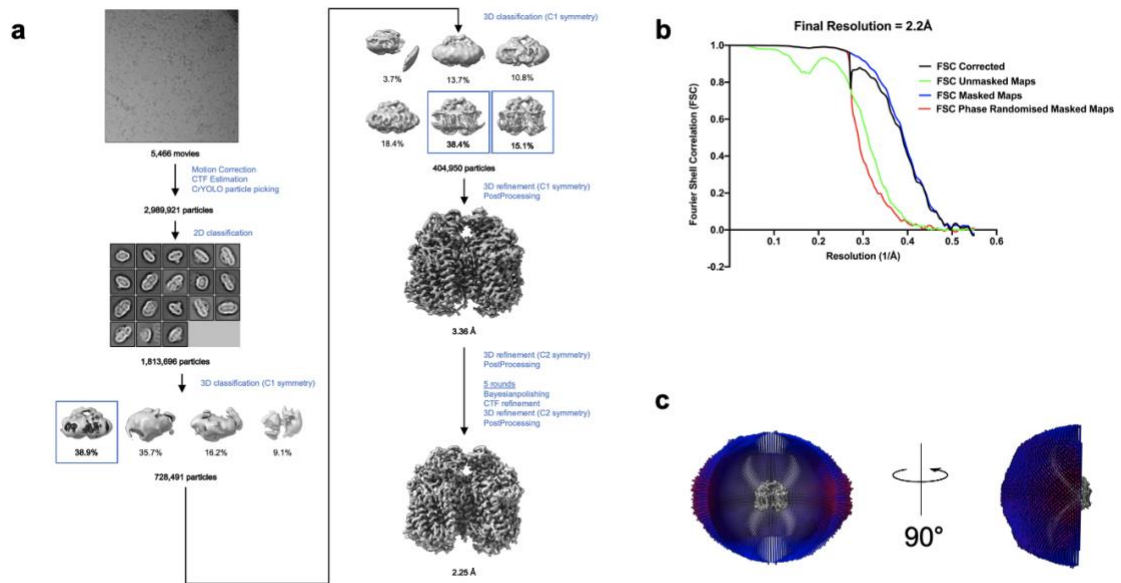
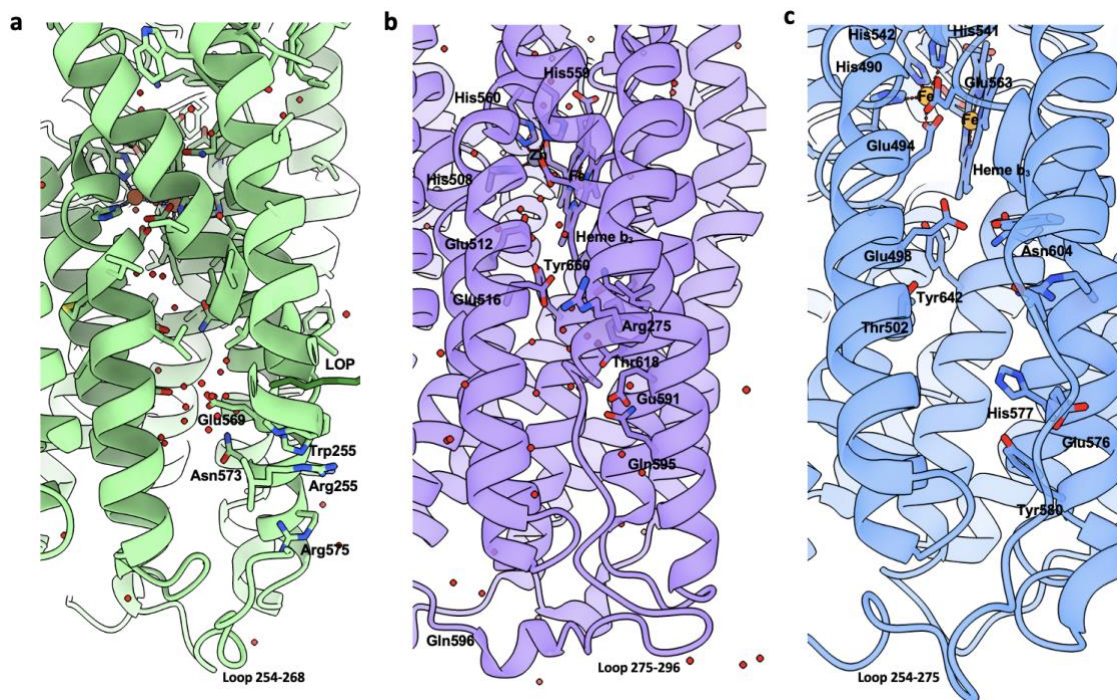


