Cryo-EM Structures of Respiratory $bc_1$-$cbb_3$ type CIII$\_$$CIV$ Supercomplex and Electronic Communication Between the Complexes

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Running title: Bacterial respiratory cytochrome $bc_1$-$cbb_3$ supercomplex

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Abbreviations

Q, quinone; QH₂, Quinol or hydroquinone; Complex III, CIII₂ or cytochrome bc₁, ubiquinol-cytochrome c oxidoreductase; Complex IV or CIV, cbb₃-type cytochrome c oxidase; cyt, cytochrome; cyt c₂, cytochrome c₂, soluble cytochrome c; cyt cₚ, cytochrome cₚ, membrane-anchored cytochrome c; cyt S-cₚ, soluble part of cytochrome c, without its membrane anchor; SC, super-complex; MS, mass spectrometry; XL-MS, cross-linking mass spectrometry; XL, cross-links; TMBZ, 3,3',5,5'-tetramethyl-benzidine; DBH₂, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; FeS, Rieske iron-sulfur protein; FeS-ED, membrane-extrinsic domain of FeS protein; b position, location of the [2Fe-2S] cluster near heme b₁; c position, location of the [2Fe-2S] cluster near heme c₁; cryo-EM, cryogenic electron microscopy; BN-PAGE, blue native polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; C-ter, C-terminus; N-ter, N-terminus; His-tag, 8-histidine tag; FLAG-tag, DYKDDDDK-tag; SEC, size exclusion chromatography; TMH, transmembrane helix; DSS, disuccinimidyl suberate; DSBU, disuccinimidyl dibutyric urea; DMTMM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride; heme-Fe, heme-iron; Eₘ, redox midpoint potential; heme cₚ₁, N-ter located c-type heme 1 of CcoP; heme cₚ₂, C-ter located c-type heme 2 of CcoP; heme cₚ, c-type heme of CcoO; SO₃²⁻, sulfite; SO₄²⁻, sulfate; RMSD, root-mean-square deviation; DDM, n-dodecyl β-D-maltoside.
Abstract

The respiratory electron transport complexes convey electrons from nutrients to oxygen and generate a proton-motive force used for energy (ATP) production in cells. These enzymes are conserved among organisms, and organized as individual complexes or combined forming large super-complexes (SC). Bacterial electron transport pathways are more branched than those of mitochondria and contain multiple variants of such complexes depending on their growth modes. The Gram-negative species deploy a mitochondrial-like cytochrome $bc_1$ (Complex III, CIII$_2$), and may have bacteria-specific $cbb_3$-type cytochrome $c$ oxidases (Complex IV, CIV) in addition to, or instead of, the canonical $aa_3$-type CIV. Electron transfer between these complexes is mediated by two different carriers: the soluble cytochrome $c_2$ which is similar to mitochondrial cytochrome $c$ and the membrane-anchored cytochrome $c_y$ which is unique to bacteria. Here, we report the first cryo-EM structure of a respiratory $bc_1$-$cbb_3$ type SC (CIII$_2$CIV, 5.2Å resolution) and several conformers of native CIII$_2$ (3.3Å resolution) from the Gram-negative bacterium *Rhodobacter capsulatus*. The SC contains all catalytic subunits and cofactors of CIII$_2$ and CIV, as well as two extra transmembrane helices attributed to cytochrome $c_y$ and the assembly factor CcoH. Remarkably, some of the native CIII$_2$ are structural heterodimers with different conformations of their [2Fe-2S] cluster-bearing domains. The unresolved cytochrome $c$ domain of $c_y$ suggests that it is mobile, and it interacts with CIII$_2$CIV differently than cytochrome $c_2$. Distance requirements for electron transfer suggest that cytochrome $c_y$ and cytochrome $c_2$ donate electrons to heme $c_{p1}$ and heme $c_{p2}$ of CIV, respectively. For the first time, the CIII$_2$CIV architecture and its electronic connections establish the structural features of two separate respiratory electron transport pathways (membrane-confined and membrane-external) between its partners in Gram-negative bacteria.
Introduction

Mitochondrial and bacterial respiratory chains couple exergonic electron transport from nutrients to the terminal acceptor oxygen (O$_2$) through a set of enzyme complexes. Concomitantly, they generate a proton motive force used for ATP synthesis and other energy-dependent cellular processes. The mitochondrial respiratory chain consists of four complexes. Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) are the entry points of reducing equivalents (NADH and FADH$_2$) derived from nutrients into the chain. They reduce the hydrophobic electron carrier quinone (Q). Reduced quinone (QH$_2$) moves rapidly within the membrane to Complex III (cytochrome (cyt) bc$_1$ or CIII$_2$) which oxidizes it and reduces the electron carrier cyt c. The reduced cyt c diffuses to Complex IV (cyt c oxidase or CIV) which oxidizes it and subsequently reduces the terminal electron acceptor oxygen to water (Nicholls and Ferguson, 2013) (Fig. 1A).

Respiratory complexes are evolutionarily conserved among organisms, but bacterial enzymes are structurally simpler than their mitochondrial counterparts, consisting mainly of the catalytic subunits. However, bacterial respiratory chains are more elaborate than those of mitochondria, since they contain various complexes forming branched pathways to accommodate their diverse growth modes (Melo and Teixeira, 2016). In facultative phototrophs, the mitochondrial-like bc$_1$-type CIII$_2$ is central to respiratory and photosynthetic electron transport pathways. CIII$_2$ is a dimer with each monomer comprised of three subunits: the Rieske FeS (FeS) protein with a [2Fe-2S] cluster, cyt b with hemes $b_h$ and $b_l$, and cyt $c_1$ with heme $c_1$ cofactors (Fig. 1A,B). The FeS protein external domain (FeS-ED) is mobile between the b (close to heme $b_l$) and c (close to heme $c_1$) positions (Darrouzet et al., 2001; Esser et al., 2006). Some species such as *Rhodobacter sphaeroides* contain a mitochondrial-like aa$_3$-type and a bacteria-specific cbb$_3$-type CIV, a monomer comprised of four subunits: CcoN with heme $b$ and heme $b_3$-Cu binuclear center, CcoO with heme $c_0$, CcoQ, and CcoP with hemes $c_{pl}$ and $c_{p2}$ cofactors (Fig. 1A,B). Other species such as *Rhodobacter capsulatus* (Khalfaoui-Hassani et al., 2016)
and pathogens like *Helicobacter pylori* and *Campylobacter jejuni* (Smith et al., 2000), *Neisseria* (Aspholm et al., 2010) have only a high oxygen affinity $cbb_3$-type CIV to support their micro-aerophilic growth.

Besides the mitochondrial-like soluble and diffusible cyt $c$, many Gram-negative bacteria contain additional electron carriers that are membrane-anchored via transmembrane domains (*e.g.*, *Rhodobacter capsulatus* cyt $c_2$ (Jenney and Daldal, 1993), *Paracoccus denitrificans* cyt $c_{552}$ (Turba et al., 1995), and *Bradyrhizobium japonicum* CycM (Bott et al., 1991)) or fatty acids (*e.g.*, *Blastochloris viridis* tetraheme cyt $c$ (Weyer et al., 1987) and *Helicobacterium gestii* cyt $c_{553}$ (Albert et al., 1998)). Conversely, Gram-positive bacteria are devoid of freely diffusing electron carriers. Instead, they may have additional cyt $c$ domains fused to their CIII$_2$ (*i.e.*, $bcc$-type) such as in *Mycobacterium smegmatis* (Kim et al., 2015) and *Corynebacterium glutamicum* (Niebisch and Bott, 2003) or CIV (*i.e.*, $caa_3$-type) such as in *Bacillus subtilis* (Winsteadt and von Wachenfeldt, 2000) and *Bacillus stearothermophilus* (Sakamoto et al., 1996). Bacterial electron carrier cyts $c$ are involved in multiple metabolic pathways. Both the diffusible cyt $c$ (*e.g.*, *R. capsulatus* cyt $c_2$ or its homologs) and the membrane-anchored cyt $c$ (*e.g.*, *R. capsulatus* $c_2$ or its homologs) electronically connect CIII$_2$ to the photochemical reaction center in photosynthesis (Daldal et al., 1986), and to CIV in respiration (Hochkoeppler et al., 1995). In species like *R. sphaeroides*, cyt $c_2$ functions in both photosynthesis and respiration, while cyt $c_3$ is restricted to respiration (Myllykallio et al., 1999).

