Supplementary Discussion

1. Background information on the Alternative Complex III

The alternative complex III (ACIII) is a multi-subunit, integral membrane protein complex found in many bacteria that catalyzes the same reaction as the bacterial bc_1 or b_6f complexes in respiratory and/or photosynthetic electron transfer chains (for reviews, see [1-5]).

$$\text{menaquinol (or ubiquinol)} + 2\text{ cyt } c_{ox} \rightleftarrows \text{menaquinone (or ubiquinone)} + 2\text{ cyt } c_{red} + 2\text{ H}^+$$

Often the ACIII is found in bacteria that lack the bc_1 or b_6f complex, but this is not always the case[2]. The genome of *Thermus thermophilus*, for example, encodes both a bc_1 complex[6] and an ACIII. Whereas there have been many studies of the bc_1/b_6f complexes[7-9], including X-ray structures[10-14], little is known about the ACIII complexes[15,16]. Although the bc_1/b_6f complexes and ACIII catalyze the same reaction, there is no structural similarity between the subunits, and the two families have independent phylogenetic histories[2]. In addition, the ACIII contains subunits that are homologous to those found in the members of complex iron-sulfur molybdoenzyme (CISM) family[17] and is one of a number of membrane-bound electron transport enzymes that contain quinone-reactive transmembrane components that lack associated redox prosthetic groups such as hemes[18].

The ACIII has been previously isolated from two organisms, both thermophilic bacteria, neither of which has a bc_1 complex: *Rhodothermus marinus*[16,19,20] and *Chloroflexus aurantiacus*[4,15,21,22]. ACIII from *R. marinus* contains seven different subunits encoded by the gene cluster *actABCDEFG* which is part of a larger putative operon that includes the genes encoding the subunits (I, II, III and IV) of the cytochrome caa_3 heme-copper oxygen reductase[16,20]. The genes encoding ACIII are frequently found clustered with genes encoding heme-copper oxygen reductases[2], mostly members of the A2 family but also with those in the A1-family (most

Flavobacteria) and C-family as well. A functional interaction between the ACIII and cyt *caa*$_3$ of *R. marinus* has been demonstrated[19,23] and the ActE subunit of ACIII, containing a single heme *c* moiety, has been demonstrated to be a direct electron donor to the oxygen reductase[23].

In *C. aurantiacus*, an anoxygenic phototroph, there are two operons encoding for the ACIII that are differentially expressed and function, respectively, in aerobic respiration or anaerobic photosynthesis[4,24]. The ACIII which is part of the cyclic photosynthetic electron transfer system has been purified and characterized[15,21,22]. This phototrophic ACIII is also made up of seven different subunits (ActABCDEFG) which are each homologues of the subunits in the *R. marinus* ACIII. The mono-heme cyt *c* subunit, ActE, is present in two copies in the *C. aurantiacus* photosynthetic ACIII[15,22] but appears to be present in a single copy in the *R. marinus* ACIII[16,20].

In the current work, ACIII is isolated and characterized from *Flavobacterium johnsoniae*, a mesophilic marine bacterium and a member of the *Bacteroidetes* phylum that has been used as a model system for studying gliding motility and the type IX secretion system associated with it[25]. Since genetic tools are available for manipulation of *F. johnsoniae*[26,27], this is a potentially valuable system for studying ACIII using molecular genetics methods not available for the organisms in which ACIII is currently studied.

2. Background on styrene-maleic acid copolymer nanodiscs

In this work, we take advantage of the natural affinity of cyt *aa*$_3$ to the Ni-NTA resin to purify the ACIII-cyt *aa*$_3$ supercomplex. The ACIII-cyt *aa*$_3$ supercomplex was solubilized and purified from membranes using a traditional detergent (Triton X-100) as well as SMA copolymer. While the use of detergents in purification of membrane proteins is extremely common and well documented[28], the SMA copolymer has only recently been used for this purpose[29-35]. The SMA

