Protein surface charge effect on 3D domain swapping in cells for c-type cytochromes

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Abstract

Many \( c \)-type cytochromes (cys) can form domain-swapped oligomers. The positively charged \textit{Hydrogenobacter thermophilus} (HT) cytochrome (cyt) \( c_{552} \) forms domain-swapped oligomers during expression in the \textit{Escherichia coli} (\textit{E. coli}) expression system, but the factors influencing the oligomerization remain unrevealed. Here, we found that the dimer of the negatively charged \textit{Shewanella violacea} (SV) cyt \( c_{5} \) exhibits a domain-swapped structure, in which the N-terminal helix is exchanged between protomers, similar to the structures of the HT cyt \( c_{552} \) and \textit{Pseudomonas aeruginosa} (PA) cyt \( c_{551} \) domain-swapped dimers. Positively charged horse cyt \( c \) and HT cyt \( c_{552} \) domain swapped during expression in \textit{E. coli}, whereas negatively charged PA cyt \( c_{551} \) and SV cyt \( c_{5} \) did not. Oligomers were formed during expression in \textit{E. coli} for HT cyt \( c_{552} \) attached to either a co- or post-translational signal peptide for transportation through the cytoplasm membrane, but not for PA cyt \( c_{551} \) attached to either signal peptide. HT cyt \( c_{552} \) formed oligomers in \textit{E. coli} in the presence and absence of rare codons. More oligomers were obtained from the \textit{in vitro} folding of horse cyt \( c \) and HT cyt \( c_{552} \) by the addition of negatively charged liposomes during folding, whereas the amount of oligomers for the \textit{in vitro} folding of PA cyt \( c_{551} \) and SV cyt \( c_{5} \) did not change significantly by the addition. These results indicate that the protein surface charge affects the oligomerization of \( c \)-type cys in cells; positively charged \( c \)-type cys assemble on a negatively charged membrane, inducing formation of domain-swapped oligomers during folding.
Keywords: c-type cytochrome; domain swapping; oligomerization; protein surface charge; lipid-protein interaction

Abbreviations used:

Ccm, cytochrome c maturation; CL, cardiolipin; cyt, cytochrome; cytS, cytochromes; DsbAss, signal peptide of periplasmic disulfide bond oxidoreductase I; E. coli, Escherichia coli; EDTA, ethylenediaminetetraacetie acid; FPLC, fast protein liquid chromatography; GdnHCl, guanidine hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HT, Hydrogenobacter thermophilus; PA, Pseudomonas aeruginosa; PAAss, signal peptide of Pseudomonas aeruginosa cyt c551; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PG, phosphatidylglycerol; PhoAss, signal peptide of alkaline phosphatase; SEC, size exclusion chromatography; SRP, signal recognition particle; SV, Shewanella violacea; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol.
1. Introduction

C-type cytochromes (cyts) are heme proteins that act as electron carriers, functioning in a variety of cellular processes [1, 2]. Many c-type cyts can form oligomers by 3D domain swapping (herein, domain swapping) [3-11]. In domain swapping, two or more identical protein monomers exchange the same domain or secondary structural elements and fold into dimers or higher oligomers whose units are structurally similar to the original monomer [12-15]. Positively charged horse cytochrome (cyt) c forms domain-swapped oligomers by treatment with ethanol, and dimeric and trimeric horse cyt c are formed by swapping the C-terminal α-helix (Fig. 1) [3]. Positively charged Hydrogenobacter thermophilus (HT) cyt c552 and negatively charged Pseudomonas aeruginosa (PA) cyt c551 also form domain-swapped dimers by treatment with ethanol; the domain swapping regions of HT cyt c552 and PA cyt c551 comprises the N-terminal α-helix and the heme (Fig. 1) [4, 5]. It has been reported that horse cyt c forms oligomers during protein folding [16, 17], and more domain-swapped oligomers are formed for higher protein concentrations during folding of horse cyt c [16]. HT cyt c552 forms domain-swapped oligomers in Escherichia coli (E. coli) cells during expression, where the structure of the dimer formed in E. coli was similar to that of the dimer obtained by the ethanol treatment [18]. The amount of HT cyt c552 oligomers increased in E. coli as the HT cyt c552 concentration was increased, whereas it decreased in the order of decrease in protein stability, indicating that domain swapping decreases in cells when the protein stability
decreases [18]. Although knowledge on domain swapping has been increasing, factors that affect domain swapping in cells remain mostly unrevealed.

**Fig. 1.** Monomeric and domain-swapped dimeric c-type cyt structures: (A) Monomeric horse cyt c (PDB ID: 1HRC), (B) dimeric horse cyt c (PDB ID: 3NBS), (C) monomeric HT cyt c552 (PDB ID: 1YNR), (D) dimeric HT cyt c552 (PDB ID: 3VYM), (E) monomeric PA cyt c551 (PDB ID: 351C), and (F) dimeric PA cyt c551 (PDB ID: 3X39). Positively and negatively charged proteins are depicted in blue and red, respectively. The two protomers of the dimers are depicted in dark and pale colors, respectively. The N- and C-termini are labeled as N and C, respectively.

Bacterial apo c-type cyt is synthesized with a signal peptide at the ribosome in the cytoplasm, and is guided into the secretary proteins by a signal peptide and transported to the periplasm [19]. At the periplasm, the signal peptide is cleaved from the polypeptide and a heme is inserted into apo c-type cyt by cyt c maturation (Ccm) proteins, resulting in the formation of
There are two types of signal peptides with different transportation mechanisms: co-translational signal peptide, which transports the polypeptide during synthesis (signal recognition particle (SRP) mechanism), and post-translational signal peptide, which transports the polypeptide after the synthesis is complete (Sec-dependent mechanism) [21, 22].