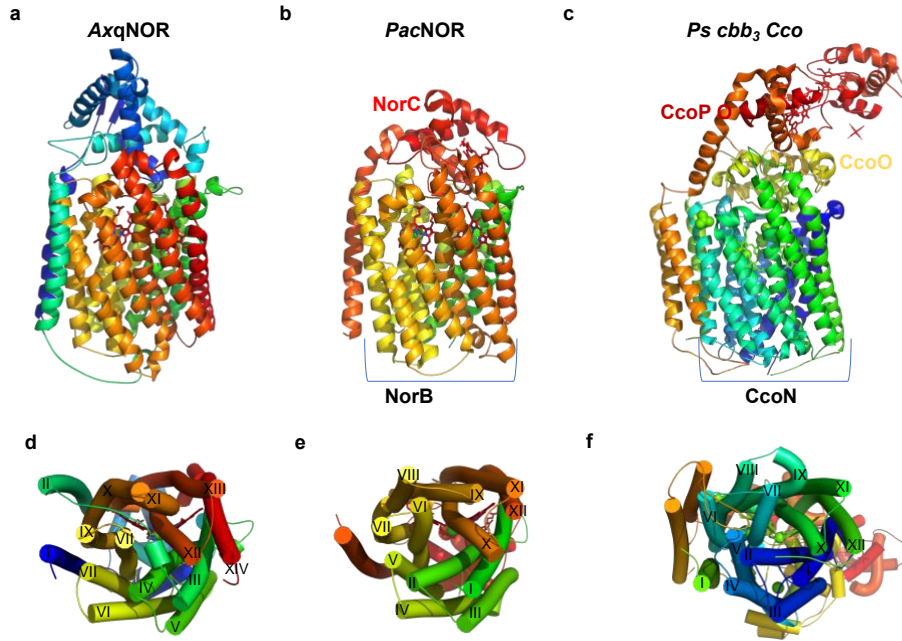
## Supplementary information



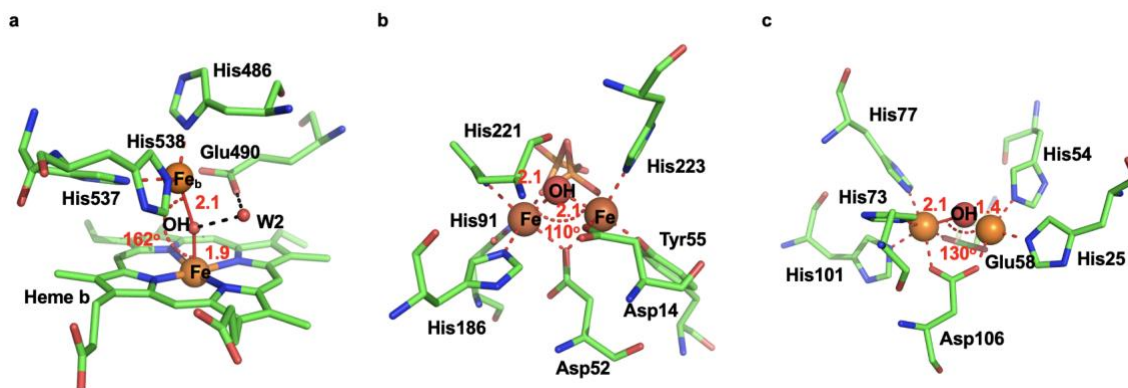
**Supplementary figure 1: CryoEM analysis of quinol-dependent Nitric Oxide Reductase (qNOR) from *Alcaligenes xylosoxidans*. (A)** Cryo-EM image processing workflow performed in RELION. Example micrograph, 2D classes, 3D classes and refined reconstructions are highlighted. **(B)** FSC curve for the final map. Final resolution was calculated using the gold-standard FSC cut-off of 0.143. **(C)** Euler angle distribution of refined particles.