copolymer is an amphipathic linear polymer with randomly placed hydrophobic styrene and hydrophilic maleic acid units. After inserting into the membrane, the anionic SMA copolymer reaches the hydrophobic core of the membrane and spontaneously forms nanodiscs with sizes ranging between 10-14 nm containing both membrane proteins and lipids. The advantage of SMA-nanodisc in the preparation of membrane proteins is that the proteins are never exposed to traditional small molecule detergents, and can be directly isolated together with lipids and proteins with which they associate within the membrane. In principle, there is less chance to lose native characteristics of proteins upon isolation. Previous work has shown that SMA nanodiscs containing membrane proteins are suitable for biochemical and biophysical studies, including negative staining electron microscopy[36] and single particle cryo-EM[30,37]. The current work is the first atomic resolution structure of a membrane protein in an SMA nanodisc using cryo-EM[38-40].

3. The aerobic respiratory chain of *F. johnsoniae*

The genome of *F. johnsoniae* contains genes that encode for protein components of a branched electron transport chain for aerobic respiration along with genes encoding enzymes required for menaquinone biosynthesis[41]. The deduced electron transport chain is shown in **Extended Data Fig. 1**. The genome encodes for both Complex I and Ndh-2, which are NADH:menaquinone oxidoreductases, along with Complex II, the succinate:menaquinone oxidoreductase. These enzymes, along with other dehydrogenases, provide multiple pathways to reduce menaquinone. There are three different oxygen reductases encoded in the genome: cyt *bd*, cyt cbb_3 and cyt aa_3. The cyt *bd* is a menaquinol:oxygen oxidoreductase, whereas both cyt cbb_3 and cyt aa_3 are cytochrome *c*:oxygen oxidoreductases that are members of the superfamily of heme-copper oxygen reductases[42]. The cyt aa_3 is a member of the A1 family of heme copper oxidoreductases[43].

There is one copy each of the genes encoding subunit I, II and IV, but there are two genes that encode different variants of subunit III (WP_012023735.1 and WP_012023736.1) and, for this reason, two variants of the assembled cyt aa_3 (vI and vII) are indicated in **Extended Data Fig. 1**. Our results indicate that only one variant (WP_012023736.1) is part of a supercomplex with ACIII.

The only enzyme that can catalyze electron transfer from menaquinol to cytochrome *c* is the ACIII[2,15,16] since this organism lacks the genes for the bc_1 complex[6] as do all members of the genus *Flavobacterium*. When cyt *bd* is expressed, the ACIII is not essential for aerobic respiration (**Extended Data Fig. 1**). The membranes of *F. johnsoniae* were analyzed using UV-visible spectroscopy and SDS-PAGE followed by staining for hemes[44] (**Extended Data Fig. 1**). The UV-vis difference spectrum between the reduced and the oxidized samples shows the presence of heme *a,* heme *b* and heme *c* in the membrane. Heme staining only reveals the two subunits from the ACIII that contain covalently bound heme *c*, indicating the absence of cyt cbb_3 and its heme *c*-containing subunits. The presence of the cyanide-insensitive cyt *bd* in the membranes is indicated confirmed by the substantial succinate-driven oxygen utilization that is resistant to 100 μM cyanide (data not shown). The UV-visible spectroscopy does not show the presence of heme *d*, so it is likely that the enzyme responsible for the cyanide-resistant activity is a variant of cyt *bd* in which heme *b* replaces heme *d* at the active site (cyt bb' or "cyanide insensitive oxidase"). Although a systematic study on the composition of the respiratory system of *F. johnsoniae* as a function of growth condition was not performed, it is clear that both cyt aa_3(vI) and cyt *bd*, but not cyt cbb_3, are present in the membranes used in this work. Our data suggest that cyt aa_3(vII) is not be present, but this was not investigated thoroughly. The growth conditions required to induce expression of cyt cbb_3 and cyt aa_3(vII) are not known.

