Supplemental Tables

Table S1. Related to Figure 1. Data collection, refinement and model statistics.

	D form	**turnover**
Data collection		
Microscope	FEI Titan Krios	FEI Titan Krios
Camera	Gatan K3 Summit	Gatan K2 Summit
Voltage (kV)	300	300
Nominal magnification	165,000x	105,000x
Calibrated pixel size (Å)	0.5316	0.828
Electron exposure (e^-/Å^2)	50.0	40.0
Exposure time total (s)	3	8
Number of frames per image	50	40
Defocus range (µm)	-0.8 – -2.2	-0.8 – -3.2
Image processing		
Motion correction software	*MotionCor2*	*MotionCor2*
CTF estimation software	*Gctf*	*Gctf*
Particle selection software	*RELION3.1*	*RELION3.0*
Micrographs (no.)	21,770	4,776
Initial particle images (no.)	1,078,960	124,092
Final particle images (no.)	178,960	54,863
Applied *B*-factor (Å^2)	-47	-62
Final resolution Relion (Å)	2.42	3.41
Final resolution denmod (Å)	2.12	-
Refinement statistics		
Initial model	6rfr	6rfr
Modeling software	*COOT, PHENIX*	*COOT, PHENIX*
Protein residues	8035	7921
Ligands	55	56
Waters	1617	-
Map CC (volume)	0.8181	0.8158
RMS deviations	0.005	0.007
Bond lengths (Å)		
Bond angles (°)	0.797	0.878
Ramachandran plot		
Outliers (%)	0.05	0.09
Favored (%)	96.88	96.46
Rotamer outliers (%)	2.88	0.56
Molprobity score	2.06	1.91
All-atom clashscore	9.81	14.92
PDB ID	**7o71**	**7o6y**

Table S2. Related to Figures 3 and 5. MD simulation setups.

	Structure for simulation setup	Charge state	Simulation time x no. of replicas
PN1	2.1 Å	Standard	400 ns x 3
PN2	2.1 Å	Propka-based	400 ns x 3
PN3	2.1 Å L384D (ND2) F396D (ND4) D397A (ND5)	Standard	400 ns x 1
PN4	2.1 Å F343 (ND4) modelled to F324 (ND2) position	Propka-based[1]	400 ns x 1
PT1	3.4 Å	Standard	400 ns x 1
PT2	3.4 Å	Propka-based	400 ns x 3

[1]ND4 charges were set to replicate the ND2 charges calculated by Propka

Table S3. Related to Figures 3 and 5. Amino acid residues with non-standard protonation states (neutral charge state of Asp, Glu and Lys and positive charge state of His) based on Propka calculation.

Subunit	2.1 Å structure	3.4 Å structure
NDUFS1	D259	D146
	D295	D295
	D377	D377
	D556	D556
	E355	E355
	E420	E420
NDUFV1	D120	H378
	D420	D120
	E121	D420
	E142	E78
	E210	E121
	E291	E210
	K276	E291
		K276
NDUFS2	D196	D196
	D331	D331
	D363	D465
	D465	E107
	E151	E151
	E376	E165
	E379	E208
	E463	E218
		E379
		E404
		E463
NDUFS3	E179	E179
	K93	E181
		E227
		E237
		K93
NDUFV2	H28	H28
	E211	E211
	K68	
NDUFS8	E186	D163
		E137
		E186
		E195
ND1	E147	D203
	E196	E101
	E206	E147
	E210	E196
	E231	E206
	K285	E231
		K285
ND2	D39	D39
	D69	D69
	K282	K282
ND3	D67	D67
	E39	E39
	E69	E69

ND4	E142 E395 K252 K299	E58 E142 E237 E395 K252
ND5	D178 D397 E80 E503 K339 K581	D501 E80 E144 E271 E401 E453 K339 K581
ND6	K182	-
ND4L	D49 E30 E66	E30 E66
NDUFA9	H76 D210 E231	D210 E260
NDUFA5	H108 E92 E109 E110	E92 E110
NDUFS4	E72 K111	K111
NDUFA12	H78 D39	H78 D39
NDUFA6	-	E82
NDUFA2	K41	H62 E46
NDUFA11	-	H160
NDUFA13	H114 D7 E77	D7 E77
NDUFS5	E53	E18 E53
NDUFA1	H29	H29 E51 E83
NDUFB11	-	E189
NDUFB8	D74 E33	D59 D74 E33
NDUFB7	E58	H49 E55 E58
NDUFAB1	-	E94 E100
NDUFB10	E39	-