**Supplementary figure 2. Comparison of the cytoplasmic part of product release channels at the dimeric interface in structurally characterized qNORs.** (a) Product release path of qNOR from *Alcaligenes xylosoxidans* (AxqNOR) from the cytoplasmic side to the binuclear centre. AxqNOR coloured in green with residues lining the proposed path in AxqNOR shown as sticks. Fe atoms and water molecules are shown as orange and red spheres, respectively. Hydrogen bonds and metal co-ordination bonds shown as black and red dashed lines, respectively. This scheme is used throughout the figure. (b) Product release channel in qNOR from *Geobacillus stearothermophilus* (GsqNOR) (PDB ID: 3AYG) coloured in purple, with residues lining its proposed proton channel highlighted as sticks. Zn shown as grey sphere. (c) Product release channel in qNOR from *Neisseria meningitidis* (NmqNOR) coloured in blue, with residues lining its proposed proton channel highlighted as sticks (PDB ID: 6L3H).

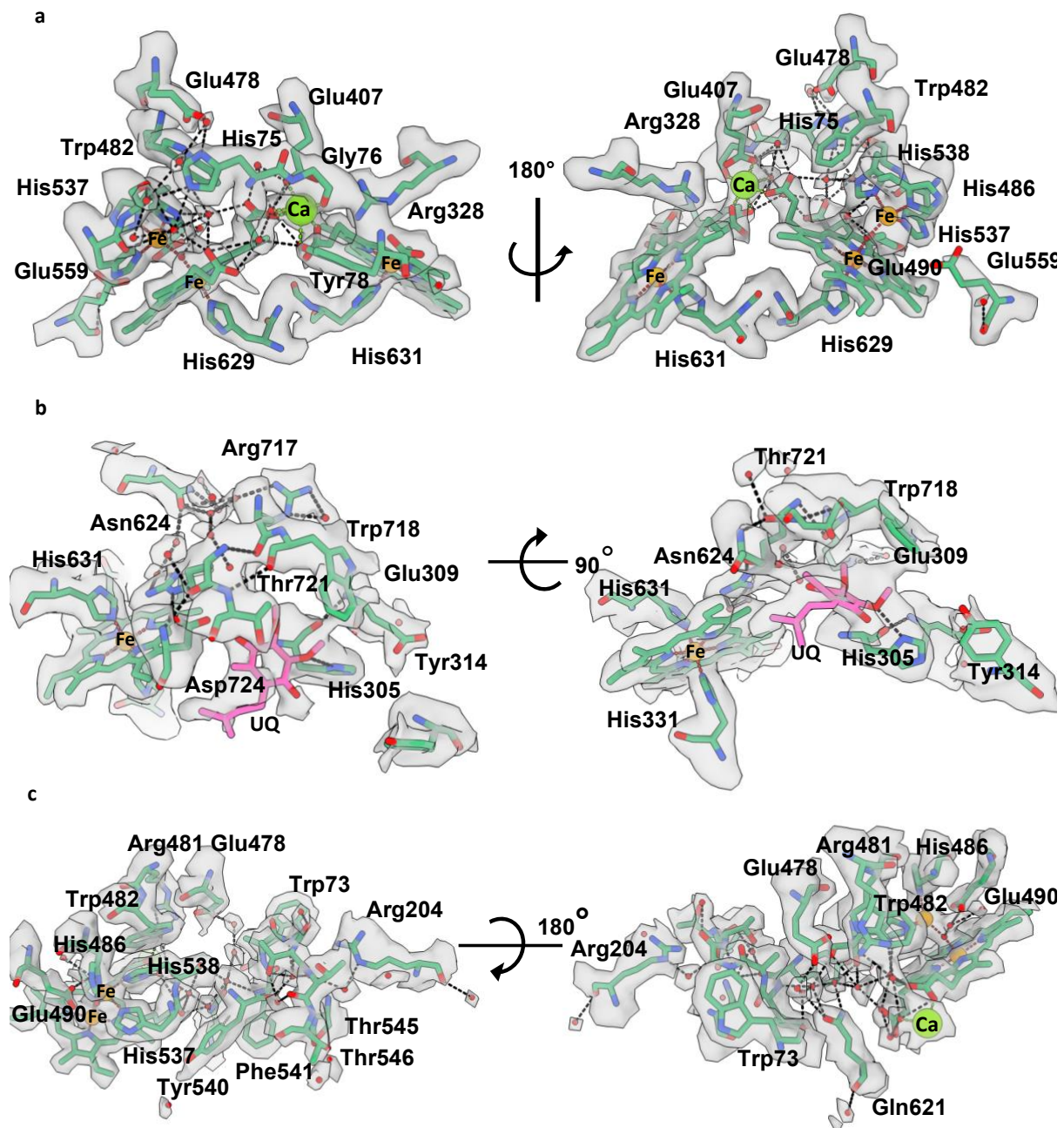


**Supplementary figure 3. Comparison of overall structures of AxqNOR, PacNOR and *Ps cbb<sub>3</sub> Cco*.** (a) Overall structure of AxqNOR viewed parallel to the membrane shown as ribbon in various colors; heme *b* and heme *b<sub>3</sub>* are shown as red sticks. Fe<sub>B</sub> and calcium ions are represented as orange and green spheres, respectively. (b) Overall structure of cNOR from *P. aeruginosa* viewed parallel to the membrane (PDB code 3WFB). The NorB and NorC subunits are shown as ribbon in various colors and red, respectively. Heme groups are shown as red sticks. Fe<sub>B</sub> and calcium ions are represented as orange and greens spheres, respectively. Average rmsd between 390 C $\alpha$  atoms of AxqNOR and NorB subunit is 4.3 Å; (c) Overall structure of *Ps cbb<sub>3</sub> cox* (PDB code 5DJQ) viewed parallel to the membrane shown as ribbon in various colors for subunit N, in yellow for subunit O; and in red for subunit P. Hemes *b* and *b<sub>3</sub>* are