Supporting Information

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SI Materials and Methods

Protein Purification. Membranes from *Aquifex aeolicus* were solubilized and the solublized proteins fractionated as described previously (1). The supercomplex was then isolated on a TSK 4000 (Tosoh) gel filtration column with buffer containing 20 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 0.05% n-dodecyl-β-D-maltoside (DDM). Cox2 was purified using a Superdex 200 (GE Healthcare) gel filtration column after the supercomplex was shifted from 0.05% (wt/vol) DDM to 0.8% (wt/vol) ß-D-octylglucoside (OG). The composition of Cox2 and of the supercomplex was determined by MALDI-MS and laser-induced liquid beam ion desorption mass spectroscopy (LILBID-MS).

MALDI-MS. MALDI-MS was used for subunit identification. The tryptic in-gel digests with reduction and alkylation of cysteines were essentially performed according to Shevchenko et al. (2). As previously described (3), MS was done using a MALDI tandem TOF instrument (Ultraflex TOF/TOF; Bruker Daltonics) and spectra analyzed using Flex Analysis v2.4 (Bruker Daltonics). If necessary, tandem MS (MS/MS) spectra were acquired on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems). Fragment ions of angiotensin were used for the calibration of MS/MS spectra achieving a precursor mass accuracy of 50 ppm and a fragment ion accuracy of 0.3 Da. MS and MS/MS data were searched against an *Aquifex* database from the National Center for Biotechnology Information (NCBI) using the Mascot database search engine v2.2 [Matrix Science Ltd. (4)]. The following search settings were applied for peptide mass fingerprint (PMF) and MS/MS ion search, respectively: enzyme, trypsin; miss cleavage sites, two; fixed modification, carbamidomethyl; variable modification, methionine oxidation; mass accuracy, 100 ppm (PMF); precursor/fragment mass accuracy, 50 ppm/0.3 Da (MS/MS). Unassigned MS/MS data were manually interpreted to create sequence tags for basic local alignment search tool (BLAST) searches on NCBI. Therefore, the most intense types of fragment ions, the N-terminal b-ions, C-terminal y-ions, and immonium ions, were used (5). Theoretical trypsin proteolysis of identified proteins and the subsequent visualization of the results were performed with Biotools v2.2 (Bruker Daltonics), which was also used to predict the peptide fragmentation patterns in MS/MS spectra.

LILBID-MS. LILBID-MS was performed as described previously (6). A user-written lab view program was used for data acquisition and analysis. The signal-to-noise ratio was improved by subtracting an unstructured background, caused by metastable loss of water and buffer molecules, from the original ion spectra. These different spectra were smoothed by averaging the signal over a preset number of channels of the transient recorder, with the smoothing interval always lying within the time resolution of the TOF mass spectrometer.

EPR Spectroscopy. X-band EPR spectra were obtained with a Bruker ESP 300E spectrometer equipped with an HP 53159A frequency counter (Hewlett Packard), ER 035 M NMR gaussmeter (Bruker, BioSpin) and a liquid helium continuous flow cryostat (Oxford Instruments). Spectra were recorded using the following parameters: microwave frequency 9.47 GHz, modulation amplitude 0.64 mT or 1 mT, modulation frequency 100 kHz. Samples were frozen in cold isopentane/methylcyclohexane (5∶1, ∼120 K) and stored in liquid nitrogen until measurement. Both Cox2 and supercomplex were recorded in the air-oxidized state without any further addition at a temperature of 10 K and microwave power of 10 mW.

Activity Assay. Cytochrome *c* oxidation/reduction and quinol oxidation were recorded with an Agilent 8453 UV-visible spectrometer at 80 °C. Horse heart cytochrome *c* (Sigma) was reduced by sodium dithionite and excess dithionite was removed by a PD10 column (GE Healthcare). Decylubiquinone (Fluka) was reduced by sodium dithionite on an Extrelut N1 column (7). The reduced cytochrome *c* and decylubiquinol were stored at −80 °C and ready to use. The reaction buffer was composed of 30 mM potassium phosphate (pH 7.0) and 0.02 mg/mL of the target enzyme. Cytochrome *c* oxidation measurements were started by injecting 56 μM reduced cytochrome *c* into the reaction buffer. Cytochrome *c* reduction was measured by applying 45 μM decylubiquinol into the reaction buffer containing 60 μM air oxidized horse heart cytochrome *c* and 1 mM potassium cyanide. Decylubiquinol oxidation was measured by adding 45 μM decylubiquinol into reaction buffer. The initial linear absorption changes of cytochrome *c* [$\Delta\varepsilon_{\text{red-ox},550\text{ nm-}540\text{ nm}} = 19\text{ mM}^{-1}\text{ cm}^{-1}$ (8)] and ubiquinol [$\Delta\varepsilon_{\text{ox-red},275\text{ nm}} = 12.5\text{ mM}^{-1}\text{ cm}^{-1}$ (9)] were used for calculating specific activities.