4. Genes encoding subunits of ACIII in *F. johnsoniae*

The gene cluster containing the genes encoding the subunits of ACIII in *F. johnsoniae* is similar to those in *R. marinus* and *C. aurantiacus*[15,20] with some differences. There are six *act* genes in the *F. johnsoniae* ACIII operon (*actABCDEF*) (**Extended Data Fig. 1**). Unlike the ACIII from *R. marinus* and *C. aurantiacus*, the ACIII operon for *F. johnsoniae* does not contain an *actG* gene. Many other organisms encoding ACIII also lack the ActG[1,2].

1) *actA* encodes a multiheme cytochrome subunit (ActA, WP 012023710.1) with the sequence containing six CXXCH heme *c* binding motifs. Using transmembrane helix prediction software (TMHMM Server v 2.0, Center for Biological Sequence Analysis, Technical University of Denmark) [45], ActA is predicted to have an N-terminal transmembrane sequence, which is probably a cleaved signal sequence, followed by a periplasmic monoheme cyt *c* domain, two additional transmembrane helices and a C-terminal periplasmic pentaheme cyt *c* domain.

2) *actB* encodes for an iron-sulfur protein (ActB, WP_012023711.1) that is expected to be in the periplasm since it has the Tat (Twin arginine translocase) signal peptide[20,22,46]. Similar to ACIIIs from other organisms, the sequence analysis identifies binding motifs for one $[3Fe\text{-}4S]^{1+/0}$ and three $[4Fe\text{-}4S]^{2+/1+}$ clusters[5].

3) *actC* encodes a protein (ActC, WP_012023712.1) predicted to have ten transmembrane helices with no motifs predicting bound prosthetic groups.

4) *actD* encodes a small protein (ActD, WP_012023713.1) with two transmembrane helices, cytoplasmic domains on both the N- and C-terminal sides of the protein, and no motifs predicting prosthetic groups.

5) *actE* encodes a periplasmic protein (ActE, WP_012023714.1) with one CXXCH motif for binding a single heme *c*. As with ActE from *R. marinus*, ActE from *F. johnsoniae* has a possible

lipoprotein signal sequence predicting a mature protein with an N-terminal lipid-modified cysteine[23].

6) *actF* encodes a protein (ActF, WP_012023715.1) which, like ActC, is predicted to have ten transmembrane helices and shows no evidence for bound prosthetic groups.

As in many other organisms[2], the ACIII genes are immediately followed by genes encoding subunits of a respiratory heme-copper oxygen reductase (**Extended Data Fig. 1**). In *F. johnsoniae*, the ACIII genes are followed by genes encoding subunits II (WP_012023716.1) and I (WP_012023717.1) of the cyt aa_3-type that is a member of the A1-family[43,47]. Subunit II is predicted to have four transmembrane helices, which is unusual (normally there are two transmembrane helices) [42,47], and a periplasmic C-terminal region that has a Cu_A motif (CXXXHXXM), indicating that the oxygen reductase is a cytochrome *c* oxidase and not a menaquinol oxidase. Subunit I is predicted to have 12 transmembrane helices and is similar to other A1-family heme-copper oxygen reductases[43,47]. The genes for the subunits III (WP_012023735.1; WP_012023736.1) and IV (WP_012023737.1) are separated by18 genes downstream of those for subunits I and II. There are two adjacent genes encoding two different variants of Subunit III (here referred to as vI and vII) and they each have unusual features. Each is predicted to have five transmembrane helices, whereas the canonical subunit III has seven transmembrane spans. Variant I (WP_012023736.1) is larger than variant II (WP_012023735.1) mostly due to a long periplasmic peptide connecting the third and fourth transmembrane spans than in variant II. Subunit IV has three transmembrane helices.

5. Protein purification and UV/visible spectroscopy

Since a structural association between the ACIII and the cyt *caa*$_3$ has been reported in *R. marinus*[19], we tested if this was also true for *F. johnsoniae*. Many A-family heme-copper oxygen reductases, such as the *F. johnsoniae* cyt *aa*$_3$, have a natural affinity for the Ni-NTA resin that is usually used for the purification of His-tagged proteins. If this were the case for the *F. johnsoniae* cyt *aa*$_3$, and if cyt *aa*$_3$ were part of a supercomplex with ACIII, then the supercomplex should bind to the Ni-NTA resin in an affinity column and be eluted upon the addition of a sufficient concentration of imidazole. This is what was observed.

