Complex structure of cytochrome c–cytochrome c oxidase reveals a novel protein–protein interaction mode

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Abstract

Mitochondrial cytochrome c oxidase (CcO) transfers electrons from cytochrome c (Cyt.c) to O₂ to generate H₂O, a process coupled to proton pumping. To elucidate the mechanism of electron transfer, we determined the structure of the mammalian Cyt.c–CcO complex at 2.0-Å resolution and identified an electron transfer pathway from Cyt.c to CcO. The specific interaction between Cyt.c and CcO is stabilized by a few electrostatic interactions between side chains within a small contact surface area. Between the two proteins are three water layers with a long inter-molecular span, one of which lies between the other two layers without significant direct interaction with either protein. Cyt.c undergoes large structural fluctuations, using the interacting regions with CcO as a fulcrum. These features of the protein–protein interaction at the docking interface represent the first known example of a new class of protein–protein interaction, which we term "soft and specific". This interaction is likely to contribute to the rapid association/dissociation of the Cyt.c–CcO complex, which facilitates the sequential supply of four electrons for the O₂ reduction reaction.

Keywords cytochrome c; cytochrome c oxidase; electron transfer complex; protein–protein interaction; X-ray crystallography

Subject Categories Membrane & Intracellular Transport; Metabolism; Structural Biology

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Introduction

Cytochrome c oxidase (CcO) is a typical αα³-type CcO, in which electrons are transferred to an active site consisting of heme a₃ and Cu₄ from Cu₄ via heme a. CcO initially accepts electrons from cytochrome c (Cyt.c) to reduce a dioxygen molecule (Ferguson-Miller & Babcock, 1996; Yoshikawa & Shimada, 2015). Electron transfer (ET) from Cu₄ to the O₂ reduction center is coupled to proton pumping across the membrane.

Extensive steady-state kinetic analyses of oxidation of ferro-Cyt.c by CcO have revealed two Cyt.c-binding sites, both of which are actively involved in catalytic turnover (Ferguson-Miller et al, 1976). Speck et al (1984) proposed a single-catalytic site model in which one binding site is the catalytic site through which electrons are transferred, whereas the other controls ET in the catalytic site. The amino-acid residues on the Cyt.c surface that interact with CcO were examined by chemically modifying basic residues of Cyt.c and observing the effect on CcO activity; these experiments revealed the critical involvement of basic residues on the Cyt.c surface (Ferguson-Miller et al, 1978; Osheroff et al, 1980). The residues that interact with CcO have been investigated more extensively by NMR studies (Sakamoto et al, 2011), which revealed that hydrophobic residues on the surface of Cyt.c make major contributions to complex formation, whereas the charged residues near the hydrophobic core refine the orientation of Cyt.c to precisely control ET. However, as noted above, analyses of the mechanism of ET between Cyt.c and CcO have been largely restricted to the Cyt.c side. With the exception of docking simulation analyses (Roberts & Pique, 1999; Sato et al, 2016), essentially no experimental information is available regarding the Cyt.c-binding surface of CcO.

Although a significant amounts of data have accumulated regarding ET from Cyt.c to CcO (Speck et al, 1984; Sakamoto et al, 2011), and the X-ray structures of mammalian CcO (PDB 5B1A) and Cyt.c (Bushnell et al, 1990; De March et al, 2014) have been determined at high resolution, the underlying mechanism of ET remains incompletely understood. A crystal structure of the complex of CcO and Cyt.c would be invaluable for mechanistic studies, but to date no structure of a Cyt.c–CcO complex has been determined other than that of αα³-type CcO from Thermus thermophilus (Lyons et al, 1990, 1993).
Two-dimensional (2D) crystals of the mammalian Cyt.c-CcO complex were prepared at higher pH (7.4–9.0) with both proteins in the oxidized state (Osuda et al., 2016), but these 2D crystals could not provide a structure of sufficient resolution to allow a detailed analysis of the interactions between these proteins. Therefore, in this study, we optimized the three-dimensional (3D) crystallization conditions for ferri-Cyt.c and oxidized CcO at high pH and solved the X-ray structure of the complex at 2.0-Å resolution. The results revealed a novel mode of protein–protein interaction mediated by three water layers.