Decylubiquinol titration measurements were started by injecting varying amounts of decylubiquinol into the reaction buffer, optionally containing 60 μM horse heart cytochrome *c*, $+/-$ 1 mM potassium cyanide.

Oxygen consumption in the presence of decylubiquinol/DTT was recorded using a Hansatech Oxygraph oxygen electrode at 40 °C as described in ref. 10. DTT, potassium cyanide, and stigmatellin were purchased from Sigma. The reaction was started by injecting 50 μL enzyme into 450 μL buffer containing 30 mM potassium phosphate buffer (pH 7.0), 0.05% DDM, 1 mg/mL asolectin, 125 μM decylubiquinol, and 5 mM DTT (final concentrations). One millimolar potassium cyanide, 40 μM stigmatellin, and 60 μM air-oxidized horse heart cytochrome *c* were applied if desired.

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2. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 1:2856–2860.
3. Galkin A, et al. (2008) Identification of the mitochondrial ND3 subunit as a structural component involved in the active/deactive enzyme transition of respiratory complex I. *J Biol Chem* 283:20907–20913.
4. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20:3551–3567.
5. Roepstorff P, Fohlman J (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed Mass Spectrom* 11:601.
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7. Palsdottir H, Hunte C (2003) Determination of cytochrome bc1 complex activity. *Membrane Protein Purification and Crystallization: A Practical Guide*, ed Hunte C, Von Jagow G, Schägger H (Academic, San Diego), 2nd Ed, pp 197–198.
8. Ouchane S, Agalidis I, Astier C (2002) Natural resistance to inhibitors of the ubiquinol cytochrome c oxidoreductase of *Rubrivivax gelatinosus*: Sequence and functional analysis of the cytochrome bc(1) complex. *J Bacteriol* 184:3815–3822.
9. Zickermann I, et al. (1996) Biochemical and spectroscopic properties of the four-subunit quinol oxidase (cytochrome ba3) from *Paracoccus denitrificans*. *Biochim Biophys Acta* 1277:93–102.
10. Abramson J, et al. (2000) The structure of the ubiquinol oxidase from *Escherichia coli* and its ubiquinone binding site. *Nat Struct Biol* 7:910–917.

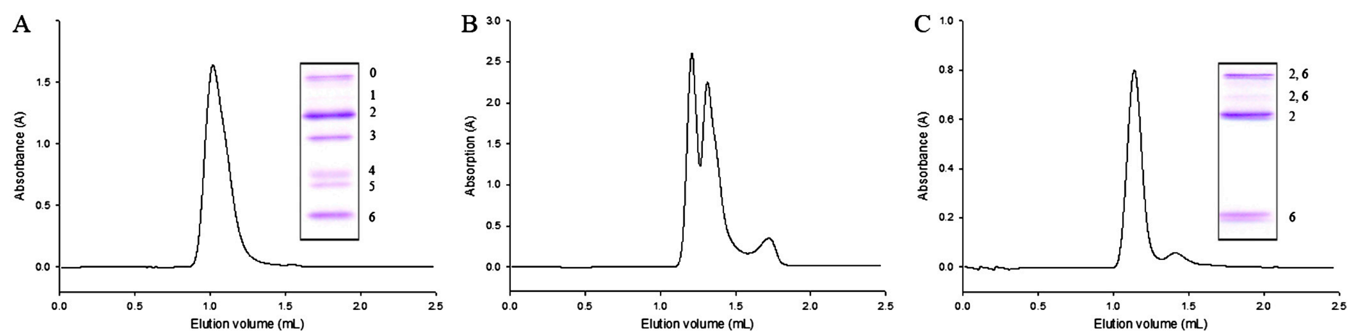


Fig. S1. Size exclusion chromatograms and subunit identification by MALDI-MS of SDS-PAGE bands. (*A*) Supercomplex; (*B*) supercomplex dissociation in detergent OG; and (*C*) Cox2. The single peaks were eluted from a Superdex 200 column (GE Healthcare) in a SMART system (GE Healthcare). Protein elution was monitored by the absorption at 280 nm. Numbers next to the protein bands in SDS-PAGE (stained by Coomassie blue) refer to the following subunits: 1, outer membrane protein C (OprC); 2, Cox2 subunit I (CoxA2); 3, cytochrome *b* (PetB); 4, cytochrome c_1 (Cyc); 5, Rieske protein (PetA); 6, Cox2 subunit II (CoxB2). The band 0 in *A* provides mixed MS peaks which can be assigned to the cytochrome bc_1 complex, Cox2, and the outer membrane protein C.

