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Formation of Domain-Swapped Oligomer of Cytochrome *c* from Its Molten Globule State Oligomer

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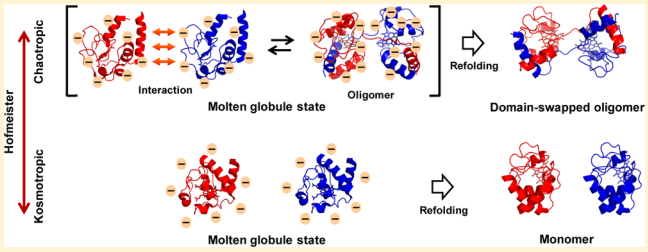
Supporting Information

ABSTRACT: Many proteins, including cytochrome *c* (cyt *c*), have been shown to form domain-swapped oligomers, but the factors governing the oligomerization process remain unrevealed. We obtained oligomers of cyt *c* by refolding cyt *c* from its acid molten globule state to neutral pH state under high protein and ion concentrations. The amount of oligomeric cyt *c* obtained depended on the nature of the anion (chaotropic or kosmotropic) in the solution: ClO_4^- (oligomers, 11% ± 2% (heme unit)), SCN^- (10% ± 2%), I^- (6% ± 2%), NO_3^- (3% ± 1%), Br^- (2% ± 1%), Cl^- (2% ± 1%), and SO_4^{2-} (3% ± 1%) for refolding of 2 mM cyt *c* (anion concentration 125 mM). Dimeric cyt *c* obtained by refolding from the molten globule state exhibited a domain-swapped structure, in which the C-terminal α-helices were exchanged between protomers. According to small-angle X-ray scattering measurements, approximately 25% of the cyt *c* molecules were dimerized in the molten globule state containing 125 mM ClO_4^-. These results indicate that a certain amount of molten globule state oligomers of cyt *c* convert to domain-swapped oligomers during refolding and that the intermolecular interactions necessary for domain swapping are present in the molten globule state.



Cytochrome *c* (cyt *c*) is a fundamental electron transfer heme protein in the mitochondrial respiratory chain. It has also been shown to play a key role in apoptosis.[1,2] Cyt *c* is globular and possesses three relatively long α-helices.[3−5] It is positively charged, and the p*I* of horse cyt *c* is 10.0 at 20 °C.[6] The heme of cyt *c* is covalently bound to Cys14 and Cys17. His18 and Met80 of cyt *c* are axially coordinated to the heme iron.[1−3] We have previously shown that horse cyt *c* forms oligomers by domain swapping its C-terminal α-helix.[7] The Met80−heme coordination bond was significantly perturbed in the domain-swapped oligomer in the oxidized state, and the dimer was shown to bind external ligands, such as cyanide ion and hydrogen peroxide.[7−9]

The molten globule state is a compact, partially folded conformation of a protein that has significant secondary structures with a slightly fluctuating or disordered tertiary structure compared with the native state.[10−17] The molten globule state has been considered as an intermediate state during folding and thus has been studied extensively to understand the stability and folding process of proteins.[10−21] The molten globule state of cyt *c* is obtained at acidic pH by an addition of salt,[22−24] alcohol,[25] or surfactant[26,27] and at alkaline pH by an addition of NaCl.[28,29] It has been reported that the tertiary conformation of the molten globule state of cyt *c* is affected by the size of the anions in the solution.[23] The heme of cyt *c* is native-like Met/His coordinated in the molten globule state containing NO_3^-, Cl^-, or Br^-, whereas the interaction between the heme propionate and Trp59 is weakened, and the heme converts to the bis-His coordinated species in the molten globule state containing ClO_4^- or I^-.[15,23,30,31]

Research on protein oligomers has gained interest, owing to their role in protein misfolding and aggregation.[32−35] Oligomerization of proteins by domain swapping has been observed in various proteins.[7−9,32−50] For example, RNase A has been shown to form domain-swapped oligomers by exchanging its N-terminal α-helix, C-terminal β-strand, or both segments between molecules.[36,40−42,48] It has been reported that RNase A partially retains its native helices in 40% acetic acid, whereas its β-sheet is fully denatured.[48] RNase A dimerized from the folding intermediate obtained during the

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dissolution process of its precipitates.[48] For cyt *c*, domain-swapped oligomers were produced by intermolecular hydrophobic interaction between the N- and C-terminal α-helices during folding from its guanidinium ion-induced denatured state.[51] It has also been shown that the charge screening effect of KCl accelerates the early polypeptide chain collapse during folding of cyt *c*.[52] However, the factors governing the domain swapping process remain unrevealed. We found that cyt *c* molecules in the molten globule state at high protein and ion concentrations form oligomers, of which a certain amount convert to domain-swapped oligomers by refolding to the neutral pH state.