shown as green sticks. (d) Arrangement of membrane-spanning helices of AxqNOR viewed from the periplasmic side. Transmembrane helices are indicated with Roman numerals in all panels. (e) Arrangement of membrane-spanning helices of *PacNOR* viewed from the periplasmic side. (f) Cytochrome *cbb<sub>3</sub>* oxidase from (PDB code 5DJQ). Subunit N with helices I ~ XII is shown in multi color. Average rmsd between 278 C $\alpha$  atoms of AxqNOR and CoxN subunit is 8 Å Helixes of subunit O and helices of subunit 3 are shown in yellow and orange-red, respectively.



**Supplementary figure 4.** Di-iron sites with bridging hydroxide ion. (a) Fe bi-nuclear site of AxqNOR. Non heme Fe is ligated by His486, His 537 and His 538 and OH ion with slightly distorted tetrahedral geometry. The hydroxide (OH) is bound in  $\mu$ -oxo configuration between two metals at 2.1 Å distance to Fe<sub>B</sub> and 1.9 Å to heme Fe, the distance between two Fe is 3.9 Å. Angle between two metal bonds is 162°. (b) Fe bi-nuclear site of Pig tartrate-resistant acid phosphatase type 5 (PDB: 5UQ6 [<https://doi.org/10.2210/pdb5UQ6/pdb>]); the distance between two Fe is 3.4 Å. (c) Fe bi-nuclear site of human Hemerythrin (PDB: 2HMQ [<https://doi.org/10.2210/pdb2HMQ/pdb>]), the distance between two Fe is 3.2 Å. Residues are shown as sticks, Fe ions as orange spheres, water molecules and OH ion as red spheres.





**Supplementary figure 5:** The cryo-EM map for key residues surrounding key elements and residues (a) AxqNOR calcium site. Cryo-EM map shown with threshold set to 0.023. (b) Key residues around the AxqNOR electron donor binding site. Ubiquinone electron donor shown as pink sticks. Cryo-EM map shown with threshold set to 0.1. (c) AxqNOR NO entry site and surrounding residues. Cryo-EM map shown with threshold set to 0.020. AxqNOR residues shown as green sticks. Cryo-EM map shown as a translucent grey surface. Waters, iron ions and calcium ions shown as red, orange and green spheres, respectively.