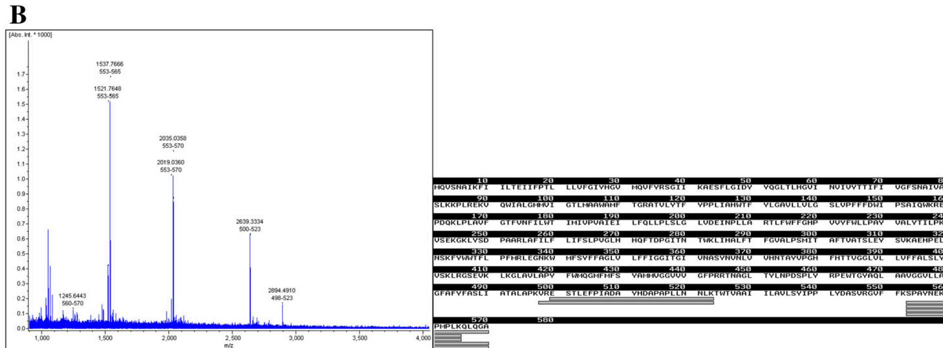
A

Gene: *coxA2*; 66.4 kDa

>gi|15607124|ref|NP_214506.1| cytochrome *c* oxidase subunit I [Aquifex aeolicus VF5]

MWVMQVSNAIKFIILTEIIFPTLLLVFGIYHGVMQVFYRSGIIKAESFLGIDYYQGLTLHGVINVIVYTTIFIVGFSNAIVAYSLKKPLREKVQWIALGMMV
IGTLMAAWAMFTGRATVLYTFYPPLIAHWTFYLGAVLLVLGSLVPFFFDWIPSAIQWKRENPDQKLPLAVFGTFVNFILWTIMIVPVAIEILFQLLPLSLG
LVDEINPLLARTLFWFFGHPVVYFWLLPAYVALYTILPKIVSEKGKLYSDPAARLAFILFLIFSLPVGLHHQFTDPGITNTWKLIHALFTFGVALPSMITAF
TVATSLEYSVKAEHPELKNSKFYWWTFLPFMRLEGNKWMFSYFFAGLVLFFIGGITGIVNASYNVNLVVHNTAYVPGHFHTTVGGLVLLVFFALSLY
MVSKLRGSEVKLKGLAVLAPYFWMQGMFMFSYAMMVGGVVVGFPRRTNAGLTYLNPDSPLYRPEWTGYAQLAAVGGVLLAIGFAFYFASLIATAL
APKVRESTLEFPIADAYHDAPAPLLNNLKTWTVAAIILAVLSYIPPLYDASVRGVFFKSPAYNEKFPMPLKQLQGAEKKEEKKELSKAEGGITQK

B



C

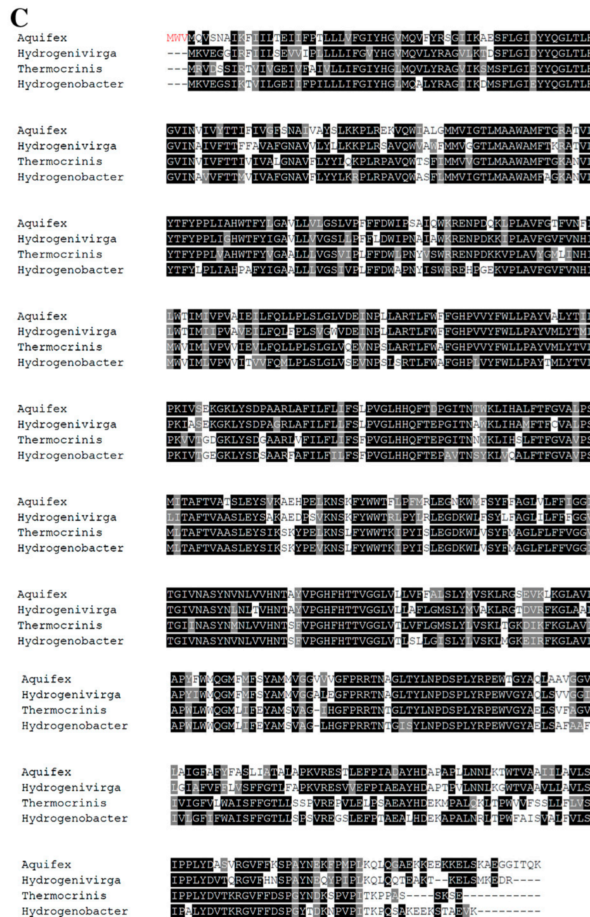


Fig. S2. Identification of sequence truncations in Cox2 subunit I (CoxA2). (*A*) CoxA2 has a calculated molecular mass of 66.4 kDa according to the database sequence. The shortened C- and N-termini are shown in red. The calculated mass of the shortened sequence fits to the Cox2 subunit I mass identified by LILBID-MS (Fig. S5). (*B*) Representative MS-spectrum of Cox2 subunit I showing the unambiguously verified C-terminal truncation. The detected signals at 1,245, 2,019, and 2,035 can only be generated by trypsin using the shortened sequence in *A*. (*C*) Sequence alignment of CoxA2 and three selected cytochrome *c* oxidase subunit I. The blast result supports the lack of three amino acids at N terminus. The selected sequences are *Hydrogenivirga specie* 128-5-R1-1 (accession no. ZP_02176550), *Thermocrinis albus* DSM 14484 (accession no. YP_003474330), and *Hydrogenobacter thermophilus* TK-6 (accession no. YP_003433137).