MATERIALS AND METHODS

Analysis of Oligomerization of cyt *c*. Oxidized horse cyt *c* (0.15−4.5 mM; Sigma-Aldrich) in the denatured state at pH 2.2 was obtained by addition of 1.5 M HCl to a cyt *c* aqueous solution. Oxidized horse cyt *c* in the molten globule state was prepared by addition of various salts (NaCl, 10 mM−1.0 M; $NaClO_4$, 25−150 mM; NaSCN, 25−150 mM; NaI, 25−200 mM; NaBr, 25−500 mM; $NaNO_3$, 25−500 mM; Na_2SO_4, 25−500 mM) to the acid denatured cyt *c* solution. The pH of the cyt *c* solution was readjusted to 2.2 with 1.5 M HCl after the addition of salts. For refolding of cyt *c* from the molten globule state, the solution of cyt *c* in the molten globule state was mixed with 100 mM potassium phosphate or Tris-HCl buffer, pH 8.0, containing the same amount of salts, using a rapid mixer (SM10003, Unisoku, Japan) (mixing dead time, 1−2 ms) at 10 °C (mixing ratio, cyt *c* solution/buffer = 1:2). Potassium phosphate buffer was used except for the $NaClO_4$-induced molten globule state (using Tris-HCl buffer), since precipitates formed when 100 mM potassium phosphate buffer, pH 8.0, was used. The pH of the solution increased to about 7.0 after the mixing procedure. The actual concentration of cyt *c* during the refolding was lower than the cyt *c* concentration in the molten globule state, since refolding of cyt *c* started during mixing of the two solutions. The solution of oxidized horse cyt *c* in the acid denatured state at pH 2.2 was also mixed with 100 mM potassium phosphate, pH 8.0, by the same method.

The amount of oligomers in the cyt *c* solution was analyzed by size exclusion chromatography with a 10/300 Superdex 75 GL gel column (GE Healthcare, Buckinghamshire) using a fast protein liquid chromatography (FPLC) system (Biologic DuoFlow 10, Biorad, CA). Conditions for the FPLC measurements were as follows: flow rate, 0.4 mL/min; monitoring wavelength, 409 nm; solvent, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C. The elution curves were fitted with a multipeak Gaussian fitting procedure (Origin 8, OriginLab Corporation, MA). The percentages of the monomer and oligomers were calculated as previously reported.[51] The amount of oligomers obtained by conversion of oxidized horse cyt *c* from the acid denatured state to neutral pH state was analyzed by the same method.

Optical Absorption and Circular Dichroism Measurements. Absorption and circular dichroism (CD) spectra of oxidized horse cyt *c* (10 μM, heme unit) were measured at 20 °C with a UV-2450 spectrophotometer (Shimadzu, Japan) and J-725 CD spectrophotometer (Jasco, Japan), respectively. Quartz cells with 1 and 0.1 cm path lengths were used for absorption and CD measurements, respectively. The cyt *c* solution was passed through a 0.45 μm filter (Millex, Millipore, MA) before the measurement. The midpoint concentrations (C_m) of the anions for the transition of cyt *c* from the acid denatured state to molten globule state were obtained by monitoring the optical absorbance at 394 nm and the mean residue ellipticity (θ) at 222 nm.

X-ray Crystallography of Dimeric cyt *c*. Dimeric horse cyt *c* was obtained by mixing a concentrated solution of oxidized horse cyt *c* (4.5 mM) in the molten globule state with 100 mM potassium phosphate buffer, pH 8.0, containing 1.0 M NaCl (1:2 mixing ratio), using the rapid mixer (SM10003, Unisoku) at 10 °C. The obtained dimer was purified by gel chromatography (Hiload 26/60 Superdex 75, GE Healthcare) using the FPLC system (Biologic Duoflow 10, Biorad). Conditions for the FPLC were as follows: flow rate, 0.8 mL/min; monitoring wavelength, 409 nm; solvent, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C.

Crystallization was carried out with the sitting drop vapor diffusion method at 277 K. Dimeric horse cyt *c* (18 mg/mL) was dissolved in 50 mM Tris-HCl buffer, pH 7.4. The dimeric cyt *c* solution (2 μL) was mixed with the reservoir solution (2 μL). The obtained droplet was equilibrated and kept at 4 °C for crystallization. The reservoir solution was 100 mM Tris-HCl, pH 8.0, containing 40% PEG 200 and 200 mM $(NH_4)_2HPO_4$.

The X-ray diffraction data were collected at SPring-8 beamline BL38B1 (Hyogo, Japan). The preliminary structure was obtained by a molecular replacement method (MOLREP), where the atomic coordinates of the structure of dimeric horse cyt *c* (PDB code 3WC8) were used as a starting model. The REFMAC program was used for structure refinement. The COOT program was used to manually correct the molecular model and pick up water molecules in the electron density map. The data collection and refinement statistics are summarized in Table S1, Supporting Information.

