curve (blue dotted line) was obtained by least-squares fitting the plots (blue sphere) of the partition coefficients (K_{av}) of standard proteins against the logarithm of their molecular weights to a linear line. Aprotinin (6.5 kDa), cyt c_{555} (9.9 kDa), ribonuclease A (13.7 kDa), ovalbumin (44 kDa), and bovine serum albumin (66.5 kDa) were used as standard proteins. The plot of the FP interface dimer (red square) is depicted with the estimated molecular weight obtained from the calibration curve. The estimated molecular weight of the FP interface dimer was 47.6 kDa, which was similar to the calculated value, 50.6 kDa.

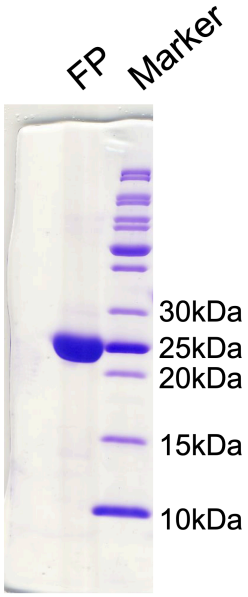

Figure S2. SDS-PAGE electrophoretogram of FP. The sample was treated with SDS buffer containing 2-mercaptoethanol as a reductant.

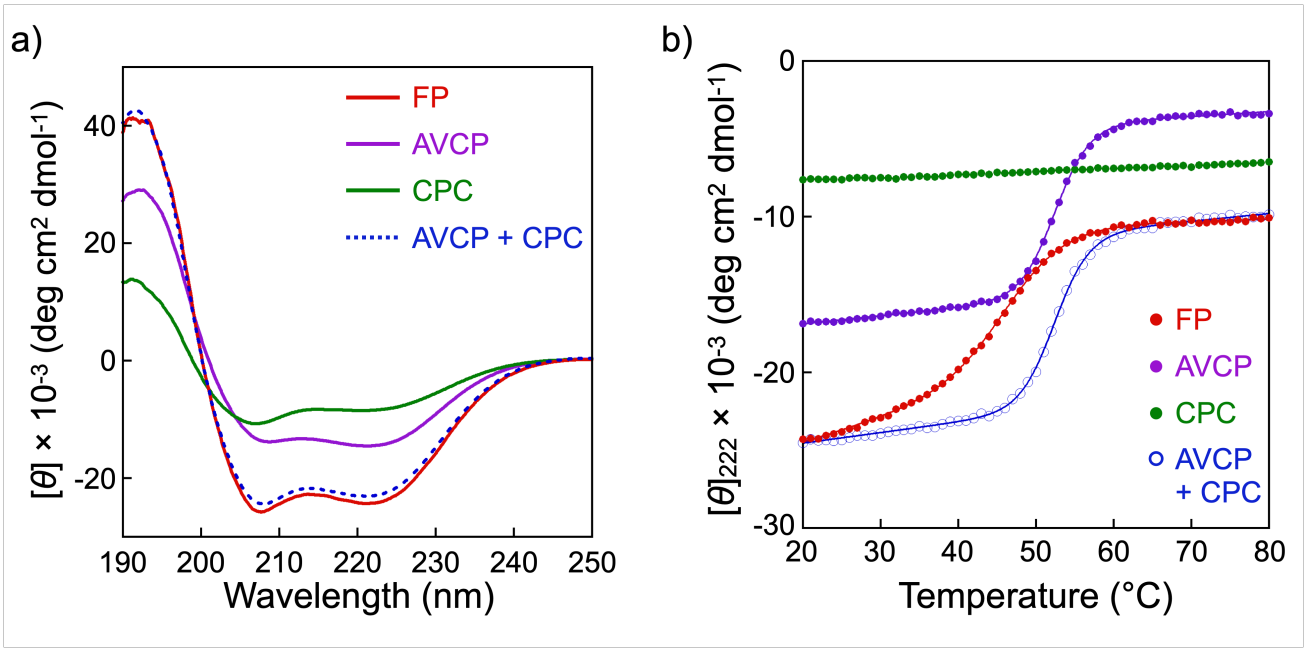

