Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supporting Information

Oligomerization Enhancement and Two Domain Swapping Mode Detection for Thermostable Cytochrome c_{552} by Elongation of the Major Hinge Loop

Chunguang Ren,[a] Satoshi Nagao,[a] Masaru Yamanaka,[a] Hirofumi Komori,[b] Yasuhito Shomura,[c,d] Yoshiki Higuchi,[c,d] and Shun Hirota*[a]

[a]*Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan*
[b]*Faculty of Education, Kagawa University, 1-1 Saiwai, Takamatsu, Kagawa 760-8522, Japan.*
[c]*Department of Life Science, Graduate School of Life Science, University of Hyogo, 3-2-1 Koto, Kamigori-cho, Ako-gun, Hyogo 678-1297, Japan.*
[d]*RIKEN SPring-8 Center, 1-1-1 Koto, Sayo-cho, Sayo-gun, Hyogo 679-5148, Japan.*

Contents

Materials and Methods

Plasmids. Plasmid pKO2 was used for expression of *Hydrogenobacter thermophilus* (HT) cyt c_{552}.[1] *Escherichia coli* strain DH5α was used for routine gene engineering. Gly residues were inserted into the sequence between Ala18 and Lys19 of HT cyt c_{552} (insG1, insG2 and insG3). Trp57 of the insG3 mutant was substituted with Lys (insG3/W57K). KOD-Plus-DNA Polymerase (Toyobo, Osaka, Japan) with forward and reverse primers (Sigma-Aldrich, St Louis, MO, USA) (Table S3, Supporting Information) was used for PCR-based mutagenesis of the pKO2 plasmid. DNA sequencing of mutant plasmids were conducted with a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA) and an ABI PRISM 310 genetic analyzer sequencing system (Applied Biosystems, Inc.). The obtained plasmids were introduced into competent *Escherichia coli* JCB387 cells.

Protein Purification and Oligomerization. Wild-type (WT) HT cyt c_{552} and its mutants were expressed in *Escherichia coli* and purified as reported previously.[2] The oxidized form of WT and mutant HT cyt c_{552} were prepared by an addition of potassium ferricyanide.

Oxidized oligomeric WT and mutant HT cyt c_{552} were prepared by dissolving oxidized WT, insG3 or inG3/W57K HT cyt c_{552} (1 mM) in 50 mM potassium phosphate buffer, pH 7.0, followed by an addition of ethanol (final concentration, 90% (v/v)) at 70 °C. After centrifugation of the solution at 8,000 g for 1 h, the precipitate was lyophilized to remove residual ethanol. The lyophilized precipitate was dissolved in 50 mM potassium phosphate buffer, pH 7.0, at 4 °C.

The oxidized insG3 mutant oligomers of HT cyt c_{552} were loaded to a gel filtration column (HiLoad 26/60 Superdex 75, GE Healthcare) with 50 mM potassium phosphate buffer, pH 7.0, using a fast protein liquid chromatography (FPLC) system (BioLogic DuoFlow 10, Bio-Rad, CA) at 4 °C to obtain the insG3 dimers. The insG3 dimers were purified with a Mono S^{TM} 5/50 GL cation exchange column using the FPLC system (BioLogic DuoFlow 10, Bio-Rad) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, at a flow rate of 0.5 mL/min. The insG3 dimers were eluted with a gradient of 0 to 250 mM Na_2SO_4 over a period of 240 min. High-order oligomers of insG3 and insG3/W57K mutants were purified with a HiLoad 26/600 Superdex 200 column (GE Healthcare) using the FPLC system (BioLogic DuoFlow 10, Bio-Rad).

The absorption coefficients of the oxidized insG3 monomer and dimers were obtained with the pyridine hemochrome method (ε = 109,000 ± 1000 $M^{-1}cm^{-1}$ at 410 nm for the monomer and dimers).[3] The concentrations of the proteins were calculated from the absorbance at 410 nm with this absorption coefficient, and adjusted to desired concentrations.