Results and Discussion

Crystallization of the Cyt.c–CcO complex

Previously, bovine CcO stabilized with n-decyl-β-D-maltoside (DM) was crystallized at a pH ≤ 6.8 and analyzed at the atomic level (Tsukihara et al., 1995, 1996). However, no 3D crystallization trial of the Cyt.c–CcO complex has been successful under crystallization conditions similar to those used for CcO at low pH. Therefore, we performed co-crystallization of Cyt.c and CcO at pH 8.0 under the same conditions used for 2D crystallization of the Cyt.c–CcO complex (Osuda et al., 2016). CcO purified from bovine heart was solubilized with DM and fluorinated octyl-maltoside (FOM), followed by addition of horse Cyt.c at a Cyt.c/CcO molar ratio of 1.2. The Cyt.c–CcO complex was then co-crystallized by the batch-wise method at 277 K (Appendix Fig S1A). Absorption spectral analysis indicated that the resultant crystals contained both Cyt.c and CcO (Appendix Fig S1B). The crystals were soaked in a crystallization solution both containing 50 μM Cyt.c and gradually increasing concentrations of the cryo-protectant ethylene glycol (EG; 40% at the final step), and then frozen in a cryo-nitrogen stream at 100 K. The addition of 50 μM Cyt.c prevented the crystal from deterioration due to release of Cyt.c molecules from the complex during soaking. These crystals diffracted X-rays to a resolution of 1.8 Å (Appendix Fig S1C and D). Statistics of the intensity data and structure refinement at 2.0-Å resolution are provided in Table 1.

Structure determination and overall structure of the Cyt.c–CcO complex

Initial phases were determined by the molecular replacement (MR) method (Rossmann & Blow, 1962) using CcO, and Cyt.c molecules were located using the \( F_o - F_c \) difference map and anomalous difference map (Appendix Fig S2A and B). Structure refinement at 2.0-Å resolution converged well: \( R/R_{free} = 0.167/0.207 \); r.m.s.d of bond lengths = 0.023 Å; r.m.s.d of bond angles = 2.0° (Table 1). The \( 2F_o - F_c \) electron-density map for the interface of Cyt.c and CcO clearly shows electron densities of side chains interacting with their counterpart proteins (Appendix Fig S2C).

The asymmetric unit of the monoclinic lattice contains a dimer consisting of two complexes of CcO and Cyt.c. The dimeric structure of CcO of the Cyt.c-CcO complex is almost identical to that of CcO crystallized in an orthorhombic lattice (Tsukihara et al., 1996). As in the CcO orthorhombic crystal (Tomizaki et al., 1999), one of the two CcO molecules in the asymmetric unit had a lower \( B \)-factor than the other, by about 7 Å\(^2\), and no significant structural difference was detected between the two complexes. Furthermore, structure refinement was performed under non-crystallographic symmetry restraint between two CcO molecules; therefore, we focused our structural descriptions on this complex.

Cyt.c is localized on the positive side of a concave surface of CcO (Figs 1 and EV1). Although \( B \)-factors of Cyt.c were significantly higher than those of CcO, all the side chains except for Lys\(^{25}\) were located in the positive density of \( 2F_o - F_c \) map. Consistent with the results of studies in which Cyt.c was chemically modified at lysyl residues, CcO interacts with the front surface of Cyt.c in a region that includes the exposed heme edge of Cyt.c (Ferguson-Miller et al., 1978; Osheroff et al., 1980). By contrast, in \( caa_3 \)-type CcO, the
propionate side of heme group of Cyt.c faces Cu₄ (Lyons et al., 2012). CCoO interacts with Cyt.c mainly via subunit II, with 94% of the contact surface of CCoO with Cyt.c belonging to subunit II, and 5 and 1% of it belonging to subunits VIb and I, respectively.

The closest inter-atomic distance between Cyt.c and any Cyt.c–CoO complex related by crystallographic symmetry is 6.9 Å. Because Cyt.c does not interact directly with any symmetry-related Cyt.c–CoO complexes (Appendix Fig S3), molecular packing in the crystal does not perturb the structure of Cyt.c in the Cyt.c–CoO complex. The CCoO and Cyt.c structures in the complex superpose well with the previously determined structures of the individual proteins, as shown in Appendix Fig S4. At the current resolution, docking of CCoO and Cyt.c results in no significant structural changes in the main chains. In Appendix Fig S5, phospholipids are depicted (sticks) along with the Cα traces of the complex (ribbons). All phospholipids detected in the crystal structure of CCoO (PDB 5B1A), but no additional lipids, are present in the Cyt.c–CoO complex. None of these phospholipids are localized near the Cyt.c-binding site; therefore, Cyt.c does not interact with phospholipids in the crystal of the complex.

