Supplementary information

Scavenging of superoxide by a membrane bound superoxide oxidase

Camilla A.K. Lundgren,[1]† Dan Sjöstrand, [1]†* Olivier Biner, [2]† Matthew Bennett,[1] Axel Rudling,[3] Ann-Louise Johansson,[1] Peter Brzezinski,[1] Jens Carlsson,[3] Christoph von Ballmoos[2]* and Martin Högbom[1]*

Affiliations:

[1] Department of Biochemistry and Biophysics, Stockholm University, 10691 Stockholm, Sweden.

[2] Department of Chemistry and Biochemistry, University of Bern, Bern, Switzerland.

[3] Science for Life Laboratory, Department of Cell and Molecular Biology, Uppsala University, Uppsala, Sweden.

† Contributed equally

* Correspondence to: hogbom@dbb.su.se (M.H.), christoph.vonballmoos@dcb.unibe.ch (C.vB.), dan.sjostrand@dbb.su.se (D.S.)

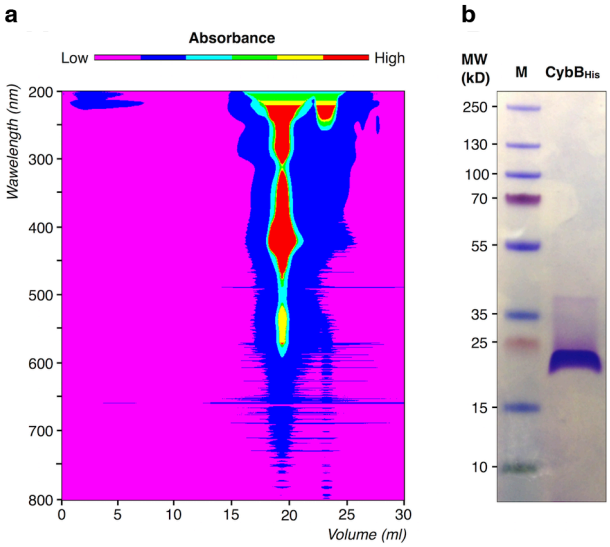
Supplementary Table 1: Measured rate constants for reaction with superoxide.

	$k_{(measured)}$ [$M^{-1}s^{-1}$]	$k_{(published)}$ [$M^{-1}s^{-1}$]
SOD		2×10^9 (ref [40])
cyt c	$(4.62 \pm 1.83)\times10^5$	6×10^5 (ref [27])
CybB (Prep. 1)	$(4.22 \pm 1.76)\times10^7$	
CybB (Prep. 2)	$(1.12 \pm 0.28)\times10^8$	

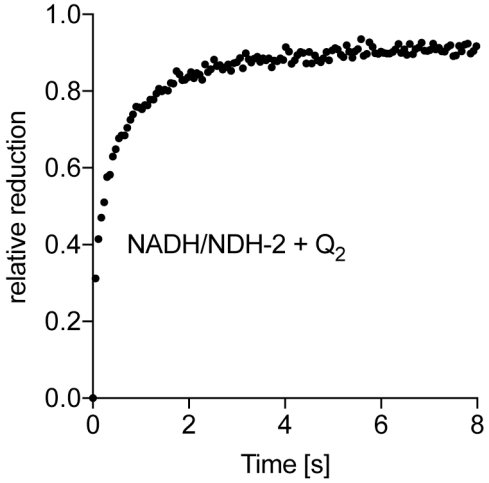
Supplementary Table 2. Crystallographic data and refinement statistics

	Refinement	Phasing
Data collection		
Space group	C 2 2 2_1	C 2 2 2_1
Cell dimensions		
a, *b*, *c* (Å)	68.05, 91.10, 97.92	68.37, 91.48, 98.32
α, β, γ (°)	90, 90, 90	90, 90, 90
		Peak
Wavelength	0.97973	0.97973
Resolution (Å)	20.0–1.97 (2.09–1.97)	50.0–1.97 (2.09–1.97)
R_{meas}	10.3 (121.2)	8.4 (97.6)
$I / \sigma I$	14.38 (2.00)	13.67 (2.09)
Completeness (%)	99.7 (99.1)	99.9 (99.3)
Redundancy	13.0 (13.2)	7.0 (6.9)
Refinement		
Resolution (Å)	20.0–1.97	
No. reflections	20754 / 1093	
R_{work} / R_{free}	0.1876 / 0.2044	
No. atoms		
Protein	1414	
Ligand/ion	132	
Water	25	
B-factors		
Protein	52.7	
Ligand/ion	50.1	
Water	46.3	
R.m.s deviations		
Bond lengths (Å)	0.0178	
Bond angles (°)	1.8294	

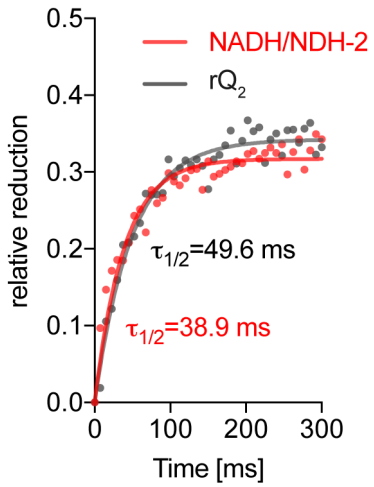
*Values in parentheses are for highest-resolution shell.