Size Exclusion Chromatography Analysis. The oligomeric HT cyt c_{552} solutions were analyzed by size exclusion chromatography (column: Superdex 75 10/300 GL, GE healthcare) using the FPLC system (BioLogic DuoFlow 10, Bio-Rad) with 50 mM potassium phosphate buffer, pH 7.0, at 4 °C. The amount of oligomers was estimated by integration of the peak

areas in the elution curves using the program Igor Pro 6.0.

Optical Absorption and Circular Dichroism Measurements. Optical absorption spectra were measured with a UV-2450 spectrophotometer (Shimadzu, Japan) using a 1-cm-pathlength quartz cell at room temperature. Circular dichroism (CD) spectra were measured with a J-725 CD spectropolarimeter (Jasco, Japan) using a 0.1-cm-path-length quartz cell at 25 °C.

X-ray Crystallographic Analysis. Crystallization of HT cyt c_{552} insG3 major and minor dimers was carried out at 277 K using the sitting drop vapor diffusion method with crystal plates (CrystalClear D Strips, Douglas Instruments, Hampton Research, CA). Oxidized insG3 major dimer was dissolved in 10 mM Tris-HCl buffer, pH 7.0, at a protein concentration of 15.4 mg/ml. Oxidized insG3 minor dimer was dissolved in 10 mM HEPES buffer, pH 7.0, at a protein concentration of 22.1 mg/ml. Droplets prepared by mixing 1 µl (2µl for the insG3 minor dimer) of the insG3 major dimer solution with 1 µl (2 µl for the insG3 minor dimer) reservoir solution were equilibrated. The best reservoir solution for the insG3 major dimer was found to be 200 mM potassium iodide, containing 15% w/v PEG 3,350. The best reservoir solution for the insG3 minor dimer was 100 mM Tris-HCl, pH 8.5, containing 200 mM sodium acetate and 30% w/v PEG 4,000. Crystals were observed after incubation at 277 K for four and three weeks for the insG3 major and minor dimer, respectively.

The diffraction data were collected at BL44XU and BL38B1 beamlines at SPring-8, Japan, for the insG3 major and minor dimer, respectively. The crystal of the insG3 major dimer treated with a cryoprotectant (200 mM potassium iodide and 40% w/v PEG 3,350) was mounted on a cryo-loop, and flash-frozen at 100 K in a nitrogen cryo-system. The crystal of insG3 minor dimer without a treatment of a cryoprotectant was mounted on a cryo-loop and flash-frozen in liquid nitrogen. The detector was MX300HE and MX225HE (Rayonix) for the insG3 major and minor dimer, respectively. The crystal-to-detector distance was 150 mm and 95 mm for the insG3 major and minor dimer, respectively. The wavelength was 0.9000 Å and 1.0000 Å for the insG3 major and minor dimer, respectively. The oscillation angle was 1.0 ° and 0.5 °, and the exposure time was 0.5 s and 2.0 s per frame for the insG3 major and minor dimer, respectively. The total number of frames was 180 and 200 for the insG3 major and minor dimer, respectively. The diffraction data were processed using the program HKL2000[4] for the insG3 major dimer, and the programs iMosflm[5] and Scala[6] for the insG3 minor dimer.

The preliminary structure was obtained by a molecular replacement method (MOLREP) using the atomic coordinates of the structure of monomeric HT cyt c_{552} (PDB code: 1YNR) as a starting model for both dimers. There were two and one dimers in the asymmetric unit of the crystal for the major and minor dimer, respectively. The Gly19–Lys22 residues of one protomer of the minor dimer were not able to place their model in the structure of the insG3 minor dimer, due to the unclear electron density. The structure refinement was performed using the program, REFMAC.[7] The molecular model was manually corrected, and water

molecules were picked up in the electron density map using the program, COOT.[8] The data collection and refinement statistics of the major and minor dimers are summarized in Tables S4 and S5, Supporting Information, respectively.

