

Supplementary Information

Dissected Antiporter Modules Establish Minimal Proton-Conduction Elements of the Respiratory Complex I

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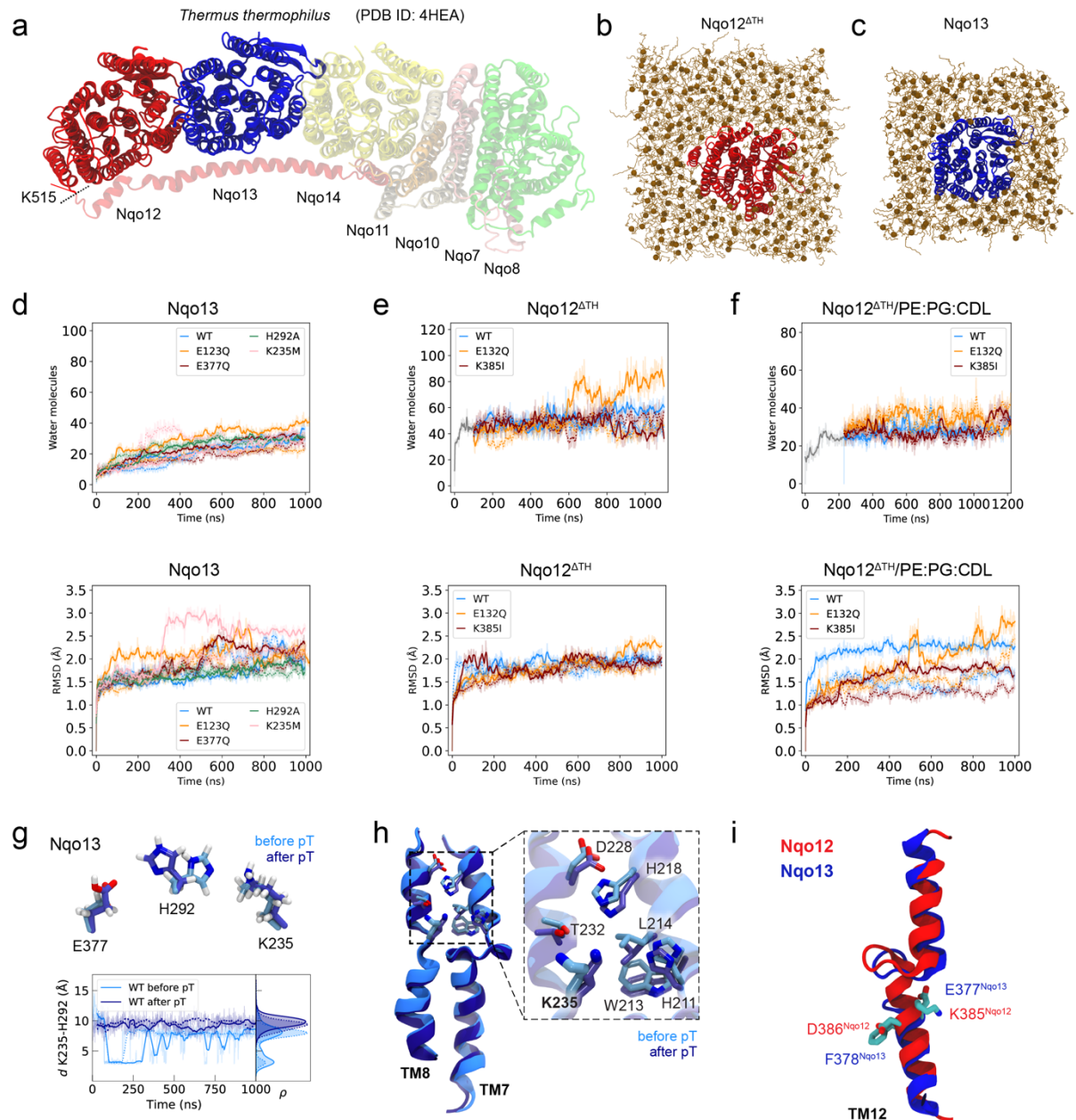
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Supplementary Methods

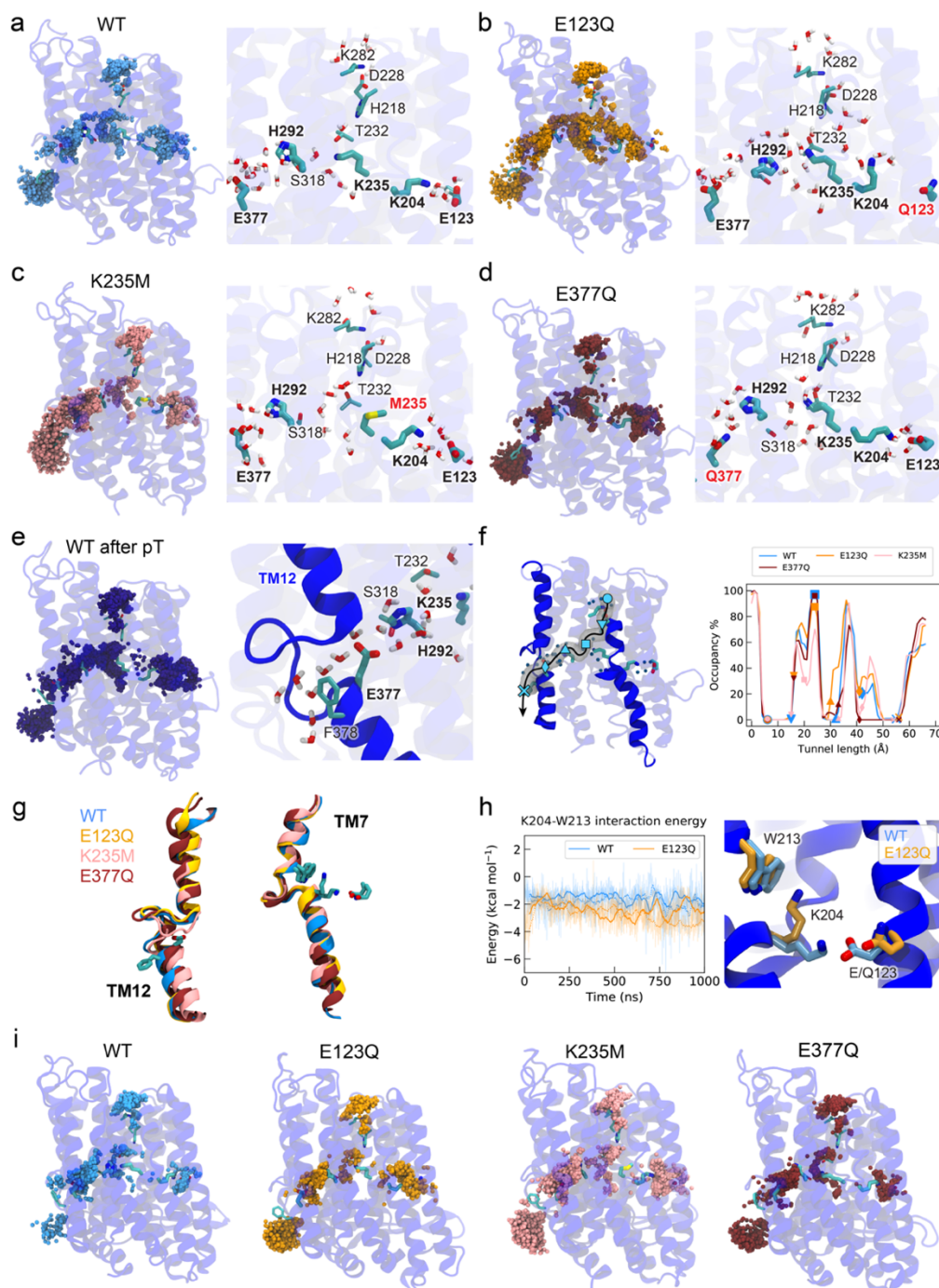
Derivation of an analytical model for ion-pair induced barrier tuning
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Supplementary Discussion

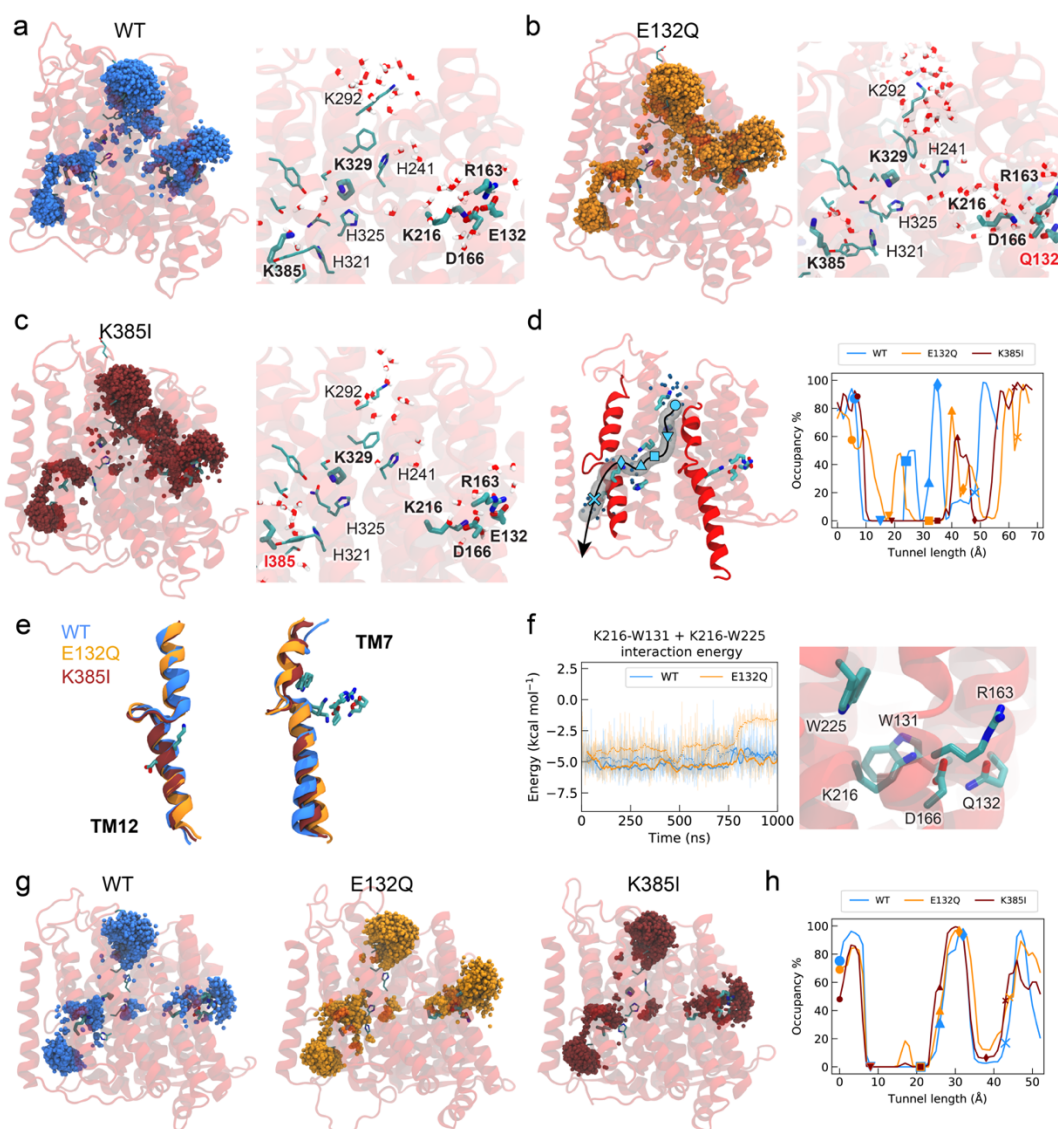
Supplementary References



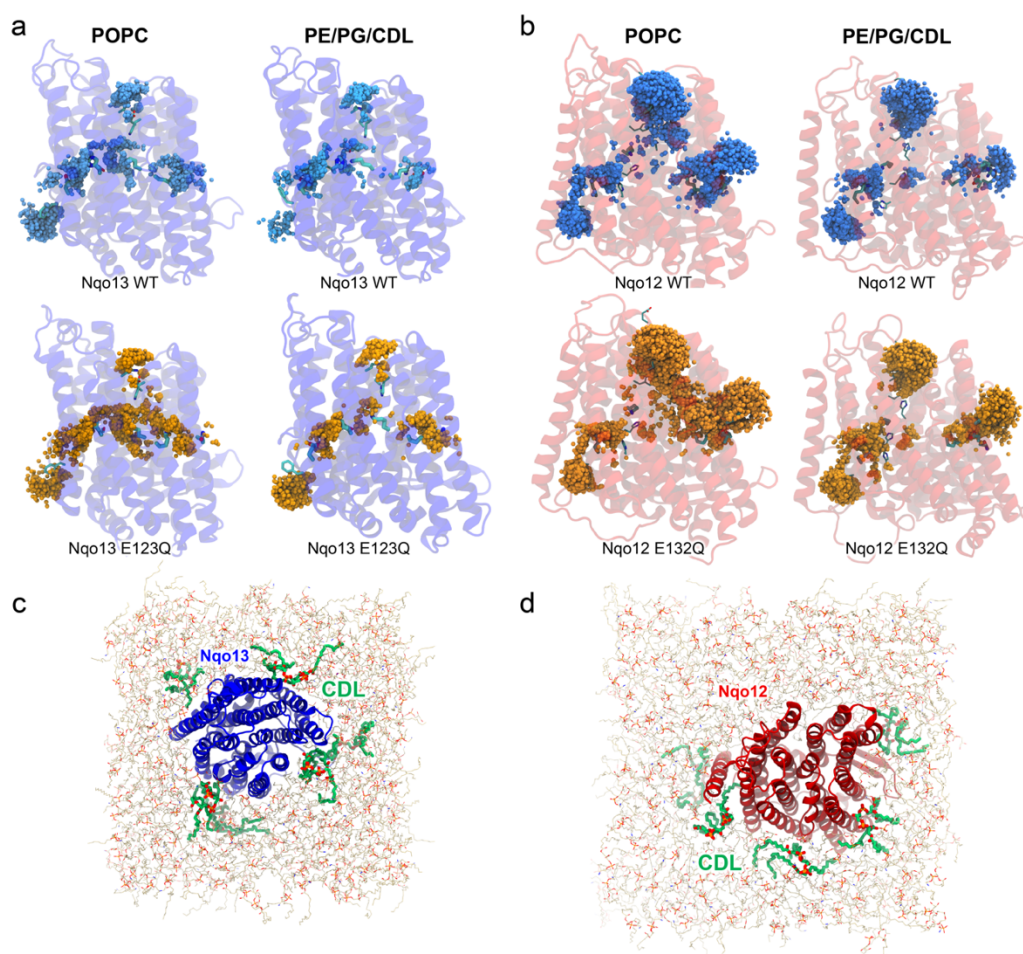
Supplementary Figure 1 | MD simulation of the dissected antiporter-like subunits. **a)** The coordinates of Nqo12 and Nqo13 were obtained from *T. thermophilus* Complex I (PDB ID: 4HEA). The transverse helix of Nqo12 was removed by truncating the protein at position 515 (Nqo12^{ΔTH}), leaving Lys515 as the new C-terminus. **b)** Nqo12^{ΔTH} and **c)** Nqo13 embedded in a POPC lipid membrane (see *Methods*). **d-f)** RMSD (*bottom*) and number of water molecules within 5 Å of proton pathway (*top*) during the MD simulations of Nqo13 and Nqo12^{ΔTH} constructs. **e, f)** Probing the effect of the membrane composition for the different Nqo12^{ΔTH} constructs with MD simulations in **e)** POPC, and **f)** POPE:POPG:CDL membranes. **g)** *Top*: conformation of residues along the lateral proton pathway in Nqo13 (light blue: Lys235⁺/His292⁰/Glu377⁻, dark blue: Lys235⁺/His292⁰/Glu377⁰). *Bottom*: Distance between Lys235 (N ϵ) and His292 (N δ) during MD simulations in Lys235⁺/His292⁰/Glu377⁻ (light blue) Lys235⁺/His292⁰/Glu377⁰ (dark blue) states. **h)** Conformational changes in conserved residues in TM7 and TM8 at the N-side proton pathway, upon proton transfer from Lys235 to Glu377 (light blue, before / dark blue, after proton transfer). **i)** Alignment of TM12 helix in Nqo12 (red) and Nqo13 (blue), showing the position of the terminal residues along the proton pathway (K385^{Nqo12}, E377^{Nqo13}) and the P-side exit (D386^{Nqo12}, F378^{Nqo13}). Data are provided in the Source Data file.