A possible electron transfer pathway from heme c to Cu₄

The concave surface consists of subunit II, which contains Cu₄, the first loading site for electrons transferred from Cyt.c. The distance between the iron atom of heme c and the copper atom of Cu₄ is 23.0 Å. The dominant ET pathway from the heme c iron to Cu₄ of CCoO was explored using the Pathways plugin for VMD (Humphrey et al., 1996; Balabin et al., 2012). The calculations suggest that the most probable ET pathway, as shown in Figs 2 and EV2, proceeds through the iron atom of heme c (Cyt.c)-Cys¹⁴ (Cyt.c)-Lys¹³ (Cyt.c)-Tyr¹⁰⁵ (subunit II of CCoO)-Met²⁰⁷ (subunit II of CCoO)-Cu₄. This ET pathway contains two short through-space jumps: one from the Nα atom of Lys¹³ (Cyt.c) to the Cα atom of Tyr¹⁰⁵ (subunit II of CCoO) (a distance of 3.6 Å), and the other from the N atom of the main chain of Tyr¹⁰⁵ (subunit II of CCoO) to the Sα atom of Met²⁰⁷ (subunit II of CCoO) (a distance of 4.0 Å). The running distance along the pathway is 41.9 Å. Chemical modification of Lys¹³ of Cyt.c induces drastic inhibition in ET activity (Ferguson-Miller et al., 1978; Osheroff et al., 1980). All vertebrate Cyt.c proteins contain Lys at the 13th residue and Cys at the 14th residue (Appendix Fig S6A); in addition, Tyr¹⁰⁵ and Met²⁰⁷ of CCoO subunit II are conserved among vertebrates (Appendix Fig S6B). These observations strongly suggest that the Cyt.c-binding site in the Cyt.c–CoO complex structure is the catalytic binding site of Cyt.c through which electrons are transferred. Compared with the pathway in ca₄a-type CCoO, only the ET from Met²⁰⁷ (subunit II of CCoO) to Cu₄ is conserved (Fig EV2) (Lyons et al., 2012).

It has been proposed that Trp¹²¹ in Paracoccus denitrificans CCoO (Trp¹⁰⁴ in subunit II of bovine CCoO) is the electron entry site from Cyt.c, based on W121Q mutation (Witt et al., 1998). The proposal was supported by some docking simulations (Roberts & Pique, 1999; Drosou et al., 2002). However, this mutation is likely to greatly influence the redox potential of Cu₄, because the side chain of Gln¹²¹ in the W121Q mutant is predicted to make hydrogen bonds with both the Sα atom of Met²²² and the S atom of Cys²⁰⁰, which coordinate to copper ions of Cu₄ (Appendix Fig S7B). Furthermore, the present X-ray structure of the complex shows that Trp¹⁰⁴ does...
not interact tightly with Cyt.c. There is a large gap between the protein surface around Trp104 and Cyt.c, in which Trp104 is separated from the closest atom of heme c by 9.0 Å (Appendix Fig S7A). Thus, the possible electron transfer pathway identified in the present X-ray structural analyses suggests a significantly more facile electron transfer than the one through the structure including Trp104 (Appendix Fig S7A).

**Catalytic binding sites**

Speck et al (Speck et al., 1984) proposed a single-catalytic site model including a catalytic site and a non-catalytic regulatory site on CcO for Cyt.c to interpret the steady-state kinetic results indicating two different Michaelis–Menten kinetics, without giving any experimental confirmation. In other words, no experimental result has disproven different Michelis–Menten kinetics, confirming. In other words, no experimental result has disproven the two-catalytic site model (Ferguson-Miller et al., 1976). Following the Speck’s definition, the above structure strongly suggests the catalytic binding site, since the Cyt.c-CcO complex shows a facile electron transfer pathway from heme c to CuA. However, following the two-catalytic site model, this retains both possibilities of the first and the second catalytic sites.

The positive side of the concave surface of CcO is negatively charged, whereas the surface area around the exposed heme edge of Cyt.c is positively charged (Fig 3A and B). Prominent intermolecular interactions in this region include six hydrogen bonds or salt bridges between CcO and Cyt.c (Fig 3C and Table 2). Lys8 (Cyt.c) interacts with Asp139 of subunit II (CcO) via a salt bridge, Glu12 (Cyt.c) forms hydrogen bonds with Asp139 (CcO subunit II), Lys13 (Cyt.c) forms hydrogen bonds with Tyr105 and Tyr121 (CcO subunit II) and a salt bridge with Asp119 (CcO subunit II), and Lys87 (Cyt.c) forms hydrogen bonds with Ser137 (CcO subunit II). These four interacting residues of Cyt.c are restricted to the molecular surface near the exposed heme edge (Fig 3B). On the basis of chemical modification and kinetic studies (Ferguson-Miller et al., 1978), three lysine residues, Lys8, Lys13, and Lys87, were predicted to interact with CcO. Recent site-directed mutagenesis and kinetics studies of Cyt.c indicated that the ET activities of K13L, K86L/K87L, and K7L/K8L mutants are significantly lower than that of the wild-type protein (Sato et al., 2016). The side chains of Lys8, Glu12, Lys13, and Lys87 of Cyt.c, as well as the side chains of Tyr105, Asp119, Ser117, Tyr121, and Asp139 of CcO subunit II, provide the physiological electron transfer complex, not an encounter complex under non-physiological conditions.