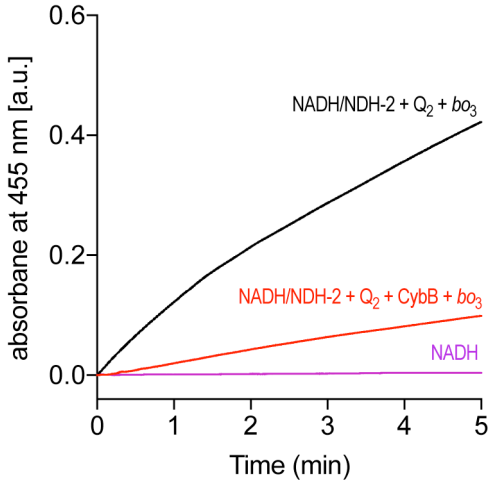
Supplementary Fig. 1. Purification of CybB. (**a**) Absorbance "heat map" (wavelength vs. time) from size exclusion chromatography of CybB with diode array detection. In addition to the standard protein UV range absorbance (i.e. tyrosine/tryptophan absorbance at 280 nm), the deep red CybB protein also absorbs in the visible light range, notably at the ~420 nm (Soret) and ~560 nm heme *b* absorbance peaks. (**b**) SDS-PAGE gel of purified CybB$_{His}$. The protein runs as a single band at its expected molecular weight (~21,8 kD). This experiment has been repeated more than 10 times with similar results.



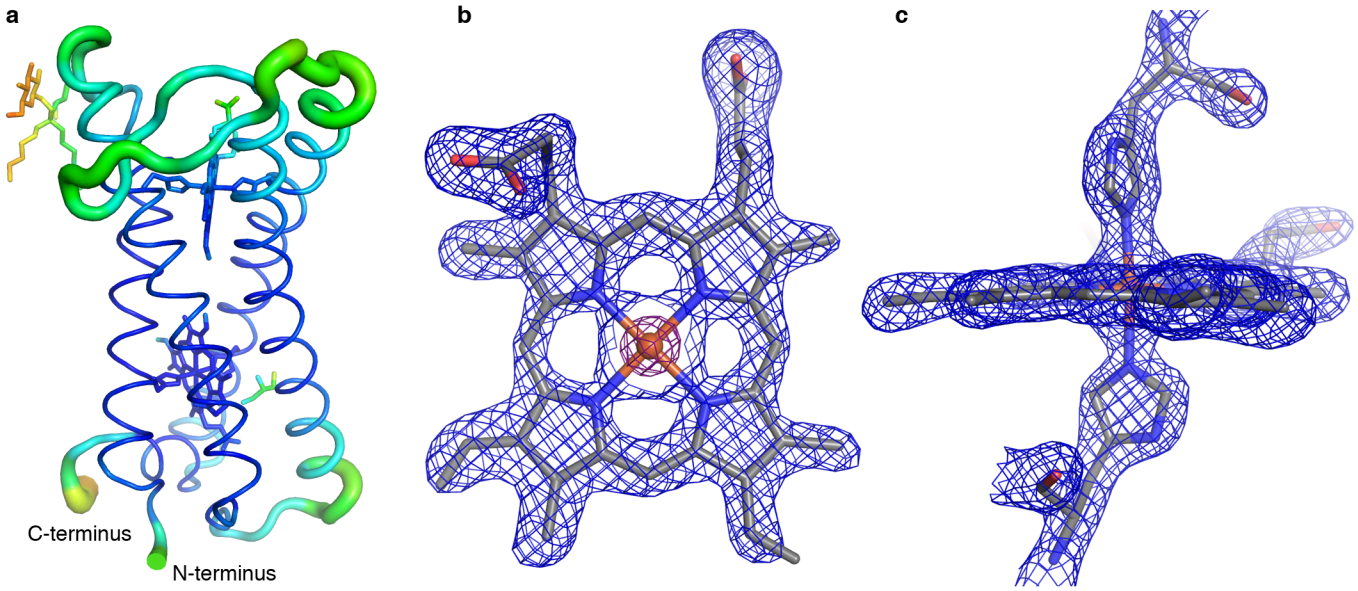
Supplementary Fig. 2. Reduction of CybB by a combination of NADH/NDH-2 and ubiquinone Q_2. NADH, NDH-2, and ubiquinone Q_2 were pre-incubated for 1 min leading to efficient production of both superoxide and reduction of ubiquinone Q_2. Mixing of the solution with detergent solubilized CybB rapidly and completely reduced the CybB (rate limited by mixing). The kinetic trace shown is a representative of at least 3 independent measurements.