AxqNOR	-----MGPYRRLWFTLI AVLAVT FAL LGFYGGEVYRQAPPI PEEVASA	43
GsqNOR	MEVNR TVSPNIQTGRKTTNSFLKS--ILIF TILISSTVLLVGGYWIFKEMAPR PKEVRSE	58
NmqNOR	-----MGQYKLLWYLLFAVLAVCF TILGMYGSEVYKAPPYPEQVVSA	43
	. : . : . : * : . : . : : * * : : : . : * : * : * *	
AxqNOR	DGTRLFGRDDILDGQTAWQSIGGMQLGSIWGHCAIQAPDWTADWLHRELMAWLDAARDA	103
GsqNOR	SGEVLMTKET IIGGQAVFQKYGLMDYGTVLGHCSYMGPDYTAELKVYTEGMQDYKAKER	118
NmqNOR	SGKVLMAKDDILAGQSAWQTTGGMEVGSVLGHCAIQAPDWTADWLHRELSAWLDLTAQQT	103
	. * * : : : * : * : : * : * : : * : * : * : * : * : * : * : *	
AxqNOR	HGRDYQQLDAPAAQLREQLKAEYRANRADAAGCKLTLSPRRAQVAQTEAYYDQLFSDA	163
GsqNOR	YNKPFADLTDEKSI IREQVIKEMRKNRYNPVTDVVLVLTDAQVYGLEKVRDYRDVFTNG	178
NmqNOR	YGKPFDEVSPPEQAVLKTRLADEYRNQSRIKEDGSSVVISDTRVKAIESILPYYHGVYGD	163
	. : . : . : : : : * * : . : : : . : . : . : * * : : . :	
AxqNOR	PALHRSRENYAMKENT-----LPDANRRRQMT HFFFWTAWAAGTEREGTSVYTNW	215
GsqNOR	DWGLKKG--LIKESDMKANRAVADSDQIQIADFFFWTAWLSTLRIGDEITYTNW	236
NmqNOR	PALQTTREHFAMKNNT-----LPSQEAAREKLFDFFFWT SWSASTNRPEDETFYTNW	215
	. : . : . : : : . : . : . : * * * : * : * : * : * : * : * * * *	
AxqNOR	PHEPLIGNHPSENVMWSIISVVVLLAGI GLLIWAFAFLR--GKEEDEPPAPARDPLTT	272
GsqNOR	PYEDAGNTMSFSAYVWWSGASVTILLIFIGIILYVYRYQLSMQEAAYAEKFPV-IDLRR	295
NmqNOR	VHEPLINNVPPTTENYMSFTSVVLLMGI GLLMWGYSFLT--KHEEVE--VPTEDPI SK	270
	* : . * : . * * * : * : * : * : : : . * * . : :	
AxqNOR	FALTPSQALGKYLFVVALFGFQVLLGGFTAHTYVGGQKFGID-----LSQWFPYSL	326
GsqNOR	QPLTPSQVAKGYFVVVALFVQTMFGALLAHTYVGGQKFGINW-----IYDILFPNI	350
NmqNOR	VQPLTPSQALGKYVPLTVALFVVQVLLGGLTAHTYVGGQKFGIDEALGFEMSDFWFPYAL	330
	* * * * * * * . . . * * * . * : : * * * * * : * : * : * : :	
AxqNOR	VRTWIIQSALFWIATGFLAAGLFLAPLNGRDPKYQKAGVDILFWALVVLVVGSGFAGNY	386
GsqNOR	AKGYHLQLAIFWIATAWLGMGIFIAPLV-GGQEPKQGLLVDLFWALVVLVGGSMIGQW	409
NmqNOR	TRTWIIQSALFWIATGFLTAGLFLAPIVNGGKDPKQAGVNFILFIIVVGGSYAGNF	390
	. : . : * * * * * * : * * : * : * : * : * * * : * : * : * * * * * :	
AxqNOR	LAIQIMPDPDLNFWLGHQGYIYVDLGRWLQIGKFGICFWLVMLRGI VPAALRTPGGDKN	446
GsqNOR	LGVNGYLGNE-WFLLGHQGWYIELGRWQIILVVGMLLWLFVFRGVKRGKRESDKGG	468
NmqNOR	FALTHILPPEFNFWFGHQGYIYLDLGRFWQLLLMVGLLLWLFMLRCTVSAFKEKGV DKN	450
	. : . : . : * : * * * * * : * : * : * : * : * : * : * : * : *	
AxqNOR	LLALLTASVGAIGLFYAGFFYGERHTLTVMEYRWVIVHLWVCGFFEVFATTALAFIFS	506
GsqNOR	LIHLLFYSAIAVPPFYIFAFFIQPDNFTMADFWRWVILHLWVCGIFEVFAVVVIGFLLV	528
NmqNOR	LLAIFVASMVGVPYAPGLFYGEKSPVAVMEYRWVIVHLWVCGFFEVFATAAFVY	510
	* : . : * . : . * * . * : : : : * : * * * : * : * * * * * : * : * :	
AxqNOR	TLGLVSRMATTASLASASFLMGGIPGTFHHLVYAGTTPVMAVGSFSALEVVPLIVL	566
GsqNOR	QLRLVTKKSTVRALYFQFTILLGSGVIGIGHYYNGSPVWIALGAVFSALEVIPLTLL	588
NmqNOR	NMGFVRRSTASTALAAAIFMLGGVPGTLHHLVYSGSTASMAI GACFSALEVVPLVLL	570
	. : * : . : . : . : : : . : * : * * * : * : * : * * * * * * * * * :	
AxqNOR	GHEAWENWRLKTRAPWMENLKWPLMCFVAVAFWNMLGAGVFGFMINPPVSLYIIGLNTT	626
GsqNOR	ILEAVEQYKMMRDGANFPYKATFWFLISTAIWNLVGAGVFGFLINLPAVSFEHQFLT	648
NmqNOR	GREAYEHWYSQHLSEWAKRLRWPLMCFVAVAFWNMIGAGVFGFLINPPI SFLYIIGLNTS	630
	* * : : : : * : * : * : * : * : * : * : * : * : * : * : * : *	
AxqNOR	PVHAHAALFGVYGFALGFTLLVLRVIRPQYALS PGLMKLAFWGLNLGLALMI FTSLLPI	686
GsqNOR	PAHGHAAAMGVYGMFAIAVLLYSLRNI V KPEAWNDKWLKFS CWMLNIGLAGMVVITLLPV	708
NmqNOR	AVHAHAALFGVYGFALGFTLLVLRVIRPQYALS PGLMKLAFWGLNLGLALMI FTSLLPI	690
	. * * * * * * : * : . * * * * * : . : . : * * * * * : * : * * * :	
AxqNOR	GLIQFHASVSEGMWYARSEAFMQDDILKTLRGRFTFGVVFLLGALAMVVQVILGLLSGK	746
GsqNOR	GILQMKFAFIHGYSRSPSFLQDDVVQNLVAVPDTIFLIGVVALLVFAIKALFHLR	768
NmqNOR	GVIQYASITHGLWYARSEEFQMEILDTLRGRFTFGVVFLLGALAMVVQVILGLLSGK	750
	* : * * . . * * * * * : * : * : * : * : * : * : * : * : * : *	
AxqNOR	-----746	
GsqNOR	KPTHGEGEELPVANHWMKDRKNSLE794	
NmqNOR	K-----751	