In cells, the expression level of a protein is affected by a rare codon and a messenger RNA secondary structure [23-25]. Translation pause caused by the rare codon or the messenger RNA secondary structure may regulate the folding of an individual domain in a multi-domain protein, where the time separation provided by the pause allows completion of the folding without interruption, thus avoiding problems in protein folding and aggregation [26-29]. Codon usage may also alter the final folded structure of the encoded protein and affect the efficiency of protein transportation [30, 31]. On the other hand, interaction of proteins with membranes has been widely studied [32-34]. This interaction may promote protein folding [35-37], misfolding [38], or unfolding [39], resulting in aggregation or oligomerization of proteins [38, 40, 41]. For example, the positively charged N-terminal of the prion protein was involved in the binding of the prion protein to a negatively charged membrane, which induces increase in the local concentration of the prion protein on the membrane and allows prion polymerization [42]. In the E. coli membrane, there are three major membrane phospholipids: zwitterionic phosphatidylethanolamine (PE), and anionic phosphatidylglycerol (PG) and cardiolipin (CL) [43]. The inner membrane of E coli cells that were grown at 37 °C consisted of ~75% PE,
~19% PG, and ~6% CL [44], demonstrating that the inner membrane is negatively charged. In this work, we investigated the effects of the protein transportation mechanism type, rare codon, and protein surface charge on the c-type cyt oligomerization by domain swapping, elucidating that the surface charge of the protein affects the oligomerization by influencing the interaction with the membrane; whereas, the protein transportation mechanism and rare codon are not essential for the oligomerization.

2. Materials and Methods

2.1 Plasmids of c-type cyts

Plasmids pkk223-3 containing the gene of HT cyt $c_{552}$, PA cyt $c_{551}$, or *Shewanella violacea* (SV) cyt $c_5$ and the gene of the signal peptide of PA cyt $c_{551}$ (PAss) at the 5′ site of each c-type cyt were gifted from Prof. Sambongi [45-47]. The horse cyt $c$ gene was amplified by polymerase chain reaction (PCR) of the pEMBL18+ plasmid containing the horse cyt $c$ gene [48]. The pkk223-3 plasmid containing the genes of horse cyt $c$ and PAss (at the 5′ site of horse cyt $c$) was prepared by substitution of the HT cyt $c_{552}$ gene with the horse cyt $c$ gene in the pkk223-3 plasmid by in-fusion mutagenesis with an In-Fusion HD Cloning Kit (TaKaRa). To obtain c-type cyts with a His-tag at the C-termini, horse cyt $c$-6His, HT cyt $c_{552}$-6His, PA cyt $c_{551}$-6His, and SV cyt $c_5$-6His genes were designed by introducing a His-tag (LVPRGSHHHHHHH) gene at the 3′ sites of the corresponding c-type cyt genes by PCR site-
directed mutagenesis. PAss was attached at the N-terminus of c-type cyt when the c-type cyt was encoded with non-optimized genes. The signal peptide of periplasmic disulfide bond oxidoreductase I (DsbAss) was used to transport c-type cyts by the SRP (co-translationally transported) mechanism, whereas the signal peptide of alkaline phosphatase (PhoAss) was used to transport c-type cyts by the Sec-dependent (post-translationally transported) mechanism. DsbAss-HT cyt c552-6His, PhoAss-HT cyt c552-6His, DsbAss-PA cyt c551-6His, and PhoAss-PA cyt c551-6His genes were designed by introducing the DsbAss or PhoAss gene at the 5’ sites and the His-tag gene at the 3’ sites of the HT cyt c552 and PA cyt c551 genes. The genes were optimized by removing rare and low-usage codons, and avoiding stable mRNA secondary structures, which were predicted with the program, Mfold [49]. The optimized genes of DsbAss-HT cyt c552-6His and DsbAss-PA cyt c551-6His were purchased from Eurofins. PhoAss-HT cyt c552-6His and PhoAss-PA cyt c551-6His genes were prepared by substituting the DsbAss gene with the PhoAss gene by PCR site-directed mutagenesis. The PCR site-directed mutagenesis was performed with a KOD-Plus-Mutagenesis Kit (TOYOBO), and the DNA sequence of the target gene was verified by an ABI 3100 Avant generic analyzer (Applied Biosystems, Inc.). The c-type cyts and signal peptides used in this study are summarized in Table 1. The amino acid sequences of the signal peptides used in this study are shown in Table S1.
Table 1

*C-type cyts used in this study.*

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<sup>a</sup>PAss, signal peptide of PA cyt<sub>c</sub>-551; DsbAss, signal peptide of periplasmic disulfide bond oxidoreductase I; PhoAss, signal peptide of alkaline phosphatase.