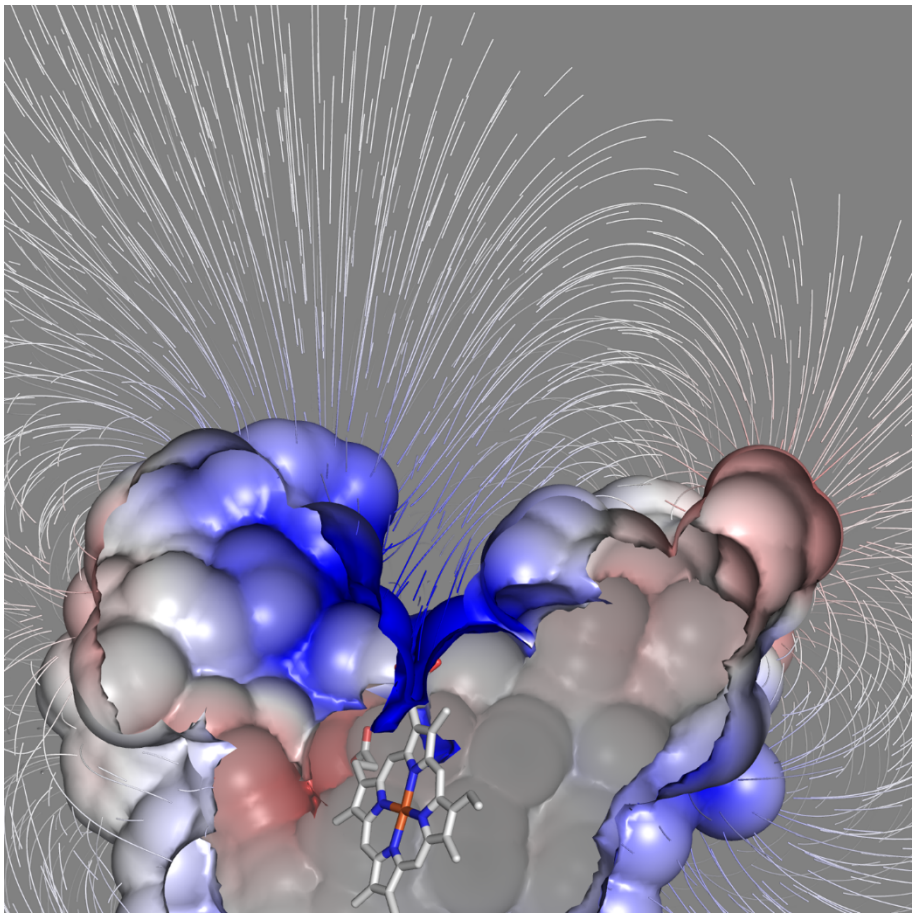
Supplementary Fig. 3. Stopped-flow kinetics of CybB reduction. Stopped-flow kinetic analysis showed that reduction of CybB with ubiquinol Q_2H_2 and NADH/NDH-2 is a fast and specific process with time constants of 40-50 ms. The kinetic trace shown is a representative of at least 3 independent measurements.



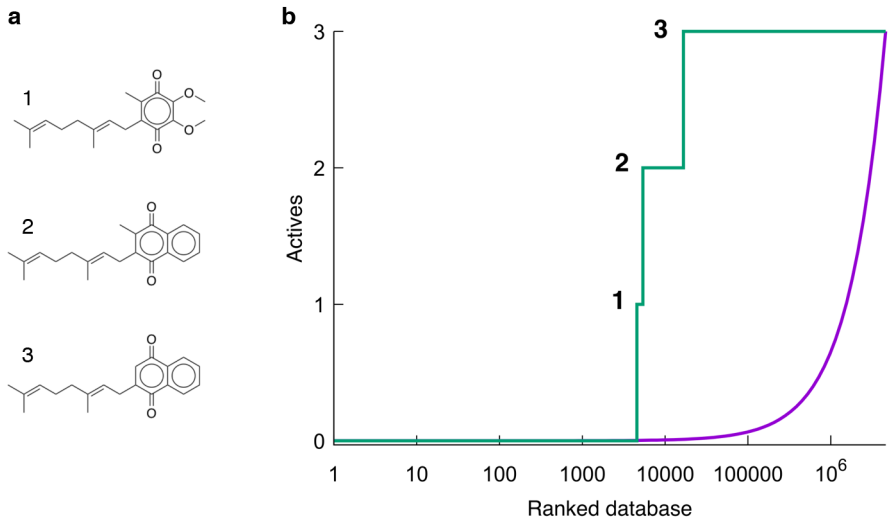
Supplementary Fig. 4. Superoxide-production assay with NADH/NDH-2. Superoxide-production was monitored by following the reduction of WST-1 at 455 nm. NADH/NDH-2 (black trace) together with ubiquinone Q_2 and bo_3 oxidase (black trace) leads to a linear production of superoxide, which is depleted by further addition of CybB (red trace). The kinetic trace shown is a representative of at least 3 independent measurements.



