Structure of yeast cytochrome c oxidase in a supercomplex with cytochrome bc1

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Cytochrome c oxidase (complex IV, CIV) is known in mammals to exist independently or in association with other respiratory proteins to form supercomplexes (SCs). In Saccharomyces cerevisiae, CIV is found solely in an SC with cytochrome bc1 (complex III, CIII). Here, we present the cryogenic electron microscopy (cryo-EM) structure of S. cerevisiae CIV in a III2IV2 SC at 3.3 Å resolution. While overall similarity to mammalian homologs is high, we found notable differences in the supernumerary subunits Cox26 and Cox13; the latter exhibits a unique arrangement that precludes CIV dimerization as seen in bovine. A conformational shift in the matrix domain of Cox5A—involved in allosteric inhibition by ATP—may arise from its association with CIII. The CIII–CIV arrangement highlights a conserved interaction interface of CIII, albeit one occupied by complex I in mammalian respirasomes. We discuss our findings in the context of the potential impact of SC formation on CIV regulation.

Cytchrome c oxidase (or complex IV, CIV) is the last enzyme in the mitochondrial electron transport chain whose activity drives oxidative phosphorylation, the process by which cells produce most of their adenosine tri-phosphate (ATP)1. CIV is a complex multi-subunit protein embedded in the inner membrane of mitochondria. Its catalytic core, conserved across kingdoms, is composed of three subunits encoded in the mitochondrial DNA. CIV catalyzes the reduction of molecular oxygen to water, conserving the released energy as coupled proton transfers across the membrane, through a mechanism that is still highly debated2. In addition, mitochondrial forms of CIV have supernumerary subunits (11 in humans), which are encoded in the nuclear genome. The supernumerary subunits might have a role in CIV assembly, stability or SC formation, and there is an increasing interest in understanding the role of specific isoforms in optimizing CIV core catalytic function in different tissues or physiological states3,4.

CIV can exist as a monomer or a dimer, or in an SC with complexes I and/or III in varying stoichiometries (I–III–IV1,2 and III1–IV1,2) with relative abundances depending on species5–9 and in mammals, tissue-type, cellular energy requirement and disease state10,11. Several structures of complex I-containing SCs have been published recently12–15 that provide details of their interaction.

Despite its lack of complex I (CI), S. cerevisiae has been extensively used as a model system for the study of mitochondrial respiratory chains, given its unique genetic amenability16. In the case of CIV, S. cerevisiae is the only system that contains the supernumerary subunits of so much interest (including isoforms), while allowing site-directed mutagenesis of the mitochondrial DNA. This uniquely permits the investigation of fundamental aspects of its catalytic mechanism as well as the identification of putative regulatory elements within supernumerary subunits. In addition, S. cerevisiae CIV is known to form SCs with cytochrome bc1 (ref. 17; or complex III, CIII), so it represents an ideal opportunity to investigate the role of SC formation on CIV activity and regulation.

High-resolution structural information for the mitochondrial form of the enzyme is limited to crystal structures of dimeric bovine CIV18 and more recently a cryo-EM structure of human CIV19 within a respirasome. Here, we present the cryo-EM structure of S. cerevisiae CIV in a III2IV2 SC at 3.3 Å resolution.

Results

Architecture of the III2IV2 SC. The III2IV2 SC was purified from S. cerevisiae mitochondrial membranes after solubilization with glyco-diosgenin (GDN) and successive metal affinity and gel filtration chromatography (Supplementary Fig. 1). The purified SC is active and reduces molecular oxygen in the presence of exogenous cytochrome c at a rate of 30.4 ± 1.3 s−1 using decylubiquinol as a reductant. When no cytochrome c was added, no oxygen reduction was observed (Supplementary Fig. 1).

The cryo-EM map (Table 1) reveals a CIII dimer at the core of the SC flanked by a CIV monomer on either side (Supplementary Fig. 2), as seen in previous low resolution models17,18. The core of the map was resolved to <3 Å, but the two CIV monomers could only be resolved to 5–8 Å, consistent with the dynamic nature of CIV within other SC structures11–13,19. To address this problem, we used subtracted experimental particle images, focusing only on masked refinements for each CIV monomer as previously described20. This procedure increased the resolution of the CIVA and CIVB monomers to 3.31 and 3.38 Å, respectively, revealing new atomic details (Supplementary Fig. 2 and Table 1). The improvement in resolution confirms that the two CIV monomers in the III2IV2 SC are identical.