2.2 E. coli growth and harvest

*E. coli* JCB387 was used as the host for the expression of *c*-type cyts [50]. The pKK223-3 plasmid containing the gene encoding *c*-type cyt was co-transformed into *E. coli* JCB387 with the pEC86 plasmid containing genes encoding cytochrome *c* maturation (Ccm) proteins. The *E. coli* JCB387 carrying both pKK223-3 and pEC86 plasmids was grown in 200 mL of Luria-Bertani medium containing 100 μg/mL ampicillin and 30 μg/mL chloramphenicol (the medium for the expression of SV cyt<sub>c</sub>-5 and SV cyt<sub>c</sub>-5-6His additionally contained 4 mL/L glycerol) at 37 °C for 23 h. The cells were harvested by centrifugation (8,000 g for 5 min at 4 °C) after culture.
2.3 Analysis of c-type cyt oligomer amount obtained from E. coli

The harvested cells that expressed His-tagged c-type cyt (horse cyt c-6His, HT cyt c552-6His, PA cyt c551-6His, SV cyt c5-6His, DsbAss-HT cyt c552-6His, PhoAss-HT cyt c552-6His, DsbAss-PA cyt c551-6His, and PhoAss-PA cyt c551-6His) were suspended in 10 mL of 100 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)–HCl buffer, pH 8.0. The E. coli suspension was placed on ice and sonicated 30 times for 2 s with 2 s intervals with an ultrasonic liquid processor (VC 505, SONICS&MATERIALS, Inc.). The sonicated sample was centrifuged (30,000 g for 20 min at 4 °C), and the supernatant was purified by Ni-affinity chromatography (HisTrap HP, GE Healthcare) using a fast protein liquid chromatography (FPLC) system (BioLogic DueFlow 10, Bio-Rad) (flow rate, 1.0 mL/min; monitoring wavelength, 280 nm and 410 nm; gradient, 25 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl and the same buffer containing 0.5 M NaCl and 0.5 M imidazole; temperature, 4 °C). The c-type cyt fractions were collected and subsequently analyzed by size exclusion chromatography (SEC; HiLoad 16/600 Superdex 75 pg, GE Healthcare) using the FPLC system (BioLogic DueFlow 10, Bio-Rad) (flow rate, 1.0 mL/min; monitoring wavelength, 280 nm and 410 nm; buffer, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C). The oligomer ratio was calculated from the peak areas in the SEC chromatogram.

2.4 Purification of non-His-tagged horse cyt c, HT cyt c552, PA cyt c551, and SV cyt c5
Oxidized horse cyt c was prepared by oxidizing horse cyt c (Wako, Japan) with 5 mM potassium ferricyanide and subsequently removing the potassium ferricyanide by ion exchange chromatography (TOYOPEARL DEAE-650C, Tosoh Co., Japan) with 50 mM potassium phosphate buffer, pH 7.0. HT cyt c552, PA cyt c551, and SV cyt c5 expressed in E. coli JCB387 were purified as reported previously [46, 47, 51]. HT cyt c552 was oxidized with 5 mM potassium ferricyanide and purified by ion exchange chromatography (HiTrap SP HP, GE Healthcare) using the FPLC system (BioLogic DueFlow 10, Bio-Rad) (flow rate, 1.0 mL/min; monitoring wavelength, 280 nm and 410 nm; gradient, 10 mM potassium phosphate buffer, pH 7.0, and the same buffer containing 0.5 M NaCl; temperature, 4 °C). PA cyt c551 and SV cyt c5 were oxidized with 5 mM potassium ferricyanide, and the potassium ferricyanide was removed by using a desalting column (PD-10 Desalting Column, GE Healthcare) with 10 mM Tris–HCl buffer, pH 8.0. Oxidized PA cyt c551 and SV cyt c5 were purified by ion exchange chromatography (HiTrap Q HP, GE Healthcare) using the FPLC system (BioLogic DueFlow 10, Bio-Rad) (flow rate, 1.0 mL/min; monitoring wavelength, 280 nm and 410 nm; gradient, 10 mM Tris–HCl buffer, pH 8.0, and the same buffer containing 0.2 M NaCl; temperature, 4 °C). After purification by ion exchange chromatography, oxidized HT cyt c552, PA cyt c551, and SV cyt c5 were purified by SEC (HiLoad 26/60 Superdex 75 pg, GE Healthcare) using the FPLC system (BioLogic DueFlow 10, Bio-Rad) (flow rate, 1.0 mL/min; monitoring wavelength, 280 nm and 410 nm; buffer, 50 mM potassium phosphate buffer, pH 7.0;
temperature, 4 °C). The concentrations of monomeric and dimeric SV cyt c5 were calculated from the absorbances of the Soret bands using the coefficients of monomeric ($\varepsilon_{410} = 123 \text{ mM}^{-1}\text{cm}^{-1}$) and dimeric ($\varepsilon_{410} = 129 \text{ mM}^{-1}\text{cm}^{-1}$, heme unit) SV cyt c5, respectively, obtained by the pyridine hemochrome method [52].

2.5 Oligomerization and purification of the dimer of SV cyt c5

Oxidized SV cyt c5 was oligomerized by mixing an oxidized SV cyt c5 solution (2 mM, 4 mL) with ethanol (36 mL) at 50 °C. The obtained solution was lyophilized to remove residual ethanol, and the obtained powder was subsequently dissolved in 10 mL of 50 mM potassium phosphate buffer, pH 7.0. After incubation of the SV cyt c5 solution at 37 °C for 1 h, the SV cyt c5 solution was analyzed by SEC (Superdex 75 10/300 GL, GE Healthcare) using the FPLC system (BioLogic DueFlow 10, Bio-Rad) (flow rate, 0.5 mL/min; monitoring wavelength, 280 nm and 410 nm; buffer, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C).