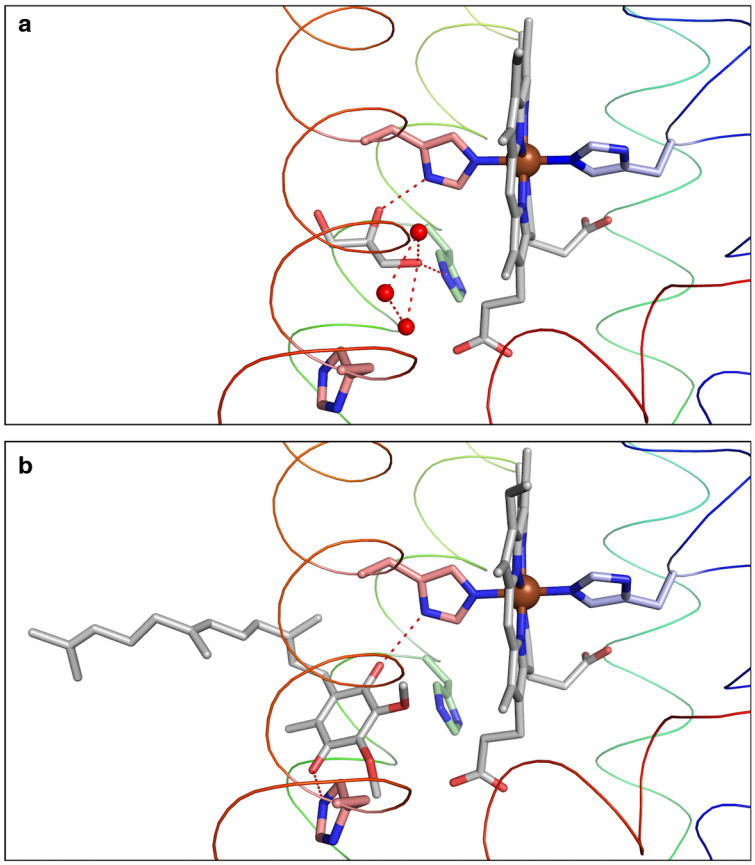
Supplementary Fig. 5. (**a**) Cartoon representation of protein structure B-factors. The highest B-factors are observed in the termini and the loop structures protruding from the membrane on the periplasmic side. The RSRZ score (14%) for the protein is worse than average for a structure at this resolution. The main reason for this is the dynamic loop structures on the periplasmic side comprising residues 106-136 where most of the residues with high RSR reside. (**b** and **c**) Electron density around hemes 1 and 2 respectively. 2Fo-Fc electron density map contoured at 2σ (blue) and 10σ (purple).



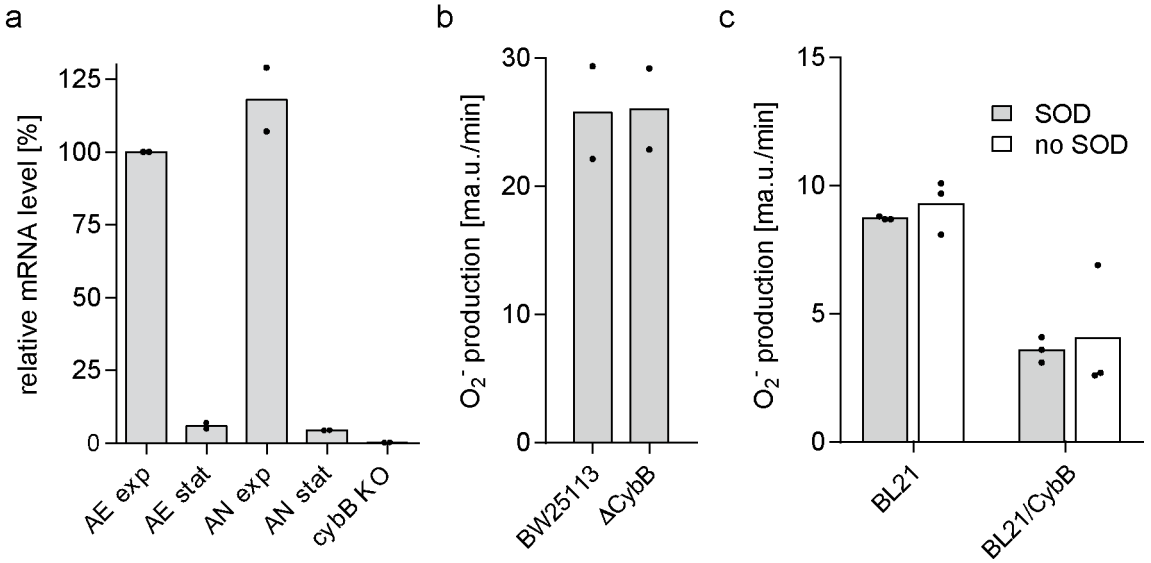
Supplementary Fig. 6. Electrostatic properties of the periplasmic surface of CybB. Electrostatic potential mapped onto the solvent accessible surface of CybB (blue positive, red negative). Electrostatic field lines are indicated. Electrostatics were calculated using APBS[54] including both hemes and displayed using the APBS plugin in PyMol.