Figure S3. a) CD spectra of FP (red), AVCP (purple), and CPC (green) in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C, and the sum of spectra of AVCP and CPC (blue dotted). b) Temperature-dependent CD ellipticities at 222 nm of FP (red), AVCP (purple), and CPC (green) in 50 mM potassium phosphate buffer, pH 7.0, and the sum of ellipticities of AVCP and CPC (blue). Scan rate was 1 ºC/min. The lines represent the fit to a two-state mode using the CDpal program. T_m values of FP and AVCP were 52.5 and 46.9 °C, respectively. The T_m value obtained from the sum spectrum of FP and AVCP was 52.5 °C. CPC did not denature below 80 °C. Concentrations of FP, AVCP, and CPC were 9, 14, and 18 µM, respectively.

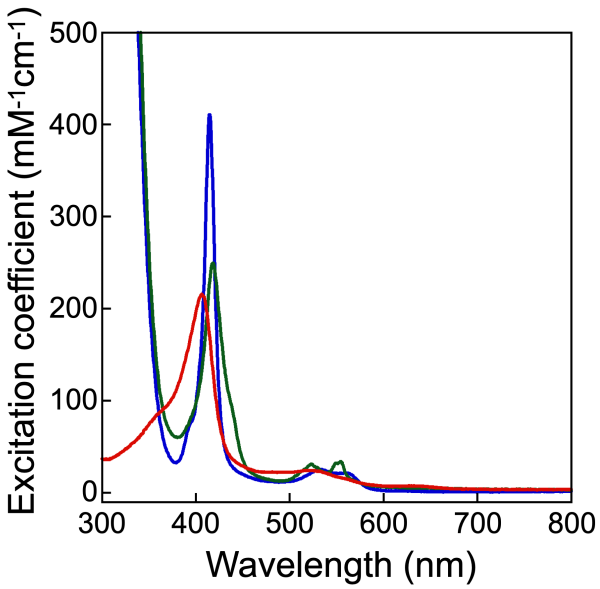

Figure S4. UV-vis spectra of the oxidised FP (red) and reduced FP obtained by adding excess dithionite under air (green) and CO atmospheres (blue) in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C. The FP concentration was 2.3 μM.