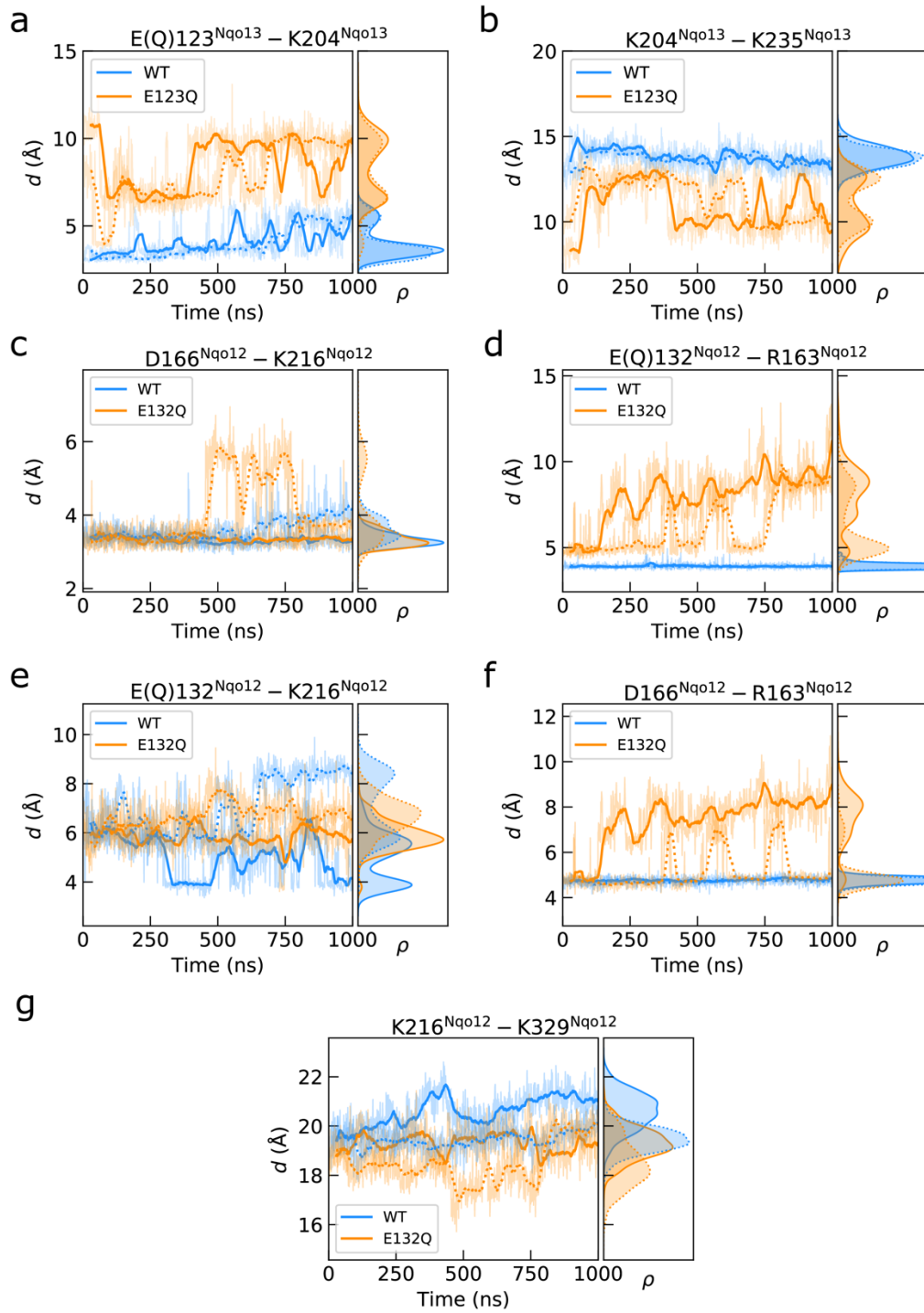
Supplementary Figure 2 | Analysis of the proton channels in Nqo13 constructs. **a-f** *Left*: Hydration along the proton transfer pathway and ion-pair region, averaged over 1 μ s MD simulations. *Right*: MD snapshot showing water connectivity and polar residues for **a**) WT before proton transfer from Lys235 to Glu377, **b**) E123Q, **c**) K235M, **d**) E377Q and **e**) WT after proton transfer from Lys235 to Glu377, with TM12 shown in solid colour (*inset, right*). **f**) Hydration level along the proton pathways for the different Nqo13 variants. Probe sites are indicated with symbols in the structure (*left*) and the hydration profile (*right*). **g**) Conformations of the transmembrane broken helices TM12 and TM7 in the different Nqo13 constructs. **h**) The conserved Trp213 stabilises Lys204 by π -cation interactions in the open ion-pair conformation. The figure shows the interaction energy between Lys204-Trp213 (*left*) and structures from MD simulations (from independent replicas) of WT and E123Q (*right*). **i**) Hydration along the proton pathways in the Nqo13 constructs (WT, E123Q, K235M and E377Q). The data is averaged over 0.5 μ s MD simulations in a PE:PG:CDL membrane (see Methods). Data are provided in the Source Data file.



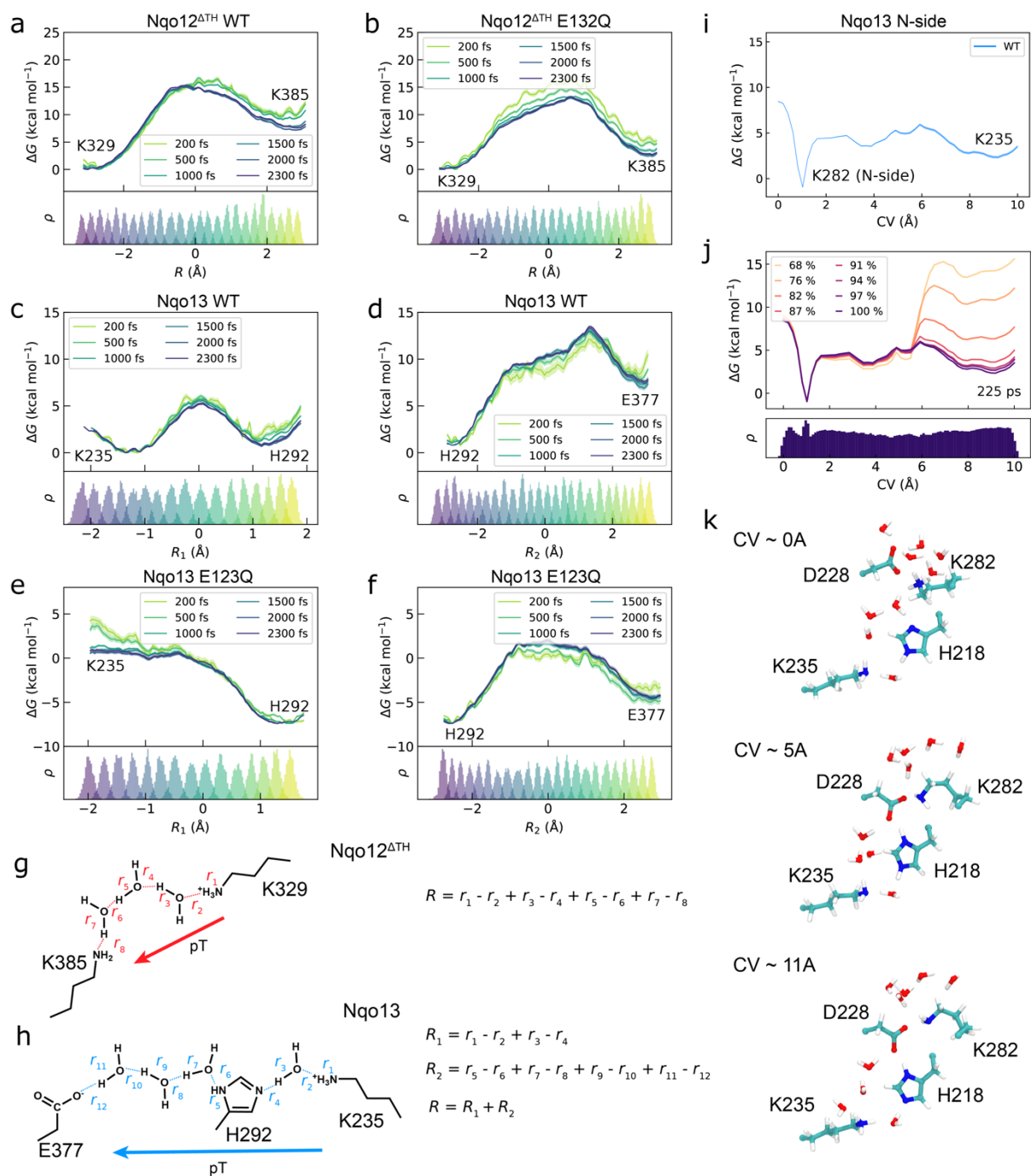
Supplementary Figure 3 | Analysis of the proton channels in Nqo12^{ATH} constructs. **a-c)** *Left:* Hydration along the proton transfer pathway and ion-pair region, averaged over 1 μs simulations in a POPC membrane. *Right:* MD snapshot showing water connectivity and polar residues in **a)** WT, **b)** E132Q, and **c)** K385I. **d)** Hydration level along the proton pathways (*left*) for the different Nqo12^{ATH} constructs. **e)** Conformation of the transmembrane broken helices TM12 and TM7 in the different Nqo12^{ATH} constructs. **f)** Trp225/Trp131-K216 interaction in WT and ion-pair mutant. **g)** Hydration along the proton pathways in the Nqo12^{ATH} constructs (WT, E132Q, and K385I). The data is averaged over 1 μs MD simulations in a PE:PG:CDL membrane (see *Methods*). **h)** Hydration profile along the proton pathway in the Nqo12^{ATH} constructs. Data are provided in the Source Data file.



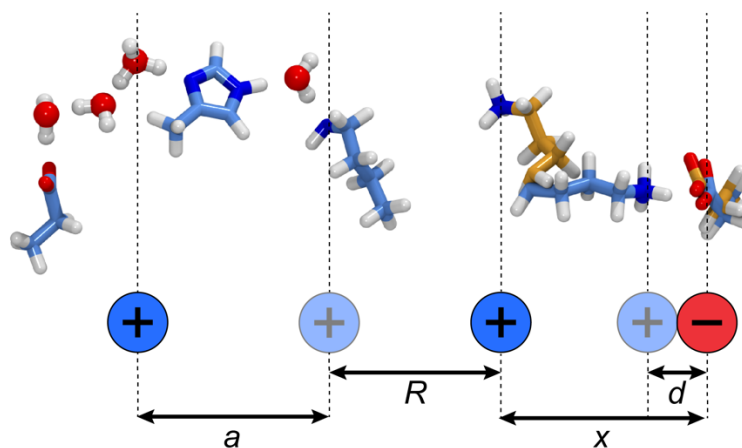
Supplementary Figure 4 | Effect of the membrane composition and protein-lipid interactions. a, b) Hydration along the proton pathways in (a) Nqo13 and (b) Nqo12^{ΔTH} constructs in a pure POPC membrane and in a PE:PG:CDL mixture for the WT (water molecules in blue) and ion-pair mutant (water molecules in orange) simulations. **c, d)** Cardiolipin (CDL) molecules stabilise the protein-lipid interface in the MD simulations with the PE:PG:CDL membrane for **c**, Nqo13 and **d**, Nqo12^{ΔTH}.



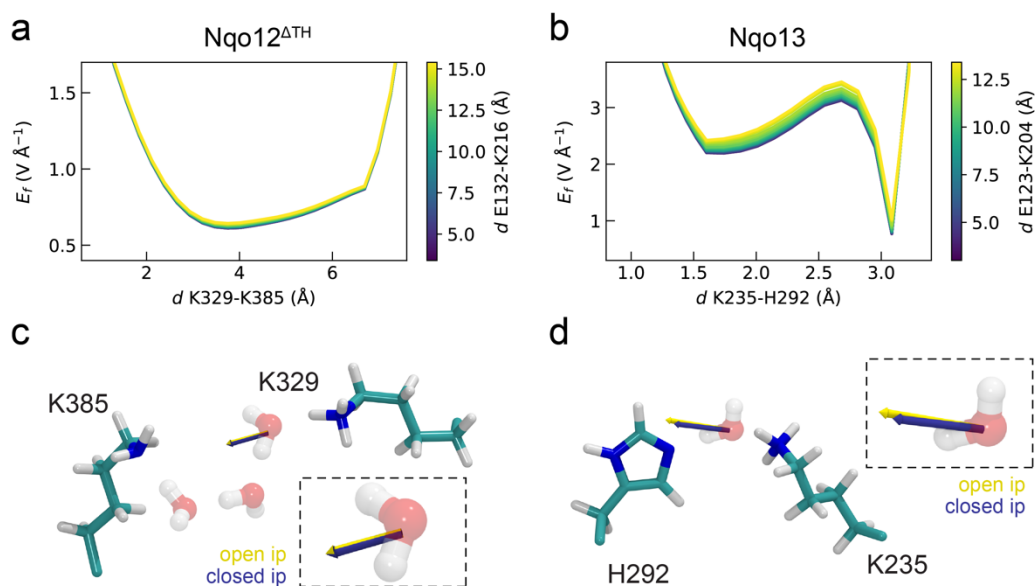
Supplementary Figure 5 | Ion-pair dynamics in Nqo13 and Nqo12^{ATH} constructs. Blue traces correspond to WT, and orange traces to ion-pair mutant constructs. Distances between **a)** E/Q123-K204 and **b)** K204-K235 in Nqo13. Distances between **c)** D166/K216, **d)** E/Q132-R163, **e)** E/Q132-K216, **f)** D166-R163), and **g)** K216-K329 in Nqo12^{ATH}. Data are provided in the Source Data file.