Supplementary Fig. 7. Docking of quinone headgroups. (**a**) 2D structures of quinone headgroups representing ubiquinone (1), menaquinone (2), and demethylmenaquinone (3). (**b**) The three quinone headgroups were seeded into a database of 4.6 million commercially available compounds that was docked to the crystal structure. Enrichment of the quinones by the binding site was quantified using a receiver operator characteristic curve (ROC) based on the docking energy scores. The number of quinones (actives or true positives) identified (y-axis) versus the ranking of the compounds in the database (x-axis) is shown. A strong enrichment of quinones (green line) compared to random selection (purple line) was obtained.



Supplementary Fig. 8: Structure-docking model comparison. (**a**) The structure of the suggested quinone-binding cavity of CybB with bound glycerol. Three waters in the cavity coordinate the glycerol molecule (all other waters were removed for clarity). It should be noted that there is some residual unmodeled density in the proximity of the modeled glycerol suggesting that there may be a larger ligand with low occupancy or a mixture of ligands bound at this site. (**b**) *In silico* generated docking model of ubiquinone-3 binding to CybB. The position and binding mode of the docked ubiquinone shows striking resemblance to the glycerol from the crystal structure. Notably, the glycerol is in approximately the same position and orientation as the upper edge of the ubiquinone ring, with two oxygens from each molecule positioned almost identically, including the quinone carbonyl oxygen coordinated by the heme-binding histidine. Moreover, the glycerol-coordinating waters occupy a similar space in the pocket as the ubiquinone ”arms” do in the docking model.



Supplementary Fig. 9. (**a**) **Relative mRNA levels of cybB under different growth conditions.** Relative mRNA levels of *cybB*, normalized to the mRNA level of a housekeeping gene (*idnT*), was determined by RT-qPCR of *E. coli* BW25113 grown under aerobic (AE) and anaerobic (AN) conditions. Shown are *cybB* mRNA levels of samples taken during exponential (exp) and stationary phase (stat). As control, *cybB* transcript levels of a *cybB* knock-out strain (KO) was determined. Shown are the mean values and the individual data points from n=2 biologically independent experiments (each measured in technical replicates).