A previous NMR study (Sakamoto et al., 2011) detected structural changes in several hydrophobic amino-acid residues of Cyt.c upon the docking of two proteins, and the authors of that study concluded that Cyt.c interacted with CcO via its non-polar surface surrounding the heme cleft, as in the cytochrome bc1 complex (Cyt.bc1–Cyt.c (Lange & Hunte, 2002) and Cyt.c-cytochrome c peroxidase (CcP) complexes (Jason et al., 2012). By contrast, our crystal structure of the Cyt.c–CcO complex has no inter-molecular interactions between hydrophobic amino acids with an inter-atomic distance < 5 Å. This is likely because NMR spectroscopy sensitively detected a small structural change undetectable by X-ray, mediated by an interaction between the residues of Cyt.c and CcO via water molecules present between the two proteins.

The ionic interaction between Lys13 (Cyt.c) and Asp119 (CcO) was predicted by a docking simulation (Roberts & Pique, 1999), and

![Figure 3. Cyt.c–CcO interaction.](image)

**Table 2. Protein–protein distances between Cyt.c and CcO.**

| Cyt.c  | CcO (subunit II) | Distances (Å) |
|-------|-----------------|---------------|
| Heme c Fe | CuA (Cu1) | 23.0 |
| Lys8 N<sub>c</sub> | Asp139 O<sub>3</sub> | 2.7 |
| Glu12 N<sub>ε</sub> | Asp139 O<sub>3</sub> | 2.9 |
| Lys13 N<sub>c</sub> | Tyr105 O<sub>n</sub> | 3.3 |
| Lys13 N<sub>c</sub> | Asp119 O<sub>3</sub> | 2.6 |
| Lys13 N<sub>c</sub> | Tyr121 O<sub>n</sub> | 3.1 |
| Lys87 N<sub>c</sub> | Ser117 O | 2.9 |
another docking simulation assigned Lys\(^8\), Lys\(^{13}\), and Lys\(^{67}\) of Cyt.c as residues interacting with CoO (Satō et al., 2016), as observed in this study. However, inconsistencies remain between the X-ray structure and the simulated structures of Cyt.c–CoO complex. The former simulation predicted that Lys\(^{57}\) (Cyt.c), which is distant from CoO in the complex structure (Appendix Fig S8), interacts with Gin\(^{103}\) and Asp\(^{159}\) of CoO subunit II. The most probable structure from the latter simulation indicated that the subunit I of CoO had a larger contact surface area with Cyt.c than subunit II of CoO, whereas in our structure most of the contact surface of CoO with Cyt.c belongs to subunit II. These inconsistencies likely arose because water molecules are present between Cyt.c and CoO, but bulk waters were removed from the surfaces of both proteins in the docking simulations.

Amino-acid residues included in the catalytic binding were assigned based on the Cyt.c–CoO complex structure equilibrated in a solution in which the enzyme exerts its normal catalytic activity (Yonetani & Ray, 1965). The interactions between Cyt.c and CoO elucidated by this crystallographic study are consistent with those revealed for the enzyme–substrate complex under turnover conditions by previous experimental studies involving chemical modifications and kinetics (Ferguson-Miller et al., 1978) or solution NMR and kinetics for complexes containing wild-type and mutant Cyt.c proteins (Sakamoto et al., 2011; Satō et al., 2016).

**Novel protein–protein interaction scheme**

Cyt.c donates electrons to CoO and (CcP) and accepts electrons from (Cyt.bc\(_1\)). We compared the interaction scheme of the Cyt.c–CcP complex with those of the Cyt.c–CcP (Pelletier & Kraut, 1992; Jasion et al., 2012) and the Cyt.bc\(_1\)–Cyt.c complex (Lange & Hunte, 2002; Solmaz & Hunte, 2008). The shortest distance between two C\(_a\) atoms of Cyt.c and CoO is 8.2 Å. By contrast, the shortest distances in the Cyt.c–CcP (PDB 4GED) and Cyt.bc\(_1\)–Cyt.c (PDB 3CX5) complexes are much shorter, 5.3 and 5.6 Å, respectively. Thus, Cyt.c in the Cyt.c–CcP complex is farther from CoO than it is from CcP and Cyt.bc\(_1\) in the corresponding complexes. Ahmed et al. (2011) compiled 179 X-ray structures of protein–protein complexes from the RSCB Protein Data Bank (Berman et al., 2000). The inter-molecular C\(_a\) distances of these 179 structures were calculated, and the distribution of the shortest distance in each complex is illustrated in Appendix Fig S9. Notably, the shortest distance in the Cyt.c–CcP complex, 8.2 Å, falls well outside the distribution. Furthermore, the contact surface areas for three ET complexes were calculated by removing surface water molecules. The area of the Cyt.c–CcP complex (222.8 Å\(^2\)) is approximately one-third that of the Cyt.c–CcP complex (615.2 Å\(^2\)), and less than one-fourth that of Cyt.bc\(_1\)–Cyt.c (1088.7 Å\(^2\)). Thus, fewer direct protein–protein interactions are involved in formation of the Cyt.c–CcP complex than either of the other two complexes. No direct interaction (< 5.0 Å) between hydrophobic residues was detected in the Cyt.c–CcP complex, whereas the other two complexes have several non-polar groups involved in their inter-molecular interactions.