Transmission Electron Microscopy. A 2 μl aliquot of oxidized oligomeric HT cyt c_{552} insG3/W57K mutant (2 μM, heme unit) dissolved in water was spread onto a carbon-coated 200-mesh copper grid (1606, JEOL) and incubated at room temperature for 10 min. After removing the solution with a filter paper, the oligomers were stained by an addition of 5% (w/w) phosphotungstic acid, pH 7.0 (adjusted by 1 M NaOH), at room temperature for 10 min. The phosphotungstic acid solution was removed with a filter paper. After repeating the addition and removal of phosphotungstic acid twice more, the sample was vacuumed 1 h in a desiccator. The transmission electron microscope (TEM) image of oligomeric HT cyt c_{552} insG3/W57K mutant was taken at 18 °C with a JEM-3100FEF microscope (JEOL) at 300 kV.

References

1. K. Oikawa, S. Nakamura, T. Sonoyama, A. Ohshima, Y. Kobayashi, S.-I. J. Takayama, Y. Yamamoto, S. Uchiyama, J. Hasegawa and Y. Sambongi, *J. Biol. Chem.*, 2005, **280**, 5527–5532.
2. (a) J. Hasegawa, S. Uchiyama, Y. Tanimoto, M. Mizutani, Y. Kobayashi, Y. Sambongi and Y. Igarashi, *J. Biol. Chem.*, 2000, **275**, 37824–37828; (b) J. Hasegawa, H. Shimahara, M. Mizutani, S. Uchiyama, H. Arai, M. Ishii, Y. Kobayashi, S. J. Ferguson, Y. Sambongi and Y. Igarashi, *J. Biol. Chem.*, 1999, **274**, 37533–37537.
3. E. A. Berry and B. L. Trumpower, *Anal. Biochem.*, 1987, **161**, 1–15.
4. Z. Otwinowski and W. Minor, *Methods Enzymol.*, 1997, **276**, 307-326.
5. A. W. Leslie and H. Powell, *Evolving Methods for Macromolecular Crystallography*, 2007, **245**, 41–51.
6. M. D. Winn, C. C. Ballard, K. D. Cowtan, E. J. Dodson, P. Emsley, P. R. Evans, R. M. Keegan, E. B. Krissinel, A. G. W. Leslie, A. McCoy, S. J. McNicholas, G. N. Murshudov, N. S. Pannu, E. A. Potterton, H. R. Powell, R. J. Read, A. Vagin and K. S. Wilson, *Acta Crystallogr. D*, 2011, **67**, 235–242.
7. A. T. Brünger, P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J.-S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Rice, T. Simonson and G. L. Warren, *Acta Crystallogr. D*, 1998, **54**, 905–921.
8. P. Emsley and K. Cowtan, *Acta Crystallogr. D*, 2004, **60**, 2126–2132.

Table S1. Root-mean-square deviation (rmsd) values between the Cα atoms of the protomers of insG3 cyt c_{552} dimers and WT cyt c_{552} monomer.[a]

	RMSD (Å)
Major dimer[b]	0.25−0.35
Minor dimer[c]	0.32−0.45

[a]There are four independent cyt c_{552} molecules in the asymmetric unit of the WT monomeric cyt c_{552} crystal, whereas there are four and two protomers in the asymmetric unit of the insG3 major and minor dimeric cyt c_{552} crystals, respectively.

[b]PDB ID: 5AUR. Residues 1−17 of a protomer and residues 24−83 in the other protomer in the same dimer were compared with the corresponding structural region of the monomer. The hinge loop (Ala18−Lys23) was excluded from the calculation.

[c]PDB ID: 5AUS. Residues 1−57 of a protomer and residues 61−83 in the other protomer in the same dimer were compared with the corresponding structural region of the monomer. The hinge loop (Gly58−Val60) and Gly19−Gly21 were excluded from the calculation. Lys22 with unclear electron density of a protomer was also excluded.