(**b**) **Superoxide production from respiring *E. coli* wt and *cybB* knockout membranes.** Superoxide production from respiring *E. coli* membranes normalized to NADH consumption. Membrane vesicles from the *E. coli* control strain BW25113 expressing wild-type levels of CybB compared with membranes isolated from the *cybB*-knockout strain. Shown are the mean values and the individual data points from n=2 biologically independent experiments (each measured in technical triplicates). (**c**) **Superoxide production from respiring *E. coli* wt and CybB overexpressing membranes.** Membrane vesicles from *E. coli* strain BL21 pLysS expressing the wild-type CybB compared with membranes isolated from CybB-overexpressing cells. Addition of SOD to the vesicles did not affect the superoxide detection by WST-1 under aerobic conditions. Shown are the mean values and the individual data points from n=3 biologically independent experiments.

```
NP_415935     ---------------------------------MENKYSRLQISIHWLVFLL--VIAAYCAMEFRGFFPRSDRP--LINMIHVSCGISILVLMVVRLLLRLKYPTPPIIPKPKPMMTGLA
YP_403372     ---------------------------------MENKYSRLQIGIHWLVFLL--VIAAYCAMEFRGFFPRSDRP--LINMIHVSCGISILVLMVVRLLLRLKYPTPPIIPKPKPMMTGLA
NP_460598     ---------------------------------MGNKYSGLQIGIHWLVFFL--VIVAYAAMELRGFAPRSYRP--WFNMTHVSCGITILLLMVARLFIRLKYPTPPIVPRPKPMMTGMA
YP_003612579  ---------------------------------MRTKYTGLQIGIHWLVFLL--VIVAYCAMEFRGFFPRTDRP--LINMIHVSCGIAILVLMVTRLLVRLKFRAPPIEPKPKAMITGLS
YP_002348217  ---------------------------------MHKKYSCLQIGIHWLVLLL--VIIAWSSIELRGFAPRSYQP--WMKMIHFSCGIAILVLMMTRILIQLRYPTPPIVPKPSPMIVGLA
NP_416483     ---------------------------------MNRFSKTQIYLHWITLLF--VAITYAAMELRGWFPKGSSTYLLMRETHYNAGIFVWVLMFSRLIIKHRYSDPSIVPPPPAWQMKAA
NP_460220     ---------------------------------MAHFSRLQITLHWLTLLL--TGIAYAAIELRGWAPKGSSVYLFMKDTHYDMGVLVWALMFLRLYLKHKYPDPVITPPPPHWQHVAA
WP_002211611  ---------------------------------MKNRYSLPQIVLHWLTLLM--IILTYLAMLLSDTVPDEERT--FVRNLHFNFGISVWLLMLVRLWLRQRNPTPAISPPLPHWQLLGV
NP_249609     ------------------------------MSWKNTESRYGSLTIALHWLTLLL--IAGVYACIELKGNFPKGSETRELLKQWHFMLGLSVFLLVWLRLLVRLATPTPRIEPAIPAWQATLA
NP_246256     MPRIIRFLLENTKLVEQACVFIDILYLVGKECDMSQKYPKPMIILHWLTLLL--VLIAY----FTGDYPPADG---LMGEIHVASGLSLAMLLLLRVGLRWHYRNQLPAHKLTRWQHIAT
NP_105468     ---------------------------------MHPTYTSTQKAIHWAVFLL--VVGLYGLTYVEELFPRGDPGRALVWWLHISFGLLLFALVVIRVGMRLALGAPGLAQEMSPLERWAA
NP_298617     ------------------------------MTLKGTAERWDWMSQALHWLIAV---LILGLGIGGLIMGNLPKTPNYFWVYTAHKSTGISVLALAVLRLLWRLYVGAPKPIAGPPVWQERVA
NP_252265     ------------------------------MSIR----RYSPSQIALHWLSAVAVLLIIALPYGADFFAGLLGGKGN--VFTLHKSLGVAVLLLTLTRLALRRIQGVPDTLEGNPDWQRAAA
NP_232647     -------------------------------MNNTKTYSKTSIALHWIVGLA--FIGVFIIGAYLSDLP-RGPEKMELVALHKSFGVLILLIALARVFWRIKEGAIPPASVMPAWQEKVA
NP_520840     ----------------MPHAARSDAAALRAPAADDGAYGKPAIALHWIIALL--IFAAFGLGLYMTDIPGFTPTKLKLYSYHKWAGITVLILAVLRVLWRLTHPAPAPVAGMPAWQQKAA
NP_105780     ----------------------------MQSITNTTTRYGWATIILHWLIGII--FIGQFALGFIMVRLTSQR-TAFELIQLHKSLGFLLLGLIILRIAWRLGNATPPLPPSVGTLERHTA
NP_385543     ----------------------------MLRNSENGFGTVTIALHWTIAVL--ILGLILAGFVMRRIEIDPGLQFSLYQWHKSFGFTALGLAAFRMLWWLVETSPAASPTLGPMEHMAA
NP_354489     -------------MQDFGDNSGRAPAAGRSWRNTPSSFGVVAIAFHWTIAVL--FVAQLALGYLMSRDSIDPVLQFNFFQYHKSIGFLVLALAVPRCLWSVFSRKPRAVDGEGPVSRFAA
NP_287191     ----------------------------MSFTNTPERYGVISAAFHWLSAII--VYGMFALGLWMVTLSYYDGWYHKAPELHKSIGILLMMGLVIRVLWRVISPPPGPLPSYSPMTRLAA
NP_460129     ----------------------------MQFKNTPQRYGVVSAALHWLTALV--VYGMFALGLWMVTLSYYDGWYHQAPEIHKSIGILLMMALIVRIIWRLYSPPPVALTSYSRLTRIGA
WP_002211010  ----------------------------MLLKNTREQFGYITIAIHWLVALT--VYALFALGLWMVTLGYYDGWYHQAPEIHKSVGCILFAVMLFRVIWRFISPPPKPLSSYNRLTRVSA
NP_249113     ----------------------------MQWRNTSSRYGVFSLFLHWGSALV--VFGLFGLGLWMRELSYYDPWYHPAPALHKSIGILLAIALLVRIVWRFVSPPPPAPANHGPLTRVAS
NP_232928     ----------------------------MKNSDS-QHYNLVTRSIHWISALV--VIGMFAVGTWMMDLSYYSEWYRTAPHWHKSVGLLLAGLTLFRLIWKALSSSPKIEG--ARWEIVAA
NP_542051     ------------------------MTQPKAIFDSPIGYGVVSRVLHWAMALM--FAWQFTS-TILHYFAKGSAIFKFFWAAHFQLGFALLLLVLLRGVWGLINCNRRPRAES--SGGRIV