Small Angle X-ray Scattering Measurements. Small angle X-ray scattering (SAXS) measurements were carried out using an UltraX18 rotating anode X-ray generator (Rigaku, Tokyo, Japan) and X-ray image intensifier CCD detector (Hamamatsu Photonics K.K., Shizuoka, Japan) as reported.[51] The X-ray tube voltage and current were 40 kV and 100 mA, respectively. In the small angle region, the scattering intensity is given by the Guinier approximation and expressed as

$$I(Q) \cong I(0) \times \exp(-1/3Q^2R_g^2) \quad (1)$$

Q (= $4\pi \sin\theta/\lambda$) is the amplitude of the scattering vector, where 2θ and λ are the scattering angle and wavelength of the X-ray, respectively. $I(0)$ represents the scattering intensity at $Q = 0$, and R_g represents the radius of gyration. The slope and *Y*-intersection of the Guinier plot [ln $I(Q)$ versus Q^2] correspond to $-1/3 \times Rg^2$ and ln $I(0)$, respectively. *Rg* and $I(0)/C$ were obtained from the Guinier plots for a series of scattering curves of different concentrations of oxidized horse cyt *c* (0.5−2.0 mM) in the molten globule state, where *C* represents the mass concentration of cyt *c*. The concentration of $NaClO_4$ in the molten globule solution was adjusted to 125 mM. The *Rg* value of infinite dilution of cyt *c* was obtained by extrapolation of the *Rg* value to zero cyt *c* concentration and defined as Rg_0. The scattering data were plotted as $I(Q)/C \times Q^2$ versus Q for the Kratky plot.

RESULTS

Oligomerization of cyt *c*. Approximately 2−20% (heme unit) of oxidized horse cyt *c* was detected as oligomers (mostly dimers) in addition to the monomers in the elution curve of size exclusion chromatography of the cyt *c* solution, which was

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obtained by refolding 2.0 mM cyt *c* from its salt-induced acid molten globule state to neutral pH state in the presence of a high concentration of ions (Figure 1). These results show that

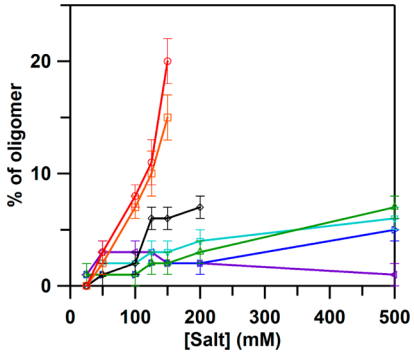

Figure 1. Amount of oxidized oligomeric horse cyt *c* obtained by refolding of cyt *c* (2.0 mM) from the molten globule state under different anions. Refolding of cyt *c* was performed by mixing the cyt *c* solution at pH 2.2 containing different salts (25–500 mM) with 100 mM potassium phosphate buffer, pH 8.0, or Tris-HCl buffer, pH 8.0, containing the same concentration of salts (1:2 mixing): $NaClO_4$ (○, red); NaSCN (□, orange); NaI (◇, black); NaBr (Δ, green); $NaNO_3$ (▽, cyan); NaCl (▷, blue); Na_2SO_4 (◁, purple). Refolding was performed at 10 °C.

cyt *c* oligomers are formed when cyt *c* is refolded from its molten globule state under high ion concentrations. The amount of oligomeric cyt *c* depended on the anion in the solution: ClO_4^- (oligomers, 11% ± 2% (heme unit)), SCN^- (10% ± 2%), I^- (6% ± 2%), NO_3^- (3% ± 1%), Br^- (2% ± 1%), Cl^- (2% ± 1%), and SO_4^{2-} (3% ± 1%) for anion concentrations of 125 mM (Figure 1 and Figure S1 of the Supporting Information); ClO_4^- (oligomers, 20% ± 2% (heme unit)), SCN^- (15% ± 2%), I^- (6% ± 2%), NO_3^- (3% ± 1%), Br^- (2% ± 1%), Cl^- (2% ± 1%), and SO_4^{2-} (2% ± 1%) for anion concentrations of 150 mM (Figure 1 and Figure S2 of the Supporting Information). However, only about 2% ± 1% (heme unit) of cyt *c* oligomerized (mostly as dimers) when refolding from its acid denatured state at pH 2.2 without addition of salts (Figures S1 and S2 of the Supporting Information).

More oligomers of cyt *c* were obtained when refolding from the molten globule state under higher ion concentrations. The amount of oligomeric cyt *c* obtained (as dimers) increased from 2% ± 1% to 16% ± 2% (heme unit) when the NaCl concentration was increased from 10 mM to 1.0 M for refolding of 3.0 mM cyt *c* from its the molten globule state (Figure 2A). The oligomer obtained also increased from 1% ± 1% to 20% ± 2% (heme unit) when the $NaClO_4$ concentration was increased from 25 to 150 mM for refolding of 2.0 mM cyt *c* from its the molten globule state (Figure 2B). Noticeably, we obtained high order oligomers for 150 mM $NaClO_4$, whereas cyt *c* precipitated upon refolding from the molten globule state in the presence of $NaClO_4$ higher than 150 mM. The amount of oligomeric cyt *c* obtained also increased when the protein concentration in the molten globule state before refolding was increased: 1% ± 1% (heme unit) ([cyt *c*], 0.25 mM) and 25% ± 2% ([cyt *c*], 4.5 mM) for refolding in the presence of 1.0 M NaCl; 2% ± 1% ([cyt *c*], 0.5 mM) and 15% ± 2% ([cyt *c*], 4.5 mM) for refolding in the presence of 125 mM $NaClO_4$ (Figure 2C,D). We obtained high order oligomers for [cyt *c*] > 2.0 mM