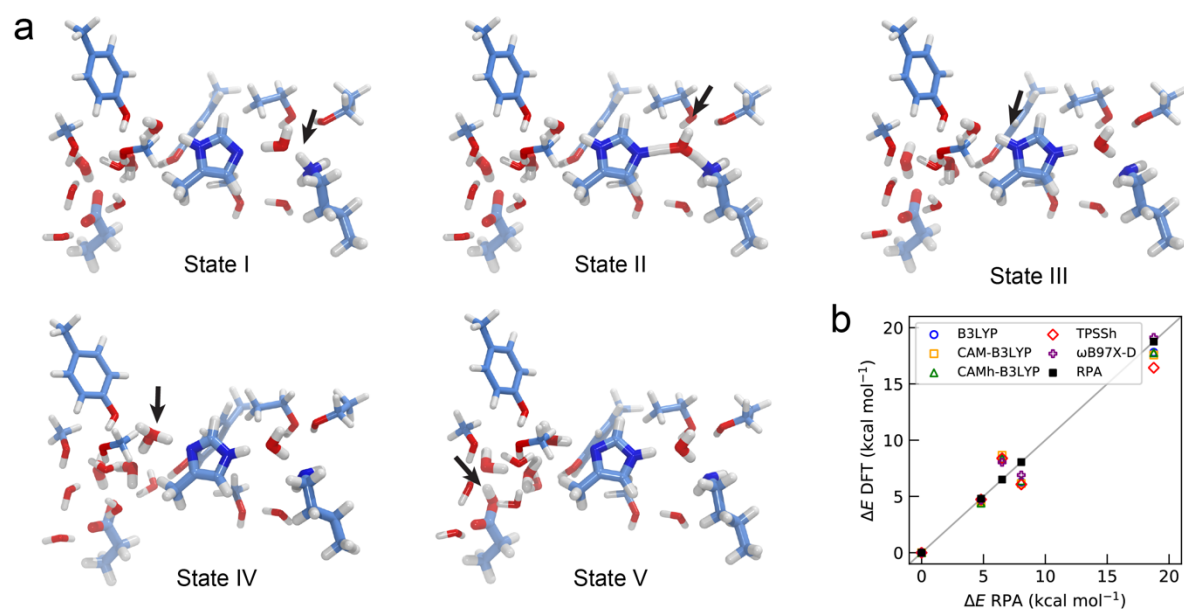
Supplementary Figure 6 | Simulation details of the QM/MM calculations. **a-f)** Convergence of free energy profiles (top) and overlap of the umbrella sampling windows (*bottom*) for the lateral proton transfer reactions in the different systems. Proton transfer from K329 to K385 in **a)** WT and **b)** E132Q variant of Nqo12^{ΔTH}. Proton transfer from **c)** K235 to H292 and **d)** H292 to E377 in WT-Nqo13. Proton transfer from **e)** K235 to H292 and **f)** H292 to E377 in E123Q -Nqo13. **g-h)** The reaction coordinate (R) for the lateral proton transfer reaction in **g)** Nqo12^{ΔTH} and **h)** Nqo13, defined as a linear combination of bond-breaking and bond-forming distances. **i)** The free energy of proton uptake from the N-side to the middle Lys235 in Nqo13, using the modified centre of excess charge (mCEC) as a reaction coordinate / collective variable (CV (Å), see *Methods*). **j)** Convergence of the MWE-QM/MM free energy profiles with increasing sampling time. **k)** Snapshots along the N-side proton transfer reaction at different CVs, from the N-side (CV = 0 Å) to the middle Lys235 (CV = 11 Å). Data are provided in the Source Data file.



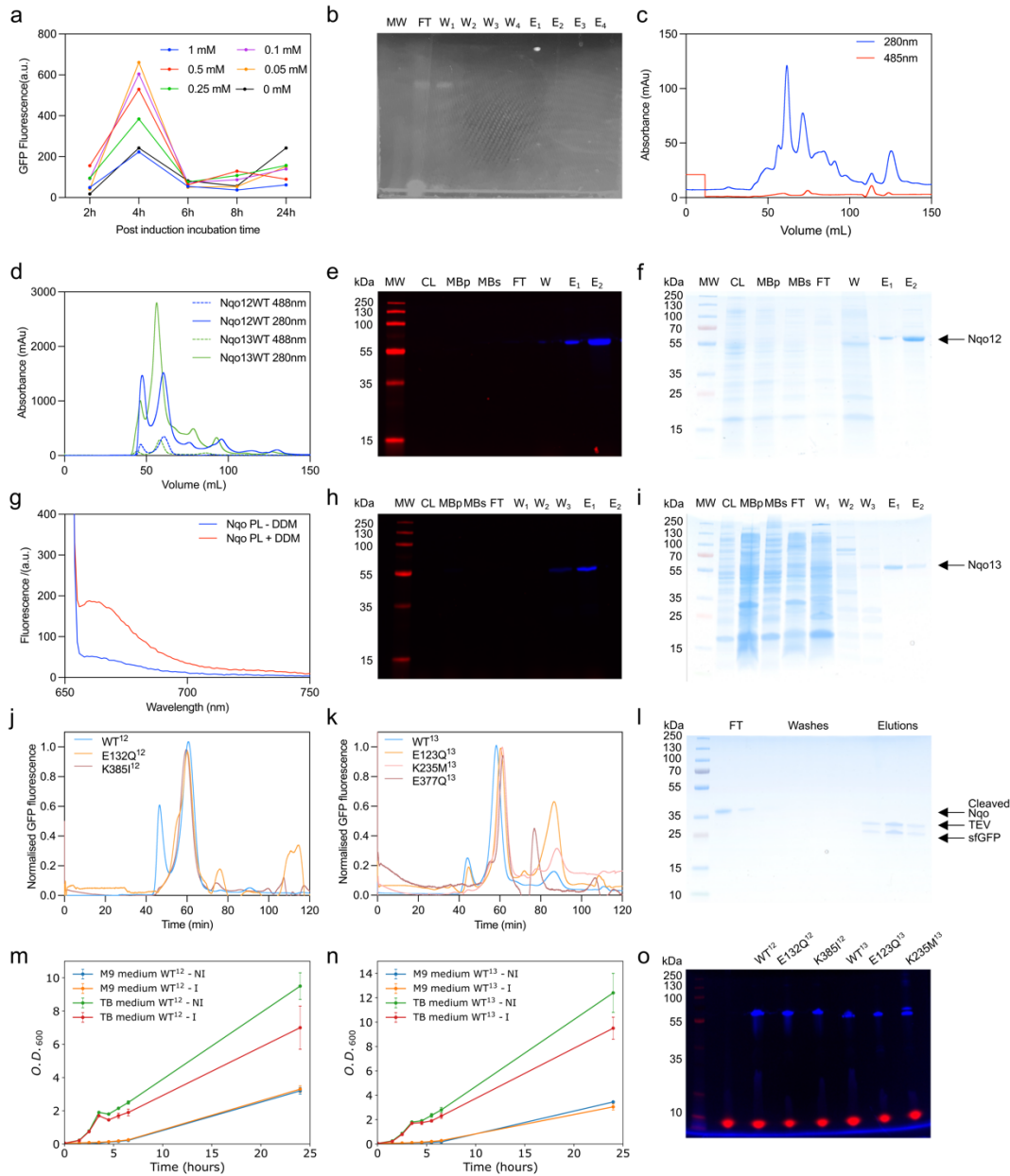
Supplementary Figure 7 | Analytical model geometry for electrostatic tuning effects. R - distance between ‘middle Lys’ and ‘ion-pair Lys’; d - distance between ion-pairs in closed conformation; x - distance between ion-pairs in the open conformation; a - position of transition state relative to the ‘middle Lys’.



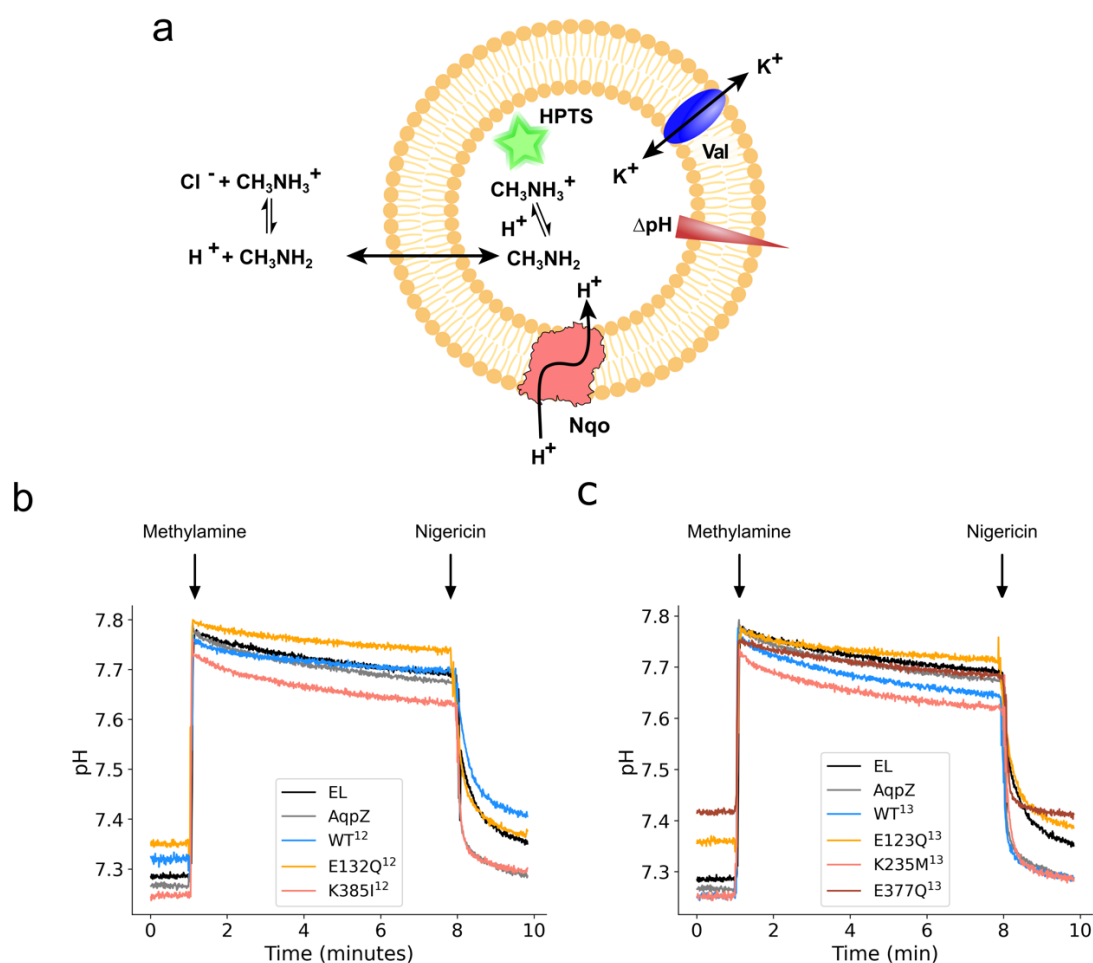
Supplementary Figure 8 | Electric field effects along the proton transfer pathways. Opening of the ion-pair induces an electric field along the proton transfer pathway in QM/MM calculations. **a)** Electric field strength along the proton transfer pathway from K329 to K385, with varying K216-E132 ion-pair distances (closed conformation in blue, open conformation in yellow) for Nqo12. **b)** Electric field strength along the proton transfer pathway from K235 to H292, with varying K204-E123 ion-pair distances (closed conformation in blue, open conformation in yellow) for Nqo13. **c-d)** Electric field vectors (closed ion-pair (blue)/open ion-pair (yellow)) for proton pathways in **c)** Nqo12 and **d)** Nqo13. Insets show a closeup of the electric field vectors. Data are provided in the Source Data file.



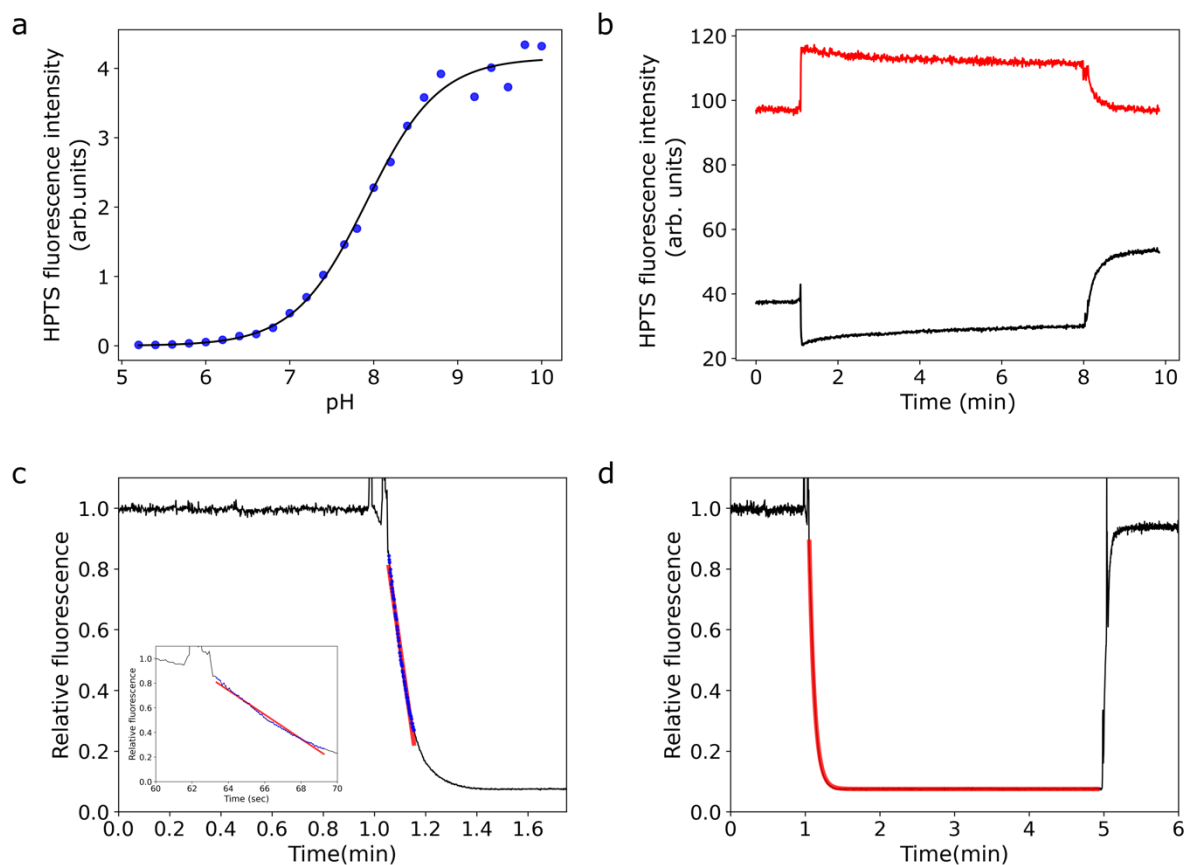
Supplementary Figure 9 | Benchmarking the proton transfer energetics. **a)** QM model systems, comprising 137 atoms, employed for the benchmarking calculations of the proton transfer reactions. The position of the proton in the different states (states I - V) is indicated by an arrow. **b)** DFT electronic energies with different functionals vs. RPA energies. See also Supplementary Table 8. Data are provided in the Source Data file.