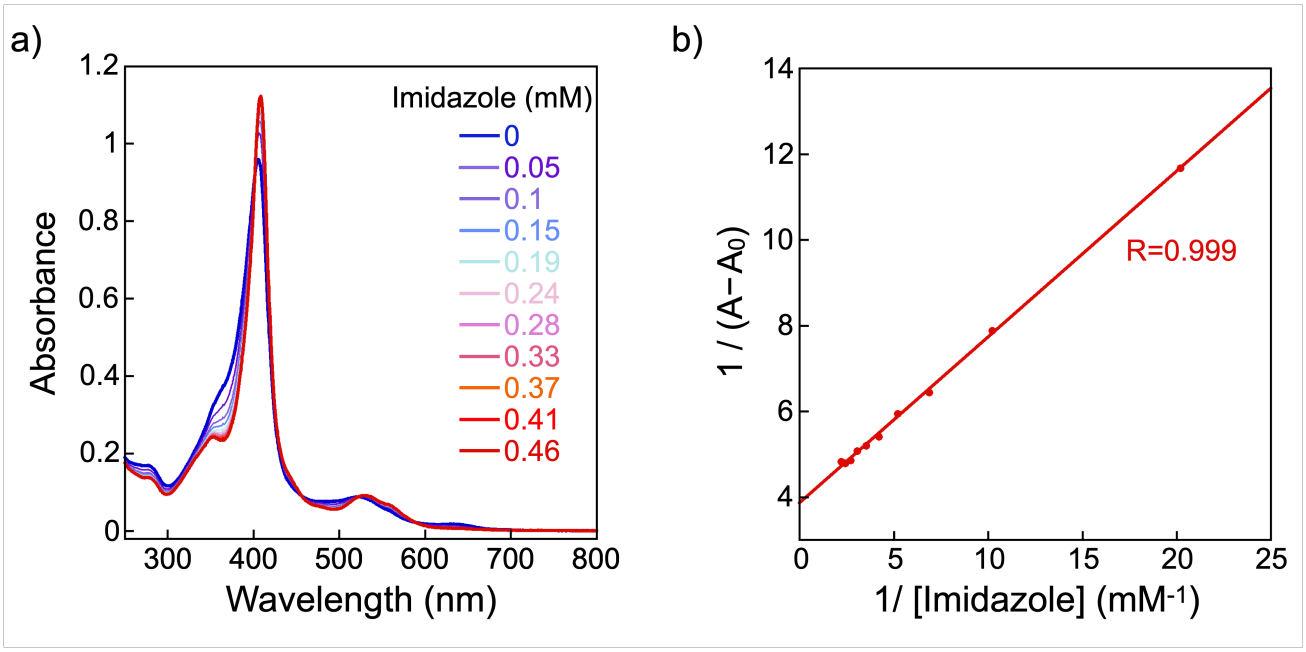

Figure S5. a) Changes in the UV-vis spectra of FP upon addition of imidazole in 50 mM potassium phosphate buffer, pH 7.0. b) Benesi–Hildebrand plots for the absorption of FP at 410.5 nm at various imidazole concentrations. The plots were analyzed by the following equation: $(A-A_0)^{-1} = (K_a(\Delta\varepsilon)[FP][Imidazole])^{-1} + ((\Delta\varepsilon)[FP])^{-1}$, where K_a represents the binding constant, [FP] and [Imidazole] represent the concentration of FP and imidazole, and $\Delta\varepsilon$ is the differential extinction coefficient obtained by subtraction of the extinction coefficient of imidazole-free FP from that of imidazole-bound FP. The FP concentration was 4.5 μM.

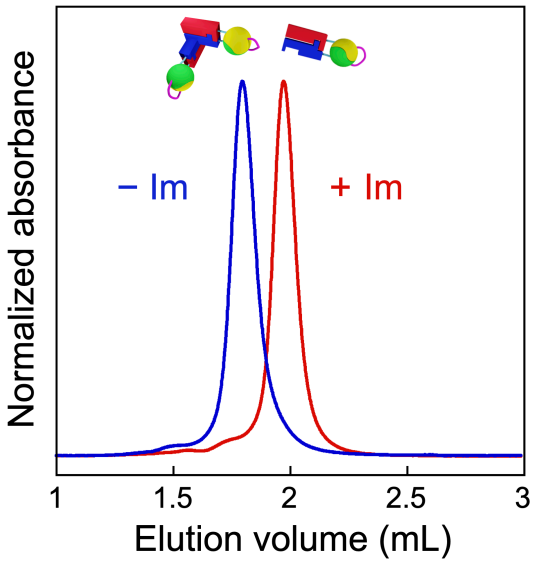

Figure S6. SEC traces of FP using Superdex 200 Increase 5/150 eluted with 50 mM potassium phosphate buffer, pH 7.0 (blue), and the same buffer containing 10 mM imidazole (red) at 4 °C. The monitoring wavelength was 410 nm. The FP concentration was 300 µM.