Table S2. Fe−His14 and Fe−Met62 (Met59 for the WT cyt c_{552} monomer) distances of the WT cyt c_{552} monomer and insG3 cyt c_{552} dimers.[a]

	Fe−His14 (Å)	Fe−Met62(Met59) (Å)
Wild-type monomer[b]	2.05–2.09	2.33–2.40
Wild-type dimer[c]	1.89	2.28
insG3 major dimer[d]	2.04–2.07	2.25–2.39
insG3 minor dimer[e]	2.04–2.05	2.34–2.35

[a]There are four independent cyt c_{552} molecules in the asymmetric unit of the WT monomeric cyt c_{552} crystal. There are four and two protomers in the asymmetric unit of the insG3 major and minor dimeric cyt c_{552} crystals, respectively.

[b]PDB ID: 1YNR. There are four independent HT cyt c_{552} molecules in the asymmetric unit of the monomeric cyt c_{552} crystal.

[c]PDB ID: 3VYM. There is one HT cyt c_{552} molecule in the asymmetric unit of the WT dimeric cyt c_{552} crystal.

[d]PDB ID: 5AUR. There are four independent HT cyt c_{552} molecules in the asymmetric unit of the insG3 major dimeric cyt c_{552} crystal.

[e]PDB ID: 5AUS. There are two independent HT cyt c_{552} molecules in the asymmetric unit of the insG3 minor dimeric cyt c_{552} crystal.

Table S3. Nucleotide sequences of the primers.

Primer	Sequence[a]
insG1-F	GGCAAGAAGGTGGGACCTGCTTACGCAG
insG1-R	AGCTTTCAGATCGTGGCAAGCCATA
insG2-F	GGAGGCAAGAAGGTGGGACCTGCTTACGCAG
insG2-R	AGCTTTCAGATCGTGGCAAGCCATA
insG3-F	GGTGGAGGCAAGAAGGTGGGACCTGCTTACGCAG
insG3-R	AGCTTTCAGATCGTGGCAAGCCATA
insG3/W57K-F	AAGGGTTCTGTTCCCATGCCTCCTCAAAAT
insG3/W57K-R	CACACCAGAACCGCCCTTCTTTATCTT

[a]Underlines indicate the nucleotides for the inserted or modified amino acids.

Table S4. Statistics of data collection and structure refinement of the insG3 cyt c_{552} major dimer (PDB ID: 5AUR).

Data collection	
X-ray source	SPring-8 (BL44XU)
Wavelength (Å)	0.9000
Space group	*P*1
Unit cell parameters	
a, *b*, *c* (Å)	32.9, 42.9, 53.6
α, β, γ (°)	86.7, 86.5, 89.8
Resolution (Å)	50.0–1.26 (1.29–1.26)
Number of unique reflections	78434 (4217)
R_{merge}[a]	0.047 (0.479)
Completeness (%)	94.7 (93.5)
<I/σ(I)>	17.3 (2.2)
Redundancy	2.0 (2.0)
Refinement	
Resolution (Å)	50.0–1.26 (1.29–1.26)
Number of reflections	62353 (2403)
R_{work}[b] (%)	18.0 (22.8)
R_{free}[b] (%)	22.9 (26.3)
Completeness (%)	83.1 (41.5)
Number of atoms in an asymmetric unit	
Protein	2474
Water	281
Heme	172
Average *B* factors (Å^2)	
Protein	21.7
Water	29.4
Heme	14.1
Ramachandran plot (%)	
Favored	99.7
Allowed	0.3
Outlier	0.0

Statistics for the highest-resolution shell are given in parentheses.

[a] $R_{merge}=\Sigma_{hkl} | I - \langle I \rangle | (\Sigma_{hkl} | I |)^{-1}$.

[b] $R_{work}=\Sigma_{hkl} | | F_{obs} | - k | F_{calc} | | (\Sigma_{hkl} | F_{obs} |)^{-1}$, k: scaling factor. R_{free} was computed identically, except where all reflections belong to a test set of 5 % of randomly selected data.