NO entry channel; heme, Fe<sub>b</sub> ligation; Ubiquinol binding; Ca binding ligands

**Supplementary figure S6A. Sequence alignment of AxqNOR, NmqNOR and GsqNOR.** Homology between AxqNOR (PDB:8BGW [<https://doi.org/10.2210/pdb8BGW/pdb>]) and NmqNOR(PDB:6L3H [<https://doi.org/10.2210/pdb6L3H/pdb>]) is 58.7%; Homology of AxqNOR and GsqNOR is 33.3%. Residues or NO entry channel are highlighted in grey; heme Fe<sub>b</sub> and F<sub>B</sub> ligands in cyan; Ubiquinol binding site in magenta and Ca binding ligands in Green; fully conserved residues are marked with \*. Alignment is performed by CLUSTRALW EBI server.

## Redox sites

<b>AxqNOR</b>	478	E	Y	W	R	W	I	V	H	L	W	-	-	V	E	G	F	535	T	F	H	H	L	Y	626	T	P	V	H	A	H
<b>NmqNOR</b>	482	E	Y	W	R	W	V	V	H	L	W	-	-	V	E	G	F	539	T	L	H	H	L	Y	630	S	A	V	H	A	H
<b>GsqNOR</b>	500	D	F	W	R	W	I	I	H	L	W	-	-	V	E	G	I	557	I	G	H	H	Y	Y	648	T	P	A	H	G	H
<b>PacNOR subunit B</b>	199	K	F	Y	W	W	V	V	H	L	W	-	-	V	E	G	V	256	T	G	H	H	Y	F	344	T	A	A	H	G	H
<b>Cytochrome bo3 subunit 1</b>	277	L	I	W	A	W	G	H	P	E	V	Y	I	L	I	L	331	W	L	H	H	F	F	416	L	I	A	H	F	H	

## Ubiquinol sites

<b>AxqNOR</b>	302	F	T	A	H	Y	T	718	W	G	R	T	F	G	D	V	V
<b>NmqNOR</b>	300	L	T	A	H	Y	T	722	W	V	R	T	A	A	D	L	I
<b>GsqNOR</b>	325	L	L	A	H	Y	Y	740	L	V	R	A	V	P	D	T	I
<b>PacNOR subunit B</b>	36	I	M	G	L	Q	Y	438	W	L	R	E	G	A	G	V	V
<b>Cytochrome bo3 subunit 1</b>	60	M	Y	I	I	V	A	496	M	I	A	A	S	G	A	V	L

