

Quinone-transporting filaments expand bioenergetic capacity in Gram-positive Bacillota

In the format provided by the
authors and unedited

Tables

Supplementary Table S1. Cryo-EM data collection, refinement and validation statistics.

	<i>Ndh-Ncp</i>	<i>Ndh-Ncp filament</i>	<i>Ndh-Ncp NADH/MK-8</i>
Data collection and processing			
Magnification (xk)	130	130	96
Voltage (kV)	300	300	300
Electron exposure (e-/Å ²)	39	39	50
Defocus range (μm)	-1.2-0.6	-1.2-0.6	-1.2-0.6
Pixel size (Å pixel ⁻¹)	0.655	0.655	0.808
Symmetry imposed	C4	C4	C4
Initial particle images (no.)	483,556	483,556	5,095,952
Final particle images (no.)	356,549	389,955	341,297
Map resolution (Å)	2.11	3.09	2.52
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	2.1-3.1	2.6-4.6	2.1-4.3
Refinement			
Initial model used (PDB code)	-	-	-
Model resolution (Å)	2.11	3.09	2.52
FSC threshold	0.143	0.143	0.143
Model resolution range (Å)	n/a	n/a	n/a
Map sharpening <i>B</i> factor (Å ²)			
Model composition			
Non-hydrogen atoms	15,768	46,296	16,084
Protein residues	2,044	6,004	2,044
Ligands	4	12	16
<i>B</i> factors (Å ²)			
Protein	87	156	95
Ligand	65	105	103
R.m.s. deviations			
Bond lengths (Å)	0.002	0.003	0.002
Bond angles (°)	0.486	0.538	0.514
Validation			
MolProbity score	1.16	1.38	1.31
Clashscore	3.71	5.50	5.66
Poor rotamers (%)	0	0.00	0
Ramachandran plot			
Favored (%)	98.57	97.63	98.72
Allowed (%)	1.28	2.37	1.28
Disallowed (%)	0.15	0.00	0.00

Supplementary Table S2. Primers, strains and plasmids used in this study.

Primer Name	Sequence 5'-3'	Purpose
Ndh:ncp Fw	CATCATGGATCCATGCCAGAAACAATCGATCAAAACAATG	Forward primer for amplification of <i>ncp-ndh</i> from <i>B. subtilis</i>
Ndh:ncp Rv	TATAATGCTAGCGATTAGTAAGCCAGGCTGAAAAGTCC	Reverse primer for amplification of <i>ncp-ndh</i> from <i>B. subtilis</i>
Mutant1 Fw	GCTGCGCTATTAAAGCCAGAAGTTGCAGCATCATTGACTGTTTGTGACCAG	Forward primer for mutation of pNdh-Ncp to generate Mutant 1
Mutant1 Rv	AATAAGATCTTGCTGGCTTTGG	Reverse primer for mutation of pNdh-Ncp to generate Mutant 1
Mutant2 Fw	GCAAGCGCGGCAGATCTTATTGCTCAGCTATTAAAGCCAGAAGTTCAAG	Forward primer for mutation of pNdh-Ncp to generate Mutant 2
Mutant1 Rv	GCTGACAGACGCATTGTTG	Reverse primer for mutation of pNdh-Ncp to generate Mutant 2
Strain or plasmid	Relevant genotype and description	Reference or source
<i>Escherichia coli</i> K12 (DH5α)	Standard laboratory <i>E. coli</i> strain for plasmid propagation and maintenance	
<i>Escherichia coli</i> K12 (C41)	Standard laboratory <i>E. coli</i> strain for recombinant protein expression	
<i>Bacillus subtilis</i> 168ca (trp+)	Strahl lab strain of <i>B. subtilis</i> for genetic modification and WT growth curve	This study
<i>Bacillus subtilis</i> 168ca (trp+) $\Delta ndh::kan$	Strahl lab strain of <i>B. subtilis</i> with <i>ndh</i> deletion	This study
<i>Bacillus subtilis</i> 168ca (trp+) $\Delta ncp::kan$	Strahl lab strain of <i>B. subtilis</i> with <i>ncp</i> deletion	This study
<i>Bacillus subtilis</i> 168	Lyras lab strain of <i>B. subtilis</i> for lipidomics experiments	This study
pNdh-Ncp	Ndh-Ncp from <i>B. subtilis</i> cloned into a modified pet22b vector which encodes an N-terminal twin strep tag	This study
pNdh-Ncp Mutant1	Ndh-Ncp from <i>B. subtilis</i> cloned into a modified pet22b vector which encodes an N-terminal twin strep tag and mutations Q14A, Q16A, Q17A, D21A	This study
pNdh-Ncp Mutant2	Ndh-Ncp from <i>B. subtilis</i> cloned into a modified pet22b vector which encodes an N-terminal twin strep tag and mutations D21A, Q22A, Q29A, E30A	This study