NP_542019     ------------------MLYRYGLMSSKTYIWDDRSGYGLVSRGFHWLMALL--FLWQFAS-AILRVFAKDMPIYSFFWSAHFQLGFALLVLALLRGVWGLMNIHRRPHKDG--AIGKLA
NP_518320     ---------------------------------MRFEPKYSLGMRALHWLVFLA--ALIAVVTIEILDAFPKGSAARAALFAAHQTMGLSVLALMVLRMLVRVGGQVPAPVSGSQ-LLERAA
consensus     ------------------------------------ky--l-iglHWlvall--via-ya---li---p--------i--iH-smGi-ilvlmvvRllwrl----p--i-----m---aa
              1........10........20........30........40........50........60........70........80........90........100.......110.......

NP_415935     HLGHLVIYLLFIALPVIGLVMMYNR--GNPWFAFG-LTMPYASEA-NFERVDSLK-----SWHETLANLGYFVIGLHAAAALAHHYFWKDNTLLRMMPRKRS------------------
YP_403372     HLGHLVIYLLFIALPVIGLVMMYNR--GNPWFAFG-LTMPYASEA-NFERVDSLK-----SWHETLANLGYFVIGLHAAAALAHHYFWKDNTLLRMMPRKRS------------------
NP_460598     HLGHLVIYLLFIALPVIGLVMMYNR--GNPWVAFG-ITMPHAAEA-NFERVDMLK-----SWHVTLANLGYFVIGLHAIAALMHHYFWKDNTLLRMMPRKRD------------------
YP_003612579  HLGHLVIYLLFIALPLIGLVMMYNR--GNDWFAFG-IAMPHAAES-NFDLADGLK-----EWHETLANLGYFVIGLHAAAALMHHYFWKDNTLLRMMPKKRQ------------------
YP_002348217  HVGHWVIYLLFIALPIIGIAILYCR--GSSWIAFG-LIMPHAEQA-NFDLADTLK-----AYHLLLANMSYFVIGLHALAALLHHYVLKDNTLLRMMPKKRG------------------
NP_416483     SLMHIMLYITFLALPLLGIALMAYS--GKSWSFLG-FNVSPFVTP-NSEIKALIK-----NIHETWANIGYFLIAAHAGAALFHHYIQKDNTLLRMMPRRK------------------
NP_460220     KLMHIALYLTFLALPLLGVAMMASG--GKSWSFFG-FTVPVFLTP-DSTLKSDIK-----RIHEMLANIGYFLIAMHAAAALFHHYIQKDDTFSRMLPGKS------------------
WP_002211611  RISHWGLYLMFLSLPILGFLTQAYG--GRTWLLLG-WQVPQWLTP-NTDVRDYLK-----QTHVFIANLGYFVIGIHGVAALYHHYIRRDDTLRRMMPGK------------------
NP_249609     RLMHYALYLMMIGLPFAGWLILSAA--GKPIPFFG-LELPPLVDK-NPDLAGQIK-----EWHETIGNAGYFLIGLHAAAALFHHFVSRDNTLVRMLPQRR------------------
NP_246256     HSVQMLLYLCLLLTPIAGYFTLTAD--ADDFMLFG-MNLPYFS------LEISLG-----KAHVILANSFIALSGVHALAALFHHVILKDNVLKSMSPH-------------------
NP_105468     KAAHLVLYALLVAIPVLGILLTWYR--GDSLSFFGLFTIPAQVSP-DRDTARFIR-----ELHSLCANSILILAGVHAAAALWHHFIRKDDVLKRMLPESTP------------------
NP_298617     NATHIMLYVLIFTIPLAGWLYDSAR-GLRTFYWFGLFEMPALVSP-NETIRGVAL-----EIHKLSFWALIGLVVLHAGAAFYHHLIQRDATLARMLPRNWFSSKS-------------
NP_252265     KAGHFLLYAVLIVMPLSGMLAG-----KRPLDLFWLVQIGPFDLP--EAVKAFAG-----GTHVTVQYLLFALILGHAAAAIWHHRVQKDDVLRAMLPQRGA------------------
NP_232647     KAVHGLLLLATLAMPLSGIAMNIG--GGRALEVFG-YTLVAAGEK-VVWLQELGA-----AVHKTAPPIIIAIVLLHIAAVLKHHLIDKDATMARMLGRG-------------------
NP_520840     EGAHIVLYLLLILAVPLTGYLLSVA--AGIKVVYLGLWELPMPFDK-SDALKEIFQ-----VAHEWLNWTMATIVVLHILAALKHHIVDSDGTLRRMLPFLR------------------
NP_105780     PLAHLALYAFQVALPLSGWALVSVSTLEIPTMPFNLFVMPNLPLTESDAAESFWA-----AAHWYLAYLGIALVALHVAAALRHHFLLRDSVLTRMITP-----------SSGGE-----
NP_385543     RATHLSLIALALLVPLSGWAVVSVSTLAIPSFYFNLIIIPHLPLPKSGTSEAFWA-----LAHAMLAYVTLGLVAIHAAAAFYHHLVRRDLVLIRMLRSGSMPRTGSTVKTSGKDDTVVE
NP_354489     RVAHSALLFLTLAVPLAGWAVASTSPLQIPSYVFDLVVVPGLPMTISDQAEAFWS-----DVHATLAYLAAVIVLLHVIAALWHHFIRRDPTLRRMLPLPDTTDNSRREISSGQ------
NP_287191     KAGHLALYLLLFAIGISGYLISTAD--GKPISVFGWFDVPATLAD-AGAQADFAG-----ALHFWLAWSVVVLSVMHGFMALKHHFIDKDDTLKRMLGKSSSDYGV-------------
NP_460129     AAGHLLLYLLLFAIIISGYLISTAD--GKPISVFGWFEIPATLTD-AGAQADIAG-----TLHLWFAWSLVIISLSHGVMALKHHFIDKDDTLKRMTGMSSSDYGAQK------------
WP_002211010  VLAHLMLYSILFCLMLSGYLISTAD--GQPINVFGWFAIPVTLAG-LPDQADTAG-----TIHLYLAWAVVVLSLLHGLAALKHHFIDGDITLKRMLGRSID------------------
NP_249113     KLGHLALYGLLLAVIVAGYLISTAD--GEPISVFGWFSVPATLSG-LPDQADVAG-----EIHLYLAWALVVFAVLHALAALKHHFVDRDPTLKRMLGRSSNDQQPR-------------
NP_232928     KSAHHLMYVGLFVLFVSGYLISTED--GRGIEVFNWFTVPGAGAL-FENQADIAG-----NIHFYTAWGLIILAGLHAVAALKHHFINRDNTLKRMLTGASK------------------
NP_542051     ALGHGLIYILMIAVPTVAMLRSYNS--GRGFSFLGMEIFAKTAVQNPAGGAPAAAVNAGLSLHGLLGWILLAVVIAHILLALAHHFIMQDNTLRYMTGRRAVS-----------------
NP_542019     VLGHLAIYVLMITIPSLALLRAYGS--GRGFSFLGMPIFAKTDIQNAMLTAPAN------ALHGPLGWVLLAAIVGHVAMALVHHFALRDDTLRYMTGHGTPN-----------------
NP_518320     RLTHGVLYVLMVGMPILGVLAIAWS--GKPIQPFGLALTLTLAQDKTLANLAK-------EVHESGATLVYIFVGLHAAAALWHEFILKDRLLRRMI----------------------
consensus     -lghlvlyllmialpv-g-lm------gkp---fg-f-mp----------d---------iH--lawlii-viglHaaaal-Hhyi-kD-tlrrMlp----------------------
              121......130.......140.......150.......160.......170.......180.......190.......200.......210.......220.......230.......
```