Membranes were solubilized with Triton X-100 or with the SMA copolymer, applied to a Ni-NTA column and washed with increasing amounts of imidazole. Regardless of the solubilizing reagent, the flow-through contains no heme *a*, but does contain heme *c* associated with ACIII. The membranes, therefore, are concluded to contain an excess of ACIII over cyt *aa*$_3$. All of the solubilized cyt *aa*$_3$ binds to the affinity resin along with some of the ACIII, but there is a population of ACIII that does not bind to the resin. The eluted heme proteins are the same for the preparation solubilized using Triton X-100 and for the preparation solubilized by the SMA copolymer (**Extended Data Fig. 1**). For the membranes solubilized with Triton X-100, the protein bound to the Ni-NTA column was washed and eluted with buffer containing the detergent DDM. For membranes solubilized using the SMA copolymer, no detergent was added to either the washing or elution buffer, and the proteins remain soluble.

The UV-visible spectra of the eluted proteins (**Extended Data Fig. 1**) show absorbance peaks typical for heme *c* and heme *a*. The Soret peak at 412 nm shifts to 418 nm upon reduction with dithionite. The α and the β maxima for heme *c* (524 nm and 552 nm, respectively) and the Soret and the visible bands for heme *a* (443 nm and at 605 nm) are clearly observed in spectra of the dithionite-reduced eluted proteins. Using the pyridine hemochrome assay as described in [48], the

presence of heme *c* and heme *a* in the protein sample was confirmed (**Extended Data Fig. 1**) and their concentrations were further quantified. The assay also confirmed that the preparation does not contain any heme *b*.

Assuming that each ACIII contains seven hemes *c* and each cyt aa_3 has two heme *a* moieties, the pyridine hemochrome assays show that the preparations eluted from the affinity column, solubilized by either Triton X-100 or SMA copolymer, contain ACIII in about 50% excess over cyt aa_3. Assuming the supercomplex has a 1:1 ratio of ACIII and cyt aa_3, as is the case for the supercomplex from *R. marinus*[19], the eluted proteins from *F. johnsoniae* consist of a 2:1 mixture of the supercomplex and free ACIII. This is supported by the cryo-EM results. Although most of the ACIII that is not part of the supercomplex is in the flow-through, there is a subpopulation of ACIII that is not part of the supercomplex that appears to bind to the Ni-NTA resin and co-elutes with the supercomplex.

6. SDS-PAGE

Samples of the purified heme protein complex from each preparation (Triton X-100 vs SMA copolymer) were analyzed by SDS-PAGE and visualized by Coomassie Blue (**Extended Data Fig. 2**). Both the detergent-solubilized and the SMA nanodisc preparations have similar molecular weight profiles and showed bands that can be putatively assigned as subunits of either ACIII or cyt aa_3. Proteins with apparent molecular weights of 48 kDa, 110 kDa, 20 kDa and 18 kDa were putatively assigned to ActA, ActB, ActD and ActE respectively. Both ActC and ActF have similar molecular weights based on their amino acid sequences and are assigned to the bands running close to 53 kDa. In the detergent-solubilized sample this is clearly seen as two bands (**Extended Data Fig. 2**) but in the SMA nanodiscs the subunits run as a single band (**Extended Data Fig. 2**).

Subunits I, II, III (variant I) and IV of cyt *aa₃* are tentatively assigned as the bands with apparent molecular weights of 67 kDa, 44 kDa, 37 kDa and 13 kDa, respectively.

The SDS-PAGE gels were also stained with TMBZ to visualize any proteins with covalent heme, i.e., heme *c*. In both the detergent-solubilized and the SMA nanodisc samples, the bands assigned as ActA and ActE are identified as containing heme, consistent with the assignment (**Extended Data Fig. 2**).