The merged map (Fig. 1) allowed us to build 44 protein subunits for the III2IV2 SC, corresponding to all 20 protein subunits of the obligatory CIII dimer, including Qcr10, which is absent from all yeast structures published so far, and 12 protein subunits for each CIV monomer; the 11 classically described subunits5,21 and the recently assigned Cox26 (refs. 23,24). The presence of all polypeptides forming the III2IV2 SC was confirmed by mass spectrometry analyses (Supplementary Table 1). Mass spectrometry also revealed the presence of the respiratory SC factors Rcf1 and Rcf2 in the final protein preparation, but those proteins are absent from the cryo-EM structure. This observation supports the role of Rcf1 and Rcf2

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Cryo-electron microscopy (cryo-EM) was used to determine the atomic resolution structure of the mammalian CIV, and the structure was compared with that of the yeast CIV.

**Table 1** | Cryo-EM data collection, refinement and validation statistics
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 III,I IV, SC (EMD-0262, PDB6HU9) | CIVa (EMD-0269) | CIVb (EMD-0268)  
---|---|---  
**Data collection and processing**  
Magnification | 130,000 |  
Voltage (kV) | 300 |  
Electron exposure (e-/Å²) | 1.645 |  
Defocus range (μm) | –1.6 to –3.6 |  
Pixel size (Å) | 1.048 |  
Symmetry imposed | C1 |  
Initial particle images (no.) | 98,968 |  
Final particle images (no.) | 44,915 |  
Map resolution (Å) | 3.35 |  
FSC threshold | 0.143 |  
Map resolution range (Å) | — |  
**Refinement**  
Initial model used (PDB code) | 1KYO, 1V54 |  
Model resolution (Å)b | 3.35 |  
FSC threshold | 0.50 |  
Model resolution range (Å) | 3.35 |  
Map sharpening β factor (Å²)c | –60.26 | –69.04 | –79.03 |  
**Model composition**  
Nonhydrogen atoms | 63,033 |  
Protein residues | 7,636 |  
Ligands | 69 |  
β factors (Å²)c | 59.9 (141.4-20.7) |  
Ligand | 54.9 (149.7-35.5) |  
R.m.s. deviations | 0.01 |  
Bond lengths (Å) | 1.32 |  
**Validation**  
MolProbity score | 2.32 |  
Clashscore | 5.62 |  
Poor rotamers (%) | 8.49 |  
Ramachandran plot |  
Favored (%) | 95.61 |  
Allowed (%) | 4.35 |  
Disallowed (%) | 0.04 |  

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*a* The model was refined against a map generated by merging the map from the three-dimensional (3D) refinement (III2IV2 SC) with the two maps generated after particle subtraction and 3D refinement focused on CIVa or CIVb.  
*b* Determined by phenix.mtrajm.  
*c* Determined by phenix. molprobity (values in parentheses denote highest and lowest values).

in yeast SC formation but suggests that they are not essential for SC stability, which is similar to findings in mammalian systems. The high resolution of the map allowed the confident modeling of 96% of all amino acids in the structure (Table 1). All prosthetic groups and metal cofactors are well defined and 44 lipids including eight cardiolipin molecules can be confidently modeled.

**Atomic resolution structure of the S. cerevisiae CIV.** The yeast CIV structure comprises 12 subunits (Fig. 2a and Supplementary Fig. 1). Cox1–3 form the catalytic core and are surrounded by nine supernumerary subunits (Cox4–9, Cox12, Cox13, Cox26). The structure of yeast CIV is remarkably similar to mammalian systems, with a calculated root mean squared deviation against bovine and human enzymes of 0.95 and 1.10 Å, respectively, but there are notable differences as discussed below. Subunit composition is also very similar: all but one S. cerevisiae subunit (Cox26) have homologs in mammals and conversely, all mammalian subunits but three (COX7B, COX8 and NDUFA4) have yeast homologs. Yeast Cox5 (homologous to mammalian COX4) exists as one of two isoforms depending on oxygen level. In normoxic conditions, almost all CIV is assembled with the Cox5A isoform, although low levels of Cox5B are also expressed. To enhance the homogeneity of our protein preparations, we used a COX5B knockout mutant strain (ΔCOX5B) that only expresses the Cox5A isoform.