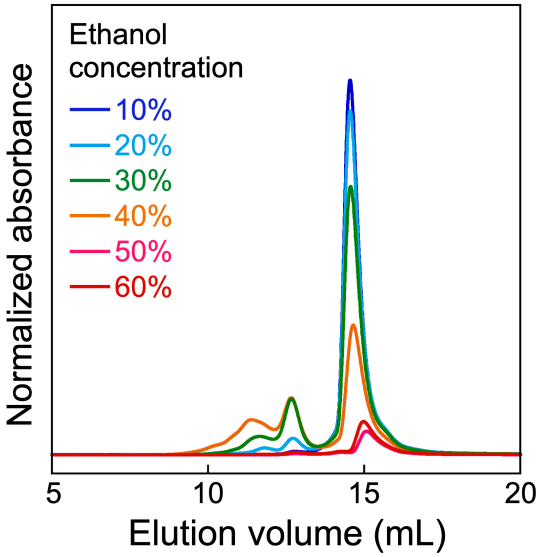

Figure S7. SEC traces of the FP solution after addition of various concentrations of ethanol, lyophilization, and residual redissolution with 50 mM potassium phosphate buffer, pH 7.0. SEC was performed using Superdex 200 Increase 10/300 column with 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM imidazole at 4 °C. The FP concentration was 250 μM after addition of ethanol. The monitoring wavelength was 410 nm.

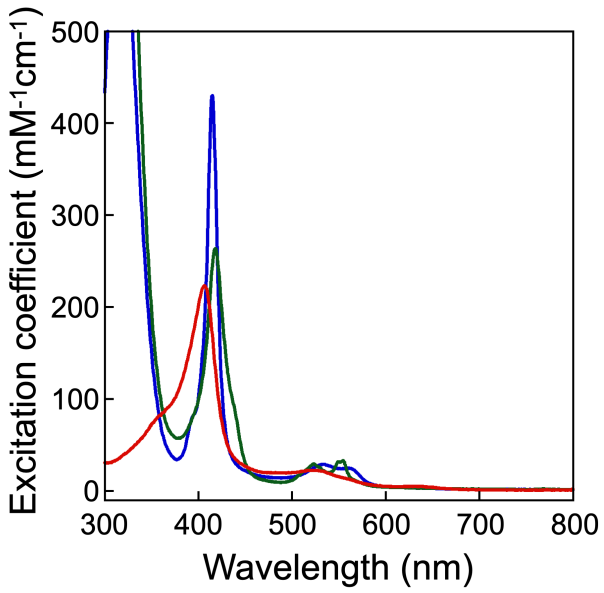

Figure S8. UV-vis spectra of oxidised (red) and reduced FP 3D-DS dimer obtained by adding excess dithionite under air (green) and CO atmospheres (blue) in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C. The FP 3D-DS dimer concentration was 1.5 μM in monomer unit.

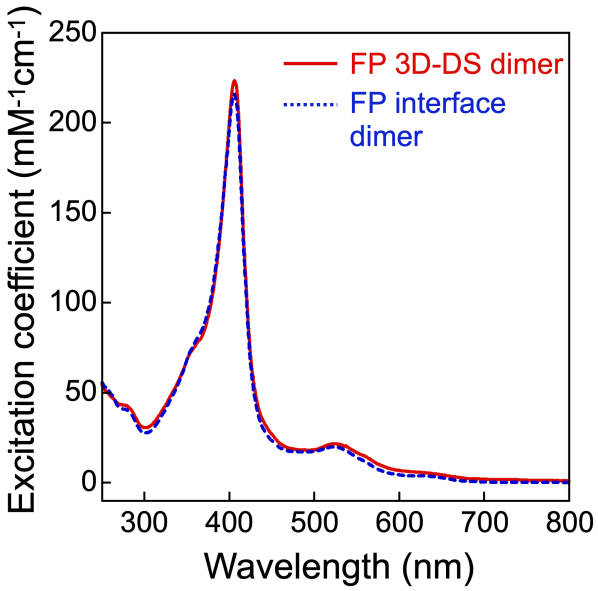

Figure S9. UV-vis spectra of the oxidised FP interface dimer (blue dotted line) and 3D-DS dimer (red solid line) in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C.