Dimeric SV cyt c5 was purified from the solution containing the monomer and oligomers with different sizes by performing SEC (HiLoad 26/60 Superdex 75 pg, GE Healthcare) several times using the FPLC system (BioLogic DueFlow 10, Bio-Rad) (flow rate, 1.0 mL/min; monitoring wavelength, 280 nm and 410 nm; buffer, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C).
2.6 X-ray crystallography of dimeric SV cyt c₅

Crystallization of oxidized dimeric SV cyt c₅ was carried out at 4 °C using the sitting drop vapor diffusion method with the crystal screening kit NeXtal Tubes PEGs Suite (QIAGEN). The protein concentration was adjusted to 1.2 mM (heme unit) in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.0. The droplets prepared by mixing 1 μL of the protein solution with 1 μL of reservoir solution were equilibrated. The best reservoir solution was found to be 20% polyethylene glycol (PEG) 3350 and 200 mM potassium nitrate. A crystal was obtained in the protein solution after incubation at 4 °C for 3 days.

The diffraction data were collected at the BL26B1 beamline of SPring-8, Japan. The crystal was mounted on a cryo-loop after a treatment of cryoprotectant (25% PEG 3350), and flash-frozen at 100 K in a nitrogen cryo-system. The detector was EIGER X 4M (Dectris). The crystal-to-detector distance was 70 mm and the wavelength was 1.0000 Å. The oscillation angle was 0.1° and the exposure time was 0.5 s per frame. The total number of frames was 1800. The diffraction data were processed using the program XDS [53].

The preliminary structure was obtained by the molecular replacement method (MOLREP) [54] using the atomic coordinates of the monomer structure of SV cyt c₅ (PDB ID: 5B6Q) as a starting model. The structure refinement was performed using the program REFMAC [55]. The molecular model was manually corrected, and water molecules were picked up in the electron density map using the program COOT [56]. The data collection and refinement statistics are
summarized in Table S2.

2.7 Preparation of liposomes from mitochondrial lipids

Horse cardiac muscle chunks (200 g) were placed in 0.6 L of 10 mM sodium phosphate buffer, pH 8.0, containing 250 mM sucrose and 0.1 mM ethylenediaminetetraacetateic acid (EDTA), and blended with a food processor (TK430, TESCOM) for 2 min. The blended sample was centrifuged at 900 g for 10 min, and the supernatant was filtered with 2 layers of gauze to remove the fat. After adjusting the pH of the solution to 7.4 with 3 M NaOH, the solution was centrifuged at 7,000 g for 10 min. The precipitate was suspended in 25 mL of 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl. The suspended solution was homogenized twice with a tissue grinder (WHEATON) and centrifuged at 7,600 g for 10 min. The supernatant was removed by a pipette, and the brown precipitate was suspended in 25 mL of 10 mM Tris–HCl buffer, pH 7.4, containing 70 mM sucrose and 210 mM mannitol. The suspended solution was homogenized twice by the tissue grinder and centrifuged at 7,600 g for 10 min. Mitochondria were obtained as a precipitate. After removing the supernatant with a pipette, mitochondria were suspended in 4 mL of 5 mM sodium phosphate buffer, pH 7.4, containing 10 mM KCl, 2 mM MgCl2, 70 mM sucrose, 0.2 mM EDTA, and 210 mM mannitol. To extract lipids from mitochondria, a mixture of chloroform (20 mL), methanol (40 mL), and pure water (12 mL) was added to the mitochondrial solution (4 mL), and the solution mixed
for 2 min. Additional chloroform (20 mL) was added to the sample solution, and the solution was mixed for 30 s. Subsequently, pure water (20 mL) was added to the sample solution, and the solution was mixed for another 30 s (the final proportion of chloroform, methanol, and water was 1:1:0.9) [57]. The sample was filtered through a filter paper on a Buchner funnel and transferred to a separatory funnel. After shaking the funnel and allowing a few minutes for complete separation and clarification of the solutions, the chloroform layer containing the mitochondrial lipids was collected.

Liposome was prepared by the thin film hydration method [58]. A thin lipid film was formed by removing the chloroform by evaporation with a rotary evaporator at room temperature. The obtained lipid film was further dried under vacuum for 3 h. The dried lipid film was hydrated in pure water (1 mL), and the obtained sample was freeze-thawed for 5 times and extruded 11 times through a 100-nm polycarbonate membrane using a Mini Extruder (AVESTIN, Inc. Canada) at room temperature, obtaining liposomes. The zeta potential of the liposomes was measured in 50 mM potassium phosphate buffer, pH 7.0, with a zeta-potential and particle size analyzer (ELSZ-1000, Otsuka Electronics Co., Ltd., Japan) at 25 °C. The obtained liposome was stored at 4 °C and used within 3 days.

2.8 Analysis of oligomerization of c-type cyts by protein refolding

C-type cyts were mixed with the liposomes constructed from mitochondrial lipids in 50
mM HEPES buffer, pH 7.0 (final concentrations of c-type cyt and lipids were 0.2 mM and 4.0 mM, respectively). The obtained solution was incubated at 4 °C for 1 day and subsequently centrifuged at 12,000 g for 1 h.

Unfolded horse cyt c, PA cyt c5,1, and SV cyt c5 were prepared by an addition of 4 M guanidine hydrochloride (GdnHCl) in 50 mM potassium phosphate buffer, pH 7.0, in the presence and absence of 1.0 mM liposome (final concentrations of c-type cyt and lipids were were 0.2 mM and 1.0 mM, respectively). Unfolded HT cyt c552 was prepared under the same conditions except for using 6 M GdnHCl. The unfolded c-type cyts were refolded by removing guanidinium ions from the solution with a desalting column (PD SpinTrap G-25, GE Healthcare) at 4 °C. The obtained solutions were incubated at 4 °C for 5 h to stabilize the oligomers, and subsequently analyzed by SEC (Superdex 75 10/300 GL, GE Healthcare) using the FPLC system (BioLogic DueFlow 10, Bio-Rad) (flow rate, 0.5 mL/min; monitoring wavelength, 410 nm; buffer, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C).