Supplementary Table S3. Classical MD simulation setups, their size, time scales, and lipid composition.

Setup	Starting structure PDBID	Number of atoms	Number of lipids in the Ncp chamber	Number of lipids at the end of Ncp tube	Number of MK7	Replica 1 (μs)	Replica 2 (μs)	Replica 3 (μs)
1	9PXL	666,368	40	-	-	1.05	1.17	1.01
2	9PXL	664,518	27	-	-	1.05	1.19	1.06
3	9PXL	665,005	27	5	-	1.01	1.10	1.11
4	9PXL	665,523	27	10	-	0.53	0.58	0.51
5	9PXL	663,776	-	-	-	1.06	1.13	1.13
6*	9PXK	666,394	27	8	4	1.00	1.00	1.00

*In this setup, initial arrangement of chamber lipids was taken from the last snapshot of the production run of setup 2 (replica 1). In the production run of setup 6, we additionally applied distance restraints between NADH-FAD and FAD-MK7 (see methods)

Supplementary Notes

Supplementary note 1. Incubation of the Ndh-Ncp with *B. subtilis* membranes.

To assess how the Ndh–Ncp complex interacts with membranes from its native host, we incubated the purified complex with *B. subtilis* membranes, followed by re-isolation and analysis of its structure and lipid content (Extended Data Fig. 4a). After incubation, membranes were removed by ultracentrifugation, and the complex was subjected to Streptactin affinity purification via its twin-Strep-II tag. Only a minor fraction of the complex bound to the Streptactin resin, with the majority recovered in the flow-through. SDS-PAGE analysis of the flow-through revealed that Ncp had undergone proteolysis during membrane incubation, likely removing the twin-Strep-II tag and preventing resin binding (Extended Data Fig. 4b). This cleaved complex was subsequently re-isolated by size exclusion chromatography, yielding a preparation with >90% of contaminating proteins removed (Extended Data Fig. 4b). Negative stain TEM confirmed that this fraction

contained intact Ndh–Ncp complexes and was largely free of membrane contamination (Extended Data Fig. 4b). This material was used for lipidomic analysis, with this procedure repeated to provide three independent replicates. In contrast, negative stain TEM of the small fraction retained on the Streptactin resin revealed extended Ndh–Ncp filaments that were associated with membrane vesicles (Fig. 3k, Extended Data Fig. 4a,g). Due to the low abundance of this fraction and the presence of membrane contamination, it was not suitable for lipidomic analysis