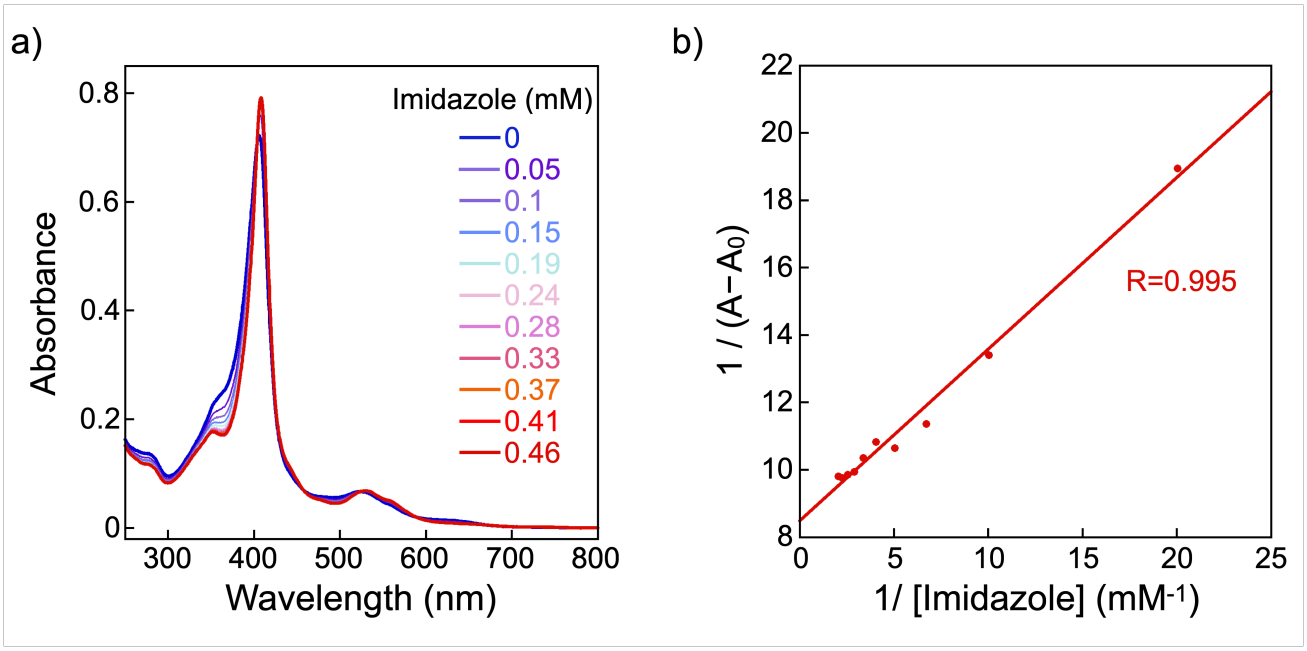

Figure S10. a) Changes in the UV-vis spectra of the FP 3D-DS dimer upon addition of imidazole in 50 mM potassium phosphate buffer, pH 7.0. b) Benesi–Hildebrand plots of the absorbance of the FP 3D-DS dimer at 410.5 nm against various imidazole concentrations. The plots were analyzed by the following equation: $(A-A_0)^{-1} = (K_a(\Delta\varepsilon)[FP][Imidazole])^{-1} + ((\Delta\varepsilon)[FP])^{-1}$, where K_a represents the binding constant and $\Delta\varepsilon$ is the differential extinction coefficient obtained by subtraction of the extinction coefficient of the imidazole-free FP 3D-DS dimer from that of the imidazole-bound FP 3D-DS dimer. The FP 3D-DS dimer concentration was 3.4 μM in monomer unit.