Cox1 and Cox2 house the prosthetic groups responsible for electron transfer and O₂ reduction, namely the dinuclear Cu₅, center, heme a, heme a₃ and CuB. All prosthetic groups are well resolved in the EM map, as well as the characteristic HPEVY ring of amino acids formed by covalent linkage between Nε2-His241 and Cε2-Tyr245 in Cox1 (Supplementary Fig. 3). Clear densities were observed at the expected magnesium and calcium/sodium binding sites in Cox1 (modeled as a Ca²⁺ ion) and the zinc binding site in Cox4. The density map also confirms the predicted positioning of the residues forming the D-, K- and H-channels described as putative proton pathways in Cox1 of A1-type oxidases.

Cox26, recently assigned as a subunit of S. cerevisiae CIV, is composed of a single transmembrane helix with a kink towards its C terminus. This arrangement enables effective hydrophobic interactions with Cox1 and Cox2 (Fig. 2b), which are further supported by two lipids held against the core of the enzyme. In the matrix, Cox26 interacts at its N terminus with Cox6 and Cox9 via a network of electrostatic and hydrophobic interactions (Fig. 2b). Deletion of Cox26 does not prevent CIV assembly, nor does it have any notable effect on its activity. However, the position of Cox26 in the SC, and the interactions it makes with the subunits above, suggest Cox26 could stabilize the region of CIV at the interface with CIII, supporting a role in formation or stability of the III₁IV₅ SC, even though no direct connection with CIII is evident from the structure.

A notable difference between yeast and mammalian CIV is the conformation of the N-terminal domain of Cox5A (Fig. 2c) that is not present in mammalian CIV. In addition, yeast CIV has a higher stability than mammalian CIV, which is similar to findings in mammalian systems.
shifted towards CIII in the S. cerevisiae SC to form protein–protein interactions further described below. This region is implicated in allosteric inhibition of CIV by ATP and Ser43, a residue that was shown by mutagenesis to contribute to this allosteric effect, is at the interface with CIII. More detailed biochemical analyses will be required to determine whether SC formation has a role in the ATP feedback inhibition mechanism.

Another difference is seen in Cox13, the most peripheral subunit of the SC (Figs. 1c and 2a,d). Cox13 is characterized by a single extended and bow-shaped transmembrane α-helix, protruding from the membrane into the matrix and the intermembrane space (IMS), with a bulge between residues Ser55 and Leu60. The N and C termini of Cox13 are oriented towards the core of the complex, interacting with Cox1 and Cox3 on the IMS and Cox4 on the matrix side. Its mammalian homolog, COX6A, has a shorter N terminus that folds back into the membrane, providing the dimerization interface observed in the bovine crystal structure (Fig. 2d). The relevance of the dimeric state of CIV is still disputed, and refinement of SC structures of various origins, including yeast, whenever the hydrophobic substrate was not completely extracted from the preparation, and it is due to the higher affinity of ubiquinone to the Qi site as compared to Qo. Apart from the presence of Qcr10 and changes resulting from interactions with CIV (see below), our model is consistent with the CIII structures described previously.

Despite low sequence identity, the transmembrane helix of Qcr10 (Fig. 3a, pink ribbon) occupies a position similar to that of subunit 11 in mammalian structures, making extensive interactions with Qcr9 and with Rip1 on the IMS side of the inner mitochondrial membrane supported by a lipid (Fig. 3a,b). In the matrix, the extended N terminus of Qcr10 interacts with Cor2 and Qcr7 of the other monomer (Fig. 3c), while in the IMS, Qcr10 extends to interact with cytochrome c, (Fig. 3d). These observations are in line with the proposed role of Qcr10 in CIII assembly and Rip1 stability.

A conformational change is observed at the N terminus of the transmembrane helix of Rip1 due to interactions with a cardiolipin that goes on to interact with Cox5A of CIV (light pink), forming part of the interface between the two complexes.

Complete structure of S. cerevisiae CIII. In S. cerevisiae, each CIII monomer consists of 10 subunits. Cytochrome b, cytochrome c1 and Rip1 form the catalytic core containing two hemes B, one heme C and a [2Fe-2S] cluster, respectively. All prosthetic groups are visible in the density map along with a molecule of ubiquinone in two apparent configurations at the Q site residing between the two cytochrome b subunits (Supplementary Fig. 4 and Fig. 3a). This is a common feature in structures of CIII of various origins, including yeast, whenever the hydrophobic substrate was not completely extracted from the preparation, and it is due to the higher affinity of ubiquinone to the Qi site as compared to Qo. Apart from the presence of Qcr10 and changes resulting from interactions with CIV (see below), our model is consistent with the CIII structures described previously.