Supplementary Fig. 10. Sequence alignment of diverse CybB homologues. Sequence alignment of a number of diverse representative sequences belonging to the COG3038 and PRK11513 groups of the Cytochrome_b_N Superfamily (NCBI accession number cl23723). Arrows indicate heme Fe-coordinating residues, red oval indicate residues potentially involved in proton transfer from the cytoplasm.

Sequences are denoted with their NCBI accession numbers. The top sequence represents E. coli SOO (NP_415935). The other sequences are homologous proteins from the following organisms: Agrobacterium fabrum NP_354489; Brucella melitensis NP_542019, NP_542051; Enterobacter cloacae YP_003612579; Escherichia coli NP_416483, NP_287191; Mesorhizobium loti NP_105468, NP_105780; Pasteurella multocida NP_246256; Pseudomonas aeruginosa NP_249113, NP_249609, NP_252265; Ralstonia solanacearum NP_518320, NP_520840; Salmonella enterica NP_460129, NP_460220, NP_460598; Shigella dysenteriae YP_403372; Sinorhizobium meliloti NP_385543; Vibrio cholerae NP_232647, NP_232928; Xylella fastidiosa NP_298617; Yersinia pestis YP_002348217, WP_002211010 WP_002211611. It should be noted that the eukaryotic B_{561}[37] belongs to different protein families (but also within the Cytochrome_b_N Superfamily). Compared to SOO, the eukaryotic B_{561} family has a different number of TM helices and opposite membrane insertion topology of the heme-coordinating helices. They also show no quinone binding site. Still, since they display strikingly analogous heme architecture and positively charged patch with an exposed heme edge, studies on their reactivity with superoxide would be interesting.