7. Mass spectrometry:

The presence of subunits from the *F. johnsoniae* ACIII and cyt aa_3 was confirmed by mass spectrometry for both the detergent-solubilized preparations and the SMA-nanodiscs (**Table S1**). The subunits in the detergent-solubilized preparation that were identified with confidence are those that contain substantial hydrophilic domains or are hydrophilic proteins. In ACIII these were the ActA, ActB, ActD, ActE, and in cyt aa_3 these were the Subunits II and III (variant I). The more hydrophobic subunits, ActC, ActF and Subunit I were not detected. Coverage was better in the analysis of the subunits present in the SMA nanodiscs with all of the expected subunits of the ACIII-cyt aa_3 supercomplex being identified with reasonable confidence (**Extended Data Fig. 2**), with the possible exception of Subunit IV of cyt aa_3. The data did not indicate the presence of variant II of subunit III in the protein eluted from the Ni-NTA resin. Variant I of cyt aa_3 is the dominant form present in the membranes of *F. johnsoniae* under the growth conditions utilized.

8. Enzyme activities

Enzyme assays were carried out to confirm that the ACIII and cyt aa_3 are functionally associated. Quinol:oxygen oxidoreductase activity showed that the supercomplexes, isolated either

by solubilization by Triton X-100 or SMA copolymer, oxidize quinol and reduce O_2, a reaction that requires functional coupling between the ACIII complex and the respiratory O_2 reductase. It has recently been reported that the ActE (monoheme cyt *c*) subunit of the *R. marinus* ACIII can donate electrons directly to the cyt *caa₃* oxygen reductase in *R. marinus* [23]. The native quinone in the membranes of *F. johnsoniae* is menaquinone-6[41,49]. Menaquinones, including water-soluble analogues such as menadiol, have lower midpoint potentials (e.g., $E_{m,7}$ = -74 mV) than ubiquinones (e.g., $E_{m,7}$ = 100 mV) [50] and are very susceptible to autoxidization [51] by O_2 ($E_{m,7}$ = 820 mV) [52], resulting in a large baseline activity in the absence of enzyme in assays of the supercomplex. Since the ACIII:cyt aa_3 preparations from *F. johnsoniae* are active with ubiquinol-1 (Q_1H_2) as substrate, which has a low rate of autoxidation, the supercomplex was routinely characterized by its Q_1H_2:O_2 oxidoreductase (ubiquinol-1 oxidase) activity. The enzyme preparations oxidize Q_1H_2 and reduce O_2 with k_{cat} values of about 15 *e* s^{-1} for the detergent-solubilized preparation and 22 *e* s^{-1} for the SMA-nanodiscs (**Extended Data Fig. 3**). These numbers are comparable to the turnover number of the cyt caa_3 from *R. marinus* using ActE as a substrate[23]. With each preparation, the oxygen consumption assay is completely inhibited in the presence of 50 μM KCN, consistent with the O_2-reacting species being the cyt aa_3 heme copper oxygen reductase[53].

The quinol:cytochrome *c* oxidoreductive activity could also be evaluated directly using horse heart cyt *c* as an electron acceptor but the turnover is much reduced to about 1.6 *e* s^{-1} using Q_1H_2 as the substrate with the detergent-solubilized preparation (**Extended Data Fig. 3**). Using MK_4H_2 as the substrate (under anaerobic conditions to avoid autoxidation) the cyt *c* reductase activity of the detergent-solubilized supercomplex increases to 20 *e* s^{-1} but only 5 *e* s^{-1} for the SMA-nanodisc preparation. A possible explanation for this could be that the ACIII in the SMA nanodiscs is not

accessible to the externally added cyt *c*. Recall that about one-third of the total ACIII in the final preparations is not part of the supercomplex.