Fig. 3 Interactions of Qcr10 with other subunits of CIII, and that of Rip1 with a lipid at the interface with CIV. a, Position of Qcr10 (pink ribbon) in the CIII structure with its N and C termini highlighted. Other subunits of CIII that interact with Qcr10 are highlighted in colors. A molecule of ubiquinone at the Qi site is shown as blue spheres. The boxes indicate specific regions of interaction that are highlighted in the other panels. b, Qcr10 (pink) forms interactions with the transmembrane helix of Rip1 (yellow) and with Qcr9 (green) at both the matrix and IMS faces of the membrane. c, The N-terminal tail of Qcr10 interacts with Cor2 (purple) and Qcr7 (cyan) in the matrix. d, Interactions of Qcr10 with Cor2 and Cytc, (gray), Rip1 (yellow) and Qcr9 (green). e, A slight shift in the N terminus of Rip1 (yellow) compared to the yeast X-ray structure (blue) accommodates interactions with a cardiolipin that goes on to interact with Cox5A of CIV (light pink), forming part of the interface between the two complexes.
The CIII–CIV interface. While the CIII dimer is symmetrical and no differences can be seen in the two CIII–CIV interfaces, the alignment of the two halves of the merged SC structure on CIII reveals a deviation of up to 3 Å at the extreme periphery of CIV (Supplementary Fig. 5). This most probably arises from the intrinsic flexibility of the SC and explains why a 3D refinement with C2 symmetry failed to improve the SC resolution (see Methods).

The CIII–CIV interface reveals protein–protein interactions on either side of the inner mitochondrial membrane and interactions via bridging lipids in the membrane region itself. The majority of interactions occur on the matrix side between Cor1 and the N terminus of Cox5A, facilitated by the conformational shift in the latter as described above (Fig. 4a). In the IMS, the C-terminal domain of Cox5A is in position to interact with both the C terminus of Qcr6 and a loop region between helices 6 and 7 of cytochrome c1 (Fig. 4b,c). Within the membrane, Cox5A contacts the N-terminal helix of Rip1 and Qcr8 via a cardiolipin molecule and another lipid modeled as phosphocholine (Fig. 4d). Two other cardiolipins indirectly support the CIII–CIV interface highlighting their crucial role in SC formation36.

Previous work indicates that mammalian COX7A2L is required for the formation and stability of the CIII–CIV SC in mammals37, which is consistent with published respirasome structures11,12. S. cerevisiae has no homolog of COX7A2L so it must follow a different mechanism of CIII–CIV SC formation than the one proposed in mammals. In addition, our work suggests that the III2IV2 SC in yeast doesn’t require any other proteins to maintain its stability, the interaction between Cox5A and Cor1, two highly conserved subunits and phospholipids being sufficient to stabilize the SC. However, no mammalian structure of the III2IV1/2 SC is currently available and, in the absence of CI, it cannot be excluded that the CIII–CIV interface is different in a III2IV1/2 SC compared to the respirasome. Mammalian CIV has an additional subunit, COX7B, in front of COX4–1 (the mammalian homolog of Cox5A). However, superimposition of the bovine CIV structure onto the yeast III2IV2 SC suggests a similar interaction between CIII and CIV as the one observed in yeast remains possible (Supplementary Fig. 6).

Discussion

The functional role of SC formation is still unclear38,39. Reactive oxygen species prevention by steric inhibition of one half of CIII by CIV in the respirasome has been proposed40. In the yeast III2IV2 SC, the symmetry of CIII is maintained and no obvious interactions are apparent that could stabilize the hinge region of Rip1, whose flexibility allows the movement of the head group domain, which is implicated in reactive oxygen species production. However, it is noteworthy that the same subunits of CIII are involved in SC formation and in all SC structures resolved so far (Fig. 5). The homologs of yeast CIII subunits Cor1, Rip1, Qcr6 and Qcr8 interact with CI in the mammalian respirasome structures. These are the same subunits that interact with CIV in our III2IV2 structure, highlighting a conservation of the CIII interaction interface, albeit one occupied by CI in mammals and by CIV in S. cerevisiae, which lacks CI. Additionally, in the CI-containing Yarrowia lipolytica