**Accession number**

Coordinates and structure factors of dimeric SV cyt c5 have been deposited in the Protein Data Bank with accession number 6K7C.

3. Results
3.1 Oligomer formation and dimer structure of SV cyt c5

Although several positively charged c-type cyts, horse cyt c [3], HT cyt c552 [4], and *Aquifex aeolicus* cyt c555 [7], are known to domain swap, only one negatively charged c-type cyt, PA cyt c551 [5], with a similar fold is reported to domain swap. Thus, we investigated oligomerization of negatively charged SV cyt c5 to elucidate that domain swapping can also occur generally *in vitro* in negatively charged c-type cyts (Fig. S1). SV cyt c5 in 50 mM potassium phosphate buffer, pH 7.0, was treated with 90% (v/v) ethanol, lyophilized, and dissolved in the same buffer. By analyzing the ethanol-treated SV cyt c5 by SEC, several peaks corresponding to the dimer (11.6 mL), trimer (10.7 mL), and higher-order oligomers (10.0 mL) were observed in the chromatogram in addition to the monomer peak (13.3 mL) (Fig. S2). These results indicate that SV cyt c5 oligomerizes by the ethanol treatment, similar to the properties in other c-type cyts [3-7, 15, 59]. However, oligomers larger than the tetramer were difficult to obtain by the ethanol treatment, similar to the oligomerization of HT cyt c552 and PA cyt c551 [4, 5], whereas a large amount of higher order oligomers was formed in horse cyt c [3].

We solved the X-ray crystal structure (PDB ID: 6K7C) of dimeric SV cyt c5 at 1.15 Å resolution to elucidate its detailed structure at the atomic level (Fig. 2). There were two independent SV cyt c5 molecules in the unit cell of the dimeric SV cyt c5 crystal. The N-terminal region (Gln1–Ser16) containing the heme was exchanged between protomers in the
SV cyt \( c_5 \) dimer, similar to the structures of PA cyt \( c_{551} \) and HT cyt \( c_{552} \) domain-swapped dimers [4, 5]. The hinge loop was constructed with 4 amino acids: Met17, Gly18, Val19, and Ala20. Met53 coordinated to the heme in the domain-swapped dimer of SV cyt \( c_5 \), but the Met53 residue originated from the other protomer to which the heme belonged. The root-mean-square deviation (rmsd) values between the C\( \alpha \) atoms of the monomer and those of the region containing the N-terminal region (Gln1–Ser16) of a protomer and the rest region of the other protomer excluding the hinge region (Met17–Ala20) in the domain-swapped dimer were calculated as 0.40 or 1.32 Å (Table S3). These results indicate that the structures were similar between the monomer and the domain-swapped dimer.

**Fig. 2.** Crystal structures of monomeric and dimeric SV cyt \( c_5 \). (A) Structure of monomeric SV cyt \( c_5 \) (PDB ID: 5B6Q). (B) Structure of dimeric SV cyt \( c_5 \) solved in this study (red and pink, PDB ID: 6K7C). The two protomers are depicted in red and pink, respectively. The Met17–Ala20 residues (hinge loop) are depicted gray. The hemes, Cys11, Cys14, His15, and Met53 are shown as stick models. The N- and C-termini are labeled as N and C, respectively. The sulfur atoms of the heme axial Met ligand and heme-linked Cys are shown in orange, and the nitrogen atoms of the heme axial His ligand are shown in blue.
3.2 Oligomerization of c-type cyts in E. coli

We have previously shown that HT cyt c552 domain swaps in E. coli possessing the HT cyt c552 expression system [18]. To investigate the domain swapping of c-type cyts in E. coli cells, His-tagged c-type cyts and Ni-affinity chromatography were used to avoid the possibility of oligomer formation and dissociation, which may occur during the purification process by ionic exchange chromatography. E. coli expressing His-tagged c-type cyt was cultured, and the monomer and oligomers of c-type cyt were purified by Ni-affinity chromatography, followed by SEC analysis. In the SEC chromatogram of horse cyt c-6His, a peak (36% of the total peak area) was observed at 65–75 mL in addition to the peak (64% of the total peak area) corresponding to the monomer at 80–90 mL (Fig. 3A, horse cyt c-6His). Similarly, a peak (25% of the total peak area) was observed at 60–80 mL in addition to the peak (75% of the total peak area) corresponding to the monomer at 82–92 mL in the SEC chromatogram of HT cyt c552-6His (Fig. 3A, HT cyt c552-6His). However, only monomer peaks were observed at 80–90 mL in the SEC chromatograms of negatively charged PA cyt c551-6His and SV cyt c5-6His (Fig. 3A, PA cyt c551-6His and SV cyt c5-6His). These results clearly show that positively charged horse cyt c and HT cyt c552 form species larger than the monomer in E. coli cells, while negatively charged PA cyt c551 and SV cyt c5 do not.
Fig. 3. SEC analysis of c-type cyts and SDS-PAGE analysis of the oligomer peak fractions in the SEC chromatograms. (A) Elution curves of c-type cyts. All the proteins obtained by culturing *E. coli* in 200 mL of Luria-Bertani media and purified by Ni-affinity chromatography were subjected to the analysis for each c-type cyt. Measurement conditions: column, HiLoad 16/600 Superdex 75 pg column; flow rate, 1.0 mL/min; detection wavelength, 280 nm (blue) and 410 nm (red); buffer, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C. (B) SDS-PAGE of horse cyt c-6His and HT cyt c552-6His fractions: (M) Protein marker, SEC fractions of horse cyt c-6His at (lane 1) 68–73 mL and (lane 2) 81–86 mL, and those of HT cyt c552-6His at (lane 3) 62–67 mL, (lane 4) 67–72 mL, (lane 5) 72–77 mL, and (lane 6) 83–88 mL.