In recent years, the co-occurrence of individual complexes together with multi-enzyme super-complexes (SCs) in energy-transducing membranes has become evident (Acin-Perez and Enriquez, 2014; Melo and Teixeira, 2016). However, the regulation and physiological role of this heterogeneity are debated (Brzezinski, 2019; Letts and Sazanov, 2017; Milenkovic et al., 2017). SCs may stabilize individual complexes, enhance catalytic efficiency through substrate/product channeling, or minimize production of harmful intermediates (*e.g.*, reactive oxygen species) to decrease cellular distress.
(Enriquez, 2016; Letts et al., 2019; Quintana-Cabrera and Soriano, 2019). Mitochondrial SCs, such as CICIII2CIV (respirasomes) or their smaller variants containing only CICIII2 (Sousa and Vonck, 2019) or CIII2CIV (Letts et al., 2016) have established molecular architectures (Gu et al., 2016; Hartley et al., 2019; Wu et al., 2016). Bacterial SCs, including these of *P. denitrificans* (Berry and Trumpower, 1985; Stroh et al., 2004), *Geobacillus stearothermophilus* (Bergdoll et al., 2016), *Bacillus PS3* (Sone et al., 1987), *M. smegmatis* (Kim et al., 2015), and *C. glutamicum* (Kao et al., 2016), have been characterized biochemically, but only the structure of the Gram positive *M. smegmatis* SC (CIII2CIV) has been reported (Gong et al., 2018; Wiseman et al., 2018).

As of yet, no respiratory SC structure has been determined for Gram-negative bacteria, the evolutionary precursors of mitochondria. Furthermore, SCs containing ancient forms of CIV (*i.e.*, *cbb*$_3$-type) representing primordial features of respiratory chains with multiple electron carriers are unknown (Ducluzeau et al., 2008). Structural studies of such SCs have been hampered due to unstable interaction between CIII$_2$ and CIV, hence their trace amounts in nature. We have overcome this hurdle using a genetic approach, yielding large amounts of SCs from the Gram negative facultative phototroph *R. capsulatus*. Here, we report the first cryo-EM structure of a respiratory *bc$_1$-cbb$_3$* type SC (CIII2CIV, at 5.2Å resolution), as well as several conformers of native CIII$_2$ (at 3.3-4.2Å resolution). We define the interaction regions of cyt $c_2$ and cyt $c_y$ within the SC by combining cryo-EM, cross-linking mass spectrometry (XL-MS) and integrative structure modeling. We propose that the membrane-bound cyt $c_y$ donates electrons to heme $c_p1$, while the diffusible cyt $c_2$ transfers them to heme $c_p2$, of CcoP subunit of CIV. For the first time, this work establishes the structural features of CIII$_2$CIV and its two distinct respiratory electron transport pathways (membrane-confined and membrane-peripheral) connecting its partners in Gram-negative bacteria.
Results

Stabilization, isolation, and composition of functional fused SCs. Earlier studies on soluble cyt c-independent electron transport pathways have indicated that in some species (e.g., *R. capsulatus* (Myllykallio et al., 2000)), CIII₂, CIV, and the membrane-anchored cyt c₇ are in close proximity to each other. BN-PAGE of membranes from a wild type strain of *R. capsulatus*, overstained for CIV-specific in-gel activity, showed barely detectable bands around ~450 kDa M, ([Fig. S1A](#)). The masses of these bands were larger than that of the CIV monomer (~100 kDa, running as ~230 kDa on BN-PAGE) or CIII₂ dimer (~200 kDa, running as >250 kDa on BN-PAGE), suggesting the occurrence of large SCs. However, these entities were of low abundance and highly unstable, rendering their study difficult. In our earlier work, translationally fusing cyt c₁ of CIII₂ to cyt c₇ had produced an active bcc-type CIII₂ (i.e., cyt bc₁-c₇ fusion) (Lee et al., 2008), suggesting that this approach might also be used to stabilize the interactions between CIII₂ and CIV.

During the assembly processes of CIII₂ and CIV, cyt c₁ interacts with cyt b to form a cyt b-c₁ subcomplex (Davidson et al., 1992), and CcoP associates with CcoNOQ subcomplex to yield an active CIV (Kulajta et al., 2006). We thought that translationally fusing the C-terminus (C-ter) of cyt c₁ to the N-terminus (N-ter) of CcoP, which are on the inner (n) side of the membrane, forming a bipartite cyt c₁-CcoP fusion protein might produce a stable bipartite bc₁-cbb₃ type SC ([Fig. 1C,D](#)). Furthermore, adding the natural 69-residue linker (L) and the 100-residue cyt c domain of c₇ to the C-ter of cyt c₁-CcoP, which is on the outer (p) side of the membrane, to form a tripartite cyt c₁-CcoP-c₇ fusion protein might yield a tripartite bc₁-cbb₃ type SC with an attached electron carrier ([right panels of Fig. 1C,D](#)). This approach (see Supplemental Information, Methods, for details) yielded fusion constructs ([Fig. S1B](#)) that functionally complemented a mutant lacking CIII₂ and CIV for photosynthesis-proficiency (i.e., CIII₂ activity) and CIV activity ([Fig. S1C](#)).
The His-tagged bipartite and Flag-tagged tripartite SCs were purified from detergent-dispersed membranes by tag-affinity and size exclusion chromatography (SEC) (SI, Methods) (Fig. 2A,B). BN-PAGE of isolated proteins showed that the A-1 and B-1 fractions contained mostly the large entities of Mr ~450 kDa range (Fig. 2A,B, insets), and SDS-PAGE revealed that they had the cyt c\textsubscript{1}-CcoP (~65 kDa) or cyt c\textsubscript{1}-CcoP-c\textsubscript{y} fusion proteins (~80 kDa) (Fig. 2C). All protein bands seen in Fig. 2C were identified by mass spectrometry (MS) (Table S3) and assigned to the subunits of CIII\textsubscript{2} and CIV. The fusion proteins also contained covalently-attached heme cofactor(s), as shown by 3,3’,5,5’-tetramethyl-benzidine (TMBZ) staining, which is specific to covalent heme containing c-type cyts (Fig. 2D). CcoQ (Mr ~7kDa) of CIV was absent in both SC preparations.

**Spectral and functional characterization of SCs.** Purified SCs were characterized by optical redox difference spectra for their total cyt b and cyt c contents. The spectra were distinct from those of CIII\textsubscript{2} (Valkova-Valchanova et al., 1998) or CIV (Gray et al., 1994), and the tripartite SC contained more heme c than the bipartite SC, due to the additional cyt c domain of c\textsubscript{y} (Fig. S2A). Both SC preparations exhibited 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DBH\textsubscript{2}):cyt c reductase activity (12.4 +/-1.8 \mu mol/mg of protein/min and 7.2 +/-2.1 \mu mol/mg of protein/min for bipartite and tripartite SCs, respectively), which is specific to bc\textsubscript{1}-type CIII\textsubscript{2} (Fig. S2B). They also had cyt c:O\textsubscript{2} reductase activity (0.46 +/-0.14 \mu mol/mg of protein/min and 2.9 +/-0.7 \mu mol/mg of protein/min for bipartite and tripartite SCs, respectively), which is specific to cbb\textsubscript{3}-type CIV (Fig. S2C). Importantly, the tripartite SC exhibited DBH\textsubscript{2} and O\textsubscript{2} dependent DBH\textsubscript{2}:O\textsubscript{2} reductase (i.e., coupled CIII\textsubscript{2}+CIV) activity (0.143 +/-0.025 \mu mol of O\textsubscript{2} consumed/mg of protein/min) without addition of a soluble electron carrier (e.g., horse heart cyt c or R. capsulatus cyt c\textsubscript{2}) (Fig. S2D), unlike the bipartite SC that required it. Remarkably, the cyt c domain of c\textsubscript{y} fused to cyt c\textsubscript{1}-CcoP transferred electrons from CIII\textsubscript{2} to CIV.
Structures of the tripartite SCs. We first focused on cryo-EM analysis of the tripartite SC preparations that were more stable and abundant than the bipartite SCs (Fig. 2B, fraction B-1). Initial 3D classes were of primarily two different sizes (Fig. S3, Box 1, left). The smaller (~180 Å length) particles were asymmetrical, and their size and shape suggested that they may correspond to a dimeric CIII2 associated with a single CIV. Focused classification and processing of the subclass containing ~62,000 particles with the highest initial resolution, and best discernable features, led to a tripartite CIII2CIV map (SC-1A, EMD-22228) at 6.1 Å resolution (Fig. S3A, see SI Methods for details), while another dataset yielded a slightly lower resolution map (SC-1B, EMD-22230) at 7.2 Å (Fig. S3B) (Table 1). The larger particles (~250 Å length, Fig. S3, Box 1) were more symmetrical and represented a dimeric CIII2 flanked by two CIV (i.e., CIII2CIV2), as expected based on two $c_1$-CcoP-$c_y$ subunits per CIII2. However these particles were rare (~5,000) and their map (SC-1C) could not be refined beyond ~10 Å resolution (Fig. S3C).