Table S5. Statistics of data collection and structure refinement of the insG3 cyt c_{552} minor dimer (PDB ID: 5AUS).

Data collection	
X-ray source	SPring-8 (BL38B1)
Wavelength (Å)	1.0000
Space group	$C222_1$
Unit cell parameters	
a, b, c (Å)	71.0, 71.0, 62.1
α, β, γ (º)	90.0, 90.0, 90.0
Resolution (Å)	30.0–1.30 (1.37–1.30)
Number of unique reflections	38911 (5621)
R_{merge}[a]	0.113 (0.369)
Completeness (%)	100.0 (100.0)
<I/σ(I)>	6.8 (2.9)
Redundancy	3.9 (3.9)
Refinement	
Resolution (Å)	30.0–1.30 (1.33–1.30)
Number of reflections	37017 (2843)
R_{work}[b] (%)	17.1 (20.0)
R_{free}[b] (%)	20.2 (21.6)
Completeness (%)	99.9 (99.9)
Number of atoms in an asymmetric unit	
Protein	1251
Water	216
Heme	86
Average B factors (Å^2)	
Protein	9.9
Water	18.6
Heme	5.6
Ramachandran plot (%)	
Favored	99.3
Allowed	0.7
Outlier	0.0

Statistics for the highest-resolution shell are given in parentheses.

[a] $R_{merge}=\Sigma_{hkl} | I - \langle I \rangle | (\Sigma_{hkl} | I |)^{-1}$.

[b] $R_{work}=\Sigma_{hkl} | | F_{obs} | - k | F_{calc} | | (\Sigma_{hkl} | F_{obs} |)^{-1}$, k: scaling factor. R_{free} was computed identically, except where all reflections belong to a test set of 5 % of randomly selected data.

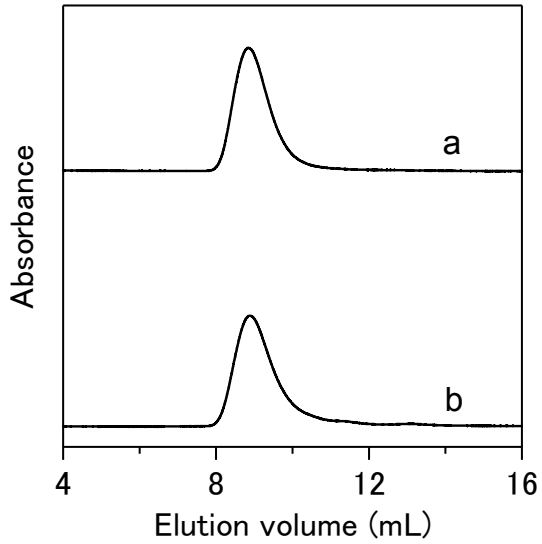

Figure S1. Size exclusion chromatograms of insG3 cyt c_{552} oligomers (a) before and (b) after incubation at 50 °C for 1 h. Measurement conditions: column, Superdex 75 10/300 GL; flow rate, 0.5 mL/min; monitoring wavelength, 410 nm; solvent, 50 mM potassium phosphate buffer; pH, 7.0; temperature, 4 °C.