The fraction at 68–73 mL in the horse cyt c SEC chromatogram was subjected to SDS-PAGE analysis (Fig. 3B). A main band corresponding to a protein sized ~14 kDa was detected in the gel. This size corresponded well to that of horse cyt c-6His, indicating that the horse cyt
c oligomers, obtained from *E. coli*, were constructed with monomeric horse cyt *c* and dissociated to the monomers by the SDS treatment. The proteins in the SEC fractions at 62–77 mL of HT cyt *c*552-6His also dissociated to HT cyt *c*552 monomers in the SDS-PAGE analysis (Fig. 3B). These characteristics were similar to those reported for domain swapping [3, 18], indicating that positively charged horse cyt *c* and HT cyt *c*552 form domain-swapped oligomers in *E. coli* cells. However, the protein transportation mechanism may affect the oligomerizations in horse cyt *c* and HT cyt *c*552, which were expressed with PAss, although the protein transportation mechanism by PAss is not known. Additionally, the genes of *c*-type cyts contained rare and low-usage codons for *E. coli*. Thus, the effect of the protein transportation mechanism and rare and low-usage codons on oligomerization of *c*-type cyts in *E. coli* are investigated in the following section.

3.3 *Effects of membrane transportation mechanism type and rare codon on c-type cyt oligomer formation*

To investigate the effect of the protein transportation mechanism and rare and low-usage codons on oligomerization of *c*-type cyts in *E. coli*, we used the co-translational DsbAss and the post-translational PhoAss signal peptides with common codons for *c*-type cyt expression. Oligomer peaks were detected at 60–75 mL in the SEC chromatograms of co- and post-translationally transported HT cyt *c*552-6His (DsbAss-HT cyt *c*552-6His and PhoAss-HT cyt
c552-6His) purified from E. coli cells, whereas no peak was observed in the oligomer region for co- and post-translationally transported PA cyt c551-6His (DsbAss-PA cyt c551-6His and PhoAss-PA cyt c551-6His) (Fig. 4). These results indicate that the membrane transportation mechanism type is also not essential for oligomerization of c-type cyts in cells. The oligomer ratios were 28% and 40% for co- and post-translationally transported HT cyt c552-6His, respectively (Fig. 4), which difference may be caused by the transportation mechanism. However, the structural and translational properties of the signal peptides may affect the oligomerization.

**Fig. 4.** SEC elution curves of co-translationally transported HT cyt c552-6His (DsbAss-HT cyt c552-6His), post-translationally transported HT cyt c552-6His (PhoAss-HT cyt c552-6His), co-translationally transported PA cyt c551-6His (DsbAss-PA cyt c551-6His), and post-translationally transported PA cyt c551-6His (PhoAss-PA cyt c551-6His). The genes of His-
tagged HT cyt $c_{552}$ and His-tagged PA cyt $c_{551}$ were optimized by removing rare and low-usage codons and avoiding stable mRNA secondary structures. All the proteins obtained by culturing *E. coli* in 200 mL of Luria-Bertani media and purified by Ni-affinity chromatography were subjected to the analysis for each c-type cyt. Measurement conditions: column, HiLoad 16/600 Superdex 75 pg column; flow rate, 1.0 mL/min; detection wavelength, 280 nm (blue) and 410 nm (red); buffer, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C.

It has been reported that rare codons may reduce the quantity and quality of synthesized proteins in *E. coli* or other organisms [23, 24, 60]. We studied the effect of gene optimization on oligomerization of c-type cyts in *E. coli* by analyzing the oligomer amount using optimized (without rare and low-usage codons) genes. The genes of His-tagged HT cyt $c_{552}$ and His-tagged PA cyt $c_{551}$ were optimized by removing rare and low-usage codons and avoiding stable mRNA secondary structures. Oligomer peaks were detected at 60–75 mL in the SEC chromatogram of HT cyt $c_{552}$-6His purified from *E. coli* cells using gene-optimized genes (Fig. 4, DsbAss-HT cyt $c_{552}$-6His and PhoAss-HT cyt $c_{552}$-6His). Since HT cyt $c_{552}$ oligomers were obtained from *E. coli* cells expressing the protein with optimized genes, as well as from those expressing the protein with non-optimized genes (Fig. 3A, HT cyt $c_{552}$-6His), rare codons are not essential for the oligomerization by domain swapping. However, the peak areas of oligomeric HT cyt $c_{552}$-6His obtained from optimized genes were 28 and 40% for co- (Fig. 4, DsbAss-HT cyt $c_{552}$-6His) and post-translationally (Fig. 4, PhoAss-HT cyt $c_{552}$-6His) transported HT cyt $c_{552}$-6His, respectively; these values were higher than that (25% of the total peak area) of oligomeric HT cyt $c_{552}$-6His obtained from non-optimized genes (Fig. 3A, HT
3.4 Effect of negatively charged liposomes on c-type cyt oligomerization during refolding