9. Blue Native (BN)-PAGE:

The existence of an ACIII-cyt aa_3 supercomplex is also suggested by analysis of the samples eluted from the Ni-NTA column using BN-PAGE (**Extended Data Fig. 2**). Equal amounts of protein from each sample were compared. The detergent-solubilized sample showed considerable smearing of bands, with a broad band above 800 kDa (**Extended Data Fig. 2**). A single sharp band is near 500 kDa, which is close to the expected size of 465 kDa for the supercomplex composed of one copy of ACIII (~305 kDa) and one copy of cyt aa_3 (~160 kDa). However, this sharp band is not a major component. The simplest interpretation of this result is that the majority of the protein in the detergent-solubilized preparation is aggregated. On the other hand, the SMA-nanodisc preparation shows a major band near 500 kDa, consistent with a 1:1 supercomplex between ACIII and cyt aa_3 (**Extended Data Fig. 2**). While this preparation appears to exist mostly as the 1:1 supercomplex, there are additional complexes present, notably a band near 1000 kDa.

10. Size exclusion chromatography (SEC)

The enzyme preparations eluted from the Ni-NTA column were also analyzed by size exclusion chromatography using Superdex 200 GL 10/300. The detergent-solubilized preparation elutes as multiple peaks (**Extended Data Fig. 2**) with the majority being in the excluded volume, consistent with considerable aggregation, since the exclusion limit is about 1300 kDa for a globular protein. The elution profile of the SMA-nanodisc preparation has two distinct populations associated with two sharp peaks in the gel filtration (**Extended Data Fig. 2**). The material which elutes first (peak

I, **Extended Data Fig. 2**) is near the exclusion limit of the column, whereas the material corresponding to the peak II remains in the included volume, consistent with an apparent molecular weight of about 500 kDa. Samples from the maximum of each peak were collected, concentrated (~6-fold) and re-analyzed by size exclusion chromatography and BN-PAGE (**Extended Data Fig. 2**). **Extended Data Fig. 2** also shows that the material collected from peak I elutes at the position from the Superdex 200 GL 10/300 column, as is also the case for material collected from peak II (**Extended Data Fig. 2**). This result indicates that the SMA-nanodiscs in the preparation are in two distinct populations that are not in rapid equilibrium with each other and do not interconvert, at least over the course of 1 or 2 days. The material eluted in peak I from the Superdex 200 GL 10/300 column corresponds to the higher molecular-weight band in the BN-PAGE analysis (**Extended Data Fig. 2**), whereas the material in peak II runs as a single band in the blue native gel with an apparent molecular weight of about 500 kDa (**Extended Data Fig. 2**). Thus, the two peaks from the gel filtration column correspond to the two bands observed in the blue native gel and these are stable particles or complexes.

The ratios of heme *c*:heme *a* and the turnover number (per mole of cyt aa_3) were also determined for the material loaded onto the Superdex column as well as the materials eluted in peak I and peak II. The results (**Extended Data Fig. 3**) show that the heme compositions as well as the Q_1H_2:O_2 oxidoreductase turnover numbers are the same before and after gel filtration chromatography, and are also the same for the materials in peak I and peak II. These two populations of particles do not represent a separation of the supercomplex from free ACIII.