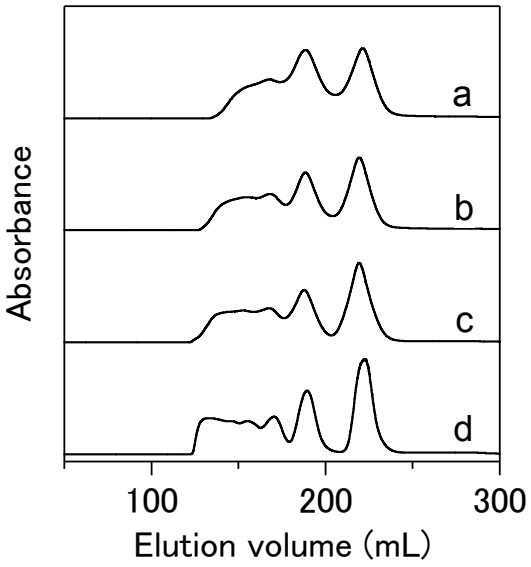

Figure S2. Size exclusion chromatograms of WT and mutant cyt c_{552} after treatment with ethanol: (a) WT, (b) insG1 mutant, (c) insG2 mutant and (d) insG3 mutant. After addition of ethanol up to 90% (v/v) to WT or mutant cyt c_{552} (1 mM) at 70 °C, the obtained precipitates were lyophilized and dissolved in buffer at 4 °C. Measurement conditions: column, Superdex 75 26/60 GL; flow rate, 0.8 mL/min; monitoring wavelength, 410 nm; solvent, 50 mM potassium phosphate buffer; pH, 7.0; temperature, 4 °C.

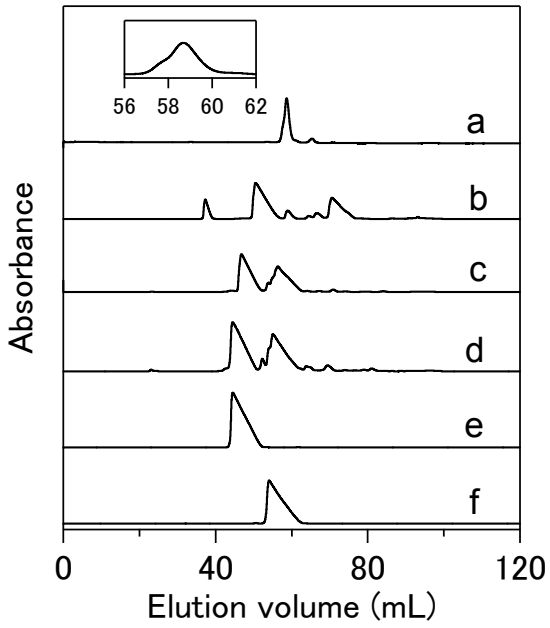

Figure S3. Cation exchange chromatograms of WT and mutant cyt c_{552} dimers: (a) WT dimers, (b) insG1 dimers, (c) insG2 dimers, (d) insG3 dimers, (e) insG3 major dimer and (f) insG3 minor dimer. Inset: Expanded chromatogram of WT cyt c_{552} dimers. Measurement conditions: column, Mono S^{TM} 5/50 GL; flow rate, 0.5 mL/ min; monitoring wavelength, 410 nm; gradient, 10 mM potassium phosphate buffer, pH 7.0, with 10 mM potassium phosphate buffer, pH 7.0, containing 250 mM Na_2SO_4; temperature, 4 °C.