The water molecules within 7 Å of both proteins of Cyt.c and CoO fall into three categories, as noted by Ahmed et al. (2011): bridging waters that interact with both proteins; non-bridging waters that interact with one but not both proteins; and non-interacting waters that are more than 3.5 Å from both proteins. In this study, interactions between waters and proteins atoms were assigned based on a distance of < 3.5 Å between the water oxygen atom and the nearest atom of the protein. As shown in Fig 4 and Appendix Table S1, more water molecules are present between Cyt.c and CoO than between Cyt.c and CcP or Cyt.bc\(_1\) and Cyt.c, and there are a total of 14 non-interacting water molecules in the Cyt.c–CcP complex. By contrast, the Cyt.c–CcP and Cyt.bc\(_1\)–Cyt.c complexes each have only four and two non-interacting water molecules, respectively. Almost the same numbers of bridging waters are in three ET complexes. Extremely fewer non-bridging waters are at Cyt.c in the both complexes of Cyt.bc\(_1\)–Cyt.c and Cyt.c–CcP than Cyt.c–CcO complex (Fig 4; Appendix Table S1). Hydrophobic

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Comparison of the distribution of water molecules between Cyt.c and each redox partner. A–C Stereo views of water molecules between proteins in Cyt.c–CcO (A), Cyt.bc\(_1\)–Cyt.c (PDB 3CX5) (B), and Cyt.c–CcP complexes (PDB 1D4E) (C). Bridging and non-interacting waters are shown in yellow and blue, respectively. Non-bridging waters interacting with Cyt.c are red spheres, and that interacting with redox partners are shown in green. Cyt.c and the redox partners are shown by wire models in red and green, respectively.
residues of Cyt.c in the Cyt.bc$_1$–Cyt.c complex and the Cyt.c–CcP complex likely contact directly with their counterpart and remove waters in part from the surface of their Cyt.c.

Any water in the Cyt.c–CcO complex has at least one hydrogen bond with a protein atom or a water molecule. Each of the bridging and non-bridging waters in the Cyt.c–CcO complex interacts, on average, with three polar atoms or waters and one non-polar atom (Appendix Table S2). The waters at CcO interact prominently with Asp, whereas those at Cyt.c interact mainly with Lys and Gln (Appendix Table S3). At least two water molecules closely contact a non-interacting water molecule. These water molecules construct a hydrogen bond network between Cyt.c and CcO (Fig EV3). The averaged B-factor of 64 waters is 62.7 Å$^2$, which is between that of CcO (37.9 Å$^2$) and that of Cyt.c (88.2 Å$^2$).

Out of 19 non-bridging waters at CcO in the complex, 14 are located at almost the same sites in the CcO crystal (PDB 5B1A), four are in slightly shifted positions, and one water is not assigned in the CcO crystal. Out of 23 non-bridging waters at Cyt.c in the complex, only five waters are present in the Cyt.c crystal structure (PDB 1HRC). This is probably because the interacting sites of Cyt.c in the Cyt.c–CcO complex are involved in the tight contacts of crystal packing in the Cyt.c crystal, which removes waters from the molecular surface upon crystallization. Because the protein volumes of Cyt.c–CcO and CcO crystals are ~30% of their unit cell volume, significantly lower than that of Cyt.c crystal, more than 40%, non-bridging water sites are common to the Cyt.c–CcO and CcO crystals.

When Cyt.c docks with CcO, both proteins preserve their main chain folds, and retain water molecules on their surfaces, and they interact with each other via the long arms of side chains (Fig 5A). On the other hand, the docking of Cyt.c and CcP or Cyt.bc$_1$ leads to the exclusion of water molecules from the surface of each protein (Fig 5B). The chemical shift-perturbed residues of ferri-Cyt.c associated with the binding of CcO (Sakamoto et al, 2011) are not affected by direct protein–protein interactions, but are influenced by indirect interactions via the water layers in the crystal structure.

The main chain folds of CcO and Cyt.c in Cyt.c–CcO complex are almost identical to those of the corresponding crystals, with C$_\alpha$ r.m.s.d. values of 0.47 and 0.41 Å, respectively. All the CcO side chain structures in the region interacting with Cyt.c are similar to those of the CcO crystal except for Asn$^{200}$ (subunit II of CcO), where the two structures are different from each other by a ~90° rotation angle around the C$_\beta$–C$_\alpha$ bond. By contrast, several side chains of Cyt.c in the interacting region have different orientations between the crystals of Cyt.c–CcO and Cyt.c, probably because of packing effects in the Cyt.c crystal, as noted above for non-bridging waters at Cyt.c.