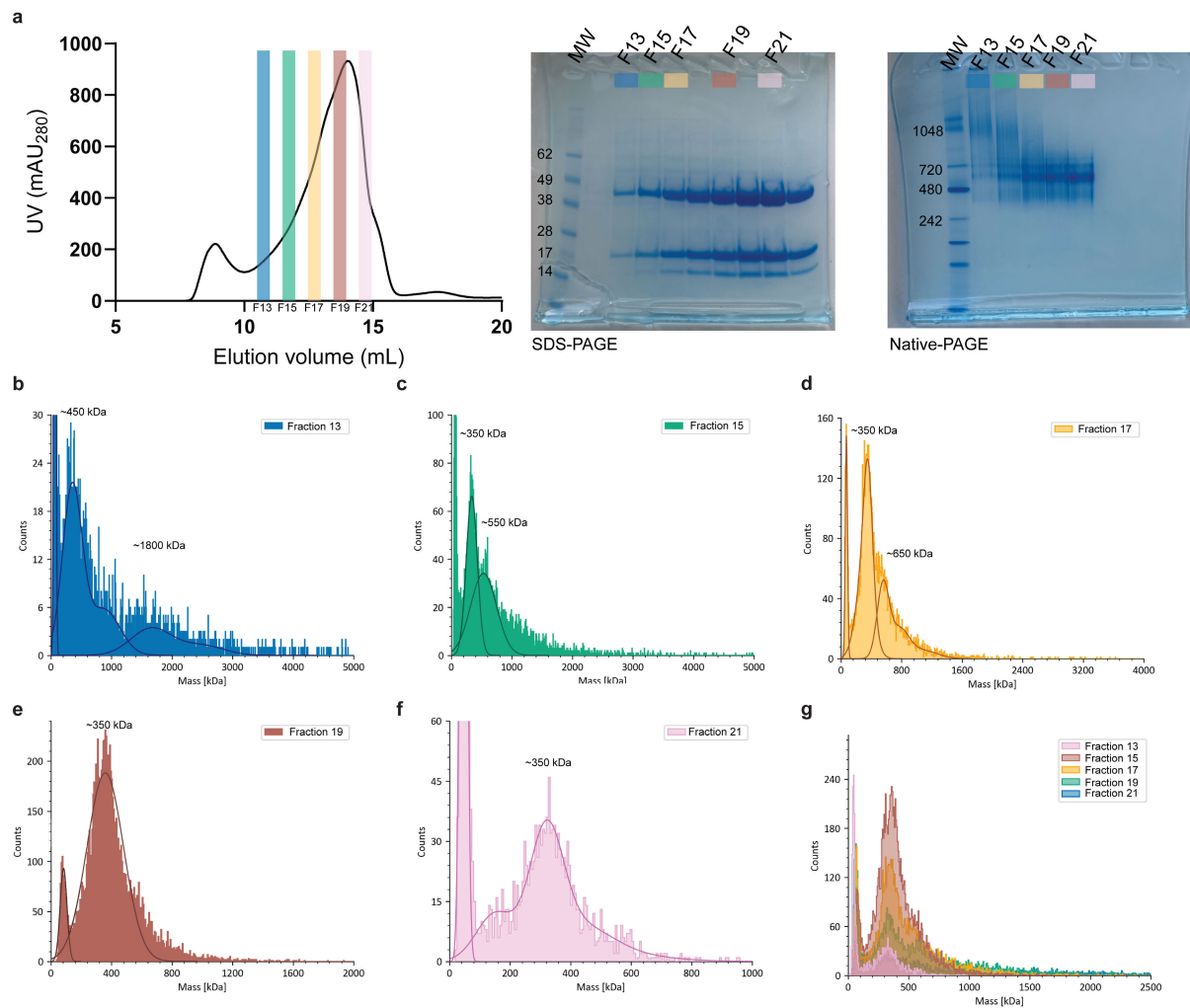
Supplementary note 2. Preparation of the Ndh-Ncp-phospholipid complex MD simulations. The Ndh-Ncp-phospholipid complex was investigated using classical atomistic molecular dynamics simulations on two cryo-EM structures of the Ndh–Ncp complex with and without substrate. Guided by cryo-EM and lipidomics data, we examined the architecture and dynamics of the lipidic interior of the tube. The FAD, NADH, and MK-7 molecules associated with the complex were restrained during these simulations to generate a mode of the holo-Ndh-Ncp-phospholipid complex (see methods).

Volume estimates suggested the Ndh-Ncp chamber could accommodate ~40–45 lipids. Accordingly, we generated a model with 40 lipids tightly packed into the Ndh-Ncp chamber and analysed its dynamics (Extended Data Fig. 5a,c, setup 1). In this configuration, lipid movement was highly constrained; which is not fully consistent with the blurred cryo-EM density observed in this region, indicating lipid mobility. We therefore constructed an alternative model where we aligned the pre-equilibrated lipid bilayer patch with the centre of the chamber, and retained the lipids populating the tube interior, resulting in 27-lipids bilayer inside the tube (Extended Data Fig. 5a, setup 2). Lipids in this new setup 2 were substantially more mobile than in setup 1 (Extended Data Fig. 5c). During microsecond-scale dynamics of setup 2, lipid head groups and hydration shells migrated toward the protein surface, exposing them to solvent, while water was expelled from the conduit, leading to a hydrophobic environment in the interior of the Ndh-Ncp complex (Extended Data Fig. 5d-f). By contrast, lipids in setup 1 remained largely immobile. These results indicate that the poorly resolved cryo-EM density is best