The \textit{R. capsulatus} cbb3-type CIV is highly homologous to that of \textit{P. stutzeri} but not identical (see Methods for details). Thus, a homology model of CIV was built using the \textit{P. stutzeri} structure (PDB: 3MK7; 3.2 Å resolution) as a template and validated (Table S7) (SI, Methods). In addition, the existing CIII2 model (PDB: 1ZRT; 3.5 Å resolution) was further refined (PDB: 6X10; 3.3 Å resolution) using our cryo-EM data (see below and Table 2). These models were fitted as rigid bodies into the maps SC1-A with a correlation coefficient $CC_{box}$ of 0.75 and SC-1B with a correlation coefficient $CC_{box}$ of 0.71 (Fig. S4A) (Table 1). The [2Fe-2S] clusters of the FeS proteins of CIII2 could be recognized closer to heme $b_L$ (b position) than to heme $c$ (c position), but had lower occupancy and resolution likely due to conformational heterogeneity (Fig. S4B). In particular, the heterogeneity of the FeS-ED in monomer A (i.e., adjacent to CIV) was more pronounced than that in monomer B (i.e., away from CIV) of CIII2. Lower resolutions of the FeS-ED portions were anticipated because of their
mobility (Darrouzet et al., 2001; Esser et al., 2006). Details of the tripartite CIII₂CIV structure are described below together with the bipartite SC, which has a higher resolution.

Superimposition of the CIII₂ portions of SC-1A and SC-1B maps showed that CIV was in different orientations in different maps (Fig. S4C). The two extreme locations of CIV with respect to CIII₂ were displaced from each other by a translation of ~3Å and a rotation of ~37 degrees (Fig. S4D, E; SC-1A in red, and SC-1B in blue). Other subclasses identified in 3D classifications showed CIV in slightly different orientations between those seen in SC-1A and SC-2B maps. This variable rotation of CIV around CIII₂ is attributed to the limited interaction interface between the CcoP (N-ter TMH) of CIV and the cyt b (TMH7) of CIII₂ (see Fig. 3C), indicating that the CIII₂CIV interface is flexible.

In the interface regions of SC-1A and SC-1B maps, additional weaker features that are not readily attributable to CIII₂ and CIV structures were also observed. Intriguingly though, no membrane-external features corresponding to cyt c domain of cᵧ, which is an integral part of the cyt c₁-CcoP-cᵧ subunit of tripartite CIII₂CIV, could be discerned in these maps.

**Structure of bipartite SC supplemented with cyt cᵧ.** In an attempt to locate the cyt c domain of cᵧ, the bipartite SC preparations devoid of it (Fig. 2A, fraction A-1) were supplemented with either purified full-length cyt cᵧ, or with its soluble variant lacking the TMH (i.e., cyt S-cᵧ) (Ozturk et al., 2008), to yield the bipartite SC+cy and SC+S-cy samples. Following SEC, the elution fractions analyzed by SDS-PAGE showed that only the intact cyt cᵧ, but not the cyt S-cᵧ, associated with the SC (Fig. S5A). Thus, the cyt c domain of cᵧ does not bind tightly to, and its TMH is required for association with, this SC.

The cryo-EM analyses of the bipartite SC+cᵧ samples were carried out as above, and yielded a map (SC-2A, EMD-22227) at 5.2Å resolution (Fig. S6A,B), with local resolutions ranging from 4.3-8.0Å (Fig. S7A,C). The homology model of CIV and the refined model of CIII₂ (PDB: 6XI0) were
fitted as rigid bodies into SC-2A with a correlation coefficient $CC_{\text{box}}$ of 0.74 ([Fig. 3A](#)) ([Table 1](#)). Comparison of SC-2A (bipartite CIII$_2$CIV) with SC-1A (tripartite CIII$_2$CIV) maps showed that they were highly similar with RMSD of 1.6 Å. They are collectively referred to as CIII$_2$CIV, irrespective of their bipartite or tripartite origins.

The dimensions of the slightly curved CIII$_2$CIV structure (~155x60x90Å, LxWxH) were consistent with a CIII$_2$ dimer associated with one CIV. On SC-2A map at 5.2Å resolution, some large aromatic side chains could be discerned ([Fig. 3B](#)), and of the TMHs seen, 34 accounted for by two FeS proteins, two cyts $b$ and two cyts $c_1$ (2, 16 and 2 TMHs per dimer, respectively) of CIII$_2$, and single CcoN, CcoO and CcoP (12, 1 and 1 TMHs, respectively) of CIV ([Fig. 3C](#)). The features corresponding to the heme cofactors of CIII$_2$CIV were readily attributed to hemes $b_1$ and $b_t$ of cyt $b$, heme $c_1$ of cyt $c_1$, and to hemes $b$ and $b_3$ of CcoN, heme $c$ of CcoO and hemes $c_{p1}$ and $c_{p2}$ of CcoP proteins. As seen with the tripartite maps, the [2Fe-2S] clusters of CIII$_2$ could be recognized closer to heme $b_t$ (in b position), but had lower resolution because of conformational heterogeneity.

An additional TMH was observed at the distal end of CIV ([Fig. 3A](#), rotated 180 degrees in [Fig. 4A](#)) close to CcoN TMH3 and TMH4 ([Fig. 3C](#)). Due to its location, this TMH (depicted in [Fig. 3](#) and [Fig. 4](#) as an *ab initio* model of the CcoN Arg25-Leu48 residues generated by I-TASSER ([Yang et al., 2015](#))) was tentatively attributed to the extra N-ter TMH (*i.e.*, TMH0) of CcoN ([Fig. 4B](#)).

The interface of CIII$_2$CIV is roughly delimited by CcoN TMH8 and TMH9, CcoP TMH, cyt $b$-TMH5 and TMH7, and cyt $c_1$ C-ter TMH of monomer A, with the closest interaction being between CcoP TMH and cyt $b$ TMH7 ([Fig. 3A,C](#)). Two highly confined inter-complex connections and two interacting TMHs of unknown identities were present at the interface ([Fig. 4A](#), red and blue TMHs). One such connection was at the *n* face of the membrane, near the cyt $c_1$ and CcoP TMHs ([Fig. 4C](#), Lys257$_{c1}$ and Thr13$_{CcoP}$). These subunits being covalently linked, the connecting feature in the map was tentatively attributed to their junction linking CIII$_2$ and CIV.
The assembly factor CcoH and cyt c₇ TMHs are located at CIII₂CIV interface. The identities of the unknown TMHs at the interface of CIII₂CIV (Fig. 3 and Fig. 4) were sought using a co-evolution based approach, RaptorX-ComplexContact (Zeng et al., 2018), predicting the residue-residue contacts in protein-protein interactions. All known single TMH containing CIV-related proteins (i.e., CcoQ subunit, CcoS and CcoH assembly factors (Koch et al., 2000) and cyt c₇ (Myllykallio et al., 1997)) were analyzed against all subunits of CIII₂ and CIV. Significant predictions of interacting residue pairs (confidence value >0.5) were observed only between CcoN (primarily TMH9) and the putative CcoH N-term TMH (Table S4). An ab initio model of CcoH TMH (Fig. S8A, residues 11 to 35) was generated by I-TASSER (Yang and Zhang, 2015), and docked onto CIV using PatchDock (Schneidman-Duhovny et al., 2005) with the predicted residue-residue contacts as distance restraints (15 Å threshold) and without using the corresponding cryo-EM maps (SI, Methods). The top scoring models converged to a single cluster around the location of the unknown TMH, close to CcoN TMH9 at CIII₂CIV interface (Fig. S8B). Close examination of the interactions between CcoH TMH and CcoN TMH9 showed that multiple co-evolutionarily conserved residues are in close contacts (Fig. S8C). Earlier studies had suggested that CcoH is near the CcoP and CcoN, to which it can be cross-linked by disuccinimidyl suberate (DSS, spacer length ~11Å) (Pawlik et al., 2010). Thus, the unknown TMH located close to CcoN TMH9 (Fig. 3C and Fig. 4C, blue TMH) was tentatively assigned to the assembly factor CcoH.