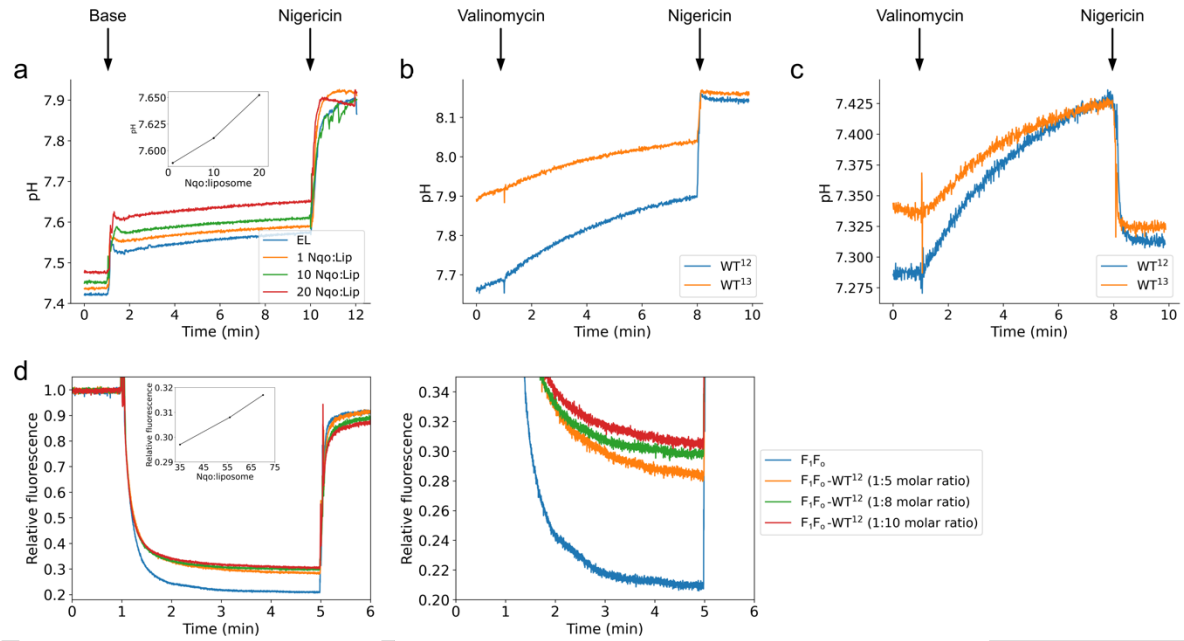
Supplementary Figure 10 | Construct design, expression, purification, and stability of dissected antiporter-like modules. **a**) Nqo12^{ATH} expression trial using BL21 strain followed by GFP in-cell fluorescence. **b**) In-gel fluorescence of Nqo12^{ATH}-sfGFP (without linker) monitoring IMAC fractions. **c**) Size-exclusion chromatography profile showing the absorbance at 280 nm and GFP excitation at 488 nm of Nqo12^{ATH}-sfGFP without linker expressed in BL21. **d**) Size-exclusion chromatography profiles showing the absorbance at 280 nm and GFP excitation at 488 nm. **e**) In-gel fluorescence and **f**) Coomassie gel of Nqo12^{ATH} following the purification fractions (CL: cell lysate, MBp: membranes pellet, MBs: membrane centrifugation supernatant, FT: flow through, W: wash, E₁: IMAC elution, E₂: SEC elution). **g**) Nqo orientation in liposomes measured using the NTA-Atto 647 N fluorescent label. **h**) In-gel fluorescence image and **i**) Coomassie gel of Nqo13 following the purification fractions (CL: cell lysate, MBp: membranes pellet, MBs: membrane centrifugation supernatant, FT: flow through, W₁₋₂₋₃: washes, E₁: IMAC elution, E₂: SEC elution). SEC profiles for **j**) Nqo12 and **k**) Nqo13, eluted at 60 mL, showing GFP fluorescence followed at 488 nm. **l**) Gel showing sfGFP removal by overnight cleavage with TEV protease. Growth tests performed in TB and minimum medium (M9) for **m**) Nqo12 and **n**) Nqo13 in induced (I) and non-induced (NI) conditions. **o**) In-gel fluorescence assessing the reconstitution efficiency of the Nqos into proteoliposomes. Data are provided in the Source Data file.



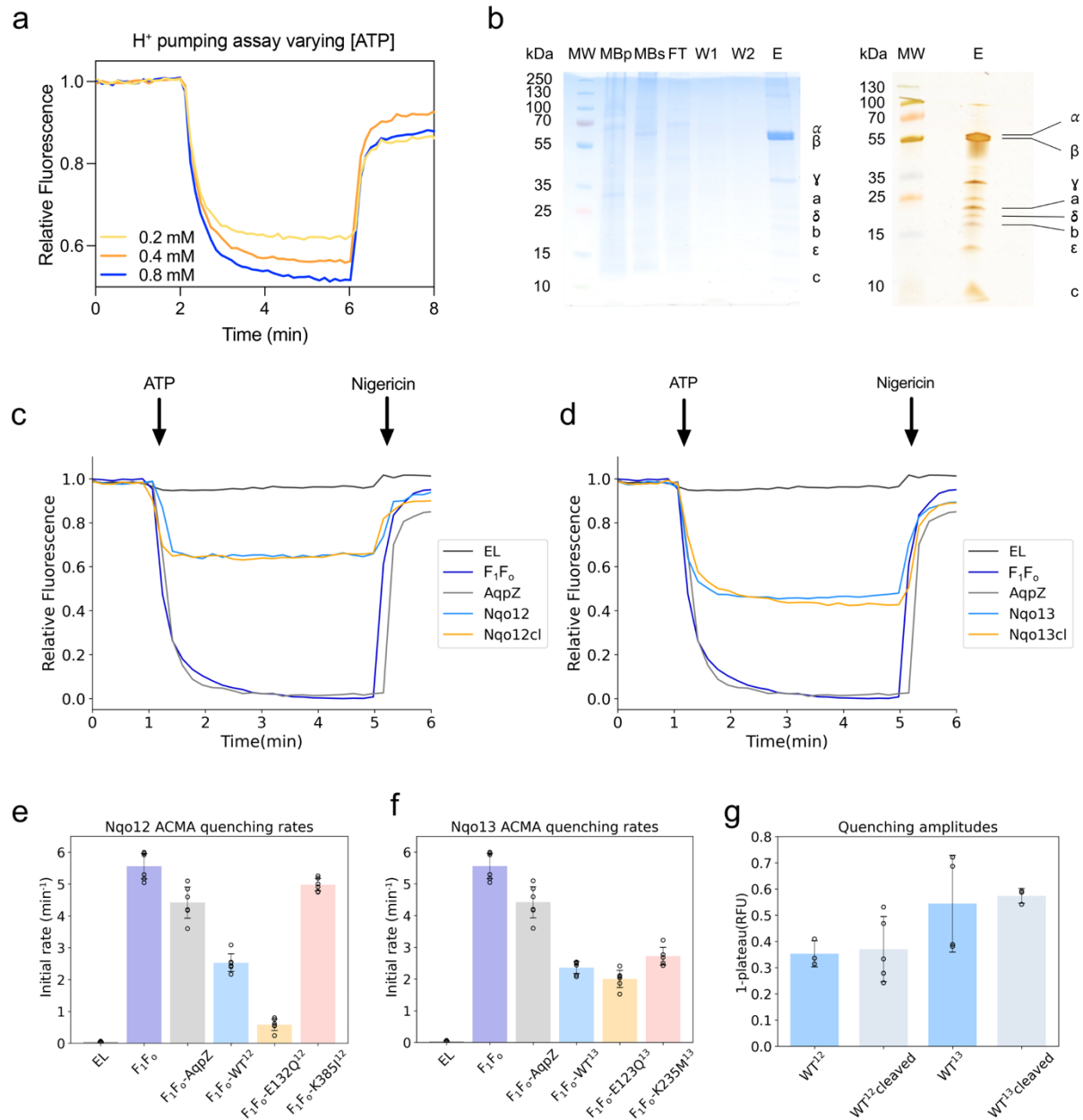
Supplementary Figure 11 | Biophysical characterisation of proton conduction properties in dissected antiporter-like modules. **a)** Proteoliposome assays for probing the proton conduction kinetics in the dissected antiporter-like subunits with pyranine (HPTS) by addition of $\text{CH}_3\text{NH}_3^+\text{Cl}^-$. Proton conduction in **b)** Nqo12 and **c)** Nqo13 proteoliposomes upon addition of 10 mM methylamine hydrochloride. Methylamine ($\text{pK}_a=10.6$) addition leads to smaller kinetic differences between the constructs as compared to the acetate ($\text{pK}_a=4.8$) assays, possibly due to the shifted equilibrium between the acid and base forms ($[\text{acid}]/[\text{base}] = 10^{3.4}$ amine vs. $10^{2.4}$ acetate at pH 7.2), secondary reactions between hydroxide ions and CH_3NH_3^+ , and/or differences in membrane permeability of the weak acid/base ($P_{\text{CH}_3\text{COOH}} = 6.9 \times 10^{-3} \text{ cm s}^{-1}$, $P_{\text{CH}_3\text{NH}_2} = 9 \times 10^{-1} - 8 \times 10^{-2} \text{ cm s}^{-1}$). Data are provided in the Source Data file.



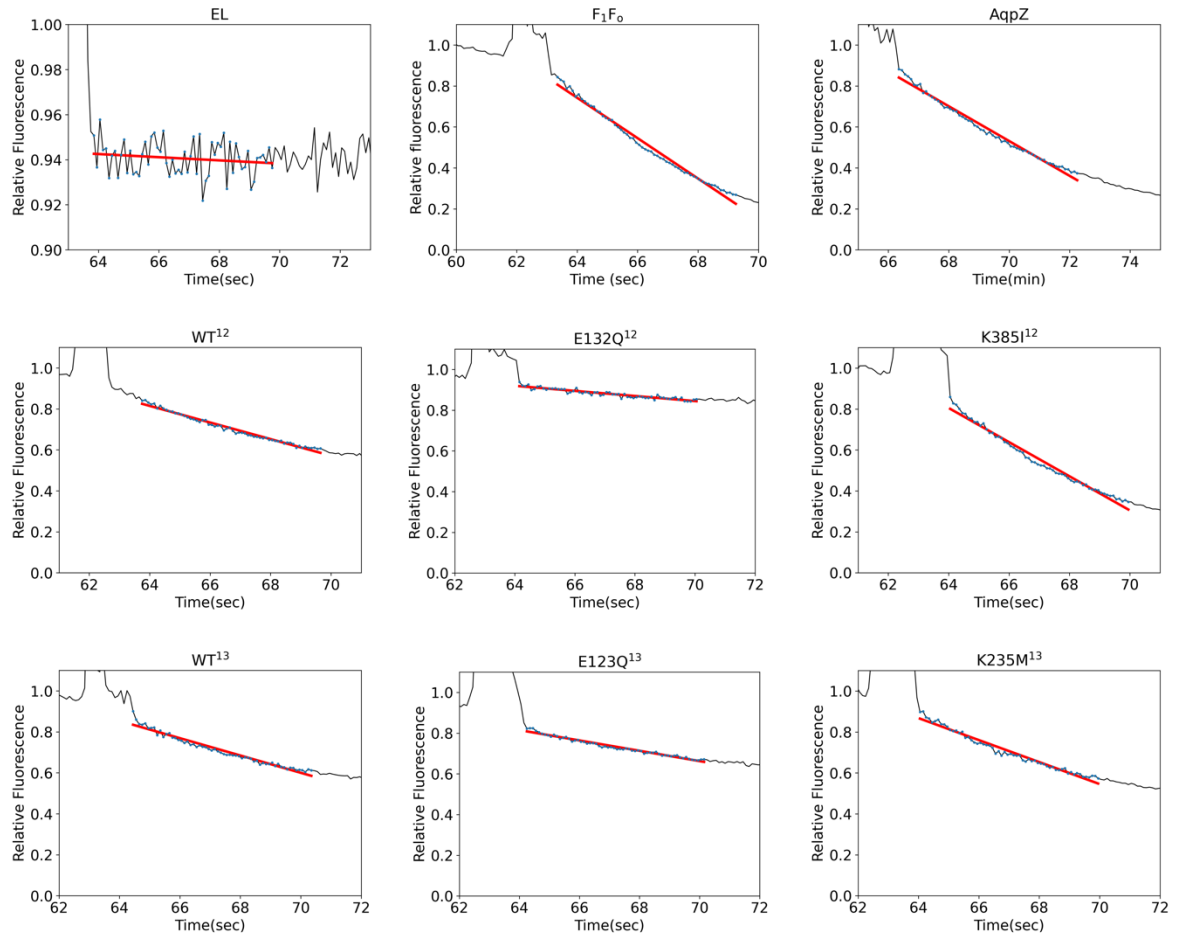
Supplementary Figure 12 | Calibration of pyranine fluorescence and data fitting. **a)** Pyranine calibration curve measured between pH 5 and 10. The higher measuring temperature (37°C) as well as the buffer conditions lead to an upshift in the pyranine pK_a from 7.2 (*cf. e.g. Ref¹⁵* for exploration of buffer effects). **b)** Raw data for pyranine fluorescence measurements following excitation at $\lambda_{ex}=404$ nm (*red*) and $\lambda_{ex}=454$ nm (*black*) and emission at $\lambda_{em}=510$ nm. ATP synthase data fitted using **c)** a linear regression or **d)** an exponential fit. Data are provided in the Source Data file.



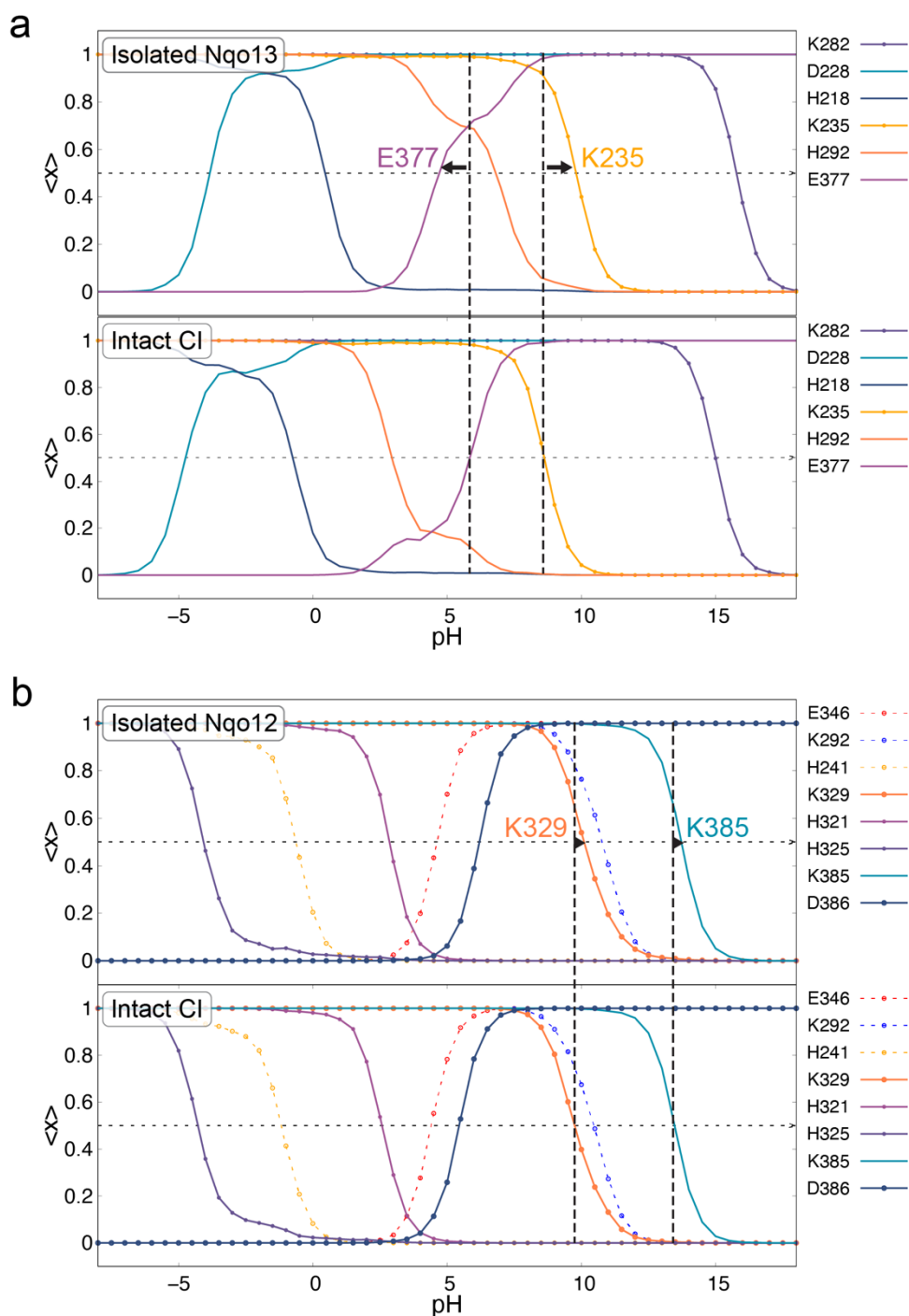
Supplementary Figure 13 | Kinetic optimisation and characterisation of proton conduction in proteoliposomes. **a)** Proton conduction monitored in the dissected antiporter-like subunits reconstituted in proteoliposomes with different Nqo-per-liposome ratio. The *pmf* was generated by addition of NaOH. *Inset*: linear relationship between the Nqo:liposome ratio and the resulting steady-state pH level upon addition of base. **b, c)** WT-Nqo12 and WT-Nqo13 mediated proton conduction induced by an external **b)** ΔpH - or **c)** $\Delta \Psi$ - gradient, followed by addition of valinomycin (at 1 min). **d) Left**: ACMA fluorescence quench upon addition of ATP with different Nqo:F₁F₀ ratios. *Inset*: linear relationship between the Nqo:liposome ratio and the resulting steady-state pH level upon addition of ATP. *Right*: Closeup of the quenching levels. Data are provided in the Source Data file.