KEY	
W	IDENTICAL MATCH
W	POSITIVE MATCH
W	NEGATIVE MATCH

## Ca binding

<b>AxqNOR</b>	73	W	G	H	G	A	Y	Q	326	L	V	R	T	W	405	G	Y	E	Y	V	557	A	L	E	V	V
<b>NmqNOR</b>	73	L	G	H	G	A	Y	Q	330	L	T	R	T	W	409	G	Y	E	Y	L	561	A	L	E	V	V
<b>GsqNOR</b>	88	L	G	H	G	S	Y	M	350	I	A	K	G	Y	427	G	W	E	Y	I	579	A	L	E	V	I
<b>PacNOR subunit B</b>	-	-	-	-	-	-	-	-	55	V	A	R	M	V	136	G	I	V	I	V	278	A	L	E	P	L
<b>PacNOR subunit C</b>	68	L	G	E	G	A	Y	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
<b>Cytochrome bo3 subunit 1</b>	38	F	G	K	W	-	-	-	101	Q	I	F	T	A	220	-	M	T	M	F	349	G	I	T	T	M
<b>Ps cbb3 cox</b>	-	-	-	-	-	-	-	-	55	R	L	R	P	L	120	S	K	E	Y	A	277	L	I	L	L	A

## NO channel

<b>AxqNOR</b>	71	S	I	W	G	H	204	R	E	G	T	S	V	T	Y	T	N	540	Y	F	A	G	T	T	619	Y	I	Q	G	L
<b>NmqNOR</b>	71	S	V	L	G	H	204	R	P	D	E	T	F	T	Y	T	N	544	Y	F	S	G	S	T	623	Y	I	Q	G	L
<b>GsqNOR</b>	86	T	V	L	G	H	225	R	I	G	D	E	I	T	Y	T	N	542	Y	Y	N	G	S	P	641	F	E	H	G	Q
<b>PacNOR subunit B</b>	0	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	261	F	W	I	G	V	P	337	Y	T	H	G	T	
<b>PacNOR subunit C</b>	66	T	L	L	G	E	136	-	-	-	-	-	-	D	T	N	-	-	-	-	-	-	-	-	-	-	-			
<b>Cytochrome bo3 subunit 2</b>	0	-	-	-	-	-	72	-	S	N	K	D	A	K	Y	S	P	336	F	T	M	G	A	G	409	V	L	H	N	S

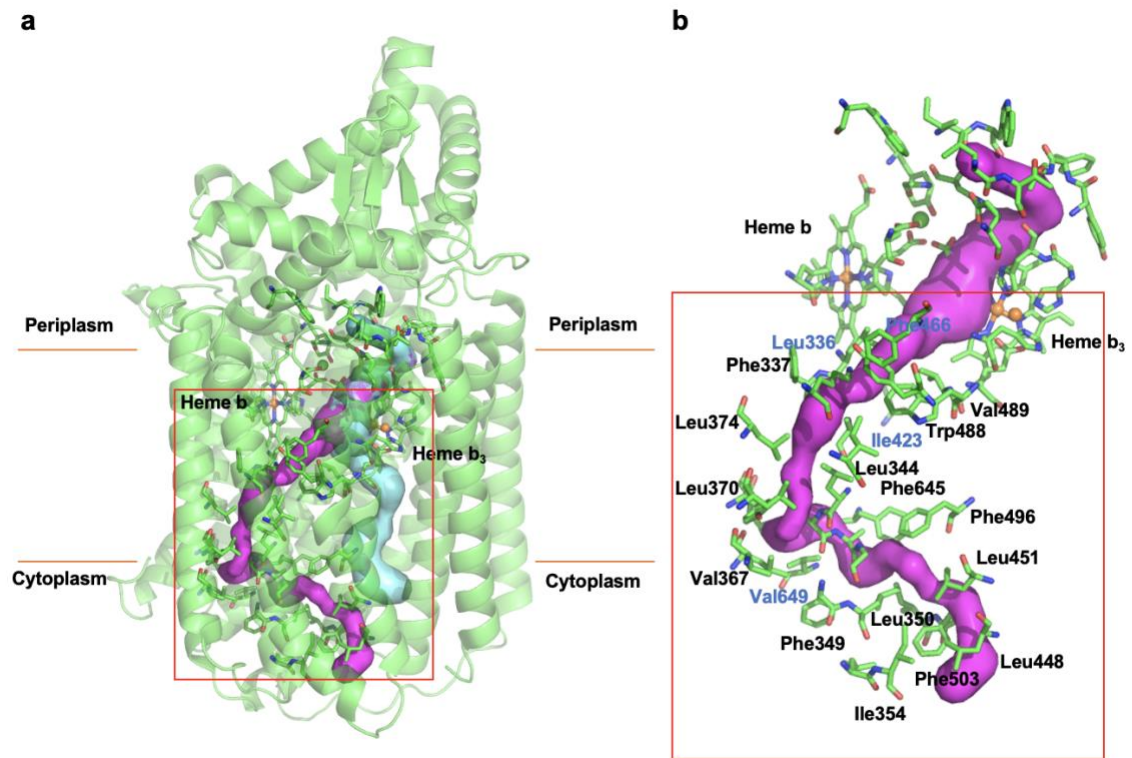
**Supplementary figure 6B. Sequence fragments conservation for AxqNOR, NmqNOR, GsqNOR, PacNOR subunits B and C and subunit 2 of COX.** Identical residues are colored in dark yellow, positive matches by light yellow. The upper sequences were aligned by pairwise alignment against AxqNOR sequence using BLAST and scored according to the BLOSUM62 score matrix. The bottom sequences were not similar enough to AxqNOR for pairwise alignment, so were aligned by multiple sequence alignment with the upper sequences using Clustal Omega. These aligned sequences were not scored by a matrix and so were only marked for identical residues.