Lipids were extracted from the mitochondria of horse heart to obtain stable, negatively charged liposomes and investigate the effect of liposomes on oligomerization of c-type cyts during folding. The zeta potential of the prepared liposome was $-33 \pm 3$ mV, confirming that the surface of the liposome was negatively charged. We investigated the interaction of c-type cyts with negatively charged liposomes by mixing c-type cyt with the negatively charged liposomes. Red precipitates of lipids were obtained when horse cyt c or HT cyt $c_{552}$ was mixed with the liposomes, whereas the precipitates were white when PA cyt $c_{551}$ or SV cyt $c_{5}$ was mixed with the liposomes (Fig. S3). These results indicated that positively charged horse cyt c and HT cyt $c_{552}$ interact with the negatively charged liposomes, but negatively charged PA cyt $c_{551}$ and SV cyt $c_{5}$ do not.

After refolding horse cyt c and HT cyt $c_{552}$ from GdnHCl-unfolded states in 50 mM potassium phosphate buffer, pH 7.0, and analyzing the protein solution by SEC, the areas of the oligomer peaks (horse cyt c, 10.2–11.5 mL; HT cyt $c_{552}$, 10.0–12.0 mL) relative to those of the monomer peaks (horse cyt c, 11.7–13.5 mL; HT cyt $c_{552}$, 12.2–14.0 mL) in the chromatograms increased about 50% and 20% for horse cyt c and HT cyt $c_{552}$, respectively, by the addition of negatively charged liposomes during folding (Fig. 5). However, the intensities
of the oligomer peaks for PA cyt $c_{551}$ oligomers (10.8–12.0 mL) and SV cyt $c_5$ oligomers (10.5–12.4 mL) in the chromatograms did not change significantly by the liposome addition. These results indicate that positively charged $c$-type cyts interact with the negatively charged liposomes during folding and that more oligomers form during refolding in the presence of liposomes, apparently due to protein assembly on the membrane.

**Fig. 5.** SEC elution curves of $c$-type cyts after refolding in the presence (red) and absence (blue) of negatively charged liposomes constructed with mitochondrial lipids. The intensities of the curves are normalized by the total area of the curve. Measurement conditions: column, Superdex 75 10/300 GL column; flow rate, 0.5 mL/min; monitoring wavelength, 410 nm; buffer, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C.

**4. Discussion**

The oligomer amounts of HT cyt $c_{552}$ expressed *in E. coli* from optimized genes (Fig. 4,
DsbAss-HT cyt \textit{c}_{552}-6\text{His} and PhoAss-HT cyt \textit{c}_{552}-6\text{His}, with co- and post-translational signal peptides, were higher than that expressed from non-optimized genes (Fig. 3A, HT cyt \textit{c}_{552}-6\text{His}), although the signal peptides were different. It has been reported that the amount of HT cyt \textit{c}_{552} oligomers increases \textit{in E. coli} when the expression of HT cyt \textit{c}_{552} is increased [18]. The amount of cyt \textit{c} oligomers also increases by increasing the protein concentration during folding [16]. The optimized genes of HT cyt \textit{c}_{552} are translated rapidly, and presumably the concentration of HT cyt \textit{c}_{552} increases in the cells; thus, chances for a HT cyt \textit{c}_{552} molecule to interact with another HT cyt \textit{c}_{552} molecule may increase in the cells, resulting in the formation of an oligomer. These results support the hypothesis that \textit{c}-type cyt oligomers are formed by intermolecular interaction during folding in the cells as well as \textit{in vitro} [16, 17].

The interaction of \textit{c}-type cyt with the membrane for oligomerization is summarized in Fig. 6. A \textit{c}-type cyt polypeptide (with a signal peptide) is transported from the cytoplasm to the periplasm by secretary proteins (Fig. 6A). Subsequently, the signal peptide is cleaved from the polypeptide, and another \textit{c}-type cyt peptide is transported to the periplasm (Fig. 6B). In the periplasm of bacteria, positively charged apo \textit{c}-type cyt molecules may assemble on the surface of a negatively charged membrane in unfolded states before heme incorporation, allowing unfolded \textit{c}-type cyt molecules to concentrate at the membrane surface (Fig. 6B). When a heme is inserted to an apo \textit{c}-type cyt by Ccm proteins, the holo \textit{c}-type cyt may form a transient oligomer with another apo \textit{c}-type cyt (Fig. 6C). In fact, apo HT cyt \textit{c}_{552} has been previously
detected in the HT cyt c\textsubscript{552} oligomer obtained from \textit{E. coli} cells possessing the HT cyt c\textsubscript{552} expression system \cite{18}. Subsequently, a heme is inserted into the apo \textit{c}-type cyt of the transient holo–apo complex, resulting in formation of a domain-swapped dimer (Fig. 6D). The surfaces of dimeric horse cyt \textit{c} and HT cyt c\textsubscript{552} were positively charged (Figs. S4A and B), and the dimers may further form higher order oligomers at negatively charged membranes, whereas the surfaces of dimeric PA cyt c\textsubscript{551} and SV cyt c\textsubscript{5} were negatively charged (Figs. S4C and D).

\textbf{Fig. 6.} Schematic view of \textit{c}-type cyt folding at the membrane surface. (A) Signal peptide (green) leads the apo \textit{c}-type cyt peptide (blue) to be transported to the periplasm by secretary proteins (orange). (B) Another \textit{c}-type cyt molecule is transported to the periplasm, and apo \textit{c}-type molecules may assemble at the membrane surface. (C) A heme is inserted to one of the apo \textit{c}-type cyt by Ccm proteins (gray), and the holo \textit{c}-type cyt may interact with an apo \textit{c}-type cyt. (D) A heme is inserted to the other apo \textit{c}-type cyt, resulting in formation of a domain-swapped oligomer. The N- and C-termini are labeled as N and C, respectively.