An important difference between the maps of the bipartite CIII₂CIV+c₇ (SC-2A) and tripartite CIII₂CIV (SC-1A) was in the features corresponding to the unidentified TMHs at the interface. These densities were barely visible in SC-1A, but highly enhanced in SC-2A (Fig. 4C), indicating higher occupancy. The observation that only the native cyt c₇ binds to bipartite SC via its TMH (not its cyt c domain, i.e., cyt S-c₇), suggested that the TMH (red in Fig. 4C), next to CcoH TMH (blue in Fig. 4C),
4C), may correspond to the membrane-anchor of cyt $c_y$. This explanation is most plausible since the bipartite CIII$_2$CIV+$_c$$_y$ samples were supplemented with full-length cyt $c_y$ while the tripartite samples contained only the fused cyt $c$ domain but not the TMH. Indeed, landmark densities corresponding to the helix-breaking Gly11 and two correctly spaced bulky sides chains of Phe15 and Tyr21 of cyt $c_y$ TMH (NH$_2$-xxxGly11xxxPhe15xxxxxTyr21-COOH) were discerned (Fig. 4D).

Additionally, some CIII$_2$CIV+$_c$$_y$ subclasses exhibited a weak feature on the $p$ side of the membrane that may reflect the cyt $c$ domain of $c_y$ (Fig. S6G, SC-2B). However, this feature could not be refined to high resolution, consistent with the weak binding of cyt $c$ domain of $c_y$ to CIII$_2$CIV (Fig. S5A). Moreover, the predominant conformation of CIV in the bipartite CIII$_2$CIV+$_c$$_y$ (Fig. S6A,B, SC-2A) shifted towards that found in SC-1A map of tripartite SC (Fig. S3A), with no major class corresponding to SC-1B. This suggested that the local interactions between the CcoH and cyt $c_y$ TMHs and CIV decreased the interface flexibility of CIII$_2$CIV (Fig. 4C).

**Cryo-EM structures of *R. capsulatus* native CIII$_2$.** During this study we noted that the bipartite SC+$_c$$_y$ samples contained large amounts of smaller particles (~110Å length, Fig. S3, Box 2) that were the size of CIII$_2$ (Fig. S6C,D). Analyses of these particles using C2 symmetry led to the map CIII$_2$ at 3.3Å resolution for native CIII$_2$ (Fig. S6E), with local resolutions ranging from 3.0 to 4.0Å (Fig. S7B,D) (Table 2). The FeS-ED parts showed a lower occupancy and resolution compared to the rest of the map, indicating conformational heterogeneity. Interestingly, when similar analyses were carried out without imposing C2 symmetry, three distinct maps (CIII$_2$ c-c, CIII$_2$ b-c and CIII$_2$ b-b) for CIII$_2$ were obtained at 3.8, 4.2 and 3.5Å resolutions, respectively (Fig. S6F). These maps were superimposable with respect to cyt $b$ and cyt $c_1$ subunits, except for the FeS-ED portions. The CIII$_2$ structures depicted by the CIII$_2$ b-b (Fig. 5A-C) and CIII$_2$ c-c (Fig. 5D) maps exhibited overall C2 symmetry, but in the former the FeS-EDs were located in b, whereas in the latter they were in c.
position (Esser et al., 2006). Notably, the third structure (Fig. S6F, CIII₂ b-c) was asymmetric, with the FeS-ED of one monomer being in c, and the other in b positions (Fig. 5E). Such asymmetric structures of native CIII₂ have been rarely seen using crystallographic approaches, although proposed to occur during QH₂ oxidation by CIII₂ (Castellani et al., 2010; Cooley et al., 2009; Covian and Trumpower, 2005). Similar low occupancy and resolution of the FeS-EDs, suggesting conformational heterogeneity, were also seen with the CIII₂CIV maps.

**Interactions of cyt c₂ and cyt cγ with CIII₂CIV.** The interaction interfaces between CIII₂CIV and its physiological electron carriers were pursued using cross-linking mass spectrometry (XL-MS) (Gotze et al., 2015; Slavin and Kalisman, 2018). First, the co-crystal structure (PDB: 3CX5) of yeast CIII₂ with its soluble electron carrier iso-1 cyt c (Solmaz and Hunte, 2008) was used as a template (homology between yeast cyt c₁ and *R. capsulatus* cyt c₁: 31% identity and 58% similarity; iso-cyt c and cyt c₂: 25% identity and 56% similarity) to model the binding of cyt c₂ on bacterial CIII₂. As the co-crystal structure contains only one iso-1 cyt c bound to one of the two cyt c₁ of yeast CIII₂, *R. capsulatus* cyt c₁ (PDB: 6XI0) and cyt c₂ (PDB: 1C2N) structures were superimposed with their counterparts on the co-crystal structure, and a model with a single cyt c₂ docked to one monomer of CIII₂ was generated. To experimentally verify this model, the protein cross-linker 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM) was used with *R. capsulatus* cyt c₂ bound to CIII₂ (SI, Methods). Multiple intra-subunit cross-links (XLs) within CIII₂CIV detected in several experiments served as controls (**Table S5** and **Fig. S9A**). High-confidence XLs were obtained using both FindXL (Kalismann et al., 2012) and MeroX (Iacobucci et al., 2018) search engines, and only those identified by both were retained. The three XLs between cyt c₁ and cyt c₂ provided distance restraints (~30Å for DMTMM) for docking cyt c₂ to CIII₂ using PatchDock (**Table S5** and **Fig. S9B**). The docking models clustered at a single region per monomer of CIII₂ (**Fig. 6A**, right), which
overlapped with the binding site of cyt c2 defined by the model generated by alignment to the yeast co-crystal structure (Fig. S9C). The distance from cyt c2 heme-Fe to cyt c1 heme-Fe is ~16.8Å for the co-crystal derived model, while comparable distances between ~13.8 - 20.4Å were obtained with the docking models. Thus, docking with Patchdock integrating XL-MS based distance restraints defined reliably, but with limited accuracy, the interaction region of cyt c2 on CIII2.

No information about the binding sites between cyt c2 and cbb3-type CIV was available, so the XL-MS with DMTMM was extended to this case. Similarly, the XLs found between the proteins (1 between cyt c2 and CcoP, and 8 between cyt c2 and CcoO) provided distance restraints for docking cyt c2 to CIV via Patchdock (Table S5). The cyt c2 docking models also clustered in a single region of CIV (Fig. 6A, left), closer to heme c_p2 (c2 heme-Fe to c_p2 heme-Fe: ~15.2 to 35.6Å) than heme c_p1 (c2 heme-Fe to c_p1 heme-Fe: ~23.0 to 42.0Å) of CcoP subunit (Fig. 7). Surface charge complementarities between the positively charged face of cyt c2 and the negatively charged likely binding regions on both CIV and on CIII2 are seen (Fig. S10A). These two cyt c2 binding regions on CIII2CIV are distant from each other (closest c2 heme-Fe on CIII2 to that on CIV is ~69Å) (Fig. 7A).

Next, the binding interactions between cyt c domain of cy and CIII2CIV were addressed using DMTMM and disuccinimidyl dibutyric urea (DSBU) as cross-linkers. Similar to DMTMM, DSBU yielded multiple intra-subunit XLs within the subunits of CIII2CIV, providing experimental controls (Table S6 and Fig. S9D). Six XLs (five cyt c_y to cyt c_1 and one cyt c_y to FeS protein) with DMTMM (Table S5) and four XLs (only cyt c_y to FeS protein) with DSBU (Table S6) were identified. Although chemically different cross-linkers were used, XLs were observed only between cyt c_y and CIII2, and not with CIV, suggesting that this cyt c domain is closer to CIII2 in CIII2CIV. Using the XLs as distance restraints (~35Å for DSBU and ~30Å for DMTMM) PatchDock generated two binding clusters for cyt c domain of c_y on each CIII2 monomer of SC. One of the clusters was on cyt c_1, overlapping with the binding region of cyt c2 (Fig. 6B), whereas the other one was located between
cyt c₁ and the FeS-ED near the inter-monomer region of CIII₂ (Fig. S11). To further support these binding locations obtained by XL-MS-based docking, we sought classes that have extra densities corresponding to cyt c domain of cy in our cryo-EM datasets, and found a minor 3D class containing ~18,000 particles (Fig. S6G), which has an extra feature between CIV and CIII that may be attributable to this domain (Fig. S11). The two docking clusters, clearly visible in top view (Fig. S11C), were more spread out compared with those of cyt c₂ (Fig. 6A, Fig. 7A,C), with the distances between cyt cy heme-Fe and cyt c₁ heme-Fe of CIII₂ monomer A being between 13.8 to 47.1Å, consistent with the weak binding of cyt c domain of cy.