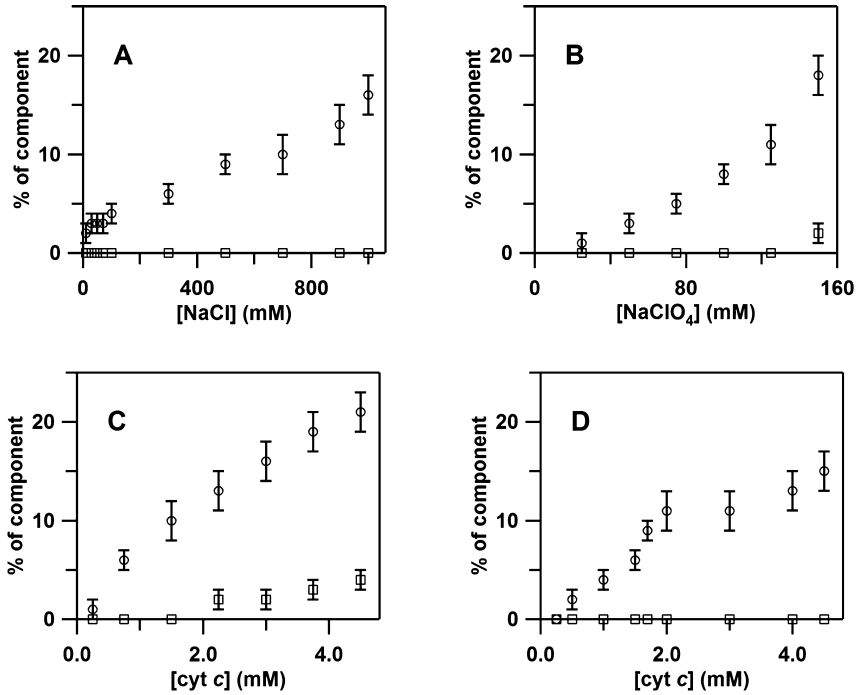

Figure 2. Amount of oxidized oligomeric horse cyt *c* obtained by refolding of cyt *c* from the molten globule state under different ion and protein concentrations: dimer (○) and higher order oligomers (□). Refolding of cyt *c* was performed by 1:2 mixing the solution of cyt *c* in the molten globule state at pH 2.2 containing (A, C) NaCl or (B, D) $NaClO_4$ with (A, C) 100 mM potassium phosphate buffer, pH 8.0, or (B, D) 100 mM Tris-HCl buffer, pH 8.0, containing the same concentration of salts. Mixing conditions: (A) [cyt *c*] (in the molten globule state) 3.0 mM, [NaCl] 0.01–1.0 M; (B) [cyt *c*] 2.0 mM, [$NaClO_4$] 25–150 mM; (C) [cyt *c*] 0.25–4.5 mM, [NaCl] 1.0 M; (D) [cyt *c*] 0.25–4.5 mM, [$NaClO_4$] 125 mM. Refolding was performed at 10 °C.

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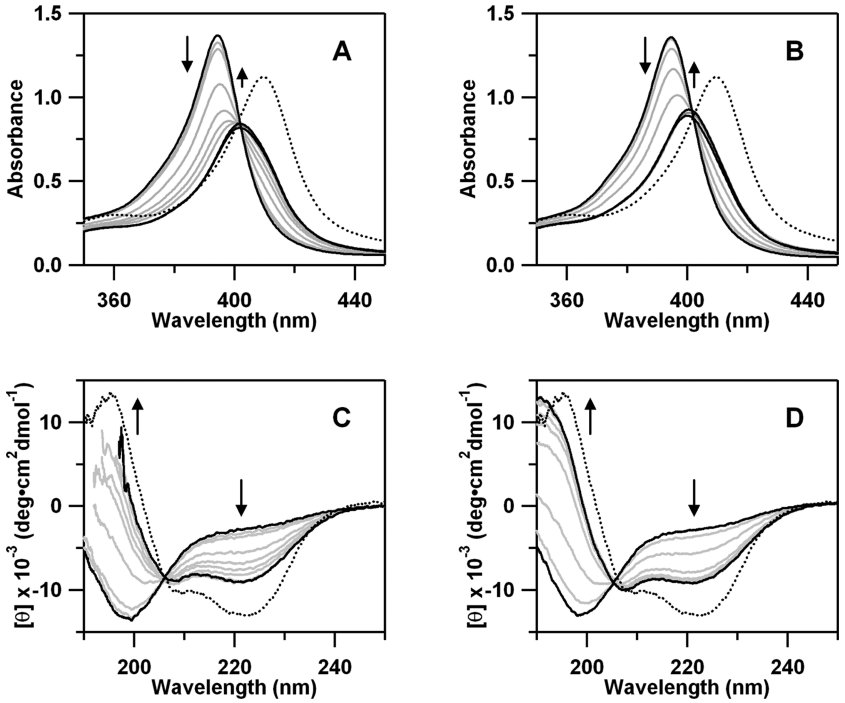