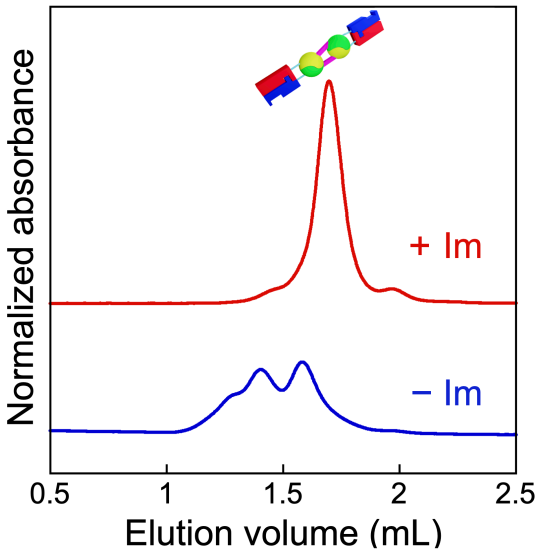

Figure S11. SEC traces of the FP 3D-DS dimer using Superdex 200 Increase 5/150 column eluted with 50 mM potassium phosphate buffer, pH 7.0, with (red) and without (blue) 10 mM imidazole at 4 °C. The monitoring wavelength was 410 nm. The FP 3D-DS dimer concentration was 25 μM in monomer unit.

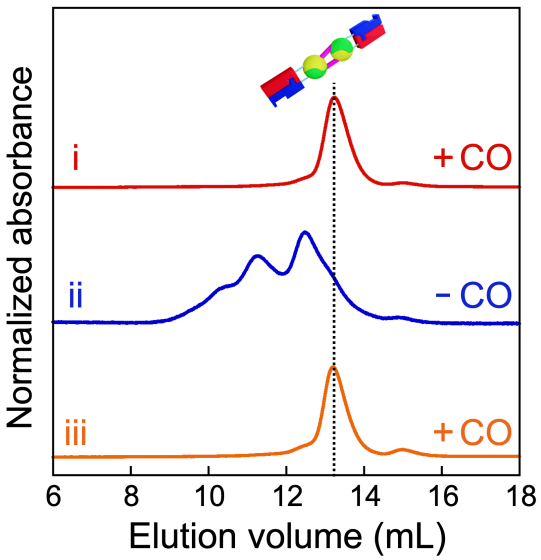

Figure S12. SEC traces of the FP 3D-DS dimer using a Superdex 200 Increase 10/300 column: (i) after reduction with excess dithionite under a CO atmosphere, (ii) after subsequent removal of dithionite in air, and (iii) after subsequent re-reduction under a CO atmosphere. Eluents were (i, iii) 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM dithionite bubbled with CO and (ii) the same buffer in air without dithionite. The monitoring wavelength was 410 nm. After performing SEC analysis in air for the oxidised sample, dithionite was added to the sample solution and CO was introduced before performing SEC analysis under CO atmosphere. The FP 3D-DS dimer concentration was 5 μM in monomer unit.