A 5'3' Frame 2

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tttctcctctttccttcctctatattttttatttgaaaaaattaattacttttgatcac
 F  S  S  F  L  P  L  Y  F  F  I  -  K  N  -  L  L  F  D  H
aaaatcacttttatcttaaatttgagctaaaacataaaacaaagaaacatattgatttc
 K  I  T  F  I  L  N  L  S  -  N  I  K  Q  R  N  I  L  I  F
aattagtttaatcttgtcagtaagttagtattacttgacaattacaagtttgaggtgaaa
 N  -  F  N  L  V  S  K  L  V  L  L  D  N  Y  K  F  E  V  K
aattaattatgtagctcataaaaactcgtgaggaggtgtgaaaaatgaatgaaaagcatg
 N  -  L  C  S  S  -  K  L  V  R  R  C  E  K  -  M  K  S  M
aacatgaggaattttcccgtcgggtacaatagcatttttatcttcatgatggtctttt
 N  M  R  N  F  S  R  R  V  Q  -  H  F  L  S  S  -  W  S  F
atgcggttctctggttcatgatttactgggttctacttgaaaggggtaaggagagatgg
 M  R  F  S  G  S  -  F  T  G  F  Y  L  K  G  G  K  E  R  W
acagggctgaaaaaacaggactaactttagctttaatacttctgcttaccttctttagct
 T  G  L  K  K  Q  D  -  L  -  L  -  Y  F  C  L  P  S  L  A
                                           frame shift
tgatcgtttacgctgcaaagggactcaagatagacattccccacgtgtgtaacggacgta
 -  S  F  T  L  Q  R  D  S  R  -  T  F  P  T  C  V  T  D  V
                   II.&III.                   I.
gaacccttccaggaagggaaactcattaaacacggggacaagaggtacgaactccacata
 E  P  F  Q  E  G  K  L  I  K  H  G  D  K  R  Y  E  L  H  I
          I.
ctcgcaaggatgtggtactttgatttcaacaaaggtgctaccgaaataaagatacccgta
 L  A  R  M  W  Y  F  D  F  N  K  G  A  T  E  I  K  I  P  V
ggttccgtagtggacatattcacgacttcaaaggatgtagttcacggtgtacatattcat
 G  S  V  V  D  I  F  T  T  S  K  D  V  V  H  G  V  H  I  H
ggaaccaattacaacgtaatggcaattcccggaactgttggttacatgaggataaagttt
 G  T  N  Y  N  V  M  A  I  P  G  T  V  G  Y  M  R  I  K  F
gaaaaacccggagtttaccacgttgtttgccacgagttctgcggtgttggtcaccatgct
 E  K  P  G  V  Y  H  V  V  C  H  E  F  C  G  V  G  H  H  A
atgcaaggaaaaataatcgttgaataa
 M  Q  G  K  I  I  V  E  -
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B 5'3' Frame 1