Figure 3. Optical absorption and CD spectra of oxidized monomeric horse cyt *c* (10 μM, heme unit) at pH 2.2 containing NaCl or $NaClO_4$: (A, B) optical absorption and (C, D) CD spectra. Spectra at (A, C) 0, 0.005, 0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5, and 1 M NaCl and (B, D) 0, 1, 3, 6, 10, 25, 50, 100, and 150 mM $NaClO_4$ are shown. Black lines represent the spectra of the solution containing (A) 0, 0.3, and 1.0 M NaCl, (B) 0, 25, and 100 mM $NaClO_4$, (C) 0 and 1.0 M NaCl, and (D) 0 and 100 mM $NaClO_4$, respectively. Dotted lines represent the spectra of native cyt *c* in 50 mM potassium phosphate buffer, pH 7.0. The spectra were measured at 20 °C.

in the molten globule state containing 1 M NaCl. However, oligomeric cyt *c* detected decreased upon increasing the concentration of SO_4^{2-} during rapid mixing of the acidic cyt *c* solution with buffer: 3% ± 1% and 1% ± 1% (heme unit) for 50−125 and 500 mM SO_4^{2-}, respectively (Figure 1).

Optical Absorption and CD Spectra of cyt *c* in the Molten Globule State Containing NaCl or $NaClO_4$. The Soret band at 394 nm of acid denatured oxidized cyt *c* red-shifted to ∼400 nm with an addition of NaCl or $NaClO_4$ at pH 2.2, corresponding to the formation of the salt-induced molten globule state (Figure 3A,B).[22] The C_m values of the transition in the absorption spectra were obtained as about 31 and 3 mM for the addition of NaCl and $NaClO_4$, respectively (Figures S3A and S4A of the Supporting Information). These values were similar to those reported previously.[53] In the presence of high concentrations of NaCl (0.3−1.0 M) or $NaClO_4$ (25−150 mM), the Soret band of cyt *c* was also observed at ∼400 nm. Negative CD bands were generated at 208 and 222 nm upon the addition of NaCl or $NaClO_4$ to the acid denatured cyt *c* solution (Figure 3C,D),[53] with similar C_m values as obtained from the absorption spectra (Figures S3 and S4 of the Supporting Information). The 208 and 222 nm bands were also observed in the CD spectra at high concentrations of NaCl (0.3−1.0 M) or $NaClO_4$ (25−150 mM) (Figure 3C,D). These results show that the molten globule state of cyt *c* was maintained at high ion concentrations, much higher than the C_m values. However, the Soret band red-shifted further, about 0.5−2 nm, and its intensity increased slightly at higher ion concentrations (NaCl, 0.5−1.0 M; $NaClO_4$, 50−150 mM) compared with that at lower ion concentrations (NaCl, 0.3 M; $NaClO_4$, 25 mM) (Figure 3A,B).

Crystal Structure of Dimeric cyt *c* Obtained by Refolding from the Molten Globule State. We performed X-ray crystallographic analysis to elucidate the structure of dimeric horse cyt *c* obtained by refolding from the NaCl-induced molten globule state. The 1.8 Å resolution structure of dimeric cyt *c* exhibited a domain-swapped structure (PDB code 3WUI), where the C-terminal α-helix was displaced from its original position in the monomer and replaced by the corresponding helix of another cyt *c* molecule (Figure 4). The present dimeric cyt *c* structure was similar to those obtained by treatment with ethanol[7] and by refolding from the guanidinium ion-induced denatured state.[51]

Oligomerization of cyt *c* in the Molten Globule State. SAXS can be applied to study oligomerization of proteins in solution, where it is difficult to obtain crystals for detailed structural analysis.[54−56] SAXS profiles of oxidized horse cyt *c* at pH 2.2 were measured under various concentrations of $NaClO_4$ up to 125 mM, since cyt *c* oligomers larger than dimers were

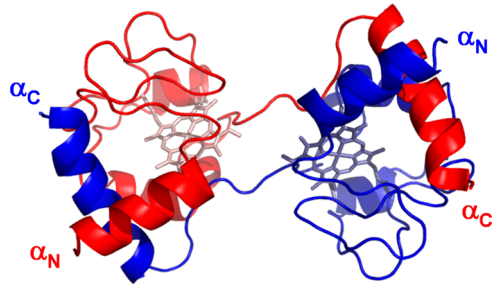

Figure 4. Crystal structure of dimeric horse cyt *c* (red and blue, PDB ID 3WUI) obtained by refolding of cyt *c* (4.5 mM) from its molten globule state under 1.0 M NaCl. The hemes are shown in pale stick models. The N- and C-terminal α-helices are labeled as α_N and α_C, respectively.