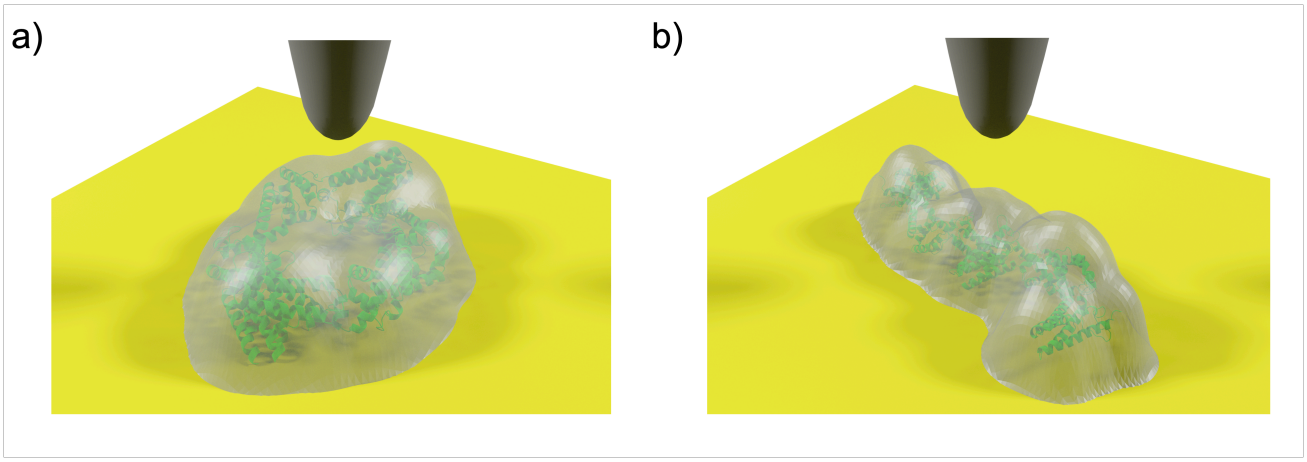

Figure S13. Schematic representation of AFM measurements for the a) cyclic and b) linear (FP 3D-DS dimer)$_2$ on a mica substrate (yellow). The cyclic and linear structures of (FP 3D-DS dimer)$_2$ were predicted by AlfaFold2 (green).

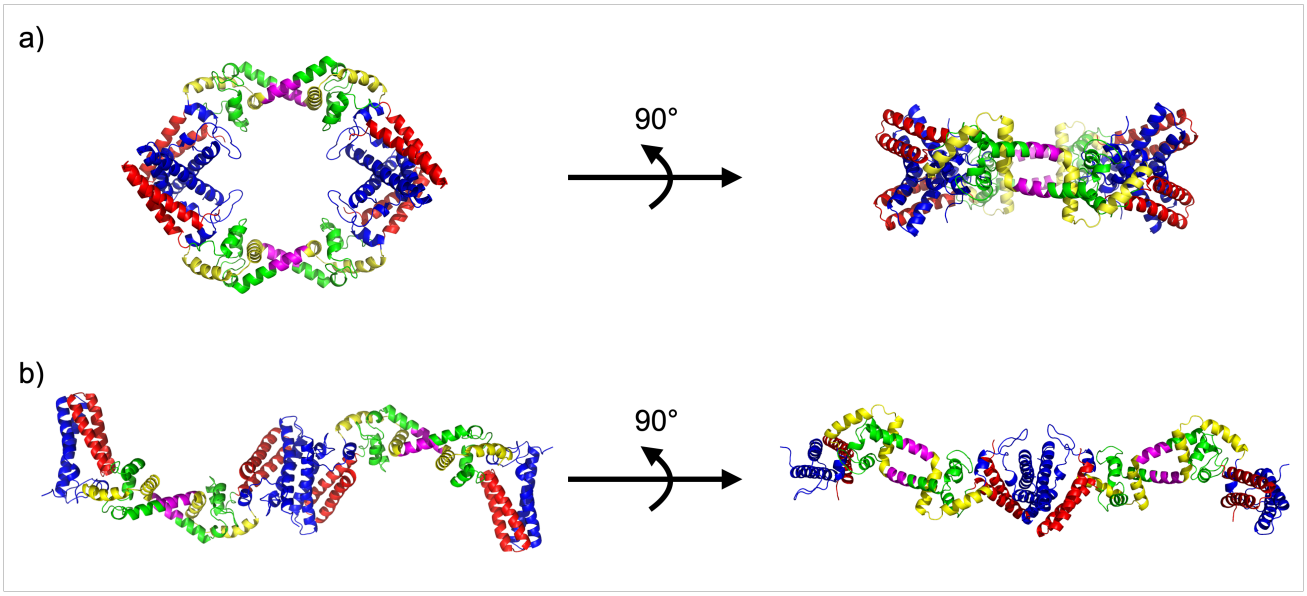

Figure S14. AlphaFold2 predicted structures of the (FP 3D-DS dimer)$_2$ which interacted intermolecularly between a) two AVCP interfaces and b) one AVCP interface. The N- and C-terminal regions of the AVCP unit are depicted in blue and red, respectively. The N- and C-terminal regions of the cyt c_{555} unit are depicted in green and yellow, respectively. The inserted α-helical linker in the CPC unit is shown in magenta.