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tttctcctctttccttcctctatattttttatttgaaaaaattaattacttttgatca
 F  L  L  F  P  S  S  I  F  F  Y  L  K  K  L  I  T  F  -  S
caaaatcacttttatcttaaatttgagctaaaacataaaacaaagaaacatattgatttt
 Q  N  H  F  Y  L  K  F  E  L  K  H  K  T  K  K  H  I  D  F
caattagtttaatcttgtcagtaagttagtattacttgacaattacaagtttgaggtgaa
 Q  L  V  -  S  C  Q  -  V  S  I  T  -  Q  L  Q  V  -  G  E
aaattaattatgtagctcataaaaactcgtgaggaggtgtgaaaaatgaatgaaaagcat
 K  L  I  M  -  L  I  K  T  R  E  E  V  -  K  M  N  E  K  H
gaacatgaggaattttcccgtcgggtacaatagcatttttatcttcatgatggtcttt
 E  H  E  E  F  F  P  S  G  T  I  A  F  F  I  F  M  M  V  F
                                                      Start
tatgcggttctctggttcatgatttactgggttctacttgaaaggggtaaggagagatg
 Y  A  V  L  W  F  M  I  Y  W  V  L  L  E  R  G  -  G  E  M
gacagggctgaaaaaacaggactaactttagctttaatacttctgcttaccttctttagc
 D  R  A  E  K  T  G  L  T  L  A  L  I  L  L  L  T  F  F  S
                    III.        II.       frame shift
ttgatcgtttacgctgcaaagggactcaagatagacattccccacgtgtgtaacggacgt
 L  I  V  Y  A  A  K  G  L  K  I  D  I  P  H  V  C  N  G  R
agaacccttccaggaagggaaactcattaaacacggggacaagaggtacgaactccacat
 R  T  L  P  G  R  E  T  H  -  T  R  G  Q  E  V  R  T  P  H
actcgcaaggatgtggtactttgatttcaacaaaggtgctaccgaaataaagatacccgt
 T  R  K  D  V  V  L  -  F  Q  Q  R  C  Y  R  N  K  D  T  R
aggttccgtagtggacatattcacgacttcaaaggatgtagttcacggtgtacatattca
 R  F  R  S  G  H  I  H  D  F  K  G  C  S  S  R  C  T  Y  S
tggaaccaattacaacgtaatggcaattcccggaactgttggttacatgaggataaagtt
 W  N  Q  L  Q  R  N  G  N  S  R  N  C  W  L  H  E  D  K  V
tgaaaaacccggagtttaccacgttgtttgccacgagttctgcggtgttggtcaccatgc
 -  K  T  R  S  L  P  R  C  L  P  R  V  L  R  C  W  S  P  C
tatgcaaggaaaaataatcgttgaataa
 Y  A  R  K  N  N  R  -  I
```

C

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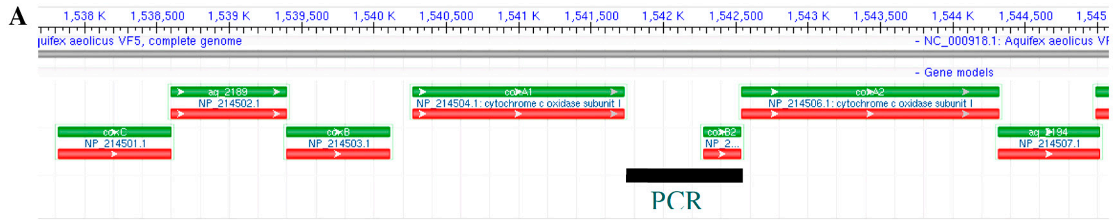
                                                      Start
                                                        atg
                                                          M
gacagggctgaaaaaacaggactaactttagctttaatacttctgcttaccttctttagc
 D  R  A  E  K  T  G  L  T  L  A  L  I  L  L  L  T  F  F  S
                    III.        II.       frame shift
ttgatcgtttacgctgcaaagggactcaagatagacattcccacgtgtgtaacggacgta
 L  I  V  Y  A  A  K  G  L  K  I  D  I  P  T  C  V  T  D  V
                   II.&III.                   I.
gaacccttccaggaagggaaactcattaaacacggggacaagaggtacgaactccacata
 E  P  F  Q  E  G  K  L  I  K  H  G  D  K  R  Y  E  L  H  I
          I.
ctcgcaaggatgtggtactttgatttcaacaaaggtgctaccgaaataaagatacccgta
 L  A  R  M  W  Y  F  D  F  N  K  G  A  T  E  I  K  I  P  V
ggttccgtagtggacatattcacgacttcaaaggatgtagttcacggtgtacatattcat
 G  S  V  V  D  I  F  T  T  S  K  D  V  V  H  G  V  H  I  H
ggaaccaattacaacgtaatggcaattcccggaactgttggttacatgaggataaagttt
 G  T  N  Y  N  V  M  A  I  P  G  T  V  G  Y  M  R  I  K  F
gaaaaacccggagtttaccacgttgtttgccacgagttctgcggtgttggtcaccatgct
 E  K  P  G  V  Y  H  V  V  C  H  E  F  C  G  V  G  H  H  A
atgcaaggaaaaataatcgttgaataa
 M  Q  G  K  I  I  V  E  -
```

Fig. S3. Correction of the annotated Cox2 subunit II (CoxB2) based on MALDI-MS data. The six frame translation was performed with the program Translate (1). The two relevant translation frames are depicted in *A* and *B*. The discovered frameshift is indicated and the real N-terminal extension is colored in red. The sequences printed in red mark the MS/MS verified regions (see Fig. 1) which showed the presence of the N-terminal extension (I) and the frameshift (II and III). The incorrectly annotated CoxB2 sequence is shown in green in *A* and *C*. (*C*) The corrected *coxB2* sequence.