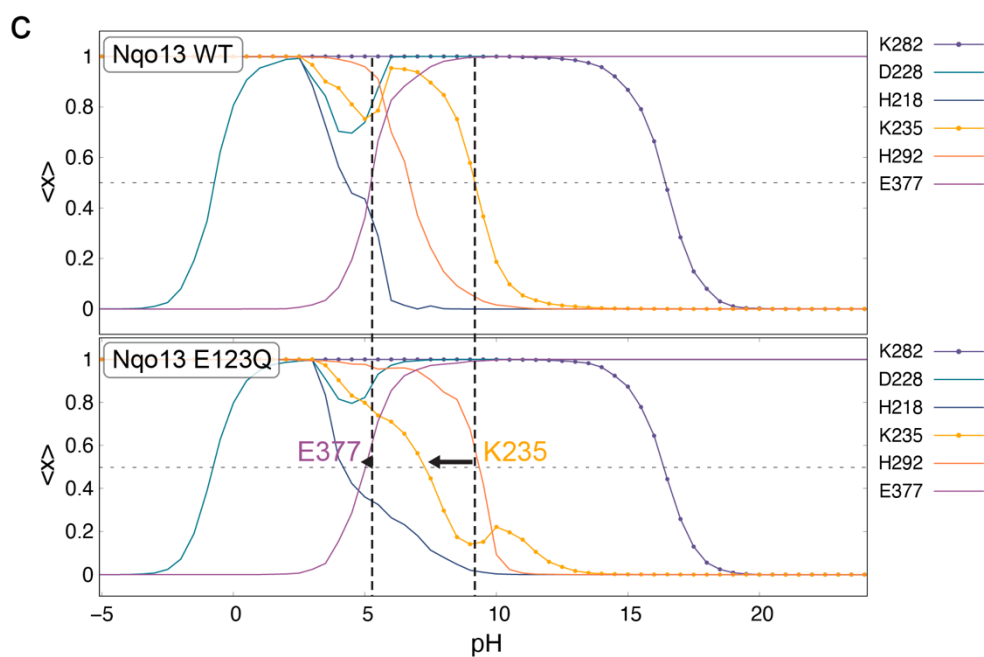
Supplementary Figure 14 | Protonation dynamics in (proteo)liposomes reconstituted with ATP synthase. a) Effect of ATP concentration on the ACMA fluorescence quenching. **b)** Coomassie gel of ATP synthase following the purification fractions (MBp: membranes pellet, MBs: membranes supernatant, FT: flow through, W1-2: washes, E: elution, and silver stained ATP synthase elution fraction. Proton conduction of **c)** Nqo12 and **d)** Nqo13 co-reconstituted with ATP synthase followed by ACMA fluorescence quenching. cl - sfGFP cleaved constructs. **e, f)** Fitted ACMA quenching rates from proteoliposome experiments with **e)** ATP synthase-Nqo12 and **f)** ATP synthase-Nqo13. Data shown are derived from independent experiments where $n = 6$ (mean \pm SD) except F₁F_o-K235M¹³ where $n=5$. **g)** Effect of cleaving sfGFP on the ACMA quenching for Nqo12 ($n_{WT12}=4$, $n_{WT12 \text{ cleaved}}=3$) and Nqo13 ($n_{WT13}=3$, $n_{WT13 \text{ cleaved}}=5$). Data are provided in the Source Data file.



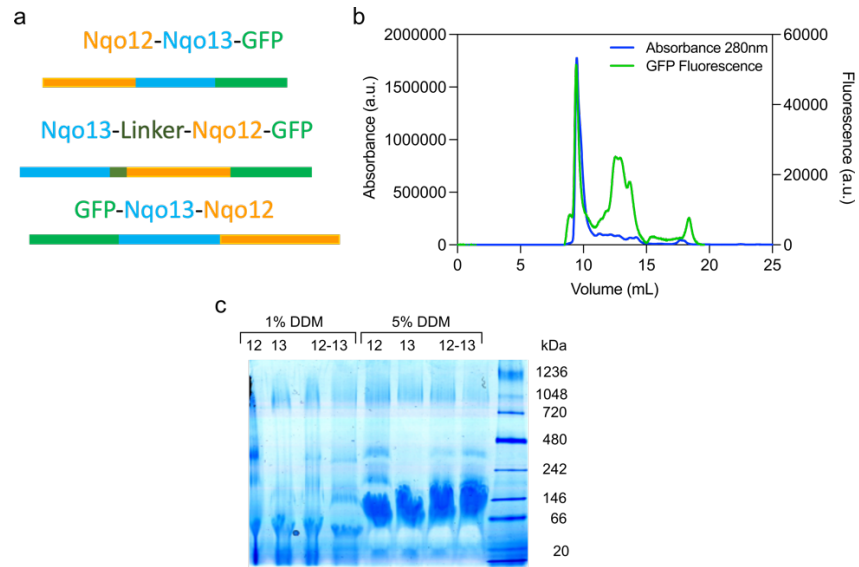
Supplementary Figure 15 | Kinetic analysis of proton conduction in F_1F_0 -Nqo proteoliposomes. Proton conduction monitored in the dissected antiporter-like subunits reconstituted in proteoliposomes, with the *pmf* generated by addition of ATP with co-reconstituted ATP synthase using a linear regression fit. Data are provided in the Source Data file.



Supplementary Figure 16 | Analysis of pK_a tuning effects in the dissected antiporter-like subunit and the intact Complex I. The figure shows the protonation probability ($\langle x \rangle$) as a function of pH. **a)** Titration curves of residues along the proton pathway for the isolated Nqo13 subunit (*top*), and Nqo13 within the intact Complex I (*bottom*), and **b)** the isolated Nqo12 subunit (*top*), and Nqo12 within the intact Complex I (*bottom*). Data are provided in the Source Data file.



Supplementary Figure 16 (contd.) | Analysis of pK_a tuning effects in the dissected antiporter-like subunit and the intact Complex I. The figure shows the protonation probability ($\langle x \rangle$) as a function of pH. **c)** Titration curves of residues along the proton pathway for the Nqo13 WT (*top*), and Nqo13 E123Q constructs, based on constant-pH MD simulations (see *Extended Methods*). Data are provided in the Source Data file.



Supplementary Figure 17 | Formation of a Nqo12-Nqo13 complex. **a)** Design of an Nqo12-Nqo13 complex constructs. **b)** FSEC profile of truncated Nqo12 (Δ A571-T583)-Nqo13-GFP complex membranes with the protein content followed at 280 nm (blue) and GFP fluorescence followed at 485 nm (green). **c)** Blue-native PAGE of Nqo12^{ATH} and Nqo13 samples reconstituted separately and co-reconstituted in liposomes with *E. coli* polar lipids, followed by solubilisation in 1% or 5% DDM. The putative Nqo12^{ATH}-13 complex was reconstituted using a protein to lipid ratio of 1:70 on the left and 1:140 on the right. Data are provided in the Source Data file.

Nqo12/T. thermophilus/1-606 1 ----MALLGT---ILLPLLGALLGLGKMRREPLPGVL---ASGLVL----ASFLLAGL----LSGGARFQA---EWL----PGIPFSLLDNLSGFLLIVTVGFLIHVYAI 92
Nuo1/E. coli/1-613 1 ----MNMLALT---IILPLIGFVLLAFSRGRWSENVAIV---GVGSVGLAALVTFAGIVDFF----ANGEQTSYQPLWTMSVGDNIGFNLVLDGLSLTMLSVTVTGFLIHMYAS 184
Nqo12/P. denitrificans/1-703 1 ----MEKVF---LFAPLIASLIALGWRRAIGKAQYL---TGTVLFLSCL----SWYFLFSDF---GVRPHIPLVDWVTVGDFHAEWAIRLDRLTAIMLIVTVTSALVHMYSL 99
NUSM/Y. lipolytica/1-655 1 ----MYNAISL---IILPCISWLFPFGGRLGVYVTRM---TSTLIITITLI----TYYFYQLLGNNNPINLELFNLYINDYLDINYFIEDALITITMLAITITSSMVHIYSI 184
NDS/M. musculus/1-607 1 ----MNIFTTSILLIFILLSPILISMSNLKIHNFPLYTTTISKFSFI----SLLPLMHFFNNMEYMITTHWVMTMSMELKMSFKTDFSSILFTSVLFWTWSIMQFSS 185
NDS/O. aries/1-606 1 ----MNLFSLSLTVLITLLTPIIAINFNTKFNTPNPLYKVTITSCAFIT---SMIPTMHFIHTGQEMIISNWHHLTIQTLKLSFKMDFDSMHFVPVLFVWTSIMEFSM 185
NDS/B. taurus/1-606 1 ----MMHFSLSLTVLITLLTPIIMMSFNTPKSNPNLYKVTIAISYAFIT---SMIPTMHFIHSGQELIISNWHHLTIQTLKLSFKMDFDSMHFVPVLFVWTSIMEFSM 185
NDS/H. sapiens/1-603 1 ----MTHTMTHTLTLTSLIPITLTVNPKNKSYPHYKSVIVASTFI---SLFPTTHMFLCQDEVIISNWHATTQTQLSLFKMDFDSMHFVPVLFVWTSIMEFSM 185
NDS/A. thaliana/1-669 1 ----HYLLI---VFLPLLGSVAGFGFRFLGSEGAIM---TTTCVSFSSIL----SLIAFYEVALGASACYLRIAPWISSEMFSDASGFLFDLSLTVMLIVTSSISLVLHYSI 181
NdhF1/T. vestitus/1-656 1 MEPLYQYAWLI---PVLPLLGLVIGFGLIAFSETTSKLR---RPSAIFIMALMAIAMGHSLTFLWSVQV---GHLPYTQMIENAAAGNLHIAMGVVIDPLAALMLVITVTVAFLVMLYSD 112

Nqo12/T. thermophilus/1-606 93 GVMGSDP-----GYSRFAYFNLFIAMMLTIVLADSVPMVFIHNEGVGLAFLLLIGFMYKNPYQVADARKAFVNNRIGDLGFMLHAILWALYGTLSISELKEAMEGLK----- 197
Nuo1/E. coli/1-613 105 WMRGEE-----GYSRFAYFNLFIASHVVLVADNLLMLYHMGVGLCSYLLIGFYTPDKNGAAMKAFVVTIVGDFLAFALFILYNELGTFLNFMVELAPAHFAD----- 210
Nqo12/P. denitrificans/1-703 100 GMAHDNDNTHDEHYKARFAYLSFTFAFMLMLVADNLLQMFHMGVGVQVASYLLIGFYKKASANAAMKAFVNNRVGDFGLLLIFGFIYMLTGSVQDFEIRFQPQLAQTEIDFL 217
NUSM/Y. lipolytica/1-655 105 GMETDP-----HQVRFSSLSMFTFMIIITVGSNVFVLVGVMEFIVGTSYLLISFWVTRLQAMKASLAVLMNRFGDAFFVYGLCVIAYVFTLNYSYTFATAYLIN-T----- 209
NDS/M. musculus/1-607 106 WMHSDP-----NINRFIKYLLFLITMLIITSANMFOLFIEGVGIMFLLIGMWYGRTDANTAAQALTYNRIIGDIGFILAMVWFLSNMNSWELQIM-----FS 204
NDS/O. aries/1-606 106 WMHSDP-----NINQFFKYLFLITMLIIVTANNLFOLFIEGVGIMFLLIGMWYGRTDANTAAQALTYNRIIGDIGFILAMWFLNLNTWDLQIF-----ML 204
NDS/B. taurus/1-606 106 WMHSDP-----NINQFFKYLFLITMLIIVTANNLFOLFIEGVGIMFLLIGMWYGRADANTAAQALTYNRIIGDIGFILAMWFLNLNTWDLQIF-----ML 204
NDS/H. sapiens/1-603 106 WMHSDP-----NINQFFKYLFLITMLIIVTANNLFOLFIEGVGIMFLLISWYARADANTAAQALTYNRIIGDIGFILALAWFILHNSWDPOQMA-----LL 204
NDS/A. thaliana/1-669 102 SMSEDP-----HSPRFMCYLSITFTFFMLMLVTDGNPLQLFIEGVGLASYLLIHFVTRLOQADKAIAKMLVNRVDFGLALGILGCTFLQTVDFTIFACASVPR---NSWIFC 211
NdhF1/T. vestitus/1-656 113 GMAHDP-----GYVRFAYLSLGGSSLLGVVSPNLVQVYVFWELVGMCSYLLIGFYDRKSAEAAKQAFVTRNRVDFGLLLMGVLFWATGTFDFAGMGDRLTTELVTGL--- 220