The B-factors of the side chain atoms of N$_i$ (Lys$^8$), N$_e$ (Gln$^{12}$), N$_f$ (Lys$^{18}$), and N$_i$ (Lys$^{25}$) of Cyt.c, which interact with residues of CcO, are 37.7, 39.3, 41.7, and 48.7 Å$^2$, respectively, significantly lower than the average B-factor of Cyt.c (88.2 Å$^2$) and as low as that of the extracellular domain (residues 91–227) of CcO subunit II (35.2 Å$^2$). By contrast, the B-factors of Cyt.c atoms increase with distance from these CcO-interacting residues (Fig 3D). Cyt.c in the Cyt.c–CcO complex undergoes a large fluctuation, using the regions interacting with CcO as a fulcrum. The variation in the B-factors in Cyt.c molecule of the Cyt.c–CcO complex is another unique feature of the ET complex. However, horse ferri- and ferro-Cyt.c and the Cyt.c molecules in the Cyt.c–CcP and Cyt.bc$_1$–Cyt.c complexes exhibit low B-factors at the heme c group (Figs 3E and EV4). The high flexibility of Cyt.c in the Cyt.c–CcO complex is likely to compensate for the entropy loss caused by introduction of more waters upon docking.

The inter-molecular interaction between Cyt.c and CcO is characterized by mutual recognition mediated by a few long arms of hydrophilic amino acids, small contact surface, a long span between the two proteins, the presence of three water layers between the two proteins, and a large fluctuation of Cyt.c that uses the regions
that interact with CcO as a fulcrum. The non-interacting water molecules in the Cyt.c–CcO complex exist in vacant spaces around the interacting amino-acid residues of both proteins (Fig 6) and closely contact with water molecules, thus providing hydrogen bond network between Cyt.c and CcO (Fig EV3). This novel mode of protein–protein interaction, which we term “soft and specific contact”, is not observed in other ET complexes (Table 3) (Shen et al., 1994; Morales et al., 2000; Müller et al., 2001; Axelrod et al., 2002; Darnault et al., 2003; Ashikawa et al., 2006; Sukummar et al., 2006; Dai et al., 2007; Hagelaeken et al., 2007; Senda et al., 2007; Nojiri et al., 2009; Fritz-Wolf et al., 2011; Hiruma et al., 2013; Yuki et al., 2013; Acheson et al., 2014; McGrath et al., 2015).

The same region of Cyt.c interacts with Cyt.bc1 in the Cyt.bc1–Cyt.c complex crystal (Lange & Hunte, 2002; Solmaz & Hunte, 2008) and with CcO in the Cyt.c–CcO complex crystal, as proposed based on the results of a chemical modification study (Rieder & Bosshard, 1980). Cyt.c receives and donates electrons through the same site via a repeated association/dissociation process. The novel mode of protein–protein interaction discovered in this study is likely to decrease the potential barrier caused by structural changes upon association/dissociation of the Cyt.c–CcO complex because conformational change of both proteins and rearrangement of surface waters are not required upon docking. Therefore, soft and specific contact between the two proteins is important for efficient donation of four electrons from Cyt.c to CcO for the O2 reduction reaction. It is remarkable that the X-ray structure of the interface of Cyt.c–CcO complex facilitating the electron transfer from heme c to CuA is greatly different from that from heme c1 to heme c, indicating the electron tranfering structures in these two complexes are specialized for the different electron transfer processes [e.g., two electron transfer from heme c1 to heme c versus four electron transfer from heme c to CuA; and different molar contents of 3:7:9 for Cyt.bc1, CcO, and Cyt.c (Hafeti & Galante, 1978)]. Further structural and functional comparisons of these complexes would develop insights in the mechanism of the energy transduction by the mitochondrial electron transfer system.

We hypothesize that there are many cases of soft and specific protein–protein interactions involved in various cellular processes. One reason why these interactions were not discovered previously may be related to the need to perform extensive searches for optimal crystallization conditions for these intrinsically unstable protein complexes, as described above. Because single-particle analysis by cryo-electron microscopy does not require a crystal, a high-resolution single-particle analysis would increase the chance of detecting soft and specific protein–protein interaction.

### Materials and Methods

#### Preparation of horse heart Cyt.c sample

For each crystallization trial, horse heart Cyt.c (Nacalai Tesque) was freshly dissolved in 15 mM sodium phosphate buffer at pH 8.0, and then dialyzed for 1 h against the same buffer to remove remaining salts. The concentration of Cyt.c was calculated from the absorption spectrum of the fully dithionite-reduced form, using $\Delta%_{550-535}$ nm = 19.2 mM$^{-1}$ cm$^{-1}$.