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detected by refolding from the molten globule state under 150 mM $NaClO_4$. A peak was observed at about 0.10 $Å^{-1}$ in the Kratky plot of cyt *c* at pH 2.2 in the presence of 25 mM $NaClO_4$, whereas no distinct peak was observed in the absence of $NaClO_4$ (Figure 5A). These results indicate that cyt *c* folded

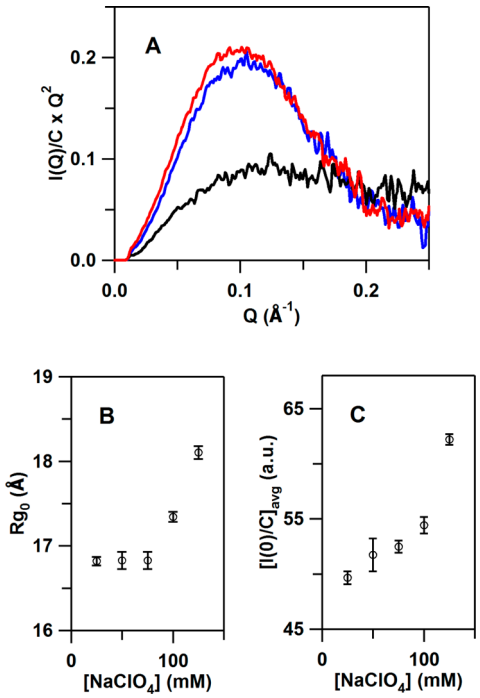

Figure 5. (A) Small-angle X-ray scattering curves of oxidized horse cyt *c* shown by Kratky plots: Acid denatured state (black) and molten globule state containing 25 mM (blue) and 125 mM (red) $NaClO_4$. Measurement conditions: [cyt *c*], 2.0 mM; [$NaClO_4$], 0, 25, and 125 mM; pH 2.2; temperature, 10 °C. (B) $NaClO_4$ concentration dependence of Rg_0. (C) $NaClO_4$-concentration dependence of $[I(0)/C]_{avg}$.

into a salt-induced molten globule state upon the addition of $NaClO_4$ to the acid denatured state, as reported previously.[57] The Rg_0 value of 16.9 ± 0.2 Å of cyt *c* in the molten globule state containing 25 mM $NaClO_4$ corresponded relatively well to that of the NaCl-induced molten globule state (NaCl 500 mM) of cyt *c* (17.4 ± 0.1 Å),[58] which has been revealed to be monomeric.[58] These results show that cyt *c* was also in a monomeric form in the molten globule state containing 25 mM $NaClO_4$. However, the peak observed in the Kratky plot of cyt *c* at pH 2.2 shifted slightly to a smaller *Q* value upon increase in the $NaClO_4$ concentration from 25 to 125 mM (Figure 5A), and the Rg_0 value increased to 18.3 ± 0.2 Å at 125 mM $NaClO_4$ (Figure 5B and Table 1).

Table 1. $NaClO_4$-Concentration Dependance of Rg_0 and $[I(0)/C]_{avg}$ of cyt *c* at pH 2.2 Obtained from the SAXS Analysis

[$NaClO_4$]	Rg_0 (Å)	$[I(0)/C]_{avg}$ (au)
25	16.9 ± 0.2	49.6 ± 0.6
50	17.0 ± 0.2	51.7 ± 1.5
75	16.8 ± 0.2	52.5 ± 0.5
100	17.3 ± 0.2	54.4 ± 0.8
125	18.3 ± 0.2	62.2 ± 0.5

The $I(0)/C$ values resulting from the Guinier analysis did not exhibit an obvious protein concentration dependence (Figure S5 of the Supporting Information). Therefore, the average value of $I(0)/C$ was calculated for 0.5−2.0 mM cyt *c* ($[I(0)/C]_{avg}$) and was used to compare the apparent molecular weight under various salt concentrations. The $[I(0)/C]_{avg}$ value of cyt *c* in the molten globule state showed a similar $NaClO_4$-concentration dependence to that of Rg_0, where $[I(0)/C]_{avg}$ increased 25% ± 1% when the $NaClO_4$ concentration was increased from 25 to 125 mM (Figure 5C and Table 1). The $I(0)/C$ value did not change significantly for different concentrations of $NaClO_4$ at pH 7.0, showing that the salt concentration dependence of the solvent density could be ignored (Figure S6 of the Supporting Information). The $[I(0)/C]_{avg}$ does not change upon a conformational change of the protein without a change of its molecular weight, since $[I(0)/C]_{avg}$ is proportional to the molecular weight of the protein, whereas the Rg_0 value changes upon the conformational change. Therefore, the higher $[I(0)/C]_{avg}$ value in the molten globule state at high salt concentrations indicated that cyt *c* oligomers were produced in the molten globule state, which contributed to a certain amount of increase in the Rg_0 value (Figure 5B and Table 1). The amount of cyt *c* molecules in the oligomeric form was estimated to be about 25% (heme unit) in the molten globule state containing 125 mM $NaClO_4$ with the assumption that all the oligomers were dimers. Although the molar fraction of the dimer in the molten globule state was larger than that of the domain-swapped dimer (11%) obtained by refolding from 2 mM cyt *c* in the molten globule state under 125 mM $NaClO_4$, these results show that a certain amount of molten state dimers convert to domain-swapped dimers during the refolding to the neutral pH state.