1 Gasteiger E, et al. (2003) ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* 31:3784–3788.



B

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Genomic DNA     1 ------------------------TTTCTCCTCTTTCCTTCCTCTATATTTTTTTATTT   35
                                          TTTCTCCTCTTTCCTTCCTCTATATTTTTTTATTT
PCR product    61 ATTCACTAGTGATTCGCGGATCCTGTTTCTCCTCTTTCCTTCCTCTATATTTTTTTATTT  120

Genomic DNA    36 GAAAAAATTAATTACTTTTTGATCACAAAATCACTTTTATCTTAAATTTGAGCTAAAACA   95
                  GAAAAAATTAATTACTTTTTGATCACAAAATCACTTTTATCTTAAATTTGAGCTAAAACA
PCR product   121 GAAAAAATTAATTACTTTTTGATCACAAAATCACTTTTATCTTAAATTTGAGCTAAAACA  180

Genomic DNA    96 TAAAACAAAGAAACATATTGATTTTCAATTAGTTTAATCTTGTCAGTAAGTTAGTATTAC  155
                  TAAAACAAAGAAACATATTGATTTTCAATTAGTTTAATCTTGTCAGTAAGTTAGTATTAC
PCR product   181 TAAAACAAAGAAACATATTGATTTTCAATTAGTTTAATCTTGTCAGTAAGTTAGTATTAC  240

Genomic DNA   156 TTGACAATTACAAGTTTGAGGTGAAAAATTAATTATGTAGCTCATAAAAACTCGTGAGGA  215
                  TTGACAATTACAAGTTTGAGGTGAAAAATTAATTATGTAGCTCATAAAAACTCGTGAGGA
PCR product   241 TTGACAATTACAAGTTTGAGGTGAAAAATTAATTATGTAGCTCATAAAAACTCGTGAGGA  300

Genomic DNA   216 GGTGTGAAAAATGAATGAAAAGCATGAACATGAGGAATTTTTCCCGTCGGGTACAATAGC  275
                  GGTGTGAAAAATGAATGAAAAGCATGAACATGAGGAATTTTTCCCGTCGGGTACAATAGC
PCR product   301 GGTGTGAAAAATGAATGAAAAGCATGAACATGAGGAATTTTTCCCGTCGGGTACAATAGC  360

Genomic DNA   276 ATTTTTTATCTTCATGATGGTCTTTTATGCGGTTCTCTGGTTCATGATTTACTGGGTTCT  335
                  ATTTTTTATCTTCATGATGGTCTTTTATGCGGTTCTCTGGTTCATGATTTACTGGGTTCT
PCR product   361 ATTTTTTATCTTCATGATGGTCTTTTATGCGGTTCTCTGGTTCATGATTTACTGGGTTCT  420

Genomic DNA   336 ACTTGAAAGGGGGTAAGGAGAGATGGACAGGGCTGAAAAAACAGGACTAACTTTAGCTTT  395
                  ACTTGAAAGGGGGTAAGGAGAGATGGACAGGGCTGAAAAAACAGGACTAACTTTAGCTTT
PCR product   421 ACTTGAAAGGGGGTAAGGAGAGATGGACAGGGCTGAAAAAACAGGACTAACTTTAGCTTT  480

Genomic DNA   396 AATACTTCTGCTTACCTTCTTTAGCTTGATCGTTTACGCTGCAAAGGGACTCAAGATAGA  455
                  AATACTTCTGCTTACCTTCTTTAGCTTGATCGTTTACGCTGCAAAGGGACTCAAGATAGA
PCR product   481 AATACTTCTGCTTACCTTCTTTAGCTTGATCGTTTACGCTGCAAAGGGACTCAAGATAGA  540

Genomic DNA   456 CATTCCCCACGTGTGTAACGGACGTAGAACCCTTCCAGGAAGGGAAACTCATTAAACACG  515
                  CATT CCCACGTGTGTAACGGACGTAGAACCCTTCCAGGAAGGGAAACTCATTAAACACG
PCR product   541 CATT-CCCACGTGTGTAACGGACGTAGAACCCTTCCAGGAAGGGAAACTCATTAAACACG  599

Genomic DNA   516 GGGACAAGAGGTACGAACTCCACATACTCGCAAGGATGTGGTACTTTGATTTCAACAAAG  575
                  GGGACAAGAGGTACGAACTCCACATACTCGCAAGGATGTGGTACTTTGATTTCAACAAAG
PCR product   600 GGGACAAGAGGTACGAACTCCACATACTCGCAAGGATGTGGTACTTTGATTTCAACAAAG  659

Genomic DNA   576 GTGCTACCGAAATAAAGATACCCGTAGGTTCCGTAGTGGACATATTCACGACTTCAAAGG  635
                  GTGCTACCGAAATAAAGATACCCGTAGGTTCCGTAGTGGACATATTCACGACTTCAAAGG
PCR product   660 GTGCTACCGAAATAAAGATACCCGTAGGTTCCGTAGTGGACATATTCACGACTTCAAAGG  719

Genomic DNA   636 ATGTAGTTCACGGTGTACATATTCATGGAACCAATTACAACGTAATGGCAATTCCCGGAA  695
                  ATGTAGTTCACGGTGTACATATTCATGGAACCAATTACAACGTAATGGCAATTCCCGGAA
PCR product   720 ATGTAGTTCACGGTGTACATATTCATGGAACCAATTACAACGTAATGGCAATTCCCGGAA  779