Supplemental Figures

Figure S1. Map validation. (A) FSC plots for resolution estimation and model validation of complex I in the D form. Gold-standard FSC plot between two half-maps separately refined in RELION3.1 (blue) (Zivanov et al., 2018) indicates a resolution of 2.4 Å (0.143 threshold). Map-to-model FSC for the final refined model and the RELION map (red) and the density modified map (green) indicate a resolution of 2.5 and 2.2 Å, respectively (0.5 FSC criterion). **(B)** FSC plots for complex I under turnover. Gold-standard FSC (blue curve): 3.4 Å, map-to-model FSC (red): 3.6 Å. **(C)** D form and **(D)** turnover cryo-EM map colored by local resolution as determined in RELION. Color scale in Å. **(E-G)** Details of the wt cryo-EM density before **(E)** and after **(F)** density modification in Phenix (Terwilliger 2020). **(G)** density modified map with fitted model. Red spheres: water molecules. The procedure improved the average resolution of the map from 2.4 to 2.1 Å. It had subtle effects in high-resolution areas, especially improving water densities (top), while improving the continuity of less well resolved features (bottom).

Figure S2. Examples of cryo-EM density of complex I at 2.1 Å resolution. (A) TMH ND2 152-171. **(B)** TMH ND2 229-249 with π-bulge. **(C)** salt bridge in ND1 with complete density for Glu26. **(D)** N-terminus of ND2 with formylmethionine, interacting with NUXM. **(E)** β sheet in NDUFS3. **(F)** Water molecules in the hydrophilic axis, labelled as in Figure S3. **(G)** FeS clusters in NDUFS1. **(H)** NADPH in NDUFA9. **(I)** Cardiolipin, coordinated by N-terminal Ala of NDUFA1; distances in Å are indicated.

Figure S3. Water molecules in the membrane arm of *Y. lipolytica* complex I. Side view of central membrane bound subunits and central subunits NDUFS2 and NDUFS7 of the Q module as shown in Figure 2 with numbering scheme for the 99 water molecules (red spheres) resolved in the transmembrane region and at the entrance of putative proton uptake pathways. The upper left and upper right panel show detailed views of ND2 and centered around TMH3 of ND6, respectively. In the lower panel an overlay with the 2.7 Å structure of (Grba and Hirst, 2020)(PDB ID: 6yj4; cartoon, gray) with water molecules in central membrane arm subunits (waters exclusively found in 6yj4, black spheres; waters exclusively found in this work, red; overlapping positions, blue) is shown.

Figure S4. Multiple sequence alignment of antiporter-like subunits ND2, ND4 and ND5 from *Y. lipolytica*. The TM helices and the β-strands are indicated. The residues highlighted in shades of blue are colored by sequence identity. Amino acid residues discussed in the text are colored; conserved ion-pair from TMH5 and TMH7a (turquoise), conserved Lys/His residues from central hydrophilic axis (green), Lys/Glu from TMH12b (olive green), Asp and Glu from TMH12b (orange) that form proton releasing route in ND5, conserved residues of KR motif, a potential proton uptake site (pink) and conserved Phe from TMH11, a potential "gating" residue (magenta). The TMH1, TMH15 and the lateral helix of ND5 have been removed. Residue Lys511 from TMH15 of ND5 (not shown) is conserved in mitochondrial sequences, and participates in P-side connection of ND5 subunit. Alignment is based on the structural alignment of three subunits, and was prepared using Jalview (Waterhouse et al., 2009).

Figure S5. Molecular dynamics simulation setup and its stability. (A) Model system of respiratory complex I in membrane-solvent environment. Protein subunits are colored as in main text Figure 1. Lipid head groups are shown as orange spheres and lipid tails are shown in transparent red. The blue box shows the periodic boundaries, which is filled with water and Na and Cl ions (omitted for clarity). **(B-E)** Root mean square deviation (RMSD, in nm) was measured for the protein backbone atoms of all subunits for different simulation setups. The starting configuration (frame 0) of the simulation was used to align the trajectories, and as the reference for the calculation.

Figure S6. Hydration in the "E channel" region and induced opening of P-side connections in ND2/4 subunits. (A) For the E channel region (ND1, hot pink; ND3, yellow; ND4L, red; ND6, orange), the volumetric mesh is shown from MD setup PN1 with all titratable residues in their charged states and **(B)** from MD setup PN2 with protonation states as in the inset table. Water molecules (red spheres) within 6 Å from residues shown in stick representation were selected from entire simulation data. The isovalue of the mesh in panels (A) and (B) is 0.20. **(C)** Effect of introducing F396D (ND4) and **(D)** L384D (ND2) *in-silico* point mutations on the hydration of the P-side regions of antiporter-like subunits. **(E)** Hydrophobic residues Phe396^{ND4} and **(F)** Leu384^{ND2} insulate the protein interior from the aqueous phase at the P side of the membrane in the wild-type enzyme. Panels (C) and (D) are from simulation setup PN3, while (E) and (F) are from PN1. Water molecules are shown within 6 Å of the mutated residues and selected residues shown in Figure 3A. The mesh was calculated by selecting water molecules within 6 Å from these selected residues from the entire simulation data. The isovalue of the mesh is 0.10 in panels C-F. Water molecules are shown in red spheres and residues in sticks using pink (ND2) and cyan (ND4) for carbon atoms, blue for nitrogen and red for oxygen. Subunit coloring follows the same scheme as Figure 3A.