#### Crystallization of the Cyt.c–CcO complex

CcO in the fully oxidized state was purified from bovine heart mitochondria (Tsukihara et al., 1995) and dissolved in 40 mM sodium phosphate buffer (pH 6.8) containing 0.2% (w/v) n-decyl-β-D-maltoside (DM) (Dojin). CcO was diluted 10-fold in 15 mM sodium phosphate buffer (pH 8.0) containing 0.7% (w/v) fluorinated octylmaltoside (FOM) (Anatrace). CcO at pH 8.0 preserved Cyt.c

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**Table 3. Shortest distance between Cα atoms and contact surface areas of ET complexes.**

| PDB | Distance (Å) | CSA* (Å²) | Resolution (Å) | References |
|-----|--------------|-----------|----------------|------------|
| 5YS | 8.24         | 222.8     | 2.00           | This study |
| 4GED | 5.28         | 615.2     | 1.84           | Jason et al (2012) |
| 3CSX | 5.57         | 1008.7    | 1.90           | Solmaz and Hunte (2008) |
| 1EE4 | 4.71         | 1157.8    | 2.30           | Müller et al (2001) |
| 1EWA | 4.10         | 1361.1    | 2.38           | Morales et al (2000) |
| 1LRB | 5.30         | 722.8     | 2.40           | Axelrod et al (2002) |
| 1OAO | 4.40         | 2248.3    | 1.90           | Darnault et al (2003) |
| 2DE5 | 4.48         | 932.2     | 1.90           | Ashikawa et al (2006) |
| 2GC4 | 4.36         | 425.2     | 1.90           | Shen et al (1994) |
| 2IAR | 4.91         | 557.1     | 1.95           | Sukummar et al (2006) |
| 2PU9 | 4.67         | 952.0     | 1.65           | Dai et al (2007) |
| 2PVG | 5.53         | 636.6     | 2.40           | Dai et al (2007) |
| 2VJ9 | 4.76         | 657.1     | 2.45           | Hagelaeken et al (2007) |
| 2YV9 | 5.11         | 690.9     | 1.90           | Senda et al (2007) |
| 2ZON | 4.06         | 533.6     | 1.70           | Nojiri et al (2009) |
| 3QFA | 4.81         | 631.7     | 2.20           | Fritz-Wolf et al (2013) |
| 3W9C | 5.96         | 595.9     | 2.50           | Hiruma et al (2013) |
| 4FA0 | 4.05         | 1433.8    | 2.09           | Yuki et al (2013) |
| 4PB | 4.29         | 798.7     | 2.05           | Acheson et al (2014) |
| 4PW9 | 4.41         | 739.1     | 2.49           | McGrath et al (2015) |

*aContact surface area was calculated with the program AREAIMOL (Lee & Richards, 1971) in CCP4.
*bCyt-c–CcO complex.
*cCyt-bc1–Cyt-c complex.
*dCyt-c–CcP complex.
oxidation activity at ~50% of the level at pH 7.0, as reflected by $V_{\text{max}}$ (Yonetani & Ray, 1965). FOM-treated Cco was concentrated using a membrane filter (Amicon Ultra Centrifugal Filters (100 KDa), Millipore). The concentration of Cco was calculated from the absorption spectrum of the fully dithionite-reduced form, using $\Delta A_{600-630\ \text{nm}} = 46.6\ \text{mM}^{-1}\ \text{cm}^{-1}$. Cco solubilized with DM and FOM was mixed with Cyt.c at a Cyt.c/Cco molar ratio of 1.2. Co-crystallization of Cyt.c and Cco was performed by the batch-wise method at 277 K; Cyt.c–Cco (100 mg/ml CcO, ~0.5 mM Cco) was mixed with ~5% polyethylene glycol (PEG) 4000, a precipitant. Rectangular plates of Cyt.c–Cco complex crystals were obtained within 1 day. The crystals were gradually soaked in a crystallization solution containing both 50 µM Cyt.c and ethylene glycol (EG) as cryo-protectant, reaching final concentrations of 40% EG and 6% PEG 4000. After cryo-protection, crystals were quickly frozen in a cryo-nitrogen stream at 100 K.

**Structure determination**

Crystal screening and X-ray experiments were carried out at beamline BL26B2 and BL44XU of SPring-8. The dataset for the structural analysis was obtained at BL44XU, equipped with an MX300HE CCD detector. The X-ray beam cross-section for X-ray diffraction experiments was 50 × 50 µm at the crystal, and the wavelength was 0.9 Å. Photon number at the sample position was 3.0 × 10^{13} photons/s. For data collection at 100 K, the crystals were frozen in a cryo-nitrogen stream. The dataset was collected with an exposure time of 1 s and a 0.5° oscillation angle over 180°. Diffraction images were processed and scaled with DENZO and SCALEPACK (Otwinowski & Minor, 1997), respectively, and the datasets from the two crystals were merged. A total of 720 images were successfully processed and scaled. The structure factor amplitude ($|F_{\text{O}}|$) was calculated using the CCP4 program TRUNCATE (French & Wilson, 1978; Weiss, 2001; Winn et al., 2011). The crystal belongs to space group $P2_1_2_1$, with unit cell dimensions of $a = 113.3$ Å, $b = 183.9$ Å, $c = 148.9$ Å, and $\beta = 102.1°$. The asymmetric unit of the crystal lattice contains two complexes of Cco and Cyt.c. The solvent content and $V_{\text{S}}$ were 65.6% and 3.58 Å$^3$ Da$^{-1}$, respectively (Matthews, 1968).