Genomic DNA   696 CTGTTGGTTACATGAGGATAAAGTTTGAAAAACCCGGAGTTTACCACGTTGTTTGCCACG  755
                  CTGTTGGTTACATGAGGATAAAGTTTGAAAAACCCGGAGTTTACCACGTTGTTTGCCACG
PCR product   780 CTGTTGGTTACATGAGGATAAAGTTTGAAAAACCCGGAGTTTACCACGTTGTTTGCCACG  839

Genomic DNA   756 AGTTCTGCGGTGTTGGTCACCATGCTATGCAAGGAAAAATAATCGTTGAATAA-------  808
                  AGTTCTGCGGTGTTGGTCACCATGCTATGCAAGGAAAAATAATCGTTGAATAA
PCR product   840 AGTTCTGCGGTGTTGGTCACCATGCTATGCAAGGAAAAATAATCGTTGAATAAACGAATT  899
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Fig. S4. DNA sequence of the *coxB2* gene. To confirm the sequence error in CoxB2, PCR was used to generate an 808 bp DNA fragment from position 1541728 to 1542535 using the oligonucleotide pairs
5′CGCGGATCCTGTTTCTCCTCTTTCCTTCCTCTATATTT3′ and 5′CCGGAATTCGTTTATTCAACGATTATTTTTCCTTGC3′.

The incorrectly annotated *coxB2* gene consists of 258 bp from 1542278 to 1542535. The pGEM-T easy vector (Promega) was used for ligation and positive clones were selected using the restriction enzyme BstZI (Promega). The isolated plasmid was sequenced by Eurofins MWG. (*A*) A scheme of the PCR amplified region in *A. aeolicus* genome; (*B*) alignment of the PCR product and the genomic DNA. The expected frameshift according to MALDI-MS (Fig. S3) was detected at position 1542187. The genomic DNA encodes an additional cysteine C (marked in red in *B*).

Fig. S5. Cox2 and supercomplex subunit identification by LILBID-MS. (*A*) Mass positions of subunits in Cox2 and the supercomplex and (*B*) assignment of Cox2 subunits in LILBID spectrum. The displayed numbers in *A* correspond to mass position of each subunit: 63.9 kDa (Cox2 subunit I), 46.9 kDa (cytochrome *b*), 28.3 kDa (cytochrome c_1 with covalent bound mono heme *c*), 19.5 kDa (Rieske protein), 16.8 kDa (Cox2 subunit II), and 5.2 kDa (Cox2 subunit IIa). Cytochrome bc_1 complex subunits were not detected in Cox2 (*A*). Single peaks at higher mass positions fit to the masses of combined Cox2 subunits: subunit I and IIa (69.1 kDa), subunit I and II (80.7 kDa), and subunit I, II, and IIa (85.9 kDa) (*B*).

Fig. S6. Redox difference pyridine hemochrome absorption spectra of extracted hemes from Cox2 (dotted line) and the supercomplex (solid line). Hemes were extracted as described in ref. 1. The extracted hemes were dissolved in 50% pyridine and redox difference pyridine spectra were recorded as described in ref. 2. Pyridine hemochrome *a* shows band at 585 nm whereas *b* at 556 and 526 nm. Ratios of 1.2:1 and 4.5:1 were calculated for heme *b*:heme *a* in Cox2 and the supercomplex, respectively. The extinction coefficients of $\Delta\epsilon_{R\text{-}O,556-540} = 23.98$ mM^{-1} cm^{-1} and $\Delta\epsilon_{R\text{-}O,585-620} = 25.02$ mM^{-1} cm^{-1} were applied for pyridine hemochrome *b* and *a*, respectively (2).

1 Lubben M, Morand K (1994) Novel prenylated hemes as cofactors of cytochrome oxidases. Archaea have modified hemes A and O. *J Biol Chem* 269:21473–21479.

2 Berry EA, Trumpower BL (1987) Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra. *Anal Biochem* 161(1):1–15.

Fig. S7. Heme staining in SDS-PAGE gels of supercomplex (lane 1) and Cox2 (lane 2). The SDS-PAGE was performed using precast NuPAGE 4–12% bistris gel (Invitrogen). Two micrograms proteins were loaded for each lane. Half of the gel was stained by Coomassie blue (*Left*) and the other half was stained by 3,3′,5,5′-tetramethylbenzidine (TMBZ) (Sigma) (*Right*) (1). No heme staining was observed at the location of Cox2 subunits I and II (black arrows) indicating the absence of covalently bound heme C in these subunits. The cytochrome c_1 subunit in the supercomplex used as a positive control was intensively stained by TMBZ (red arrows), but the same staining was not visible in Cox2, confirming that Cox2 is free from cytochrome c_1. Free hemes were stained at the very bottom of the gel. The unreleased hemes due to subunit aggregation were stained in both Cox2 and supercomplex as higher molecular weight bands.