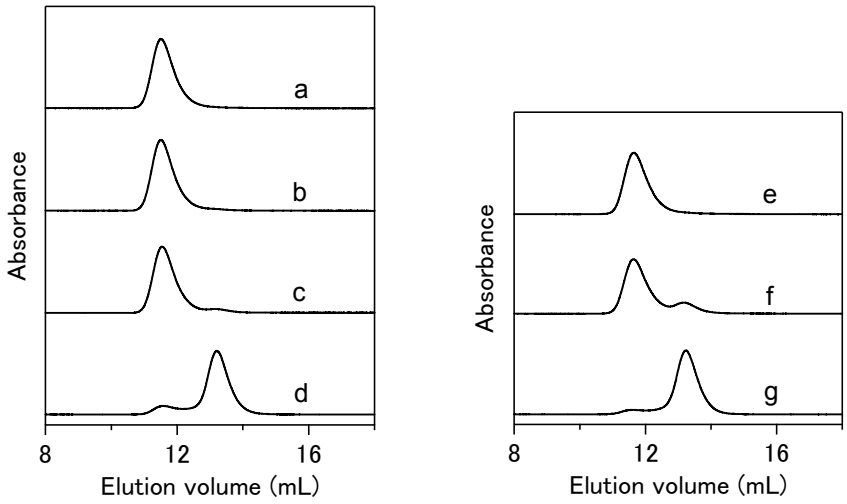

Figure S4. Size exclusion chromatograms of insG3 cyt c_{552} dimers after incubation at various temperatures. Major dimer (a) before and after incubation for 0.5 h at (b) 70 °C, (c) 80 °C and (d) 90 °C. Minor dimer (e) before and after incubation for 0.5 h at (f) 70 °C and (g) 80 °C. Measurement conditions: column, Superdex 75 10/300 GL; flow rate, 0.5 mL/min; monitoring wavelength, 410 nm; solvent, 50 mM potassium phosphate buffer; pH, 7.0; temperature, 4 °C.

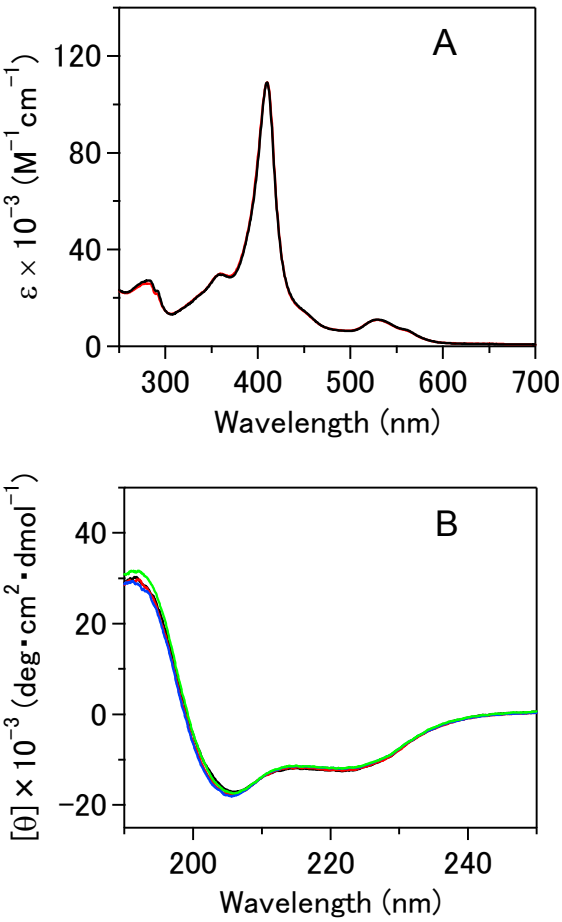

Figure S5. Optical absorption and CD spectra of oxidized WT cyt c_{552} monomer (black), insG3 cyt c_{552} monomer (red), insG3 cyt c_{552} major dimer (blue) and insG3 cyt c_{552} minor dimer (green): (A) absorption and (B) CD spectra. The concentration of the protein was calculated from the intensity of its Soret band. Measurement conditions: sample concentration (heme unit), (A) 16−17 μM and (B) 10 μM; buffer, 50 mM potassium phosphate buffer; pH, 7.0; temperature, (A) room temperature and (B) 25 °C.