Patchdock mediated docking of cyt c domain of cy was also performed with the same XLs as above but using the conformers of native CIII₂ with differently located FeS-EDs (Fig. 5C-E, CIII₂ b-b, c-c and b-c). The data showed that when the FeS-EDs are in c position (CIII₂ c-c), the docking models gathered as a single cluster on cyt c₁, slightly displaced towards the FeS-ED of the same monomer (Fig. S12A-C). However, when the FeS-EDs are in b position (CIII₂ b-b), such models were more spread out (Fig. S12D-F). The third model with one FeS-ED in c and the other in b positions showed the expected clustering pattern depending on the local FeS-ED conformation. As in the SC both FeS-EDs appear to be in the b position, we assume that the docking pattern of cyt c domain of cy is like that seen with CIII₂ b-b. Thus, the relatively spread docking position observed with SC (Fig. 7, Fig. S11) was attributed to variable conformations of the FeS-EDs on CIII₂. Furthermore, since heme c₁, and not the FeS protein, is the electron exit site of CIII₂ (Crofts et al., 2008; Osyczka et al., 2005), the cluster on cyt c₁ was taken as the productive binding region of cyt c domain of cy.

Examination of all pertinent distances between the cofactors of CIII₂CIV (Fig. 7A) indicates that the binding region of cyt c domain of cy near heme c₁ of CIII₂ is far away from the expected electron entry point(s) of CIV. The large distance (~50.8Å) separating cyt c₁ heme-Fe of CIII₂ monomer A from CcoP cₚ₁ heme-Fe (the closest compared with heme cₚ₂ of CIV) renders it impossible to define a
location for cyt $c_y$ close enough to heme $c_1$ reducing it, and heme $c_{p1}$ oxidizing it, to sustain productive electron transfer from CIII$_2$ to CIV. This distance constraint, the inability to resolve the cyt $c$ domain of cyt $c_y$ by cryo-EM, and the higher frequency of XLs to CIII$_2$ strongly infer that the cyt $c$ domain of $c_y$ must oscillate to carry out soluble carrier-independent electron transfer within CIII$_2$-CIV to couple QH$_2$ oxidation to O$_2$ reduction (Fig. 8).
Discussion

Prior to this work, no structural information was available on any bacterial \textit{cbb}$_3$-type CIV containing SC, or on its interactions with its physiological redox partners. Here, we describe the first cryo-EM structures of CIII$_2$CIV, a \textit{bc}$_1$-\textit{cbb}$_3$ type respiratory SC from the Gram-negative, facultative phototroph \textit{R. capsulatus}. We also define the likely binding regions of the electron carriers cyt \textit{c}$_2$ and cyt \textit{c}$_y$ to CIII$_2$CIV, and report the structures of both homo- and hetero-dimeric conformers of native CIII$_2$. Although X-ray based structures of bacterial \textit{bc}$_1$-type CIII$_2$ are available, native CIII$_2$ heterodimers have not been observed frequently. Similarly, only a single structure, that of \textit{P. stutzeri} (Buschmann et al., 2010), was available for \textit{cbb}$_3$-type CIV. Members of this subfamily of heme-Cu:O$_2$ reductases are widespread among bacteria and essential for major micro-aerobic processes, including anaerobic photosynthesis, nitrogen fixation, symbiosis and bacterial infection (Khalfaoui-Hassani et al., 2016).

Unlike the obligate CIII$_2$CIV$_2$ SC of \textit{Actinobacteria}, which is rigid and devoid of a free electron carrier (Gong et al., 2018; Wiseman et al., 2018), the \textit{R. capsulatus} facultative CIII$_2$CIV is naturally of low abundance and flexible, limiting its structural resolution. The dual function of bacterial CIII$_2$ interacting with both the photochemical reaction center in photosynthesis, and cyt \textit{c} oxidase in respiration, may necessitate this natural plasticity to allow swift metabolic adaptations. Similar flexibilities have also been seen with the yeast and human SCs (Sousa and Vonck, 2019).

Isolation of CIII$_2$CIV was only possible using a genetically modified strain carrying a translational fusion between CIII$_2$ and CIV (SI, Methods). Despite the complexity of translocation, maturation and assembly processes of multi-cofactor containing membrane complexes, this fusion approach is of general use. Our fused SC preparations were compositionally heterogeneous, containing mixtures of CIII$_2$CIV$_2$, CIII$_2$CIV and CIII$_2$ particles. The basis of this heterogeneity is unclear, though it may stem from subunit sub-stoichiometry, incomplete assembly, or higher susceptibility to degradation during sample preparations. Insertion of different spacers at the cyt \textit{c}$_1$-CcoP fusion junction, overexpression
of the subunits and the related assembly components could not overcome the heterogeneity (SI, Methods). Consequently, structural studies required extensive data collections and limited structural resolutions, but allowed analyses of fragmented particles.

**Structures of CIII\textsubscript{2}CIV.** The structures of the tripartite CIII\textsubscript{2}CIV or bipartite CIII\textsubscript{2}CIV+c\textsubscript{y} at sub-nanometer resolution (~5.2 to 7.2Å) were highly similar. Limited protein-protein interaction between the subunits of CIII\textsubscript{2} and CIV was seen at the interface where the TMHs of cyt c\textsubscript{y} and CcoH were located (Fig. 4), limiting the flexibility of CIII\textsubscript{2}CIV. Another helix-like feature found at the exterior edge of CIV was attributed to the extra N-ter helix (TMH0) unique to *R. capsulatus* CcoN. However, due to the limited resolutions of the structures, these attributions are tentative. Limited resolution also precluded identification of non-protein constituents at the CIII\textsubscript{2}CIV interface. In this respect, *R. capsulatus* lacks cardiolipin, often implicated in SC stability (Arias-Cartin et al., 2012). Instead, it produces ornithine lipid that can mimic cardiolipin upon dimerization (Aygun-Sunar et al., 2006). Ornithine lipid-less mutants contain very low amounts of CIV and CIII\textsubscript{2}, and if any SC is unknown.

Previously, neither the exact location nor the mobility of cyt c\textsubscript{y}, which is the basis of the “soluble carrier-independent” electron transfer from CIII\textsubscript{2} to CIV, were known. The SC structure shows that locking the N-terminal TMH of cyt c\textsubscript{y} at the interface allows mobility of its cyt c domain (Fig. 8). The linker region attaching the TMH to cyt c domain remains unresolved, but is long enough to allow oscillations between CIII\textsubscript{2} and CIV. Earlier studies with *R. capsulatus* cyt c\textsubscript{y} had shown that a full-length linker is needed for rapid (<~50 µsec) electron transfer from CIII\textsubscript{2} to the photosynthetic reaction center in photosynthesis (Myllykallio et al., 1998). In contrast, a shorter linker (~45-residue instead of 69) is fully proficient for respiratory electron transfer to CIV (Daldal et al., 2001).

**Structures of bacterial native CIII\textsubscript{2}.** In native CIII\textsubscript{2} conformers, different positions of the [2Fe-2S] cluster bearing FeS-EDs were seen. Crystallographic structures have often depicted bacterial CIII\textsubscript{2} as symmetrical homodimers (Berry et al., 2004; Esser et al., 2006; Xia et al., 2008). These structures
were obtained in the presence of inhibitors constraining FeS-EDs near heme \( b_L \) or used mutants stabilizing it on cyt \( b \) surface. Alternatively, they contained crystal contacts restricting the FeS-ED movement (Esser et al., 2008). To our knowledge, no native heterodimeric CIII\(_2\) structure of bacterial origin with different conformations of its FeS-EDs has been reported. Only recently, the cryo-EM structures of mitochondrial SCs with different maps for CIII\(_2\) FeS-EDs have been reported (Letts et al., 2019; Sousa et al., 2016). Thus, native CIII\(_2\) is not always a symmetric homodimer, and the FeS-ED of each monomer is free to move independently from each other, which has functional implications. The Q-cycle models describe the mechanism of CIII\(_2\) catalysis by two turnovers of a given monomer (Crofts and Berry, 1998; Crofts et al., 2008; Osyczka et al., 2005). The mobility of the FeS-ED between the b and c positions is essential for QH\(_2\) oxidation, and the different positions of the FeS-ED protein are often attributed to different catalytic steps (Esser et al., 2006). Emerging asymmetric structures of bacterial and mitochondrial native CIII\(_2\) obtained by cryo-EM in the absence of inhibitors or mutations, combined with the well-established inter-monomer electron transfer between the heme \( b_L \) of the monomers (Lanciano et al., 2013; Lanciano et al., 2011; Swierczek et al., 2010; Yu et al., 2002), start to provide a glimpse into plausible “heterodimeric Q cycle” mechanism(s) (Castellani et al., 2010; Cooley, 2010; Cooley et al., 2009), at least when CIII\(_2\) is a part of SCs. Accordingly, CIII\(_2\) may cycle between homo- and hetero-dimeric conformations in regards to its FeS-EDs during catalysis. These mechanistic implications remain to be studied.