Figure S7. Water content in central hydrophilic axis of antiporter-like subunits. The left and right panels show number of water molecules from selected residues in central hydrophilic axis from PN1 and PN2 simulations, respectively. Data from PN2 simulations is closer to structural water content (shown by dotted lines). The three MD replicas are shown for each simulated state in different colors. The bold lines are running average over 20 simulation frames.

Figure S8. Membrane bending and lipid-protein interactions. Lipid bilayer bends and adjusts according to protein structure in MD simulations of complex I. **Inset** shows the unique lipid-protein architecture near putative proton exit route in ND5 subunit, in part induced by tilted TMHs of accessory subunits NDUFB3 and NDUFB2.

Figure S9. Overall conformational changes in complex I under turnover. (A) Sideview of *Y. lipolytica* complex I (deactive, gray; turnover, blue), **(B)** view from the backside, **(C,D)** same views as in (A,B) for an overlay of closed (pink, PDB ID: 6zkc) and open (green, PDB ID: 6zkd) state of ovine complex I (overlayed on subunit ND1), **(E)** same orientation as in (A) showing detailed view of the membrane arm, orange arrows indicate relative movement of P_D module, the orange box encloses an area with weak cryo-EM density for complex I under turnover, **(F)** orange surface highlights protein structure for which cryo-EM density is weak or missing for complex I under turnover, including a section of the long N-terminal extension of NDFUS2, the C-terminus of NDUFA9 and TMH4 and the TMH3-4 loop of ND6, **(G)** segmented map of ND6 from the 2.1 Å complex I map, **(H)** fitted model, **(I)** 3.4 Å map of ND6 under turnover (this work), **(J)** 4.5 Å map, EMD-4385 (Parey et al., 2018); G-J colored from blue (N-terminus) to pink (C-terminus). Under turnover, density for the TM3-4 loop and for TM4 is weak, indicating mobility of these regions, while TM1-3 and TM5 including the π-bulge region of TM3 are rigid.

Figure S10. NADH oxidation and Q reduction site of complex I. (A) Topology of electron transfer reactions in the peripheral arm, **(B)** density for substrate NADH and the primary electron acceptor FMN in the NADH oxidation site, selected residues participating in NADH binding are shown in stick representation. **(C)** Q reduction site of *Y. lipolytica* complex I captured during steady state activity (DBQ, yellow; compare Figure 4) overlayed with complex I from sheep (salmon, PDB ID: 6zkc) and **(D)** with complex I from *T. thermophilus* (gray, PDB ID: 6i0d). In *Y. lipolytica* and ovine complex I the distance between the tyrosine residue near cluster N2 and Q is too long for a hydrogen bond. A different mode of Q binding is observed in complex I from *T. thermophilus* where Q is bound in a tyrosine – histidine ligation.

Figure S11. Hydration bridge between acidic residues of ND3 and ND4L. (A) Overlay of two MD simulation snapshots showing two conformations of Ile62 of ND6 subunit. Change in conformation of Ile62 and surroundings either closes **(B)** or opens **(C)** the route leading to water-influx in the dry region connecting conserved acidic residues of ND3 and ND4L subunits. The volumetric mesh (0.2 isovalue) is calculated over entire simulation trajectory (setup PN1, simulation replica 3 for panel B and simulation replica 2 for panel C).

Movie 1. Cryo-EM density (grey mesh) and model (protein in stick representation; inorganic sulfur, iron and water shown as spheres) close to FeS cluster N2 (colors compare Figure 1).

Movie 2. Global conformational changes in respiratory complex I of *Y. lipolytica*. The morph shows the change between complex I in the D form and complex I under turnover conditions. The P_D module slightly moves towards the matrix side and towards the P_P module. There are significant conformational changes at the junction of peripheral arm and membrane arm, most notably in ND1.

Movie 3. Conformational changes in ND1 and in the Q binding site. The movie starts with a still image that shows labeling of selected residues. The morph between complex I in the D form and complex I under turnover conditions shows bending of TMH4 of ND1, conformational changes of the ND1 TMH5-6 loop and connected changes in and around the Q binding site. The following short sequence displays the position of the Q molecule in the turnover structure. The movie continues with restoration of the initial state.

References for Supplement

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Waterhouse, A.M., Procter, J.B., Martin, D.M., Clamp, M., and Barton, G.J. (2009). Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics *25*, 1189-1191.
Zivanov, J., Nakane, T., Forsberg, B.O., Kimanius, D., Hagen, W.J., Lindahl, E., and Scheres, S.H.W. (2018). New tools for automated high-resolution cryo-EM structure determination in RELION-3. eLife *7*, e42166.