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**Fig. 4 | Protein–protein interactions between CIV and CIII.** a,b, View from the matrix side of protein–protein interactions involving Cor1 (white) and Cox5A (pink). Residues that make inter-subunit interactions are indicated and their interactions are shown as dashed lines. c, Interactions between cytochrome c (Cytc, brown) and Qcr6 (pale green) with Cox5A (pink) in the IMS. d, A cardiolipin molecule (CDL, yellow) and a phosphocholine (PCF, gray) within the membrane interacts with residues of Cox5A (pink), Rip1 (pale blue) and Qcr8 (blue).
respirasome, where the CII symmetry is apparently maintained, the suggested CIII–CIV arrangement is similar to the one observed in the yeast III,IV, SC. This suggests that SC formation may serve to stabilize the active CIII monomer(s) by other proteins.

Finally, modulation of CIV activity in response to energy requirements by differential expression of isoforms such as Cox5 in yeast (COX4 in mammals) and COX7A in mammals has been recognized. From the yeast structure presented here and that of mammalian respirasomes, it seems that these subunits form a substantial part of the interface between proteins in SCs. Therefore, biochemical and biophysical studies investigating these isoforms, and the effect of allosteric sites identified within supernumerary subunits on CIV core catalytic subunits, must take into account SC formation. With homologs of many mammalian supernumerary subunits, extensive genetic amenability and in light of the SC structures now available, S. cerevisiae offers a powerful system to study how these factors modulate CIV activity and respiratory SC formation.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41594-018-0172-z.

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**Author contributions**

A.M. designed and supervised the research. B.M. produced the yeast mutant strain. A.M.H. did all the protein work with contribution from Y.Z. A.C.-O. and S.A. performed mass spectrometry analysis. N.L. and A.M.H. performed all microscopy work. A.M.H. and N.P. processed the cryo-EM images. N.P. built the model with inputs from A.M. and A.M.H. A.M., A.M.H. and N.P. wrote the manuscript with contributions from all authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Methods Yeast strain and cell growth. A ΔCOX5B S. cerevisiae strain only expressing the Cox5A isoform of CIV (v adle2 leu2 trpl ura3 cox5B::KanMX4) with a six-histidine-terminus at the C terminus of COX13 was constructed from W303-1B-14. Yeast cells were grown in YPD (1% yeast extract, 2% peptone and 2% galactose) medium at 28°C in 21 baffled flasks with shaking at 200 r.p.m. as described in Meunier et al.14. Cells were harvested in late log phase by centrifugation at 6,500 × g for 5 min at 4°C. Cells were washed by resuspension in 50 mM KPi, pH 7.0 and centrifuged again. Cell pellets were stored at −80°C until use.

Preparation of mitochondrial membranes. Mitochondrial membranes were prepared essentially as described previously14. Briefly, thawed yeast cells were resuspended in 30 ml 650 mM D-mannitol, 50 mM KPi, 5 mM EDTA, pH 7.4 containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Glass beads (425–600μm diameter) were added and cells were broken by mechanical lysis using a bead-beater cell disruptor. Cell debris was removed by centrifugation at 5,600 × g for 20 min at 4°C, and the supernatant was centrifuged at 120,000 × g for 50 min at 4°C to harvest the mitochondrial membranes. The membranes were then resuspended and homogenized in 50 mM KPi, 100 mM KCi, 10 mM MgCl2, 150 μM CaCl2, 0.1 mM PMSF, pH 7.4 and centrifuged as in the previous step. The pellet was then washed by cycles of resuspension/centrifugation in 50 mM KPi, 2 mM EDTA, 0.1 mM PMSF and pH 7.4, until the measure of the absorbance of the supernatant at 260 nm was below 1. Finally, the membranes were resuspended in a minimal volume of 50 mM HEPES, pH 8.0 and stored at −80°C until use.