11. Electron Paramagnetic Resonance (EPR) analysis

Both ACIII and cyt aa_3 contain redox active metal centers which can be probed using EPR spectroscopy. **Extended Data Fig. 3** shows the EPR spectrum of the air-oxidized detergent-solubilized supercomplex at 10K. The most intense feature of the spectrum is in the g = 2 region (**Extended Data Fig. 3**), which consists of a sharp isotropic g = 2.01 signal and a much broader signal with $g_z = 2.17$, $g_{x,y} = 1.96$-2.03. This broad EPR signal is typical of the binuclear Cu_A center observed in cytochrome *c* oxidases[54,55]. Despite the overlap of the $g_{x,y}$ signal of Cu_A with the sharp g = 2.01 peak, our assignment is supported by the temperature-dependence of the EPR spectra (**Extended Data Fig. 3**). In contrast to the Cu_A peaks at 3250 Gauss and 3368 Gauss, the amplitude of the sharp g = 2.01 peak diminishes more sharply with temperature and is only observable below 30K. When the sample is reduced by dithionite, all the EPR signals disappear (data not shown). On the other hand, when the sample was oxidized by ferricyanide, the signals from the Cu_A and the iron-sulfur cluster(s) persist. The sharp g = 2.01 signal is tentatively assigned to come from the oxidized form of a $[3Fe\text{-}4S]^{1+}$ cluster. From its amino acid sequence, ActB is predicted to contain one $[3Fe\text{-}4S]^{1+/0}$ cluster and up to three non-HiPIP $[4Fe\text{-}4S]^{2+/1+}$ clusters. Since the oxidized $[4Fe\text{-}4S]^{2+}$ cluster is diamagnetic and EPR silent[54], the observed EPR signal from the oxidized enzyme is assigned to a single $[3Fe\text{-}4S]^{1+/0}$ cluster. Similar observations have been reported for the ACIII from *R. marinus* and the related Qrc complex from *Desulfovibrio vulgaris*[16,56], both of which contain homologues of ActB. In addition to the signal observed from the $[3Fe\text{-}4S]^{1+/0}$ cluster, rhombic EPR signals from low-spin ferric hemes are also observed with principle g values at around 2.9, 2.3 and 1.5 (10x-magnified view in **Extended Data Fig. 3**) with the air-oxidized, detergent-solubilized sample **(Fig. S10A)**. These g values satisfy the empirical rule of $g_z^2+g_y^2+g_x^2 = 16$ for low-spin ferric heme[57].

CW EPR measurements were also carried out with the supercomplex preparation in SMA-nanodiscs. The EPR spectrum of the air-oxidized SMA-nanodisc preparation was collected at 10K (**Extended Data Fig. 3**). Using the signal from low-spin heme as an internal standard, the signal around g = 2 from the $[3Fe\text{-}4S]^{1+/0}$ cluster is about 5-fold more intense in the SMA-nanodisc preparation. We interprete this as an indication that the iron sulfur clusters are better preserved in the SMA preparation. Another contributing factor is that the hemes appear to be incompletely oxidized in the air-oxidized SMA sample based on UV-vis spectra (data not shown) and, thus, EPR silent. Signals from the low-spin ferric hemes, although weaker, are clearly resolved and their individual g values are shown in **Extended Data Fig. 3**. Above 10K, the signal from iron-sulfur clusters in the g = 2 region of the spectrum (**Extended Data Fig. 3**) is the same as that observed in the detergent-solubilized supercomplex, assigned to the $[3Fe\text{-}4S]^{1+}$ cluster. Below 10K, the EPR spectrum from the $[3Fe\text{-}4S]^{1+}$ cluster changes in a manner not observed with the detergent-solubilized preparation. Further work will be needed to determine the meaning of this observation.

In sum, the EPR spectra qualitatively show the presence of Cu_A, multiple low spin hemes, and at least one $[3Fe\text{-}4S]^{1+/0}$ cluster, features that together can be attributed to either ACIII or cyt aa_3.

12. Metal Analysis

Similar to what has been reported for the ACIIIs from *R. marinus* and *C. aurantiacus,* the ACIII from *F. johnsoniae* do not show any traces of molybdenum, indicating the absence of a molybdenum cofactor in the protein[15,20]. Interpretation of the Fe and Cu contents require knowing the concentrations of ACIII and cyt aa_3 in the preparations, which were determined by the pyridine hemochrome analysis of the hemes, assuming 7 hemes *c* per ACIII and 2 hemes *a* (a + a_3) per cyt aa_3. The total iron-content (by ICP-MS) of the detergent solubilized preparation and the SMA

nanodiscs are 21 ± 0.5 and 20± 1 mol Fe per mol of ACIII, respectively. Subtracting the Fe attributable to the cyt *aa₃* that is present in each preparation, this yields 20 ± 0.5 and 19± 1 mol Fe per mol of ACIII in the detergent-solubilized and the SMA-solubilized preparations, respectively. Sequence analysis of ACIII indicates binding motifs for three [4Fe-4S] clusters, one [3Fe-4S] cluster and 7 hemes *c* in ACIII[5], summing to 22 Fe per ACIII which is slightly more than what is measured. The ACIII from *R. marinus* was found to have ~ 21 mol Fe per mol of enzyme[20], while in *C. aurantiacus*, the ACIII has 17 mol Fe per mol of ACIII[15].