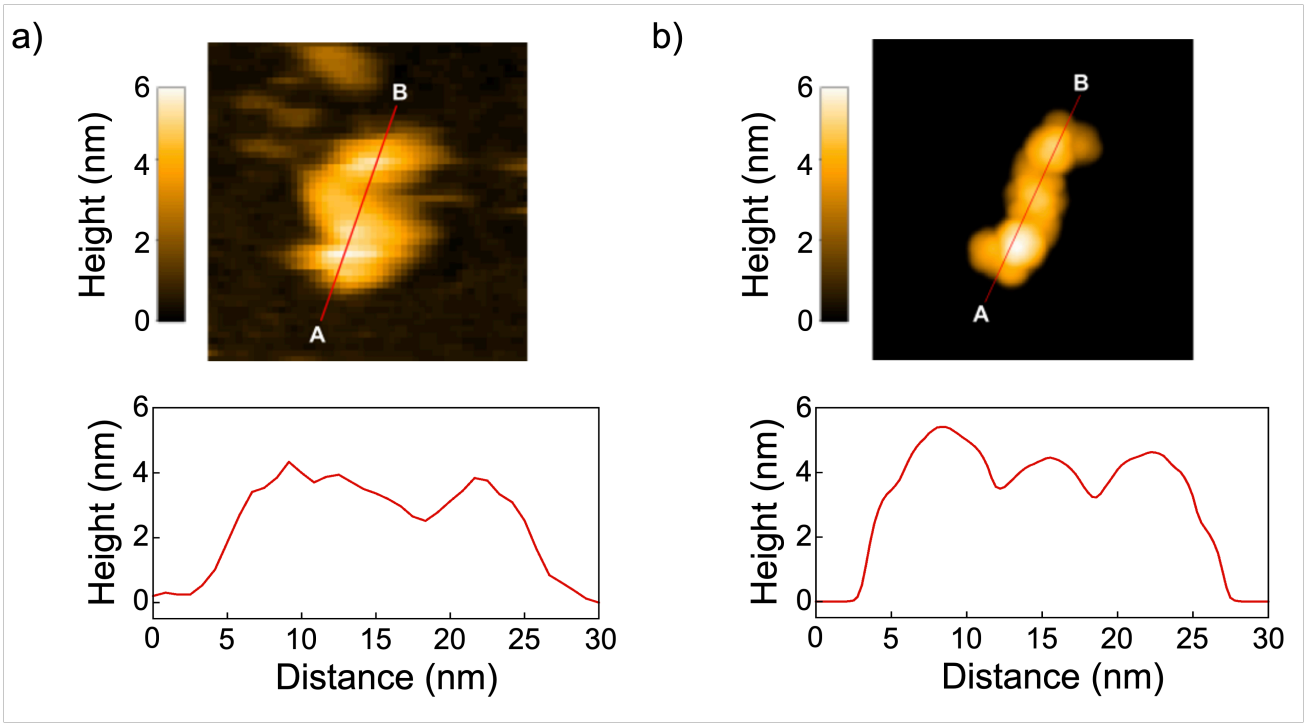

Figure S15. AFM images (top) and sliced heights (bottom) of the linear (FP 3D-DS dimer)$_2$ at room temperature: a) Representative and b) simulated images. The FP 3D-DS dimer concentration was 25 nM in monomer unit.

Supplementary Movie

Movie S1. AFM movie of (FP 3D-DS dimer)$_2$ at room temperature. The FP 3D-DS dimer concentration was 25 nM in monomer unit. The movie was captured at a scanning speed of 0.1 s/frame. Scan area: 50 nm × 45 nm. Pixel size: 61 × 55 pixels^2.