Cco was initially located in the unit cell at 3.0-Å resolution by the molecular replacement (MR) method (Rossmann & Blow, 1962) using the program MOLREP in CCP4 (Collaborative Computational Project 4, 1994) with the fully oxidized Cco structure, previously determined at 1.8-Å resolution (PDB 2DYR) (Shinzawa-Itoh et al., 2007), as a model. Cyt.c was located following the MR search at 3.0-Å resolution using horse Cyt.c (PDB 1HRC) (Bushnell et al., 1990) as a model, as in the previous case. Initial phases up to 5.0-Å resolution were calculated with atomic parameters determined by MR and extended to 2.0-Å resolution by density modification (Wang, 1985) coupled with non-crystallographic symmetry averaging (Bricogne, 1974, 1976) using the CCP4 program DM (Cotwan, 1994). The resultant phase angles ($\phi_{\text{MR/DM}}$) were used to calculate the electron-density map (MR/DM map) with Fourier coefficients $|F_{\text{O}}|$ exp($\phi_{\text{MR/DM}}$) and the anomalous difference electron-density map with Fourier coefficients $|F_{\text{O}}|^* - |F_{\text{O}}|$ exp($i(\phi_{\text{MR/DM}} - \pi/2)$), where $|F_{\text{O}}|$ is the observed structure amplitude and $|F_{\text{O}}|^* - |F_{\text{O}}|$ is the Bijvoet difference in $|F_{\text{O}}|$. The anomalous difference electron-density map indicated the Fe, Cu, and Zn positions, including the heme irons of Cyt.c. The structural model of Cyt.c–Cco was built in the MR/DM map. The structure was refined using the program REFMAC (Winn et al., 2001; Murshudov et al., 2011) at 2.0-Å resolution. Bulk solvent correction and anisotropic scaling of the observed and calculated structure amplitudes and TLS parameters were incorporated into the refinement calculation. The anisotropic temperature factors for iron, copper, and zinc atoms were imposed on the calculated structure factors. Because the two crystallographically independent monomers packed differently in the crystal, each monomer of Cco was assigned to a single TLS group in the REFMAC refinement. The quality of the structural refinement was characterized by the $R$ and $R_{\text{free}}$ values. $F_{\text{o}}$–$F_{\text{c}}$ maps were calculated with Fourier coefficients $(|F_{\text{o}}| - |F_{\text{c}}|) \exp(i\alpha_c)$, where $|F_{\text{o}}|$ and $\alpha_c$ are the calculated structure amplitude and phase, respectively, obtained in the structural refinement. Out of 3822 amino-acid residues, 56 residues of Cco could not be located in the electron-density maps of the Cyt.c–Cco complex. A total of 33 residues of Cco have multiple conformations. The structure refinement was well converged: $R = 0.167$ and $R_{\text{free}} = 0.207$. The root-mean-square deviations (r.m.s.d.) of bond lengths and angles from their ideal values were 0.023 Å and 2.0°, respectively.

Electrostatic potential was calculated separately for Cyt.c and subunit II of Cco using APBS (Baker et al., 2001). Accessible surface area was calculated with the program AREAIMOL (Lee & Richards, 1971) in CCP4, using a probe with radius 1.4 Å.

**Electron transfer pathway calculation**

The possible ET pathways from the heme c iron to CuA of Cco were explored by an empirical method, the Pathways plugin for VMD (Humphrey et al., 1996; Balabin et al., 2012), which evaluates the donor-to-acceptor tunneling coupling ($T_{\text{DA}}$) value for each pathway. We attached hydrogen atoms based on the charmm22 force field (MacKerell et al., 1998), and performed energy minimization with respect to the hydrogen atoms, which enabled us to positively identify the hydrogen bonds. To evaluate the diversity of the possible ET pathways, we generated 200 candidates, and found that most of the identified ET pathways and their $T_{\text{DA}}$ values were similar. In fact, the top 100 solutions contained the route between Lys$^{13}$ (Cyt.c) and Tyr$^{105}$ (Cco). Accordingly, we took the ET pathway with the most efficient $T_{\text{DA}}$ value as the final solution.

**Accession numbers**

Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession code 6500.

**Expanded View** for this article is available online.

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Author contributions
KS-I, SY, and TT designed the research; SS, KS-I, JB, SA, AS, EY, YK, MT, SY, and TT performed the research; SS, KS-I, and SA performed protein purification and crystallization experiments; SS, KS-I, JB, SA, AS, EY, and TT performed X-ray diffraction experiments and analyzed X-ray data; JK and MT carried out molecular dynamics simulations; SS, KS-I, AS, MT, SY, and TT wrote the manuscript; and all authors discussed and commented on the results and the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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