The copper contents of the detergent-solubilized and SMA-nanodisc preparations were also determined. For the detergent-solubilized sample, 3± 0.1 mol Cu per mol of cyt aa_3 was obtained, accounted for by the two coppers from Cu_A and one from Cu_B[58]. The SMA-nanodisc sample, however, showed a low concentration of 1± 0.1 mol Cu per mol of supercomplex, despite the higher turnover of the enzyme (**Extended Data Fig. 3**). This was not investigated further.

13. Redox titration of the detergent-solubilized supercomplex

UV-visible spectro-potentiometric redox titrations of the hemes in the supercomplex were carried out at pH 7.0 (**Extended Data Fig. 3**). Only the detergent-solubilized sample was examined, since the SMA-nanodisc requires a more alkaline pH to prevent precipitation during the course of the experiment. The apparent midpoint potentials for the *c*-type hemes of ACIII and *a*-type hemes of cyt aa_3 were determined by monitoring the absorption as a function of solution potential at 553 nm and 606 nm, respectively, and that each component could be fit to the single-electron Nernst equation. The redox titration of the hemes *c* (**Extended Data Fig. 3**) reasonably fits to four distinct E_m values: -254 mV, -155 mV, +154 mV and +326 mV versus the normal hydrogen electrode (NHE). The intensity ratio of the absorption changes of the four redox

components is roughly 1:1:2:3, respectively, consistent with seven cyt *c* components. Monitoring heme *a* absorption, the titration could be fit to two components with E_m values of +331 and +439 mV with a ratio of 1:1 (**Extended Data Fig. 3**). The results from the potentiometric titration match well with the heme predictions from the sequence and the calculations from the pyridine hemochrome assay for both types of hemes. The E_m values for the hemes *c* are similar to those seen for the ACIII from *C. aurantiacus*[15].

14. Summary of the major points from the biochemical analysis of the SMA-solubilized supercomplex:

A) There are two variants of cyt aa_3 that have distinct versions of subunit III, and only variant I is present in the membranes analyzed. Subunit III of the cyt aa_3 in the supercomplex lacks two of the canonical seven transmembrane helices.

B) The SMA copolymer solubilizes the entire functional supercomplex in a good yield. Unlike the detergent-solubilized preparation, which exists as aggregates in solution, the SMA-nanodisc preparation has discrete populations, about 70% contain a 1:1 supercomplex of ACIII:cyt aa_3 and the remaining 30% contain ACIII without cyt aa_3. The supercomplex has an expected 48 transmembrane helices, which is much larger than the 36 transmembrane helices in the trimeric AcrB protein from *E. coli*, which has been the largest complex previously reported in an SMA-nanodisc[36].

C) The SMA-nanodisc preparation contains particles of different size that are not in rapid equilibrium with each other. This was shown by isolating material from different peaks following gel filtration chromatography and re-analyzing the material either by SEC or by BN-PAGE. Cryo-EM and spectroscopic data show that the proteins within each size population of

nanodiscs appear to be the same. The difference in the size of the two populations of nanodiscs is not due to different type or amount of protein encapsulated within the SMA-nanodiscs but appears to be a property of the SMA-nanodiscs. This issue remains to be clarified. Non-equilibrating populations of SMA-nanodiscs have also been reported for a preparation of yeast cytochrome *c* oxidase[59].

D) The Fe content of both the detergent-solubilized and SMA-solubilized protein is 10% to 15% lower than expected, suggesting that some of the Fe-S clusters are not intact.

E) The SMA-nanodisc preparation is suitable for analysis by UV-visible as well as EPR spectroscopies.

References

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