**Electronic communication between CIII\(_2\)CIV partners.** Earlier, binding interactions between CIII\(_2\)CIV and its physiological electron carriers were unknown. Here we defined the likely interaction regions between the cyt \( c_2 \) or the cyt \( c_y \) and CIII\(_2\)CIV (Fig. 8). The CIII\(_2\)CIV structure indicates that the distances separating heme \( c_1 \) of CIII\(_2\) monomer A and hemes \( c_{p1} \) and \( c_{p2} \) of CIV are too large (Fig. 7) for direct microsecond scale electronic communication (Moser et al., 1992) to sustain the turnover
rate of CIII<sub>2</sub>CIV. Thus, even when CIII<sub>2</sub> and CIV form a SC, a freely diffusing cyt c<sub>2</sub> or a membrane-anchored mobile cyt c<sub>y</sub>, is required for QH<sub>2</sub>:O<sub>2</sub> oxidation.

The binding region of cyt c<sub>2</sub> on CIII<sub>2</sub> was identified earlier (Solmaz and Hunte, 2008), but that on CIV was unknown. The binding location of cyt c<sub>2</sub> on CIV determined in this study, the redox midpoint potentials (E<sub>m</sub>) of the cofactors and the distances separating them (Fig. 7A) suggest that cyt c<sub>2</sub> would confer electrons to the closer heme c<sub>p2</sub>, rather than the more distant heme c<sub>p1</sub>, of CcoP. This will then initiate canonical electron transfer via heme c<sub>p1</sub>, heme c<sub>o</sub> and heme b to heme b<sub>3</sub>-Cu<sub>b</sub> site for O<sub>2</sub> reduction (Brzezinski and Gennis, 2008; Wikstrom et al., 2018) (Fig. 8). For purified R. capsulatus proteins, the E<sub>m</sub> value of cyt c<sub>2</sub> is ~350 mV (Myllykallio et al., 1999), while those of CIII<sub>2</sub> heme c<sub>i</sub> and CIV heme c<sub>o</sub> are ~320 mV (Valkova-Valchanova et al., 1998) and ~210 mV (Gray et al., 1994), respectively. The E<sub>m</sub> values of R. capsulatus CIV hemes c<sub>p1</sub> and c<sub>p2</sub> are unknown, but based on similar E<sub>m</sub> values of heme c<sub,o</sub> for B. japonicum (200 mV) and R. capsulatus (210 mV), they are expected to be close to those of B. japonicum c<sub>p1</sub> (~300 mV) and c<sub>p2</sub> (~390 mV) (Verissimo et al., 2007).

In the case of cyt c<sub>y</sub>, its interaction region on CIV remains less well defined. Of the two binding regions of cyt c<sub>y</sub> on CIII<sub>2</sub>, that on cyt c<sub>1</sub> was taken as the most likely functional site. This binding region on cyt c<sub>1</sub> is close to that of cyt c<sub>2</sub>, but cyt c<sub>y</sub> has less complementary surface charges (Fig. S10B), consistent with its weaker binding to CIII<sub>2</sub>CIV. Anchoring cyt c<sub>y</sub> to the membrane, next to its redox partners, might have enhanced its electron transfer efficiency while minimizing its electrostatic interactions with its partners.

The distance separating the redox centers is a major factor that controls the rate of electron transfer (Moser et al., 1992). The binding region of cyt c<sub>y</sub> domain of c<sub>y</sub> on CIII<sub>2</sub> suggests that reduced cyt c<sub>y</sub>, upon its movement to CIV, might preferentially convey electrons to the closer heme c<sub>p1</sub> than heme c<sub>p2</sub> of CcoP (Fig. 8). If so, then under physiological conditions, heme c<sub>p1</sub> would be the primary receiver of electrons derived from QH<sub>2</sub> oxidation by CIII<sub>2</sub>, forming a fully membrane-confined electronic
wiring within CIII2CIV. In contrast, cyt c2 carries electrons from heme c1 to heme c_p2 via free diffusion. Significantly, this membrane-external pathway might accommodate electrons not only from QH2 but also from other donors distinct from CIII2. As such, reduction of cyt c2 during methylamine oxidation (Otten et al., 2001), or degradation of sulfur containing amino acids, converting toxic sulfite (SO3^{2-}) to sulfate (SO4^{2-}) by sulfate oxidase (Kappler and Dahl, 2001) might provide electrons to CIV, contributing to cellular energy production.

In summary, for the first time, the architecture of CIII2CIV SC along with its dynamics and interactions with its physiological redox partners established salient structural features of two distinct respiratory electron transport pathways (membrane-confined and membrane-external) that operate between CIII2 and CIV in Gram-negative bacteria.
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**Data deposition**

The following *R. capsulatus* structures and the corresponding cryo-EM maps are deposited to PDB and EMDB with the accession codes listed in the table below:

| Structure  | PDB  | map          |
|------------|------|--------------|
| CIII₂      | 6XI0 | EMD-22189    |
| CIII₂ c-c  | 6XKT | EMD-22224    |
| CIII₂ b-c  | 6XKU | EMD-22225    |
| CIII₂ b-b  | 6XKV | EMD-22226    |
| SC-2A      | 6XKW | EMD-22227    |
| SC-1A      | 6XXK | EMD-22228    |
| SC-1B      | 6XKZ | EMD-22230    |

The raw XL-MS data deposited to PRIDE repository (http://www.ebi.ac.uk/pride/archive/) with the dataset identifier PXD020038
Table 1: Statistics of data collection and 3D reconstruction of CIII<sub>2</sub>CIV SC. Six and two individual datasets were combined for the tripartite (~<em>c</em><sub>y</sub>, fused <em>c</em><sub>y</sub> domain of <em>c</em><sub>y</sub>) and the bipartite (+<em>c</em><sub>y</sub>, supplemented with native <em>c</em><sub>y</sub>) SCs, respectively. For the combined datasets, the parameters for data collection were identical, except the exposure time. For all combined datasets, the range of exposure times and the corresponding dose rates are provided.

### Data Collection

| Sample         | Tripartite CIII<sub>2</sub>CIV~<em>c</em><sub>y</sub> | Bipartite CIII<sub>2</sub>CIV + <em>c</em><sub>y</sub> |
|----------------|-----------------------------------------------|-----------------------------------------------|
| Number of micrographs | 17390                                         | 17680                                         |
| Electron microscope      | FEI Titan Krios                              | FEI Titan Krios                              |
| Voltage (kV)            | 300                                           | 300                                           |
| Electron detector       | K2                                            | K3                                            |
| Pixel size (Å)          | 1.32                                          | 1.36                                          |
| Defocus range (μm)      | 0.4 - 4.0                                     | 0.4 - 4.0                                     |
| Frames per movie        | 40                                            | 80                                            |
| Total dose (e/Å²)       | 40                                            | 40                                            |
| Exposure time (s)       | 9 - 15                                         | 3.0 - 3.2                                     |
| Dose rate (e/Å²·s<sup>-1</sup>) | 2.7 - 4.4                                   | 12.5 - 13.2                                  |
| Dose/frame (e/pix)      | 1.74                                          | 0.93                                          |

### 3D Reconstruction

| Map Name | SC–1A | SC-1B | SC-2A |
|----------|-------|-------|-------|
| Particles | 61934 | 87026 | 14978 |
| B-factor | -184  | -684  | -87   |
| Resolution range (Å) | 4.5 - 9 | 4.8 - 9 | 4.3 – 8 |
| Final resolution (Å) | 6.09 (7.14) | 7.20 (8.85) | 5.18 (7.35) |
| at FSC 0.143 (0.5) | EMD-22228 | EMD-22230 | EMD-22230 |

### Model Refinement

| PDB coordinate entry | 6XKK | 6XKZ | 6XKW |
|----------------------|------|------|------|
| Phenix CC<sub>box/mask/volume</sub> | 0.75/0.64/0.64 | 0.71/0.59/0.58 | 0.74/0.61/0.63 |
| Refinement statistics | Models were obtained by fitting the high-resolution model of CIII<sub>2</sub> (PDB: 6XI0) and the homology model of CIV (Table S7) into the maps. See these tables for refinement statistics. |
Table 2: Statistics of 3D reconstruction and model refinement of CIII₁. The datasets of the bipartite CIII₂CIV + c₇ SC (Table 1) were used for the 3D reconstruction of CIII₂. The model (PDB: 6X10) was refined in map CIII₂ (EMD-22189) and then used for rigid body fitting in maps CIII₂ c-c (EMD-22224), CIII₂ b-c (EMD-22225) and CIII₂ b-b (EMD-22226).