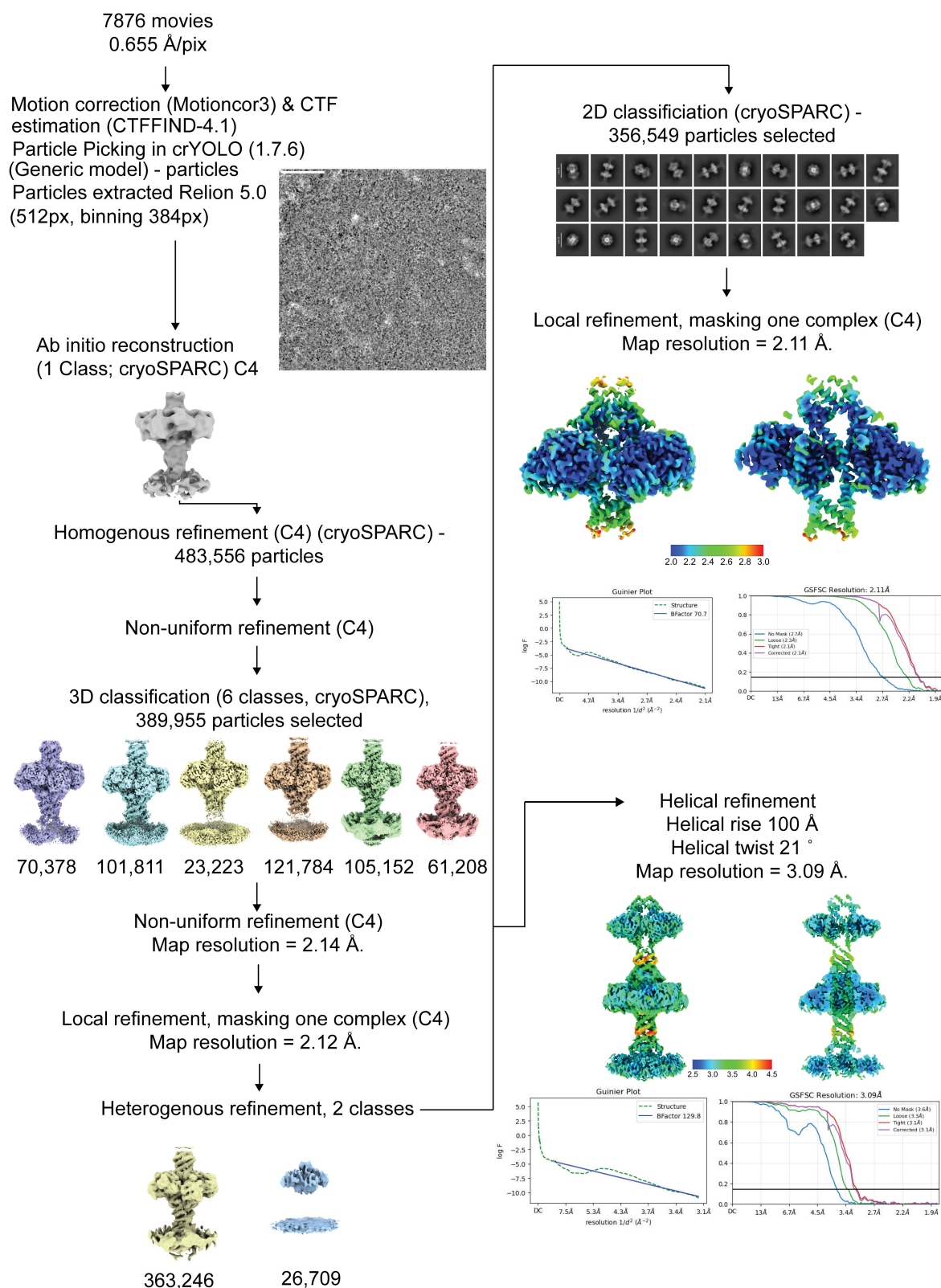
represented by the smaller number of lipids modelled in setup 2, which we used as the basis for all subsequent simulations.