Nqo12/T. thermophilus/1-606 198 --NPDLLALAGLLFLFVAGVSAQIPIMVWLPDAMAGTPVSAIHAAHMTAGVYLIASSFLYSVLPDVSYAIAVVGLLIAYGALSAGFQTDIKKIVAYSTISQLGYFLAAGVG 313
Nuo1/E. coli/1-613 211 --GNMMLMWATMLLGGVAGVSAQLPQLTADAMAGTPVSAIHAAHMTAGVYLIAHGLFLMTPVELHVGIVGAVLLIAGFAALVQTDIKRVLAYSTISQIGYFLALGVQ 326
Nqo12/P. denitrificans/1-703 218 WRDWAANLLGLFLFVAGVSAQLLHTWLPDAMEGTPVSAIHAAHMTAGVFLVCMSPLEYFADAKNFVIVIGATIAFFAATVGLVQNDIKRVIAYSTISQLGYFVAAVG 335
NUSM/Y. lipolytica/1-655 210 ----DLVLIMLALFIAMASQFGLHNWLPDAMEGTPVSAIHAAHMTAGVYLIAHGLFLMTPVELHVGIVGAVLLIAGFAALVQTDIKRVLAYSTISQIGYFLALGVQ 326
NDS/M. musculus/1-607 205 NNNDNLPLMG--LIIAATGSAQFGLHPWLPDAMEGTPVSAIHAAHMTAGVFLVCMSPLEYFADAKNFVIVIGATIAFFAATVGLVQNDIKRVIAYSTISQIGYFLALGVQ 326
NDS/O. aries/1-606 205 NPNDNLPLMG--LIIAATGSAQFGLHPWLPDAMEGTPVSAIHAAHMTAGVFLVCMSPLEYFADAKNFVIVIGATIAFFAATVGLVQNDIKRVIAYSTISQIGYFLALGVQ 326
NDS/B. taurus/1-606 205 NPNDNLPLMG--LIIAATGSAQFGLHPWLPDAMEGTPVSAIHAAHMTAGVFLVCMSPLEYFADAKNFVIVIGATIAFFAATVGLVQNDIKRVIAYSTISQIGYFLALGVQ 326
NDS/H. sapiens/1-603 205 NPNDNLPLMG--LIIAATGSAQFGLHPWLPDAMEGTPVSAIHAAHMTAGVFLVCMSPLEYFADAKNFVIVIGATIAFFAATVGLVQNDIKRVIAYSTISQIGYFLALGVQ 326
NDS/A. thaliana/1-669 212 NMRLNALSILCILFIAGVSAQIGLHTWLPDAMEGTPVSAIHAAHMTAGVFLVCMSPLEYFADAKNFVIVIGATIAFFAATVGLVQNDIKRVIAYSTISQIGYFLALGVQ 327
NdhF1/T. vestitus/1-656 221 -LSPSLAAILAIVLFLPVASQFPLHVLPDAMEGTPVSAIHAAHMTAGVFLVCMSPLEYFADAKNFVIVIGATIAFFAATVGLVQNDIKRVIAYSTISQIGYFLALGVQ 327

Nqo12/T. thermophilus/1-606 314 AYWVALFVFTFAFFKALLFLASGVVIALG--GEIDVRKMGGWKHLQTRWHALIGALALGGL-----PLL-SGFWSKDAIJAATLTYPFGGVGYVAGLVAVLAM 415
Nuo1/E. coli/1-613 327 AWDAAFILMTFAFFKALLFLASGVVIALG--HEINIFKMGGKRKSIPLVLYLCLVGGAAALSAL-----PLVTAGFFSKDEILAGAMA---NGHINLMVAGLVGAFM 427
Nqo12/P. denitrificans/1-703 336 YSAAAMFLLTHAFFKALLFLAGSVVIAHAM-----HEIDMRNYGGLRKKIPLTFWAMHITFATITVGIPLTHLGF-AGFLSKDAIIESAYG---SGYAFNVLIAACF 440
NUSM/Y. lipolytica/1-655 324 ANYLALFHLGHFAFFKALLFMSAGVIHISLN-----ESIDIRTYGGLLSYLPYTYICITIASLSHAM-----PGL-TGYTYKDIIESYGYISYISNVVYIAYL 421
NDS/M. musculus/1-607 321 QPHLAFILHCTFAFFKALLFMSAGVIHISLN-----DEIDRKMGGELKIMPTSSCLVIBSLALITM-----PFL-TGFYSKDLIEASINTNT---NAWALLITL 421
NDS/O. aries/1-606 321 QPHLAFILHCTFAFFKALLFMSAGVIHISLN-----DEIDRKMGGELKIMPTSSCLVIBSLALITM-----PFL-TGFYSKDLIEASINTNT---NAWALLITL 421
NDS/B. taurus/1-606 321 QPHLAFILHCTFAFFKALLFMSAGVIHISLN-----DEIDRKMGGELKIMPTSSCLVIBSLALITM-----PFL-TGFYSKDLIEASINTNT---NAWALLITL 421
NDS/H. sapiens/1-603 321 QPHLAFILHCTFAFFKALLFMSAGVIHISLN-----DEIDRKMGGELKIMPTSSCLVIBSLALITM-----PFL-TGFYSKDLIEASINTNT---NAWALLITL 421
NDS/A. thaliana/1-669 330 NYSVSVFLMNHFAFFKALLFLASGVVIAHMS-----DEIDRKMGGELASSPFLTYAMHLIGSLILIGF-----PFL-TGFYSKDLIEATYKTIISGNFAFMGLSVSL 432
NdhF1/T. vestitus/1-656 338 ASYAGFLHMTFAFFKALLFLGSGGVVHMEGVGNPDLAIDMRNYGGLRKYMITGATFLVGLCLATSGV-----PPF-AGFWSKDEILGAVFHA---NPAWLLTLWTAGL 444

Nqo12/T. thermophilus/1-606 416 AMRMWFLVFLGEERGH-----HHPEAPPVNMWPNHNLALGSLAGVLAIPHLPNVLPEFLKPALAE-----VEA 482
Nuo1/E. coli/1-613 428 YFRMIFIVFHGKEQIH-----AHAKVGVTHSLPLVLITL-STFVGLALVLP-PLQGVLPQTTEL-----GFDWLDYKVFVKPFLGIAWLL-----KRDPLNSMMN 570
Nqo12/P. denitrificans/1-703 441 YSMRLIFLTFYKGKRGD-----HDHAHESPVMPTIPLGVLAIGAVFAGHMVYG-PFGDGHKVTYFYHIAGAHHEAAEGEEAEHATAEAPVEHAVADTATAEAEAAEA 588
NUSM/Y. lipolytica/1-655 428 YSMKILYLTFTYSNPNNN-----TIT-----YNAHESNYITLPMFLIAIFAMFAGILKD-IYLGVTGDT-----YHPSNFAKFSSTLLGYYPITMHRAPYMNLSMSQKSASSL 563
NDS/M. musculus/1-607 422 YSMRIYFVMTKPRFPP-----LISINENDPLMNPVKRALFSGIFAGFVSYNIPTSPITVLT-----FNYPSNTFKFSNLLGYYPITMHRAPYMNLSMSQKSASSL 563
NDS/O. aries/1-606 422 YSTRIIFALLGQPRFPT-----LNIINENNPLINSIKRLIGSLFAGFIIISNNIPPTIPQMT-----FNYPSNTFKFSNLLGYYPITMHRAPYMNLSMSQKSASSL 563
NDS/B. taurus/1-606 422 YSTRIIFALLGQPRFPT-----LNIINENNPLINSIKRLIGSLFAGFIIISNNIPPTIPQMT-----FNYPSNTFKFSNLLGYYPITMHRAPYMNLSMSQKSASSL 563
NDS/H. sapiens/1-603 422 YSTRMILLTLTGQPRFPT-----LTNIINENNPLLNIPKRALGSLFAGFIIITNNISPASFPQT-----FNYPSNTFKFSNLLGYYPITMHRAPYMNLSMSQKSASSL 563
NDS/A. thaliana/1-669 433 YSFRLLFLTLVPTNSF-----GRD-----ISRCHDAPIPMAIPLILALGSLFVGYLAKD-MMIGLGTNF-----AKAIQPLQFSLHKWYFDELVEAVFKGRCRLARQVE---VDYNNVGDVGN 614
NdhF1/T. vestitus/1-656 445 YMFYMYMTFEGKFRNVPPERQEHDDHSHHAAPHPESPTWMTPLVLVLAIPSTLIGVGTPT-F-NN---LFEVFIHAPGEEK-----VA----- 524

Nqo12/T. thermophilus/1-606 483 HHLSLGAEWGLIA-----LSAAVALLGLWAGVF-----FFQ-----RKV-FPA-WYLAFAEASREAFVDRAYNALIVNPLKALAEALFY---GDRGLLSGYFG 566
Nuo1/E. coli/1-613 489 SMLTEITSGVVA-----VUGILLAAWLGLKR-----TLVTSI-ANSAPGR-LGTWYNAW-----GFDWLDYKVFVKPFLGIAWLL-----KRDPLNSMMN 570
Nqo12/P. denitrificans/1-703 447 EHAEIAAPVGGAIYHMPDNHIM-DEAHAPAWV---KVSPPVAMVLGLITAWTFYIANPSLPRL---AAHEPALYRFLLNKMYDFEIEYFIVRPKAWGL-RVLKMGDGDVADGTIN 657
NUSM/Y. lipolytica/1-655 448 -----VGTHI---LPNNFSYFDEFTSITQFY---KLPLISAILVSLIVVL---NEFFAIVFNLNKKYINTVYSIFNQKLVSDQLNHIIIFKGLVTSGN-IAHVDVKGSLVRL 589
NDS/M. musculus/1-607 482 -----MP---WFLKMTALITVILGFLIALELNNLTMLKS---MNKANPYSFSTLLGFFSPITMHRAPYMNLSMSQKSASSL 563
NDS/O. aries/1-606 482 -----MP---HYLKMTALITVILGFLIALEISNTHTYK---FNYPSNTFKFSNLLGYYPITMHRAPYMNLSMSQKSASSL 563
NDS/B. taurus/1-606 482 -----IP---LYLKALAVTFLGLLTALDNLNLTMLK---MKSPLCTFYFSNHLGFYPSITHTRTIPYGLGLTSQNLPLLLDLTLWEKLLP 563
NDS/H. sapiens/1-603 493 -----WANSLLVLPKNEILAESEFAPTII---KLPIPLFSTLGAFAVYVNVNVDQFRAF-QTSTFCNRLYSFFNKRWFDDQLVNDPLVRSFLRGYVFEALDKGAEIILGP 599
NdhF1/T. vestitus/1-656 525 -----EHAVDLTEFLILGSSVGLMGITVVAALMYLKGITPSPQAT---AKAIQPLQFSLHKWYFDELVEAVFKGRCRLARQVE---VDYNNVGDVGN 614

Nqo12/T. thermophilus/1-606 567 L---GGAARSLGGQLARLQTYGLRYVALFVLGALLGLVMRW----- 606
Nuo1/E. coli/1-613 571 I---PAVLSRFAGKGLLSSENGYLRWYVASHMIGAVVLLALMLVR----- 613
Nqo12/P. denitrificans/1-703 658 GVAMGLIP-RLTRAARVQSGYLFHYAFAMVLGIVGLITWMMRGH----- 703
NUSM/Y. lipolytica/1-655 590 VGINRLLN-KASYNVINLSNTRSSLSNMSMLITITVLSLLL-----VLVMNVNFIIVIPVLSILYILFS-- 655
NDS/M. musculus/1-607 564 KSTSTLH--TNMTTLTNOKGLIKLYFMSFILINILITILYSINLE----- 607
NDS/O. aries/1-606 564 KTISLAQ--MKMSTTITSOKGLIKLYFSLITILISTLLNFHE----- 606
NDS/B. taurus/1-606 564 KTISLAQ--MKASTLVNOKGLIKLYFSLITILISTMLNFHE----- 606
NDS/H. sapiens/1-603 564 KTISQHQ--ISTSIITSTQKGMILKLYFSFFPLTLITLIT----- 603
NDS/A. thaliana/1-669 600 YGISYTFR-RLAERISQLOSGFYHYAFAMLLGLTLVTFCCMDSLSLWMDNRLSFLILI---VSSFYTKSSQE 669
NdhF1/T. vestitus/1-656 615 LT---GFTVMVTGEGIKYLNQGRAQFYALVILAVLGFVIFSQT----- 656

Supplementary Figure 18 | Sequence conservation in Nqo12 and Nqo13. Multiple sequence alignment (MSA) of Nqo12 and Nqo13 from different species. The MSA was performed with ClustalΩ.¹⁶ Residues with up to 50% sequence conservation are coloured by the ClustalX colouring scheme.

Nqo13/T. thermophilus/1-469 1 -----MVLAVLLPVVFGAL-----L LLLGLPRALGVLGAGLSFLLNLYLFLTHPGG-----VAHAFQAPLLPG-----AGVYWAFGLGSLALFFLTIALTV 82
NuoM/E. coli/1-509 1 -----MLL PWLILTPFGGFLC-----WQTERFGVKYPRWIALITMGLTLALSLQLWQGGYSLTQSAQIPQWQSEFDMPIWR-----FGISYTHLADGLSLMLVVL TGLLG 98
Nqo13/P. denitrificans/1-513 1 -----MTNLLSIITFLPIVAAI1-MALFLRGQDEAAARNAKWALLTTTATFVLSFLVLRFDPA-----NTGQFQVEDHA--WIM-----GVCYKMGVDGSLVFLVLTTFMM 96
Nu4M/Y. lipolytica/1-486 1 -----MFLTSILLSSLYLFNRLLAWQGNVHKFYLFASNL LLLFVVLVYNFTNF-----SNSFQNFELFNSLNPFGLSNSDISNGLLFGDGLSLFTILLTVLLI 96
ND4/M. musculus/1-459 1 -----MLKII LPSLM-----LLPLTWLSSPKKTWNTVTSYSFLSLSLSTLLWQ-----TDENYKNFSNMFFSDSLSTPLIILTAWLL 73
ND4/O. aries/1-459 1 -----MLKYI IPTMM-----LMPLTWLSKNMNIWNTHTSLSLSLSTLLNQ-----FGDNLNFSLLFFSDSLSTPLIILTAWLL 73
ND4/B. taurus/1-459 1 -----MLKYI IPTMM-----LMPLTWLSKNMNIWNTHTSLSLSLSTLLNQ-----FGDNLNFSLLFFSDSLSTPLIILTAWLL 73
ND4/H. sapiens/1-459 1 -----MLKLIVPTIM-----LLPLTWLSKNMNIWNTHTSLSLSLSTLLNQ-----INNLFSCSPTFSSDPLSTPLIILTAWLL 73
ND4/A. thaliana/1-495 1 MLEHFCECYFNL-SGLILCPVLGSII-L-LFIP---NSRIRLIRLIGLCASLITFLYSVLVLIQFDS--TAKFQFVESLR--WLPY-----ENINFLYGLDGLSLFVLTITFLI 101
NdhD1/T. vestitus/1-529 1 -----MSTFPWLTTIILFPVIAALAI--PFI-PDPTGKGRIRWALAVGLIDFALIVYAFNTFYD---LNTPGHQLWESYD--WIPF-----IGLRWSVGADGLSMLPILITLGTIF 99