Many proteins can form oligomers by domain swapping \cite{61}. SV cyt c\textsubscript{5} formed oligomers \textit{in vitro} similar to horse cyt \textit{c}, HT cyt c\textsubscript{552}, and PA cyt c\textsubscript{551}. However, positively charged horse cyt \textit{c} and HT cyt c\textsubscript{552} formed oligomers \textit{in E. coli}, whereas negatively charged PA cyt c\textsubscript{551} and SV cyt c\textsubscript{5} did not (Fig. 3). Positively charged \textit{c}-type cyts may interact with the negatively
charged liposomes during refolding, resulting in an increase in the amount of oligomers (Fig. 5). Domain-swapped dimers of other positively charged proteins have also been obtained from *E. coli* cells [62, 63]. Thus, interaction of a positively charged protein with the negatively charged membrane may help proteins to domain swap in *E. coli* and cells containing negatively charged membranes.

5. Conclusions

We found that negatively charged SV cyt *cs* forms domain-swapped dimers *in vitro*, similar to other *c*-type cyts. Positively charged *c*-type cyts oligomerized in cells, whereas negatively charged *c*-type cyts did not. These results show that the surface charge of *c*-type cyt plays an important role in domain swapping in cells; positively charged *c*-type cyt may accumulate on the negatively charged membrane during folding and induce domain swapping. Domain-swapped oligomers may be obtained from *E. coli* cells easier for positively charged proteins compared to negatively charged proteins, owing to the interaction of positively charged proteins at the negatively charged membranes in the cells. These results provide a new view on domain swapping in cells.

Conflicts of interest

All the authors declare no competing interests.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://doi.org/XXXXXX

References


[26] Chartier M, Gaudreault F, Najmanovich R. Large-scale analysis of conserved rare codon
clusters suggests an involvement in co-translational molecular recognition events.


[41] Stangl M, Schneider D. Functional competition within a membrane: lipid recognition vs.


Supplementary Data

Protein surface charge effect on 3D domain swapping in cells for c-type cytochromes

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\textbf{Table S1.} Amino acid sequences of the signal peptides. p. S6

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\textbf{Table S3.} Root-mean-square deviation values for the C\textalpha{} atoms between the monomer and dimer. p. S8
Fig. S1. Electrostatic surface potentials of (A) horse cyt c (PDB ID: 1HRC), (B) HT cyt c552 (PDB ID: 1YNR), (C) PA cyt c551 (PDB ID: 351C), and (D) SV cyt c5 (PDB ID: 5B6Q) depicted using PyMOL. Blue is electropositive and red is electronegative.
Fig. S2. SEC elution curves of SV cyt c5. (A) Elution curve after an addition of 90% (v/v) ethanol, subsequent lyophilization, and dissolution with buffer. (B) Elution curve of monomeric SV cyt c5. (C) Elution curve of dimeric SV cyt c5. The intensities of the curves are normalized by the highest absorbance. Measurement conditions: column, Superdex 75 10/300 GL column; flow rate, 0.5 mL/min; detection wavelength, 280 nm (blue) and 410 nm (red); buffer, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C.
**Fig. S3.** Precipitates obtained after incubation of (A) horse cyt c, (B) HT cyt c₅₅₂, (C) PA cyt c₅₅₁, and (D) SV cyt c₅ with negatively charged liposomes constructed from mitochondrial lipids at 4 °C for 1 day, followed by centrifugation (12,000 g, 1 h, 4 °C).
**Fig. S4.** Electrostatic surface potentials of (A) dimeric horse cyt c (PDB ID: 3NBS), (B) dimeric HT cyt c$_{552}$ (PDB ID: 3VYM), (C) dimeric PA cyt c$_{551}$ (PDB ID: 3X39), and (D) dimeric SV cyt c$_{5}$ (PDB ID: 6K7C) depicted using PyMOL. Blue is electropositive and red is electronegative.
Table S1
Amino acid sequences of the signal peptides used in this study.

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<th>Signal peptide</th>
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<tr>
<td>PAss</td>
<td>MKPYALLSLLATGTLLA QGA WA</td>
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<tr>
<td>DsbAss</td>
<td>MKKIWLALAGLVLAFSASA</td>
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<tr>
<td>PhoAss</td>
<td>MKQSTIALALLPLLFTPVT KA</td>
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### Table S2
Statistics of data collection and structure refinement of the SV cyt c5 dimer (PDB ID: 6K7C).

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<tr>
<td><strong>Unit cell parameters</strong></td>
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<td>(a, \beta, \gamma) (°)</td>
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<td><strong>Completeness (%)</strong></td>
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Statistics for the highest-resolution shell are given in parentheses.

\(R_{merge}=\sum_{hkl} | I - <I> | (\sum_{hkl} | I |)^{-1}\).

\(R_{work}=\sum_{hkl} | | F_{obs} | - k | F_{calc} | (\sum_{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 S3
Root-mean-square deviation (RMSD) values for the Cα atoms between the monomer and dimer.

<table>
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<tr>
<td>Unit 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.40</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1–16 residues of protomer 1 and 21–76 residues of protomer 2.

<sup>b</sup> 1–16 residues of protomer 2 and 21–76 residues of protomer 1.