Membrane solubilization and SC purification. Membranes were diluted in 50 mM HEPES, 150 mM NaCl, 1 mM PMSF, pH 8.0 to a CIII concentration of 45 mM and protein complexes were solubilized for 1 h on ice by the addition of 1% GDN (Anatrace). After solubilization, 350 mM NaCl (to make 500 mM final) and 5 mM imidazole are added. Insoluble material was removed by centrifugation at 120,000 × g for 30 min at 4°C. Solubilized proteins were then loaded overnight in a cold room at a ratio of approximately 0.6 mL per 1 mL using a peristaltic pump onto a 5 mL HisTrap HP column (GE Healthcare) previously equilibrated with two column volumes of 50 mM HEPES, 500 mM NaCl, 5 mM imidazole, 0.05% GDN and pH 8.0. After loading, the column was washed with 3 column volumes of 50 mM HEPES, 500 mM NaCl, 5 mM imidazole, 0.05% GDN and pH 8.0, and then with 5 column volumes of 50 mM HEPES, 150 mM NaCl, 0.05% GDN, 5 mM imidazole and pH 7.2. Bound proteins were eluted with 50 mM HEPES, 150 mM NaCl, 0.05% GDN, 100 mM imidazole and pH 7.2. The eluted proteins were concentrated by centrifugation using 100 KDa molecular weight cut-off centrifugal concentrators (GE Healthcare). The resulting sample was then further purified by gel filtration, using an Äkta Pure 25 (GE Healthcare) operated at 4 °C with UV–visible difference spectra were recorded using a Polara microscope operated at 300kV and equipped with a Quantum energy filter (Gatan) with a post-GIF K2 Summit direct electron detector (Gatan) operating in counting mode. The primary data were collected using a Titan krios microscope (Thermo Fisher) operated at 300 kV and equipped with a Quantum energy filter (Gatan) (electron Bio-Imaging Center, Diamond Light Source). The images were collected with a post-GIF K2 Summit direct electron detector (Gatan) operating in counting mode at a nominal magnification of ×130,000, corresponding to the pixel size of 1.04 Å. An energy slit with a width of 0.25 eV was used during data collection. The dose rate on the specimen was set to 6.58 electrons per Å² per s and a total dose of 52.64 eÅ per Å² was fractionated over 32 frames. Data were collected using EPU software (Thermo Fisher) with a nominal defocus range set from ~1.6μm to ~3.6μm. A total of 2,740 micrographs were collected.

Image processing. Frame alignment and exposure weighting were performed with MOTIONCOR22. Contrast transfer function parameters of the motion-corrected micrographs were estimated with CTFFINED4.1. Micrographs were screened manually to remove those with excessive specimen drift, overfocus or ice defects. 98,968 particles were selected from the 2,634 remaining micrographs using reference-free particle picking with Gautomatch v.0.53 (written by K. Zhang, https://www.mrc-lmb.cam.ac.uk/kzhang/Gautomatch/), using a 360 × 360 Å² box size. Particles were sorted using several iterations of reference-free two-dimensional classification in cryoSPARC30, resulting in a final particle number of 52,257. An initial model was build using ab initio reconstruction in cryoSPARC, which was refined using heterogeneous refinement in RELION v.3.0. The III IV SC accounted for 44,915 (86%) of the particles present (14% represented the IIIIV SC) and these particles were then used for homogeneous refinement in RELION v.3.0 resulting in a 3.31 Å resolution map, based on the FSC-gold standard. Local resolution was calculated using ResMap29, revealing a range of resolutions in the map, with the core of CIII resolved to 2.9–3.2 Å, whereas the peripheral edges of the two CIV proteins were resolved to 5–8 Å.

To increase the resolution of the two CIV monomers, we used a particle subtraction approach30. In short, a soft mask was generated around IIIIV, and used to subtract density from the particles, resulting in a new set of particles that was used for focussed 3D refinement of CIVA. This process was repeated with a second soft mask around IIIIV, to refine CIVb. This resulted in an increased resolution of the two CIV monomers to 3.31 Å and 3.38 Å, with a homogeneous distribution of resolution throughout the protein (Supplementary Fig. 2). The two CIV maps were then aligned to the original map of the SC and a merged map was generated using UCSF Chimera28.