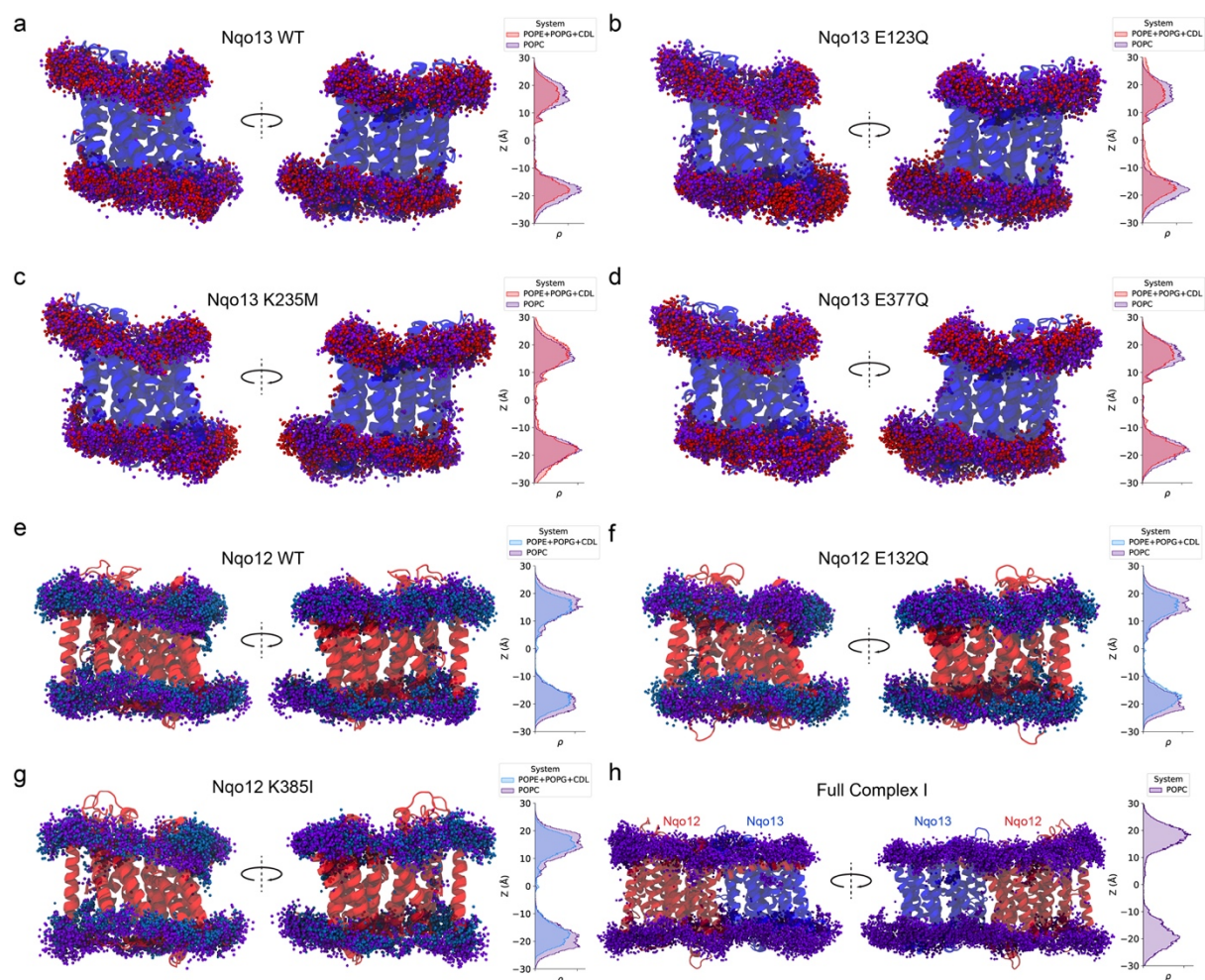
Nqo13/T. thermophilus/1-469 83 L G A L V A-----RVEGRFLGLALLMGLLGLFAARDLVYVVFPAALIPALLMLYLGGEGR-----TRALYTFVFLYLVGSLPMLAAVLGARLLSGSPPTFL-----E--- 178
NuoM/E. coli/1-509 99 VLAVLCSWKEI-----EKYQGFHNLMLLGVIGVFLADIMFLFFFMMVPMYFLLIALGHKASDGKTRITAAATKFFIYQASGLVNLIAIALVVFVHYNAT--GWTFMYE--- 208
Nqo13/P. denitrificans/1-513 97 P T I L S T W Q V Q---DKVKEYNIAFLVLEGLMIGVFTALDVLVLYFPEAGLIPMLFIIGWGGKDR---IYASFKFFLYFLGSLVNLVAMIAMYRMAGTIDPTILLTFDFPSEN 205
Nu4M/Y. lipolytica/1-486 97 P T I L L G N W Y N I---NFSNLYYTLVLAIGLVILLNFHALDYISFYLFPEATLPLFLIHLIHYGSSDS-----ERASFYVLMFLSGSLFMLLSIVVISVLNNTNF-----INHN 198
ND4/M. musculus/1-459 74 P L M L M A S Q N H L K K N N V L Q K Y T S M L S L Q I L L M T F S A T E L I M F Y I L F E A T I P T L I I T R W G N Q T E-----RLNAGIYFLFYLTIGSTPLIALILIQNHVGTNLNMLSFTHT--- 185
ND4/O. aries/1-459 74 P L M L M A S Q N H L K K N L A R K K L F T S M L I L L Q L F L M T F T A T E L I F F Y I M F E A T I P T L I I T R W G N Q T E-----RLNAGIYFLFYLTIGSTPLIALILIQNHVGTNLNMLSFTHT--- 185
ND4/B. taurus/1-459 74 P L M L M A S Q N H L K K N L A R K K L F T S M L I L L Q L F L M T F T A T E L I F F Y I M F E A T I P T L I I T R W G N Q T E-----RLNAGIYFLFYLTIGSTPLIALILIQNHVGTNLNMLSFTHT--- 185
ND4/H. sapiens/1-459 74 P L T I M A S Q R H L S E P L S R K K L Y L S M L I S L Q I S L M T F T A T E L I M F Y I F F T T L I P T L A I T R W G N Q P E-----RLNAGIYFLFYLTIGSTPLIALILYTHNTLGLSNIILLTLTAQE--- 215
ND4/A. thaliana/1-495 102 P T C I L V G W S G M---RSYGKEYTIAFLICEFLMAVEFCLDILLFYVFPSPVLPMPFIIGVWGSQRQ---KIKAAQYFFLYLTIGSTPLIALILIFQTGTIDLOIILLTFEFS--- 210
NdhD1/T. vestitus/1-529 100 T L I A L A W P V T---L-KPRLFYFLMLAMYGQIAYEAVQDMLVYFLAWLELIPVYLLIANGGHKR-----QYATKFIYLTIGSTPLIALILVAGLAMAFYGDVTSFDM-----Q--- 199

Nqo13/T. thermophilus/1-469 179 -DLLAHLPEEEAAVWFVLFALFAFAITPLFLHAWLPPOQHNPSPGLADALSTLYVGVFAFFFAIPLAPEGFAQAQGLLFLAALSALYGAWWFAAKDFTLTLAGLSHMGVA 296
NuoM/E. coli/1-509 209 -ELLNTPMSSGVEYLLMLGFFIAFAVMPVPLHGWLPDAHSQAPTAGSDVLGAILLTAAYGLLRFSLPLFPNASAEFAPIAMWLGVIGIFYGAWMAFAQTDINRLIATSVSHMGV 326
Nqo13/P. denitrificans/1-513 206 FRLLGMTVGGQMQLFLFAFFASFAVMPMPVHTWLDPAHQAPTAGSVLLAAVLHGGYGLRFLSLPMFPVASGVAQPYVFWLSAIAIVYTSLVALAQSDMKVIAIASSVAHMGV 324
Nu4M/Y. lipolytica/1-486 199 LFLVS---LDLQTIWLGLFAIMVKTPLFFIHWLVVHVSSEPLAGSMILAGLLALAYAILRLLPLLCEAQLYTPMIYIISLLTIILSLATLRQDLKVIATSSISHMGIA 313
ND4/M. musculus/1-459 186 -----LDASWSNLLWLACMMAFLIMPLYGVHLWLKPAHVEAPIAGSMILAAILLKGGYGMIRISIIIDPLTKYMAYPFII-LSLWGMIMTSSICLRQTDLSLIAVSSVSHMALV 297
ND4/O. aries/1-459 186 -----MPNSWSNTPMMLACMMAFMVMKMPLYGLHLWLKPAHVEAPIAGSMVLAAILKGGYGMIRITILLNPIITDFMAYPFIM-LSLWGMIMTSSICLRQTDLSLIAVSSVSHMALV 297
ND4/B. taurus/1-459 186 -----VHNSWSNVFMMACMMAFMVMKMPLYGLHLWLKPAHVEAPIAGSMVLAAILKGGYGMIRITILLNPIITDFMAYPFIM-LSLWGMIMTSSICLRQTDLSLIAVSSVSHMALV 297
ND4/H. sapiens/1-459 186 -----LSNSWANNLMLAYTAFMVMKMPLYGLHLWLKPAHVEAPIAGSMVLAAILKGGYGMIRITILLNPLTKHMAYPFLV-LSLWGMIMTSSICLRQTDLSLIAVSSVSHMALV 297
ND4/A. thaliana/1-495 211 -----RRQIFLWIAFFASFAVMPVHVIWLPDAHQEAPTAGSVILAGILLKFGTYGFLRFSPIMFPEATLCFTPFIIYLSAIAIYTSITTLRQDLKVIATSSISHMGV 319
NdhD1/T. vestitus/1-529 200 -TLAAQDYALGFQLLYAGFLVAYGVLLTPVPLITWLPDAHQEAPTAPVHMLLAGILLKGGYGMIRITILLNPIITDFMAYPFIM-LSLWGMIMTSSICLRQTDLSLIAVSSVSHMALV 317

Nqo13/T. thermophilus/1-469 297 ALGVFSGTPEGANGGLYLAAASGVYTGGLFLA-GRLYERTGTLEIGRYRGLAQASPLAALALILFLAMVGLPGLSGFPPEFTLLLGAYKAS-----PWLAAFLSLVIAASAAAL 407
NuoM/E. coli/1-509 327 LIAITYGSLQAYQGAIVIQMIAHLSAAGLFLC-GQLYERIHTRDRMRMGGLWSKMKWLPALSLFFAVATLGMPTGNGFVGFEFMLFGSFQVV-----PVITVISTFGLVFASVYSL 437
Nqo13/P. denitrificans/1-513 325 TMGVFAANQIGVDGAIQMLSHGFIAGLFLCV-GVLYDRMHTREIDAYGGLVNRMPAYAVFMFFTHMANVGLPGTSGFVGFEFLTMGVFRVD-----TWVALVATSGVILSAAAL 435
Nu4M/Y. lipolytica/1-486 314 ILGVCNSTSLGIYGSIVLGVAHGFVSPALFLVGGILLDRYHIRIYNNYKQVLTTPYQATYIIILSFANIGTPLTGNFTGEFLSQGGFIRN-----PIIGGISCISVLAAAIQL 425
ND4/M. musculus/1-459 298 IASIMIQTPWSFGATMLMIAHGLTSSMLFCLA-NSNVERIHSRTIMARGLQMVPLMATWMLMASLANLALPPSINLMGELFTTMSLFSWS-----NFTIILMGINIIITGMSM 408
ND4/O. aries/1-459 298 IVALIQTTPWSYMGATMLMIAHGLTSSMLFCLA-NSNVERIHSRTIMARGLQTLPLMAWMLLASLNLALPPTINLIGELFVWMTFSWS-----NITIIILMGVNVITALVSL 408
ND4/B. taurus/1-459 298 IVALIQTTPWSYMGATMLMIAHGLTSSMLFCLA-NSNVERIHSRTIMARGLQTLPLMAWMLLASLNLALPPTINLIGELFVWMTFSWS-----NITIIILMGVNVITALVSL 408
ND4/H. sapiens/1-459 298 VTAIIQTTPWSFTGAVILMIAHGLTSSMLFCLA-NSNVERIHSRTIMARGLQTLPLMAFWMLLASLANLALPPTINLIGELFVWMTFSWS-----NITIIILMGVNVITALVSL 408
ND4/A. thaliana/1-495 320 TIGMFSPIQIGGSIILMLSHGLVSSALFLCV-GVLYDRHKTRLVRYGGLVSTMPNSTIFFSFTLANMSLPGTSFSGFEFLVLVGAFORN-----SLVATLAAALGMILGAAYSL 430
NdhD1/T. vestitus/1-529 318 LIGIASFTNLGMSGAVLMQVSHLIGASLEFLV-GATDHTHTILEEMCGVQMKKIFAMFTACSLASLALGMSGFVALHMFVIGFATSDAYSLPFRVIVFLAAVGVLTPTIYL 435

Nqo13/T. thermophilus/1-469 408 TAFQKTFWEEGSG-----VKDLAGAVGFALLSVLALLLMGVFGYFARGHLPLAEAFKLLGGGA----- 469
NuoM/E. coli/1-509 438 AMLHRYAVFGKAKSIASQ-ELPGMSLRELFMILLVLLVLLGFYROPILDTSHSAIGNIQQWFVNSVTTTRP----- 509
Nqo13/P. denitrificans/1-513 436 WLYRRVTLGLQLIKE-SLK-SITDTPPRRWVFIPIAMTILGVYRPLVTDVTGPAVAALVQDYNQSQAPAVATAQASH----- 513
Nu4M/Y. lipolytica/1-486 426 KLTNKL-TGSISS-YMH-RTNDVTIREKFINMILISTIGICQIMYNNLYWTVNNYIYII----- 486
ND4/M. musculus/1-459 409 YMIITTQRGKLTNHI---NLQPSHTRELTLMALHMIPLILLTTSKLITGLTM----- 459
ND4/O. aries/1-459 409 YMLITTQRGKHTHIN---NILPSFTRENALMSLHMLPLLLSLNPKIILGPLY----- 459
ND4/B. taurus/1-459 409 YMLIMTQRGKYTYHIN---NISPSTRENALMSLHMLPLLLSLNPKIILGPLY----- 459
ND4/H. sapiens/1-459 409 YMFTTTQWGLTHIN---NMKPSFTRENTLMFMHLSPIILLSLNPKIITGFS-- 459
ND4/A. thaliana/1-495 431 WLYNRVVSGLKPD-FLH-KFSDLNGREVFIIFPLVGLVWVGVPKVFDCMHTSVSNLVQHGKFH----- 495
NdhD1/T. vestitus/1-529 436 SMLREIFYGPENKELVEHEALVDAEPREVFIIACLVPIIGIGLYKLLTQIDYATGQVITARAREVPTLAQQTQEPGLGILPMVAPQLKANQA 529

Supplementary Figure 18 (contd) | Sequence conservation in Nqo12 and Nqo13. Multiple sequence alignment (MSA) of Nqo12 and Nqo13 from different species. The MSA was performed with ClustalΩ.¹⁶ Residues with up to 50% sequence conservation are coloured by the ClustalX colouring scheme.



Supplementary Figure 19 | Hydration analysis of the protein-lipid interface in the isolated antiporter constructs and in the full Complex I. Left insets show a snapshot of the hydration ensemble with water molecules within 3 Å of protein and lipids. Right insets show the distribution of water molecules along the Z axis. The analyses were performed on the last 500 ns of the respective MD simulation, with structures extracted every 10 ns. **a-d)** MD simulations of the Nqo13 constructs. **a)** WT, **b)** E123Q, **c)** K235M, and **d)** E377Q. **e-g)** MD simulations of the Nqo12^{ΔTM} constructs. **e)** WT, **f)** E132Q, and **g)** K385I. **h)** MD simulations of the full Complex I, showing water molecules around the protein-lipid interface at the Nqo12/Nqo13 subunits (based on simulation data from Ref. 7). Data are provided in the Source Data file.

Supplementary Table 1 | Steady-state pH levels and kinetics of proton transfer based on proteoliposome experiments using a weak acid/base or by ATPase-driven proton transport.

	Potassium acetate			Methylamine hydrochloride		
Construct	Plateau pH	SD	<i>n</i>	Plateau pH	SD	<i>n</i>
EL	7.05	0.013	3	7.69	0.018	3
AqpZ	7.04	0.017	6	7.68	0.011	3
WT ¹²	7.09	0.028	6	7.70	0.002	3
E132Q ¹²	7.16	0.017	6	7.74	0.002	3
K385I ¹²	7.05	0.013	6	7.63	0.003	3
WT ¹³	7.11	0.007	6	7.65	0.008	3
E123Q ¹³	7.14	0.002	3	7.67	0.004	3
K235M ¹³	7.05	0.011	6	7.64	0.009	3
E377Q ¹³	7.05	0.014	6	7.70	0.011	3

ATP synthase						
	Linear fit					
Construct	avg. k_1	SD	<i>n</i>	Plateau ΔF^*	SD	<i>n</i>
EL	0.04	0.02	6	0.96	0.01	6
AqpZ	4.42	0.49	6	0.09	0.01	6
F ₁ F _o	5.56	0.40	6	0.07	0.01	6
WT ¹²	2.53	0.28	6	0.33	0.04	6
E132Q ¹²	0.58	0.18	6	0.71	0.06	6
K385I ¹²	4.98	0.19	6	0.13	0.02	6
WT ¹³	2.36	0.19	6	0.32	0.03	6
E123Q ¹³	2.00	0.27	6	0.43	0.04	6
K235M ¹³	2.72	0.28	5	0.31	0.03	5

*Extrapolated from a single exponential fit.