**Supplementary figure 7. Putative product release pathway in AxqNOR.** (a) Location of the putative product release path from binuclear active site to the cytoplasm is shown in purple and based on CAVER 3.0 analysis. Water channel is shown in cyan. (b) Hydrophobic residues surrounding the channel and heme molecules are shown as sticks. Iron and Ca ions are represented by orange and green spheres. Part of the channel directed to the cytoplasm is indicated by the red square. Majority of the residues lining the path are strictly conserved. Residues with black labels are strictly conserved; residues with blue labels are semiconserved.

**Supplementary Table 1: Cryo-EM data collection, refinement and validation statistics**

<b>Data Accession</b>	
PDB	8BGW
EMDB	16041
<b>Data Collection</b>	
Microscope	FEI Titan Krios
Voltage (kV)	300
Detector	Falcon 4
Energy filter slit width (eV)	10
Nominal magnification	130k
Pixel size (Å/pixel)	0.91
Defocus range (µm)	-0.9 - -2.7
Exposure time	6.11
Frames	44
Exposure rate (e <sup>-</sup> pixel <sup>-1</sup> s <sup>-1</sup> )	4.61
Electron exposure (e <sup>-</sup> /Å <sup>2</sup> )	34.90
Dose per frame (e <sup>-</sup> /Å <sup>2</sup> )	0.8
Micrographs collected	5466
<b>Reconstruction</b>	
Software	RELION 3.1
Particles used in refinement	404,950
Symmetry	C2
Overall resolution when FSC=0.143 (masked) (Å)	2.2
Map sharpening B-factor (Å <sup>2</sup> )	-47.68
<b>Model Refinement</b>	
Software	REFMAC5
Non-hydrogen atoms	12845
Protein residues	11649
Ligands	746
Average B factors (Å <sup>2</sup> )	61.128
Protein	63.29
Ligands and water	91.95
R.M.S. deviations	
Bond length (Å)	0.012
Bond angle (Å)	1.649
Ramachandran statistics (%)	
Outliers	0.27
Allowed	5.47
Favoured	94.26
MolProbity score	2.36
ClashScore	15.19
Poor rotamers (%)	2.41
Model vs. Map FSC	0.854

**Supplementary Table 2: Comparison of Ca ligands and bond distances in AxqNOR, its mutant and *PacNOR*.**

Ca ligands in AxqNOR ( <i>PacNOR</i> )	2.2 Å AxqNOR <b>8BGW</b>	3.9 Å AxqNOR <b>6QQ5</b>	3.3 Å AxqNOR Variant <b>6QQ6</b>	2.7 Å <i>PacNOR</i> <b>300R</b>
Water	2.3	-	-	2.1
OH Tyr78(73*)	2.5	2.6	2.7	2.6
O Gly76(71*)	2.3	2.4	2.8	2.3
O2A Heme b <sub>3</sub>	2.3	2.6	2.1	2.6
O1D Heme b	2.2	2.3	2.5	2.4
O2D Heme b	(3.7)	2.7	2.5	(3.8)
OE2 Glu407(135)	2.1	2.5	2.4	2.8
OE1 Glu407(135)	2.2	2.9	3.0	2.6
* residues numbers for <i>PacNOR</i>				