Simulations also revealed an abrupt conformational change at the N-terminal end of the Ncp tube, where partial collapse closed the opening. This is unlikely to be physiological, especially considering this region is associated with the C-terminal end of a neighbouring Ncp upon filament formation. To prevent this, we introduced five lipids into the region, which stabilised it in a conformation closer to the cryo-EM structure (Extended Data Fig. 5a, setup 3). The addition of these lipids left unoccupied space between the N-terminal region of Ncp and the main chamber of the Ndh-Ncp complex, which we filled with five additional, all lipids oriented tail-first toward the tube end (Extended Data Fig. 5a, setup 4). During equilibration of this model, ~1–2 lipids spontaneously flipped, reorienting their head groups toward solvent while expelling water from the chamber. These simulations resulted in a stable model of the Ndh-Ncp-phospholipid complex, containing 30 lipids in the chamber and 5 at the N-terminal end (Extended Data Fig. 5a, setup 6), which was used for a final round of 1 μ s (x3) simulations resulting in the model discussed in this paper.

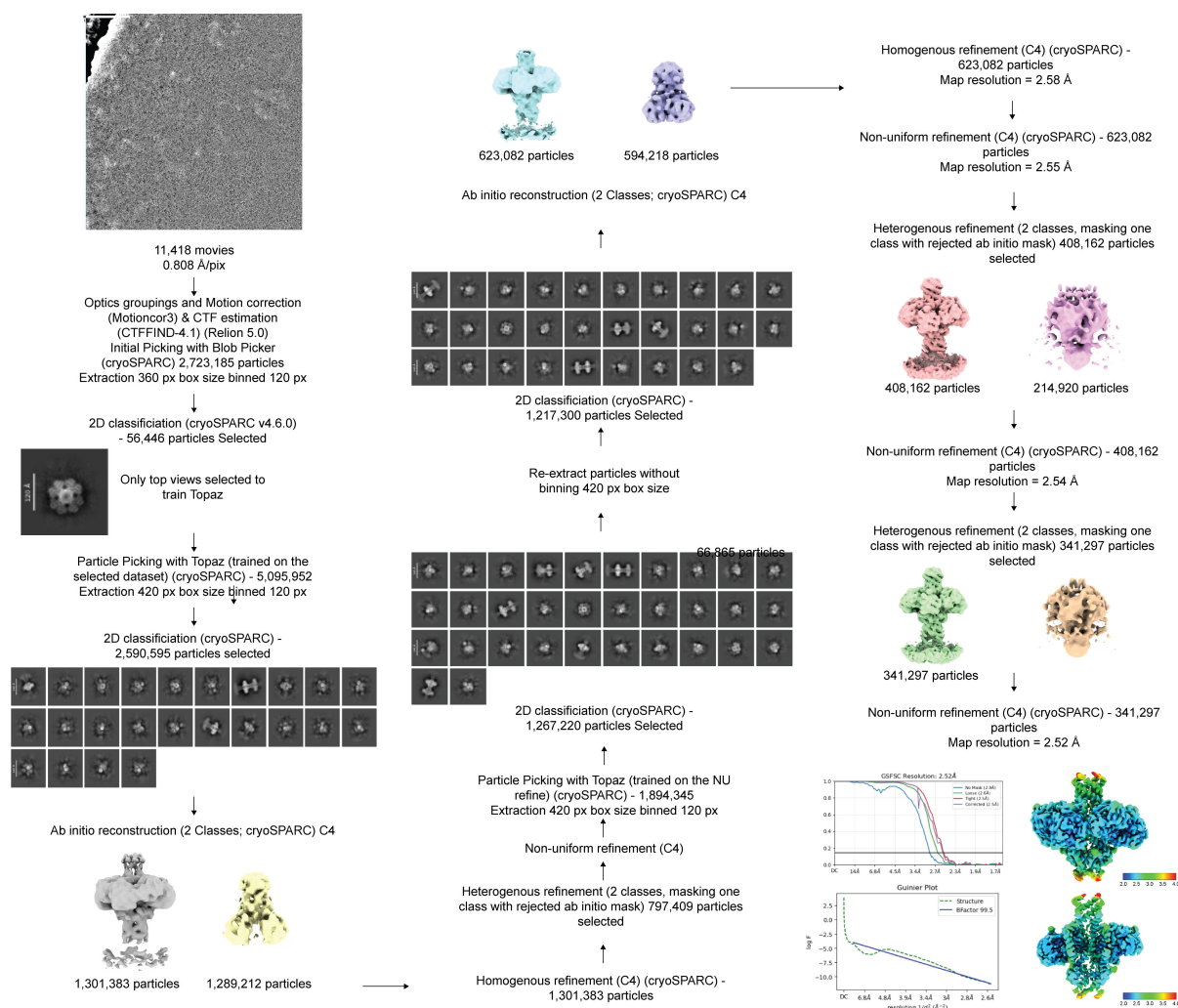
Supplementary Figures:



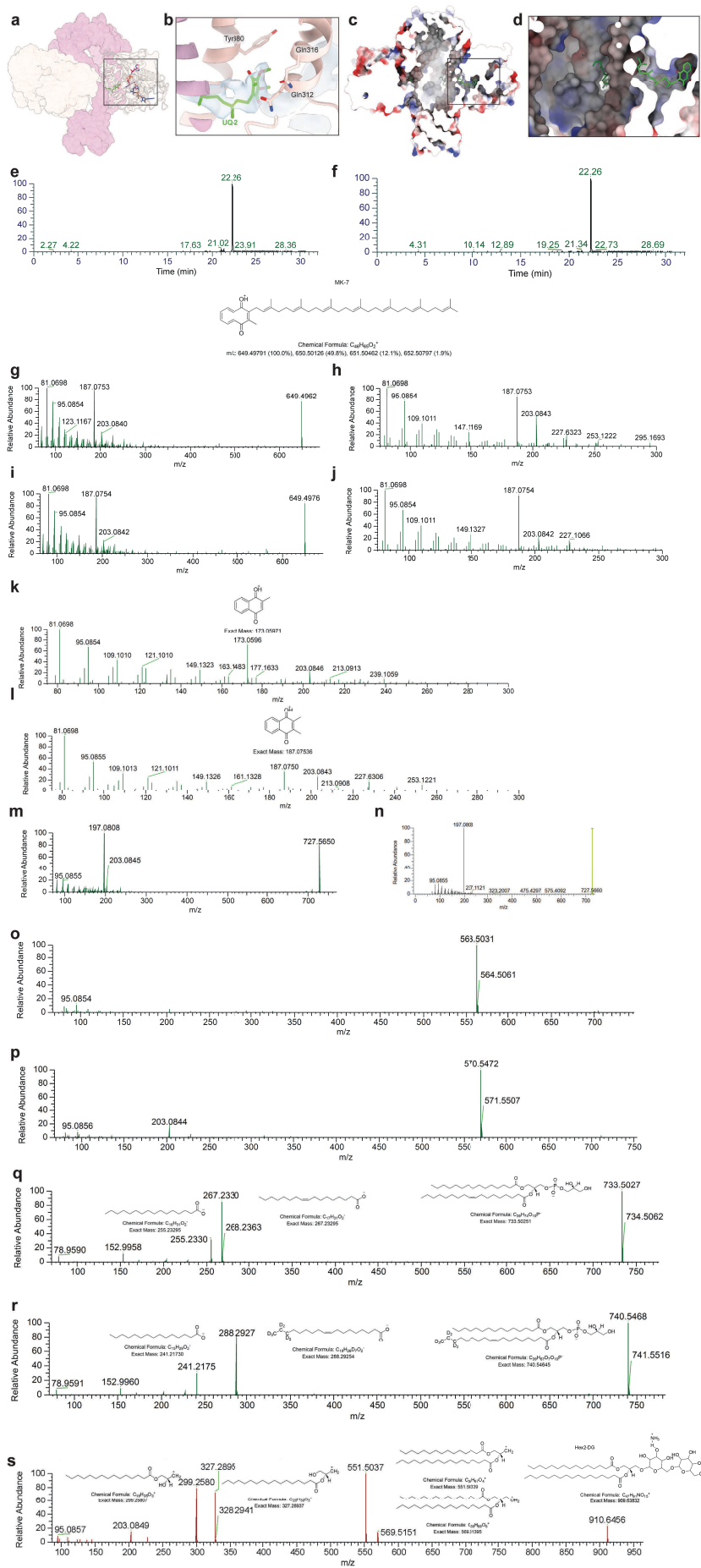
Supplementary Figure 1. Analysis of the size distribution of purified the Ndh-Ndh complex. a, SEC analysis of purified Ndh-Ncp showing fractionation of the extended elution profile, and SDS-PAGE and native-PAGE analysis of these fractions. **b-g,** mass photometry of the fractions from panel a, showing a shift towards lower molecular weight species across the SEC peak.