### Data Collection
See Table 1, bipartite CIII₂CIV + c₇

#### 3D Reconstruction

| Map Name          | CIII₂ | CIII₂ c-c | CIII₂ b-c | CIII₂ b-b |
|-------------------|-------|-----------|-----------|-----------|
| ED conformation   | b – b | c - c     | b - c     | b - b     |
| Symmetry          | C2    | C2        | C1        | C2        |
| Particles         | 37997 | 35069     | 26254     | 37710     |
| B-factor          | -33   | -71       | -50       | -54       |
| Resolution range (Å) | 3.0 - 4.0 | 3.25 - 4.5 | 3.65 - 5.0 | 3.0 - 4.0 |
| Final resolution (Å) at FSC 0.143 (0.5) | 3.30 (3.70) | 3.75 (4.15) | 4.18 (4.72) | 3.47 (3.89) |
| Phenix CCbox/mask/volume | 0.70/0.81/0.76 | 0.74/0.78/0.75 | 0.74/0.71/0.70 | 0.70/0.76/0.72 |
| EMDB map entry    | EMD-22189 | EMD-22224 | EMD-22225 | EMD-22226 |

#### Model Refinement

| PDB coordinate entry | 6X10 | 6XKT | 6XKU | 6XKV |
|----------------------|------|------|------|------|
| Model composition    |      |      |      |      |
| Non-hydrogen atoms   | 13079| 13079| 13079| 13079|
| Protein residues     | 1678 | 1678 | 1678 | 1678 |
| Heme groups          | 6    | 6    | 6    | 6    |
| [2Fe-2S] clusters    | 2    | 2    | 2    | 2    |
| MolProbity Score     | 2.23 | 2.28 | 2.29 | 2.22 |
| Clash score*         | 10.59| 11.77| 12.15| 10.21|
| Rotamer outliers (%) | 0.08 | 0.08 | 0.16 | 0.08 |
| C-beta deviations    | 1    | 1    | 1    | 1    |
| RMSD                 |      |      |      |      |
| Bond lengths (Å)     | 0.007| 0.008| 0.008| 0.007|
| Bond angles (°)      | 0.971| 1.174| 1.182| 0.976|
| Ramachandran plot    |      |      |      |      |
| Outliers (%)         | 0.42 | 0.36 | 0.30 | 0.42 |
| Favored (%)          | 83.17| 83.11| 83.05| 83.17|

*Hydrogen atoms were not considered for clash score.
**Figure Legends**

**Figure 1. Schematic representation of fused SCs.**

A. Oxidation of QH\(_2\) to Q by CIII\(_2\) and reduction of O\(_2\) to H\(_2\)O by CIV. A bifurcated electron transfer reaction conveys one electron from QH\(_2\) to the [2Fe-2S] cluster of the FeS protein (FeS, yellow), and another electron to hemes \(b_L\) and \(b_H\) of cyt b (periwinkle). The FeS protein transfers the electron from its [2Fe-2S] cluster to heme \(c_1\) on cyt c (green). The movement of reduced FeS protein from heme \(b_L\) to heme \(c_1\) and the electron transfer from heme \(b_H\) to Q from the pool to form a SQ (semiquinone) are not shown for clarity. An electron carrier cyt c (\(c_2\) or \(c_3\)) receives the electron from heme \(c_1\) and delivers it to CIV. The electron arriving to CIV reaches the heme-Cu (Cu-\(b_3\)) site, where O\(_2\) is reduced to H\(_2\)O, via the hemes \(c_{p1}\) and \(c_{p2}\) of CcoP (P, light blue), \(c_o\) of CcoO (O, dark green) and heme \(b\) of CcoN (N, purple).

B. *R. capsulatus* genes relevant to the construction of fused SCs. The *petABC* encodes the structural genes of the \(bc_1\)-type CIII\(_2\) subunits, the FeS protein (*petA*, yellow), cyt b (B, periwinkle) and cyt c (C, green). The *ccoNOQP* encodes the structural genes of the \(cbb_3\)-type CIV subunits, the CcoN (ccoN, purple), CcoO (O, dark green), CcoQ (Q, grey) and CcoP (P, light blue). The *cycY* gene (red) encodes the membrane-anchored cyt c\(_y\), and its 30-residue transmembrane helix (TMH), 69-residue linker (L) and 100-residue cyt c (cyt c) domain are indicated. Heme cofactors of \(b\)- and \(c\)-type cyts are indicated by black and white asterisks, respectively, and diamond and dot designate the [2Fe-2S] cluster and Cu atom, respectively.

C. Plasmid-borne genetic fusions. The bipartite fusion (left) is formed by linking in-frame the 3'-end of *petC* to the 5'-end of *ccoP*, and the tripartite fusion (right) is obtained by adding in-frame the linker and cyt c domain of *cycY* to the 3'-end of *ccoP*. Colors and cofactor symbols are as in A, and the His (H) and Flag (F) affinity tags (dark purple) are added at the 3'-end of the bipartite and tripartite fusion subunits, respectively.

D. Schematic depiction of bipartite (left) and tripartite (right) super-complexes (SC). The bipartite SC encodes a \(bc_1\)-type CIII\(_2\) dimer fused on each side to
a His-tagged \( cbb_3 \)-type CIV. The tripartite SC also contains the Flag-tagged cyt \( c \) domain of \( c_y \) (red) at the end of CcoP (blue).

**Figure 2. Purification and characterization of bipartite and tripartite SCs.** The SEC elution profiles of the bipartite SC (A) and tripartite SC (B) are shown. In each case, the fractions 1 and 2 were analyzed by 4-16% Native PAGE (insets) and silver staining, and only the fractions A-1 and B-1 were used for cryo-EM studies. The bands at \( \sim 450/480 \) kDa corresponding to bipartite and tripartite SCs are indicated by arrows. C. Fractions A-1 and B-1 were separated by SDS-PAGE, silver stained, and protein bands identified by mass spectrometry. The fused bipartite \( c_1\text{-CcoP} \) (A-1) and tripartite \( c_1\text{-CcoP}-c_y \) (B-1) subunits are indicated by arrows. Note the absence of \( c_1 \) and CcoP subunits in both cases. D. Peak A-1 and DDM-dispersed membranes from wild type \( R. \) capsulatus (WT) were analyzed by SDS-PAGE/TMBZ to reveal the covalently attached heme cofactors. The tripartite construct (B-1) is virtually identical to A-1, except that the \( c_1\text{-CcoP} \) band is replaced by \( c_1\text{-CcoP}-c_y \). All \( c \)-type cyts are labeled, and the additional band indicated by * corresponds to a proteolytic cleavage product of the \( c_1\text{-CcoP} \) fusion subunit.

**Figure 3. Cryo-EM structure of CIII\(_2\)CIV.** A. Side view of CIII\(_2\)CIV. The structures of CIII\(_2\) (PDB: 6XI0, refined in map CIII\(_2\) (EMD-22189) starting with PDB: 1ZRT), and the homology model of \( cbb_3 \)-type CIV obtained using \( P. \) stutzeri \( cbb_3 \) structure (PDB: 3MK7) as a template, were fitted into the cryo-EM map SC-2A depicted in transparent grey. All subunits are colored and labelled as indicated, and the additional feature at the edge of CcoN subunit of CIV, indicated by an arrow, corresponds to the extra N-ter TMH (TMH0, light purple) specific to \( R. \) capsulatus. The large arrow points out the CcoH/\( c \) helices in red/blue. B. Representative regions of the cryo-EM map showing the map quality and model fitting. The TMH2 and TMH10 of CcoN (left) shows clearly heme \( b \) and
some bulky side chains. Only the protein backbone and hemes $b_L$ and $b_H$ are resolved between the TMH2 and TMH4 of cyt $b$ (center) (see Fig. 5B for comparison with CIII$_2$ map at 3.3 Å). Large side chains are clearly visible between the TMH4 of monomer A and TMH4 of monomer B of CIII$_2$ ($4_A$ and $4_B$, respectively; right). C. Top view of CIII$_2$CIV TMHs depicted as cylinders and colored as in A. The TMHs of cyt $b$ (only CIII$_2$ monomer A) and CcoN of CIV are numbered, and the TMHs of the FeS protein (yellow), cyt $c_1$ (green), CcoO (dark green), CcoP (light blue), CcoH/c$_y$ (blue/red with an arrow) and CcoN TMH0 (light purple) are shown.