DISCUSSION

Oligomerization by domain swapping has been reported for various proteins, where the corresponding structural regions are exchanged between molecules.[7,9,36−50] RNase A forms different domain-swapped dimers by swapping a single N-terminal α-helix[36] or C-terminal β-strand.[40] The domain-swapped oligomers of RNase A are formed at high temperatures from its completely denatured state.[48,59] Domain swapping by complete unfolding has also been suggested for cyanovirin-N, Stefin A, and p13suc1.[60−62] Different multimeric domain-swapped structures have been observed for cyanovirin-N, suggesting trapped folding intermediates.[63] For cyt *c*, a transient dimer has been detected by SAXS measurements during refolding.[54] We have demonstrated that domain-swapped oligomers of cyt *c* are formed during folding from its guanidinium ion-induced denatured state by intermolecular hydrophobic interaction between the N- and C-terminal α-helices.[51] In the present study, a significant amount of oligomers of cyt *c* were formed by refolding from its acid molten globule state to neutral pH state (Figure 1), and dimeric cyt *c* obtained by the refolding from the molten globule state showed a domain-swapped structure (Figure 4). It has also been reported that cystatin forms a domain-swapped dimer from its reduced, molten globule state.[37] These results show that domain-swapped oligomers of proteins may form by folding from the molten globule state.

The Soret band at 394 nm of acid denatured cyt *c* red-shifted to 400 nm upon the addition of ions, due to formation of the molten globule state (Figure 3A,B).[22,30,31,64] However, a further red-shift of the Soret band and a slight increase in its intensity

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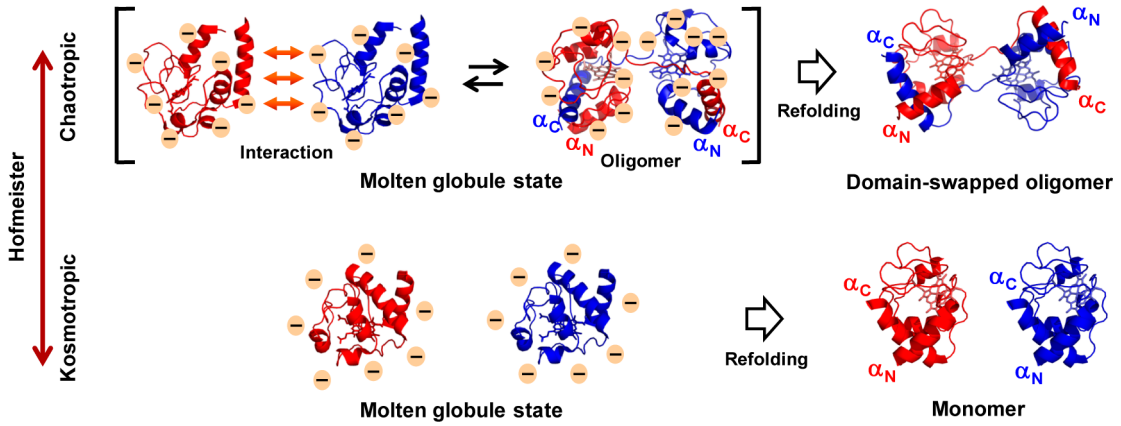

Figure 6. Schematic view of refolding of horse cyt *c* from its molten globule state. The two cyt *c* molecules are shown in red and blue colors. Ions are shown in pale orange circles. Structures of monomeric (PDB entry 1HRC) and dimeric horse cyt *c* (PDB entry 3WUI) are shown.

were observed for cyt *c* upon the addition of higher concentrations of NaCl (0.5−1.0 M) or $NaClO_4$ (50−150 mM) to its molten globule state (Figure 3A,B). We attribute these changes in the Soret band to the oligomerization of cyt *c* in the molten globule state, which has also been revealed by the SAXS experiments (Figure 5).