Model building. The three individual maps described above were used for all model building using real space refinement (Table 1) in Coot40. A high-resolution crystal structure of dimeric CIII (PDB KYO30) and a yeast homology model model28 were used as starting references for model building. All maps displayed clearly interpretable features such as bulky side chains, metal clusters, heme ligands, cardiolipin and ubiquinone. These features enabled unambiguous assignment of amino acids for all chains, except some flexible N and C termini. Notably, the C terminus of Rip1 of CIII has weak density (residues 95–215), and this is attributed to all yeast CIII crystal structures, possibly due to the high composition of charged
residues in this region. Additional densities in the map indicated the presence of long carbon chains that were modeled as di-palmitoyl-phosphatidylethanolamine (PEF), diacyl-glycerophosphocholine (PCF) and cardiolipin (CDL) molecules on the basis of map interpretation and similarities with previous structures where these ligands were found. Lipid tails were truncated according to the density maps. The three models (dimeric CIII and two CIV monomers) were then individually refined using the real space refine tool in Phenix56, using secondary structure restraints. Geometry definitions for the ligands were defined from values in the CCP4 ligand library57. Additional bond and distance restraints were implemented on specific molecules on the basis of previously published high-resolution structures. For initial refinement in Phenix, Ramachandran and rotamer constraints were also used. The models were then visually inspected in Coot for additional corrections. A final real space refinement was performed in Phenix by disabling rotamer constrains resulting in an increase of the model-to-map fit. To confirm the validity of the map, a final real space refinement was performed to the complete III2IV2 SC using the merged map described above. The final model contains 7,636 protein residues and 69 ligands. For the CIII dimer we modeled 4 B-hemes and 1 ubiquinone molecule in the 2 cytochrome b subunits, 1 C-heme for each cytochrome c1 subunit, 1 [2Fe-2S] iron-sulfur cluster for each Rip1 subunit, 8 cardiolipin molecules, 12 PEF and 6 PCF molecules. For each CIV we modeled a heme a, heme a3, CuA, and CuB in Cox1 as well as one calcium and one magnesium ion. In Cox2 we modeled a dinuclear CuA center and in Cox4 one zinc ion. Each CIV contains additionally eight PEF molecules and one PCF molecule. Refinement and model statistics are summarized in Table 1. Map and molecule representations in the figures were prepared by PyMOL (https://pymol.org/) and UCSF Chimera.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All relevant data are included in the manuscript and/or are available from the corresponding authors upon reasonable request. Cryo-EM maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession codes EMD-0262 (III2IV2 SC), EMD-0269 (CIVA) and EMD-0268 (CIVB). The coordinates of the atomic model of the III2IV2 SC built from a combination of the three maps have been deposited in PDB under accession code 6HU9.

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|     | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | EPU used at Diamond Light Source to collect cryo-EM movies |
|-----------------|----------------------------------------------------------|
| Data analysis   | MaxQuant software for analysis of raw mass spectroscopy data; |
|                 | Motioncor2 to align frames and weigh exposure; |
|                 | CTFIND4.1 to estimate CTF parameters; |
|                 | Gautomatch v0.53 for reference-free particle picking; |
|                 | cryoSPARC for reference-free 2D classification and ab-initio reconstruction; |
|                 | RELION v3.0 for reconstruction of cryo-EM density maps; |
|                 | ResMap for calculation of resolution range in the maps; |
|                 | UCSF Chimera 1.12, Pymol 1.8.5.0, Eman2 and Coot 0.8.9.1 (CCP4) to examine density maps, build atomic structures and prepare figures; |
|                 | Phenix.real_space_refine (1.13_2998) to refine atomic structures; |
|                 | Phenix.mtriage (1.13_2998) to validate atomic structures; |
|                 | Phenix.mrmerge (1.13_2998) to validate atomic structures; |
|                 | OriginPro 2015 for linear fitting of O2 reduction traces and to prepare figures. |

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Three cryo-EM maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession codes EMD-0262 (III2IV2 SC), EMD-0269 (CIV 1), EMD-0268 (CIV 2). The coordinates of the atomic model of the III2IV2 SC built from a combination of the three maps have been deposited in the Protein Data Bank (PDB) under accession code 6HU9.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size  No statistical methods were used to predetermine sample size
Data exclusions  A fraction of the acquired cryo-EM movies were discarded because of excessive specimen drift, overfocus or ice defects.
Replication  Sample preparation at the quality for cryo-EM specimen preparation was repeated three times and final UV/visible difference spectra, gel filtration elution profile and BNPAGE gels were obtained in each case. Activity measurements were performed in duplicate on two of these preparations. Cryo-EM data collection and structure calculation were not repeated.
Randomization  Samples were not allocated into experimental groups as this was not relevant to the aim of the study.
Blinding  Blinding was not relevant to our study and the data analysis was ab initio.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a  Involved in the study
☒  Unique biological materials
☒  Antibodies
☒  Eukaryotic cell lines
☒  Palaeontology
☒  Animals and other organisms
☒  Human research participants

Methods

n/a  Involved in the study
☒  ChiP-seq
☒  Flow cytometry
☒  MRI-based neuroimaging