1 Thomas PE, Ryan D, Levin W (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal Biochem* 75:168–176.

Fig. S8. EPR spectra of Cox2 and the supercomplex. (*A*) Air saturated pure Cox2 and (*B*) air saturated supercomplex. Both spectra were recorded in the air-oxidized state without any further addition at a temperature of 10 K, microwave power of 10 mW, and modulation amplitude of 0.64 mT. Under these conditions the copper signal of the Cox2 sample is power saturated and slightly cut. The inserted figures show field regions of low spin hemes recorded separately at the same temperature and microwave power as above, but with the modulation amplitude increased to 1 mT, sweep time was doubled and five scans were accumulated for each spectrum.

```
P.denitrificans    1 QDVLGDLPVIGKPVNGGMNFQPASSPLAHDQQWLDHFVLYIITAVTIFVCLLLLICIVRF  60

A.aeolicus           ------------------------------------------------------------

P.denitrificans   61 NRRANPVPARFTHNTPIEVIWTLVPVLILVAIGAFSLPILFRSQEMPNDPDLVIKAIGHQ 120
                                     ++        L L+ +  F   I++ ++ +  D    +  +  +
A.aeolicus         1 ----------------MDRAEKTGLTLALILLLTFFSLIVYAAKGLKIDIPTCVTDV--E  42

P.denitrificans  121 WYWSYEYPNDGVAFDALMLEKEALADAGYSEDEYLLATDNPVVVPVGKKVLVQVTATDVI 180
                      +   +    G        E   LA   Y  D    AT+  + +PVG  V +  T+ DV+
A.aeolicus        43 PFQEGKLIKHG----DKRYELHILARMWYF-DFNKGATE--IKIPVGSVVDIFTTSKDVV  95

P.denitrificans  181 HAWTIPAFAVKQDAVPGRIAQLWFSVDQEGVYFGQCSELCGINHAYM--PIVVKAVSQEK 238
                     H   I        A+PG +  +    ++ GVY   C E CG+ H  M   I+V+
A.aeolicus        96 HGVHIHGTNYNVMAIPGTVGYMRIKFEKPGVYHVVCHEFCGVGHHAMQGKIIVE------ 149

P.denitrificans  239 YEAWLAGAKEEFAA 252

A.aeolicus           --------------
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Fig. S9. Sequence alignment of *A. aeolicus* Cox2 subunit II and *Paracoccus denitrificans* cytochrome aa_3 oxidase subunit II. All residues coordinated with Cu_A center are conserved in *A. aeolicus* Cox2 (highlighted in green colors in red box). Most residues belong to the potential cytochrome *c* binding site are conserved in *A. aeolicus* Cox2 (highlighted in blue colors in black box), which supports the possibility of horse heart cytochrome *c* binding. The selected sequences are: *P. denitrificans* subunit II (accession no. 1QLE_B) and *A. aeolicus* subunit II (CoxB2, accession no. JN655694). The alignment was done by Geneious Basic 4.8.5 noncommercial license (1).

1 Drummond AJ, et al. (2011) Geneious v5.4, Available at http://www.geneious.com.

Table S1. Subunit identification by MALDI-MS

Protein sample	Identified subunits	Genes	Protein score	Precursor mass	Identified peptide sequence	Ions score
Supercomplex						
	Outer membrane protein c	*oprC*	94	—	—	—
	Cytochrome *c* oxidase subunit I	*coxA2*	22	1537.7613	SPAYNEKFPMPLK + Ox	74
	—	—	—	2639.3226	ESTLEFPIADAYHDAPAPLLNNLK	85
	Cytochrome *b*	*petB*	96	—	—	—
	Cytochrome c_1	*Cyc*	132	—	—	—
	Rieske protein	*petA*	174	—	—	—
	Cytochrome *c* oxidase subunit II	*coxB2*	46	—	—	—
Cox2						
	Cytochrome *c* oxidase subunit II	*coxB2*	60	—	—	—
	Cytochrome *c* oxidase subunit I	*coxA2*	14	1537.7664	SPAYNEKFPMPLK + Ox	87
	—	—	—	2639.3435	ESTLEFPIADAYHDAPAPLLNNLK	82
	Cytochrome *c* oxidase subunit I	*coxA2*	22	1537.7666	SPAYNEKFPMPLK + Ox	76
	—	—	—	2639.3334	ESTLEFPIADAYHDAPAPLLNNLK	90
	Cytochrome *c* oxidase subunit II	*coxB2*	105	—	—	—

Protein scores greater than 44 are significant ($p < 0.05$). Insignificant hits are verified with MS/MS experiments. Ions scores greater than 15 are significant ($p < 0.05$). Ox, oxidized methionine.