The $I(0)/C$ values resulting from the Guinier analysis did not exhibit an obvious protein concentration dependence (Figure S5 of the Supporting Information). The number of ClO_4^- ions interacting with each cyt *c* molecule in the molten globule state may decrease when the concentration of cyt *c* increases, which may cause decrease in the interaction between cyt *c* molecules. This effect may serve as a counterbalance to the effect of increase in the protein concentration on the $I(0)/C$ value. Approximately 25% (heme unit) of cyt *c* formed dimers in the molten globule state containing 125 mM $NaClO_4$ according to SAXS experiments, whereas approximately 11% (heme unit) of domain-swapped dimers was obtained by refolding from the molten globule state under 125 mM $NaClO_4$ (Figure 1). However, only about 5% ± 1% of cyt *c* (heme unit) was observed as dimers when the refolding from the acid denatured state was performed by mixing 2 mM cyt *c* at pH 2.2 with 100 mM Tris-HCl, pH 8.0, containing 187.5 mM $NaClO_4$ at 1:2 ratio ($NaClO_4$ concentration after mixing was 125 mM) (Figure S7 of the Supporting Information). These results show that a certain amount of cyt *c* oligomers in the molten globule state convert to domain-swapped oligomers and that the intermolecular interaction for domain swapping is already present in the molten globule state. The amount of oligomers obtained by refolding from the molten globule state may decrease compared with that of the molten globule state oligomer, since not all of the oligomers in the molten globule state may contain the native-like interaction between the N-terminal and C-terminal helices.

More oligomers of cyt *c* formed from the molten globule state containing high concentrations of anions in the following order: $ClO_4^- \approx SCN^- > I^- > NO_3^- \approx Br^- \approx Cl^- > SO_4^{2-}$ (Figure 1). This order essentially followed the Hofmeister series. According to the Hofmeister series, chaotropic anions (e.g., ClO_4^- and SCN^-) known as "water structure breakers" destabilize the protein at neutral pH, whereas kosmotropic anions (e.g., SO_4^{2-}) are "water structure makers" and stabilize the protein.[65] A relatively large amount of oligomeric cyt *c* (15−20%, heme unit) formed by refolding from the molten globule state in the presence of high concentrations of chaotropic anions, such as ClO_4^- or SCN^- (150 mM), whereas the amount of oligomers obtained decreased (5−7%, heme unit) by refolding in the presence of high concentrations of moderate chaotropic anions, such as I^- (200 mM), Br^- (500 mM), or NO_3^- (500 mM) (for 2.0 mM cyt *c*) (Figure 1). Large anions have the tendency to be weakly hydrated,[23] and chaotropic anions are less hydrated compared with kosmotropic anions.[66,67] Weakly hydrated chaotropic anions may interact with the nonpolar surface groups of proteins, whereas well hydrated kosmotropic anions may bind to the polar side chains of proteins via ion-pairing (Figure 6).[66] NMR and thermodynamic measurements along with molecular dynamics simulations suggested that the large weakly hydrated chaotropic anions (I^-, ClO_4^-, and SCN^-) interact with the polypeptide backbone of the protein, whereas slightly more hydrated Cl^- binds weakly and well hydrated SO_4^{2-} is repelled from the polypeptide backbone.[67] In fact, large chaotropic anions (I^- and ClO_4^-) are reported to weaken the tertiary interaction between Trp59 and the heme propionate of cyt *c* in the molten globule state.[15,23,30,31] Therefore, chaotropic anions may produce a stronger intermolecular interaction between cyt *c* molecules in the molten globule state and induce more oligomers of cyt *c* in its molten globule state compared with moderate chaotropic and kosmotropic anions, which resulted in more formation of domain-swapped oligomers by refolding from the molten globule state containing chaotropic anions (Figure 6). However, the amount of oligomeric cyt *c* detected decreased when the solution conditions were changed from pH 2.2 to neutral pH by rapid mixing in the presence of high SO_4^{2-} concentrations (200−500 mM), since SO_4^{2-} stabilizes the native structures of proteins and thus cyt *c* was in the native form at pH 2.2 in the presence of high SO_4^{2-} concentrations before the mixing (Figure S8 of the Supporting Information).[31] These results strongly support the hypothesis that cyt *c* oligomers in the molten globule state induce formation of domain-swapped oligomers.

CONCLUSION

Domain-swapped dimeric cyt *c* was formed by refolding from the molten globule state. The amount of oligomers obtained by refolding from the molten globule state increased when the cyt *c* and ion concentrations were increased. More oligomers were obtained by refolding from the molten globule state containing chaotropic anions compared with the molten globule state containing moderate chaotropic or kosmotropic anions.

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Approximately 25% (heme unit) of the cyt *c* molecules were dimerized in the molten globule state containing 125 mM $NaClO_4$, and about 11% (heme unit) were obtained as domain-swapped dimers upon refolding from the molten globule state. These results show that a certain amount of domain-swapped oligomeric cyt *c* is produced from the molten globule state oligomers under high ion concentrations, where the amount of oligomers depended on the chaotropic or kosmotropic nature of the anions in the molten globule state.

■ ASSOCIATED CONTENT

Supporting Information

Crystallographic statistics, size exclusion chromatographs, optical absorption spectra, SAXS analyses, and CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

cyt *c*, cytochrome *c*; FPLC, fast protein liquid chromatography; CD, circular dichroism; SAXS, small-angle X-ray scattering

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