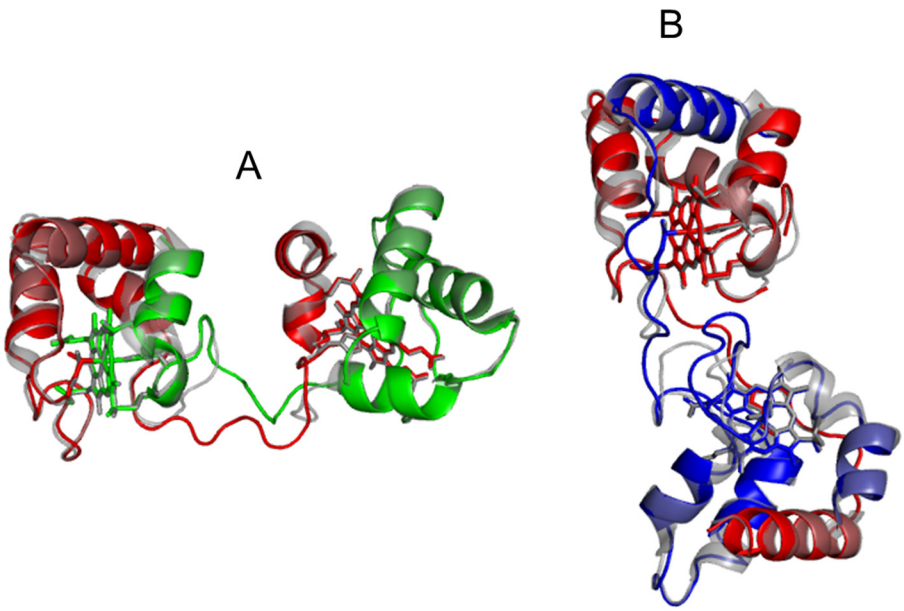

Figure S6. Overlapped views of the (A) insG3 cyt c_{552} major dimer (PDB ID: 5AUR) (red and green) or (B) insG3 cyt c_{552} minor dimer (PDB ID: 5AUS) (red and blue) structures with the WT monomer (PDB ID: 1YNR) (gray) structures. His14, Met62 (Met59 for the monomer) and the hemes are shown as stick models

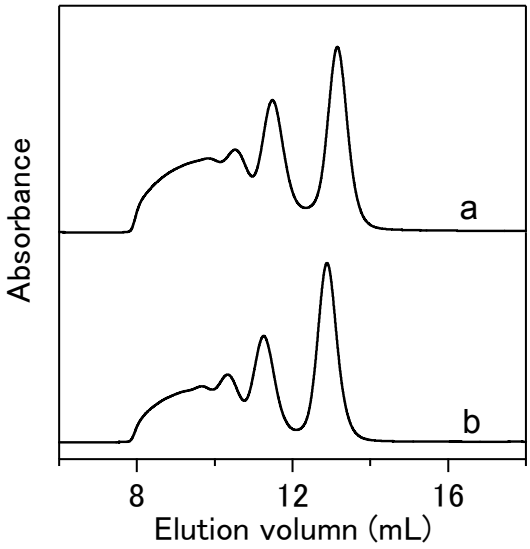

Figure S7. Size exclusion chromatograms of (a) insG3 and (b) insG3/W57K cyt c_{552} after treatment with ethanol. After addition of ethanol up to 90% (v/v) to insG3 cyt c_{552} and insG3/W57K cyt c_{552} (500 μM) at 70 ºC, the obtained precipitates were lyophilized and subsequently dissolved in buffer at 4 ºC. Measurement conditions: column, Superdex 75 10/300 GL; flow rate, 0.5 mL/min; monitoring wavelength, 410 nm; solvent, 50 mM potassium phosphate buffer; pH, 7.0; temperature, 4 °C.

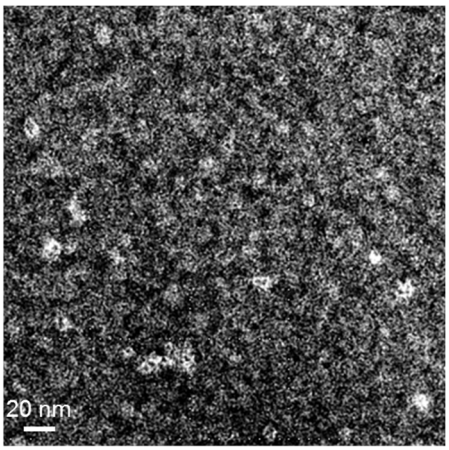

Figure S8. TEM image of insG3/W57K cyt c_{552} oligomers. Measurement conditions: protein, 2 μM (heme); staining reagent, 5% (w/w) phosphotungstic acid (pH 7.0); grid, carbon-coated 200-mesh copper grid; voltage, 300 kV; temperature, 18 °C.