**Figure 4. Extra features seen in the cryo-EM map of CIII$_2$CIV.** A. The CIII$_2$CIV structure fitted into the map SC-2A (transparent grey) is shown with the same subunit colorings as in Fig. 3A, but rotated by 180° for the back view of CIII$_2$CIV interface. The two extra TMHs at the interface are attributed to those of CcoH (blue) and cyt $c_y$ (red). An additional TMH at the edge of CIV is attributed to the predicted N-ter TMH of CcoN (named TMH0, light purple), and depicted as an *ab initio* model generated by I-TASSER server. B. Enlarged view of the region linking CcoN TMH1 (dark purple with N-ter Ser57) to the predicted N-ter TMH0 (light purple). The connection between the two TMHs (dashed line) is not resolved. C. Enlarged view of CIII$_2$CIV interface. The view is slightly rotated relative to A for better visibility of CcoP TMH in the background (light blue). For clarity, only CcoN TMH9 is shown next to CcoH (blue) and cyt $c_y$ (red) TMHs. The fusion region between cyt $c_1$ and CcoP is shown at the bottom, with the C-ter of cyt $c_1$ (green) and the N-ter (resolved portion in the map) of CcoP (light blue), and their respective terminal residues (Lys257$_{c1}$ and Thr13$_{CcoP}$) are indicated. The 12 N-terminal CcoP residues connecting these two chains (dashed line) are not clearly resolved. D. Enlarged view showing close interaction between the CcoH and cyt $c_y$ TMHs. Characteristic features of cyt $c_y$ TMH (NH$_2$-Gly11xxxPhe15xxxxTyr21-COOH) are used to
determine the registration. The helix break induced by Gly11, and the bulky sidechain densities for Phe15 and Tyr21 are clearly visible.

**Figure 5: Structures of native CIII₂ conformers with their FeS proteins in different positions.**

A. Cryo-EM map CIII₂ b-b with both FeS proteins in b position. B. Representative region of map CIII₂ b-b demonstrating map quality and model fitting. TMH2 and TMH4 of cyt b with hemes b_L and b_H are shown. C-E. Maps and models showing different conformations of the FeS proteins. In each case, the left panels show the CIII₂ structure fitted into the corresponding maps (Fig. S6F) with the subunit colorings (cyt b in periwinkle, cyt c₁ in green, and the FeS protein in yellow) as in Fig. 3. The right panels depict the top half of the models with the membrane-external domain of cyt c₁ omitted for better visibility of the positions (b - b, c – c and c – b) of FeS-EDs, and the [2Fe-2S] clusters are shown as adjacent yellow-red spheres and indicated by arrows. C. Structure of native CIII₂ with both FeS-EDs in b position (map CIII₂ b-b, EMD-22226; PDB: 6XKV). D. Structure of native CIII₂ with both FeS-EDs in c position (map CIII₂ c-c, EMD-22224; PDB: 6XKT). and E. Structure of native CIII₂ with one FeS-ED in c and one in b position (map CIII₂ b-c, EMD-22225; PDB: 6XKU).

**Figure 6: Binding regions of cyt c₂ and cyt c₁ on CIII₂CIV.** The binding regions were defined by XL-MS guided docking, and the subunits of CIII₂CIV are colored as in Fig. 3, except that the monomer B of CIII₂ is shown in light grey for clarity. Only binding regions on monomer A are shown. A. Cyt c₂ (PDB: 1C2N) was docked onto CIII₂ and CIV using Patchdock with the DMTMM generated XLs as distance restraints, and yielded one cluster of models on CIV and one per monomer of CIII₂. B. A model of cyt c domain of c₁, generated using *P. denitrificans* cyt c₅₅₂ structure (PDB: 3M97) as a template (RMSD between template and model: 0.2 Å) was docked on CIII₂ as in A, except that both DMTMM and DSBU generated XLs provided distance restraints.
Two binding clusters for cyt c domain of c$_{2}$ per monomer of CIII$_{2}$ were found. These two clusters are located behind each other on a side view, but they are clearly visible on top views (Fig. S11C, labelled 1 and 2). Here, only cluster 1 which is closer to cyt c$_{1}$ and overlapping with the binding region of cyt c$_{2}$ is shown. In all cases, the top 10 representative models are shown to depict the clusters of binding models. No binding region for cyt c$_{y}$ on CIV could be defined since no XL was found between these proteins.

**Figure 7. Organization of CIII$_{2}$CIV cofactors and redox partners.** A. The hemes and [2Fe-2S] clusters are shown inside the transparent cryo-EM map SC-2A of CIII$_{2}$CIV (EMD-22227) with the same subunit colors as in Fig. 3: hemes b$_{L}$ and b$_{H}$ (periwinkle), heme c$_{1}$ (green), hemes c$_{p1}$ and c$_{p2}$ (light blue), heme c$_{o}$ (dark green), hemes b and b$_{3}$ (purple). The [2Fe-2S] clusters are shown as yellow-red spheres. In all cases the distances (heme-Fe to heme-Fe) between the heme cofactors are indicated. The positions of docked cyt c$_{2}$ and cyt c domain of c$_{y}$ are indicated as orange (heme c$_{2}$) and red (heme c$_{y}$) spheres, respectively, representing their heme-Fe atoms. All heme-Fe atoms corresponding to the top 50 docking positions for cyt c$_{2}$ on CIV are shown as solid (< 25Å) or transparent (> 25Å) spheres, depending on their distances to heme c$_{p2}$. In the case of CIII$_{2}$, only the docking positions of cyt c$_{2}$ and cyt c$_{y}$ on monomer A and between the monomers A and B are shown, omitting those located entirely on monomer B. The TMH of cyt c$_{y}$ is shown in red at CIII$_{2}$CIV interface. B. The heme-Fe atoms of all 50 cyt c$_{2}$ models docked onto CIV are plotted in function of their distances from the heme c$_{p1}$ and heme c$_{p2}$, with the Fe atoms within 25Å shown as solid spheres, and those beyond 25Å as transparent spheres, as indicated. The vast majority of heme-Fe atoms of docked cyt c$_{2}$ models are closer to heme c$_{p2}$ than heme c$_{p1}$ of CIV (above the diagonal line). C. Top view of the map shown in A is presented to better visualize the distribution of the docked cyt c
domain of cyt cy on monomer A and between the monomers A and B. In all cases, the heme-Fe atoms are depicted by spheres and colored as indicated above and on the figure.

**Figure 8. Proposed cyt c2 and cyt c, binding regions of CIII2CIV and electron transfer pathways.**

The likely binding regions of cyt c2 and cyt c domains of cyt cy (orange and red ellipsoids, respectively), defined by XL-MS guided docking, are depicted by the distributions of their heme-Fe atoms on the transparent map SC-2A of CIII2CIV. Only the positions that are within 25Å of heme c1 of CIII2 or heme cP2 of CIV are indicated. The CIII2 and CIV cofactors together with the TMH of cyt cy are shown as in Fig. 7. The linker region (indicated by dotted or dashed lines) between the TMH and the cyt c domain of cyt cy is not resolved in the cryo-EM map. The proposed electron transport pathways are shown by thicker black arrows: upon QH2 oxidation by CIII2, cyt cy which is integral to CIII2CIV receives an electron from heme c1. It then moves (double-headed dashed red arrow) to an undefined binding region (dashed oval with cyt cy?) on CIV, where it delivers the electron to the nearest heme cP1 of CIV. Similarly, cyt c2 which is peripheral to CIII2CIV also receives an electron from heme c1, diffuses away to reach CIV and conveys it to heme cP2. The canonical electron transfers occurring from QH2 to heme c1 in CIII2, and from heme cP1 to O2 in CIV, are indicated by thinner arrows. The double headed dashed black arrow depicts the movement of the [2Fe-2S] of FeS protein from the b position (b-pos, in black) to the c position (c-pos, in grey) in CIII2 during QH2 oxidation. Electron equilibration between the two heme bL of CIII2 is indicated by double arrows, and the electron transfer steps subsequent to heme bH1 reduction are not shown for the sake of clarity.
A  e⁻ transfer from CIII to CIV

B  Genes on chromosome

C  Gene fusions on plasmid

D  CIII-CIV super-complexes (SC)

Figure 1. Steimle et al.,
Figure 2. Steimle et al.,
Figure 3. Steimle et al.,
Figure 4, Steimle et al.
Figure 5. Steimle et al.
Figure 6. Steimle et al.,
Figure 7. Steimle et al.,
Figure 8. Steimle et al.,

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\begin{align*}
\text{CIV} & \quad \text{A} \quad \text{Cl\textsubscript{II}2} \quad \text{B} \\
\text{c\textsubscript{2}} \quad \text{c\textsubscript{1}} \quad \text{c\textsubscript{0}} \quad \text{c\textsubscript{p2}} \quad \text{c\textsubscript{p1}} \quad \text{c\textsubscript{1}} \quad \text{c\textsubscript{y}} \quad \text{c\textsubscript{2}} \\
\text{½} \text{O}_2 \quad \text{H}_2\text{O} \quad \text{QH}_2 \quad \text{b}_L \quad \text{b}_H \quad \text{b}_L \quad \text{b}_H \quad \text{b}_L \quad \text{b}_H \\
\end{align*}
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