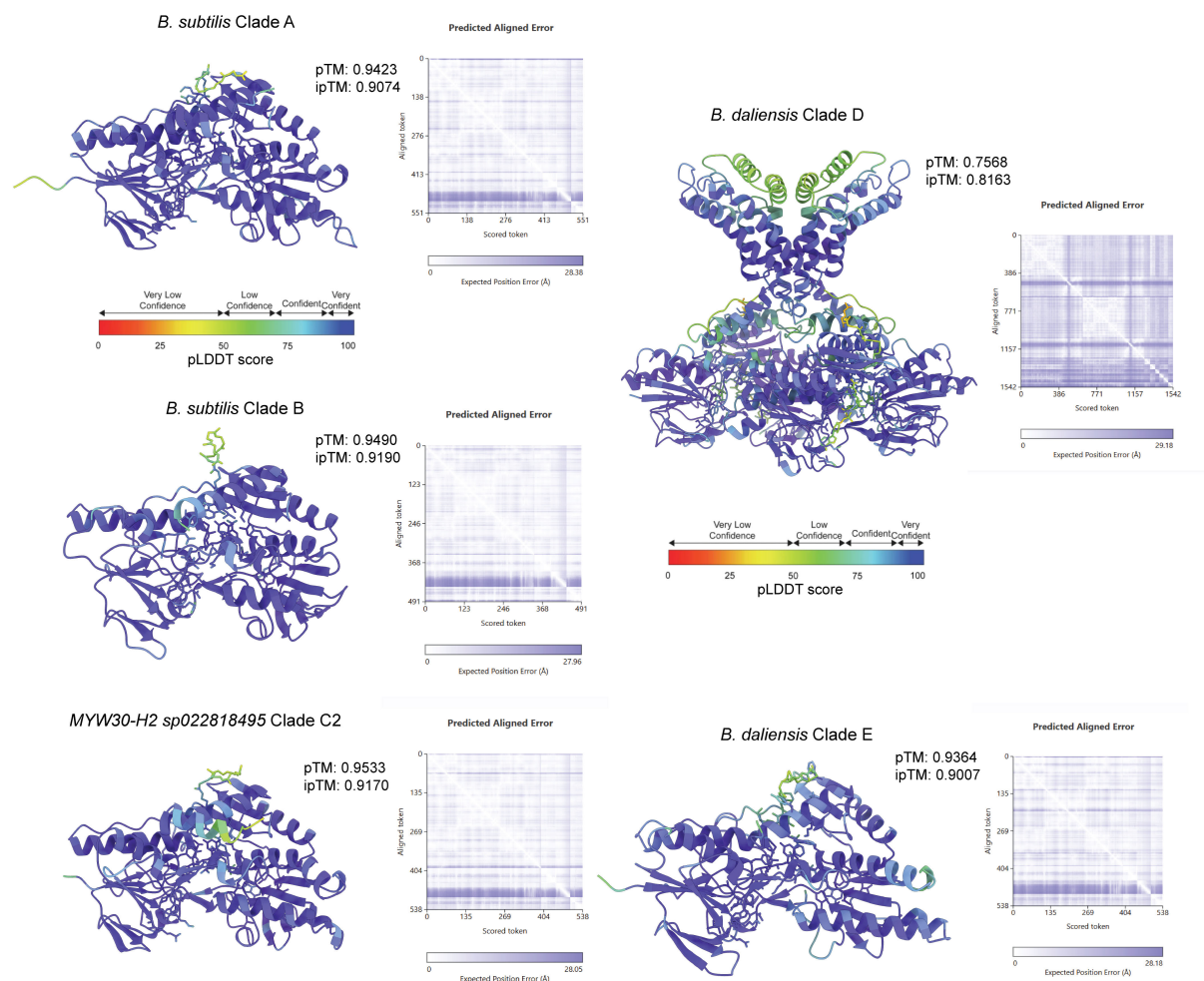
Supplementary Figure 2. Ndh-Ncp cryo-EM data processing workflow. Beginning with 7876 movies, the data was processed in Relion 5.0, crYOLO and cryoSPARC, resulting in two maps, one for the filament at 3.09 Å global resolution and one Ndh-Ncp unit at 2.11 Å global resolution.



Supplementary Figure 3. Ndh-Ncp NADH-MK-8 data processing workflow. Beginning with 11418 movies, the data was processed in Relion 5.0 and cryoSPARC, resulting in a map of 2.52 Å.



Supplementary Figure 4. Ncp quinone and lipid association. **a and b**, Cartoon representation of Ndh-Ncp with UQ-2 modelled into the quinone binding site (green). Density is shown around UQ-2. **c**, Electrostatic surface representation of Ndh-Ncp cut away down the middle vertically showing the internal tube and the MK-8 molecules interfacing into the tube. **d**, A zoom of a MK-8 molecule (with the tail length truncated based on density) showing the entrance to the Ndh quinone binding site from the Ncp tube. **e-f**, Extracted ion chromatograms for $m/z = 649.4960$ - 649.4998 for Ndh-Ncp treated with *B. subtilis* membrane (**e**) and MK-7 standard (**f**). Corresponding MS/MS spectra for the Ndh-Ncp treated with *B. subtilis* membrane (**g and h**) and MK-7 standard (**i and j**). The structure of MK-7 (inset). **k and l**, MS/MS of the feature putatively identified as the ammonium adduct of DMK-8 ($m/z = 720.57141$) in the Ndh-Ncp treated with *B. subtilis* membrane sample (**k**) and the ammonium adduct of the MK-7 standard (**l**). (Note that in neither case do we observe significant product ions above $m/z = 300$. The spectra are extremely similar. The two spectra differ in the MK-7 standard containing a product ion at $m/z 187.075$ consistent with protonated 2,3-dimethyl-1,4-napthoquinone which is absent in the putative DMK-8 spectra. Instead, the spectra for the putatively identified DMK-8 contains a product ion at $m/z = 173.0597$ which is consistent with protonated 2-methylnaphthalene-1,4-dione, the demethylated analogue of 2,3-dimethyl-1,4-napthoquinone.) **m and n**, MS/MS spectra for a Ndh-Ncp treated with *B. subtilis* membranes sample at $m/z = 788.6346$ corresponding to the putatively identified Coenzyme Q8 (UQ-8) (**m**). A screenshot showing the authentic UQ-8 MS/MS spectra from the mzCloud mass spectrometry database (**n**). **o and p**, MS/MS of the protonated ion of PE 33:1 in Ndh-Ncp treated with *B. subtilis* membrane ($m/z 703.515206$) (**o**) and the protonated ion of the PE 33:1(d7) standard (**p**). In both cases we observe essentially a single product ion corresponding to the loss of the phosphoethanolamine headgroup. **q and r**, MS/MS of the deprotonated ion of PG 33:1 in Ndh-Ncp treated with *B. subtilis* membrane ($m/z 733.5022$) (**q**) and the deprotonated ion of the PG 33:1(d7) standard (**r**). **s** MS/MS of the ammonium adduct of the putatively identified Hex2-DG 15:0_17:0. Ndh-Ncp after *B. subtilis* membrane incubation is used as the example for comparison to standards.



Supplementary Figure 5. Quality estimates for the Chai1 models of NDH-2 enzymes. NDH-2 enzymes modelled in Figure 5 shown here with colour coding to demonstrate model confidence, indicated with the pLDDT key showing confidence levels. Predicted alignment error (PAE) plots are shown on the right of each model, demonstrating the predicted error of the model, with purple showing less error and white higher error.