Supplementary Table 2 | Orientation of Nqo modules in proteoliposomes by NTA-Atto 647N labelling.

Construct	Orientation ratio (outward/inward)
WT¹²	31/69
E132Q¹²	18/82
K385I¹²	27/73
WT¹³	29/71
E123Q¹³	23/77
K235M¹³	21/79
E377Q¹³	28/72

Supplementary Table 3 | Predicted pK_a s in the dissected antiporter-like subunit relative to the intact Complex I. The pK_a computed based on PDB ID:6I0D⁶, and ΔpK_a s of the isolated Nqo13 / Nqo12 constructs relative to WT intact CI based on MD ensemble (see *Supplementary methods*).

Nqo13	pK_a X-ray, CI full	pK_a X-ray, 13 only	ΔpK_a X-ray CI full - 13 only	ΔpK_a MD: CI full - WT/Nqo13	ΔpK_a MD: CI full - E123Q/Nqo13	$\Delta\Delta pK_a$ MD: WT - E123Q
K282	15.0	15.8	-0.8	-4.1	0.4	+4.4
D228	-4.7	-3.8	-0.9	-2.6	0.5	+3.1
H218	-0.7	0.5	-1.2	-4.8	2.2	+7.0
K235	8.6	9.8	-1.2	-2.4	-1.3	+1.1
H292	2.9	6.8	-3.9	-2.5	-1.9	+0.6
E377	5.8	4.7	+1.1	-5.3	-1.6	+3.7

Nqo12	pK_a X-ray, CI full	pK_a X-ray, 12 only	ΔpK_a X-ray CI full - 12 only	ΔpK_a MD: CI full - WT/Nqo12	ΔpK_a MD: CI full - E132Q/Nqo12	$\Delta\Delta pK_a$ MD: WT - E132Q
E346	4.4	4.6	-0.8	-0.1	+0.6	+0.7
K292	10.5	10.8	-0.3	+0.6	+0.4	-0.2
H241	-1.2	-0.6	-0.6	-3.8	-0.9	+2.9
K329	9.7	10.1	-0.4	-2.2	+1.8	+4
H325	-4.3	-4.1	-0.2	+0.0	+2.8	+2.8
H321	2.6	2.9	-0.3	+2.3	+3.4	+1.1
K385	13.5	13.7	-0.2	+4.7	+6.7	+2.0
D386	5.4	6.2	-0.8	-2.0	-0.4	+1.6

Supplementary Table 4 | List of MD simulations.

Simulation	System	Time (ns)	Modelled state	Dimensions (Å)	N atoms	N water molecules
S1	Nqo13-WT	1000	Lys235 ⁺ /His292 ⁰ /Glu377 ⁻	83 x 81 x 89	59400	10900
S2	Nqo13-WT	1000	Lys235 ⁺ /His292 ⁰ /Glu377 ⁻	83 x 81 x 89	59400	10900
S3	Nqo13-WT	1000	Lys235 ⁰ /His292 ⁰ /Glu377 ⁰	83 x 81 x 89	59400	10900
S4	Nqo13-WT	1000	Lys235 ⁰ /His292 ⁰ /Glu377 ⁰	83 x 81 x 89	59400	10900
S5	Nqo13-E123Q	1000	Lys235 ⁺ /His292 ⁰ /Glu377 ⁻	83 x 81 x 89	59400	10900
S6	Nqo13-E123Q	1000	Lys235 ⁺ /His292 ⁰ /Glu377 ⁻	83 x 81 x 89	59400	10900
S7	Nqo13-K235M	1000	His292 ⁰ /Glu377 ⁻	83 x 81 x 89	59400	10900
S8	Nqo13-K235M	1000	His292 ⁰ /Glu377 ⁻	83 x 81 x 89	59400	10900
S9	Nqo13-E377Q	1000	Lys235 ⁺ /His292 ⁰	83 x 81 x 89	59400	10900
S10	Nqo13-E377Q	1000	Lys235 ⁺ /His292 ⁰	83 x 81 x 89	59400	10900
S11	Nqo13-WT in PE:PG:CDL	500	Lys235 ⁺ /His292 ⁰ /Glu377 ⁻	100 x 100 x 105	97900	19200
S12	Nqo13-WT in PE:PG:CDL	500	Lys235 ⁺ /His292 ⁰ /Glu377 ⁻	100 x 100 x 105	97900	19200
S13	Nqo13-E123Q in PE:PG:CDL	500	Lys235 ⁺ /His292 ⁰ /Glu377 ⁻	100 x 100 x 105	97900	19200
S14	Nqo13-E123Q in PE:PG:CDL	500	Lys235 ⁺ /His292 ⁰ /Glu377 ⁻	100 x 100 x 105	97900	19200
S15	Nqo13-K235M in PE:PG:CDL	500	His292 ⁰ /Glu377 ⁻	100 x 100 x 105	97900	19200
S16	Nqo13-K235M in PE:PG:CDL	500	His292 ⁰ /Glu377 ⁻	100 x 100 x 105	97900	19200
S17	Nqo13-E377Q in PE:PG:CDL	500	Lys235 ⁺ /His292 ⁰	100 x 100 x 105	97900	19200
S18	Nqo13-E377Q in PE:PG:CDL	500	Lys235 ⁺ /His292 ⁰	100 x 100 x 105	97900	19200
S19	Nqo12 ^{ATH} -WT	1000	Lys329 ⁺ /Lys385 ⁺	101 x 103 x 95	98000	19400
S20	Nqo12 ^{ATH} -WT	1000	Lys329 ⁺ /Lys385 ⁺	101 x 103 x 95	98000	19400
S21	Nqo12 ^{ATH} -E132Q	1000	Lys329 ⁺ /Lys385 ⁺	101 x 103 x 95	98000	19400
S22	Nqo12 ^{ATH} -E132Q	1000	Lys329 ⁺ /Lys385 ⁺	101 x 103 x 95	98000	19400
S23	Nqo12 ^{ATH} -K385I	1000	Lys329 ⁺	101 x 103 x 95	98000	19400
S24	Nqo12 ^{ATH} -K385I	1000	Lys329 ⁺	101 x 103 x 95	98000	19400
S25	Nqo12 ^{ATH} -WT in PE:PG:CDL	1000	Lys329 ⁺ /Lys385 ⁺	109 x 107 x 109	125300	28200
S26	Nqo12 ^{ATH} -WT in PE:PG:CDL	1000	Lys329 ⁺ /Lys385 ⁺	109 x 107 x 109	125300	28200
S27	Nqo12 ^{ATH} -E132Q in PE:PG:CDL	1000	Lys329 ⁺ /Lys385 ⁺	109 x 107 x 109	125300	28200
S28	Nqo12 ^{ATH} -E132Q in PE:PG:CDL	1000	Lys329 ⁺ /Lys385 ⁺	109 x 107 x 109	125300	28200
S29	Nqo12 ^{ATH} -K385I in PE:PG:CDL	1000	Lys329 ⁺	109 x 107 x 109	125300	28200
S30	Nqo12 ^{ATH} -K385I in PE:PG:CDL	1000	Lys329 ⁺	109 x 107 x 109	125300	28200
TOTAL		26 μs				

Supplementary Table 5 | List of designed primers.

Oligonucleotide name	Sequence (5' → 3')
Nqo12 ^{ATH} extraction F	AAC TT TAAGAAGGAGATATACCATGGCGCTTCTCGGGACGATTCTC
Nqo12 ^{ATH} extraction R	ACCTTGGAAGTATAAAATTTTCCTTGCGCTGGAAGAAGACGAAACCCG
Nqo13 extraction F	ATGGTGGTGCTGGCGGTTTC
Nqo13 extraction R	GCAGCAGAACCGCCAGCACCACCATGGTAT
Nqo12 ^{ATH} E132Q F	ATGTTTCATCGGCTGGCAGGGGGTGGGCCTG
Nqo12 ^{ATH} E132Q R	CCAGCCGATGAACATCACC
Nqo13 E123Q F	TTTTCTTTCAGGCGGCGCTGATTCC
Nqo13 E123Q R	CCGCCTGAAAGAAAACGTAGAACACCAGCAGATCAC
Nqo13 K235M F	TGTACATGGTGGGTGTTTTTTCGTTCTTTC
Nqo13 K235M R	CACCCACCATGTACAGGGTGCCCAG
Nqo13 E377Q F	TCCGGGTCAATTCCTGACCCTGCTGG
Nqo13 E377Q R	AGGAATTGACCCGGAACCGCTC

Supplementary Table 6 | Sequences of studied Nqo constructs.

Construct name	Sequence
Nqo12^{ATH} WT	MALLGTILLPLLGFALLGLFGKRMREPLPGVLASGLVLASFLLGAGLLLSG GARFQAEWLPGPFSLLLDNLSGFMILLIVTGVGFLIHVYAIGYMGGDPGY SRFFAYFNLFIAMMLTLVLADSYPMFIGWEGVGLASFLFIGFWYKNAQY ADSARKAFIVNRIGDLGFMLGMAILWALYGTLSISELKEAMEGPKLPNDLL ALAGLLFLGAVGKSAQIPLMVWLPDAMAGTPVSALIHAATMVTAGVYL IARSSFLYSVLPDVSYAIAVVGLLTAAYGALSFAFGQTDIKKIVAYSTISQLG YMFLAAGVGAYWVALFHVFTHAFFKALLFLASGSVIHALGGEQDVRKMG GLWKHLQPTRWHALIGALALGGLPLLSGFWSKDAILAATLTYPFGGVGF YVGALLVAVLTAMYAMRWVFLVFLGEERGHHPHEAPPVMLWPNHLL ALGSVLAGYLALPHPLPNVLEPFLKPALAEVEAHHLSLGAEWGLIALSAA VALLGLWAGVFFQKGSAGSENLYFQGQFSKGEELFTGVVPILVELDG DVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTYGVCQ CFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGD TLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRH NIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHM VLEFVTAAGITHGMDLEYKTSHHHHHHHHHH*
Nqo12^{ATH} E132Q	MALLGTILLPLLGFALLGLFGKRMREPLPGVLASGLVLASFLLGAGLLLSG GARFQAEWLPGPFSLLLDNLSGFMILLIVTGVGFLIHVYAIGYMGGDPGY SRFFAYFNLFIAMMLTLVLADSYPMFIGWEGVGLASFLFIGFWYKNAQY ADSARKAFIVNRIGDLGFMLGMAILWALYGTLSISELKEAMEGPKLPNDLL ALAGLLFLGAVGKSAQIPLMVWLPDAMAGTPVSALIHAATMVTAGVYL IARSSFLYSVLPDVSYAIAVVGLLTAAYGALSFAFGQTDIKKIVAYSTISQLG YMFLAAGVGAYWVALFHVFTHAFFKALLFLASGSVIHALGGEQDVRKMG GLWKHLQPTRWHALIGALALGGLPLLSGFWSKDAILAATLTYPFGGVGF YVGALLVAVLTAMYAMRWVFLVFLGEERGHHPHEAPPVMLWPNHLLA LGSVLAGYLALPHPLPNVLEPFLKPALAEVEAHHLSLGAEWGLIALSAAV ALLGLWAGVFFQKGSAGSENLYFQGQFSKGEELFTGVVPILVELDGD VNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTYGVCQCF SRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTLV NRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIE DGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMVLL EFVTAAGITHGMDLEYKTSHHHHHHHHHH*
Nqo12^{ATH} K385I	MALLGTILLPLLGFALLGLFGKRMREPLPGVLASGLVLASFLLGAGLLLSG GARFQAEWLPGPFSLLLDNLSGFMILLIVTGVGFLIHVYAIGYMGGDPGY SRFFAYFNLFIAMMLTLVLADSYPMFIGWEGVGLASFLFIGFWYKNAQY ADSARKAFIVNRIGDLGFMLGMAILWALYGTLSISELKEAMEGPKLPNDLL ALAGLLFLGAVGKSAQIPLMVWLPDAMAGTPVSALIHAATMVTAGVYL IARSSFLYSVLPDVSYAIAVVGLLTAAYGALSFAFGQTDIKKIVAYSTISQLG YMFLAAGVGAYWVALFHVFTHAFFKALLFLASGSVIHALGGEQDVRKMG GLWKHLQPTRWHALIGALALGGLPLLSGFWSKDAILAATLTYPFGGVGFY VGALLVAVLTAMYAMRWVFLVFLGEERGHHPHEAPPVMLWPNHLLAL GSVLAGYLALPHPLPNVLEPFLKPALAEVEAHHLSLGAEWGLIALSAAVA LLGLWAGVFFQKGSAGSENLYFQGQFSKGEELFTGVVPILVELDGDV NGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTYGVCQCF SRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTLV VNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHN IEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMV LEFVTAAGITHGMDLEYKTSHHHHHHHHHH*
Nqo13 WT	MVVLAVALLPVVFGALLLGLPRALGVLGAGLSFLLNLYLFLTHPGGVAHA FQAPLLPGAGVYWFAGLDGLSALFFLTIALTVFLGALVARVEGRFLGLAL LMEGLLLGLFAARDLLVFYVFEEAALIPALLMLLYYGGEGRTRALYTFVLF TLVGSPLMLAAVLGARLLSGSPTFLELLELLAHPLQEEAAFWVFLGFALAF AIKTPFLPLHAWLPPFHQENHPSGLADALGTLKYGVFAFFRAIPLAPEG FAQAQGLLLFLAALSALYGAWVAFAAKDFKTLLAYAGLSHMGVAALGVFS GTPEGAMGGLYLLAASGVYTGGLFLLAGRLYERTGTLEIGRGRGLAQSAP GLAALALILFLAMVGLPGLSGFPGEFLTLLGAYKASPWLAALFLSVIASAA YALTAFAQKTFWEEGGSGVKDLAGAEWGFALLSVLALLLMGVFPGYFARG LHPLAEFAKLLGGENLYFQGQFSKGEELFTGVVPILVELDGDVNGHKFS VSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTYGVCQFSRYPDH MKRHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTLVNRIELK GIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGSVQ LADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMVLEFVTA AGITHGMDLEYKTSHHHHHHHHHH*
Nqo13 E123Q	MVVLAVALLPVVFGALLLGLPRALGVLGAGLSFLLNLYLFLTHPGGVAHAF

	<p>QAPLLPGAGVYWAFGLDGLSALFFLTIALTVFLGALVARVEGRFLGLALLM EGLLLGLFAARDLLVFYVFFQAAALIPALLMLYLYGGEGRTRALYTFVLFTLV GSLPMLAAVLGARLLSGSPTFLLDLLAHPLQEEAAFWVFLGFALAFAIKT PLFPLHAWLPPFHQENHPSGLADALGTLYKVGVF AFFRFAIPLAPEGFAQ AQGLLLFLAALSALYGAWVAFAAKDFKTLLAYAGLSHMGVAALGVFSGTP EGAMGGLYLLAASGVYTGGLFLLAGRLYERTGTLEIGRGRGLAQSAAPGLA ALALILFLAMVGLPGLSGFPGEFTLLGAYKASPWLAALAFLSVIASAAAYAL TAFQKTFWEEGGSGVKDLAAGWGFALLSVLALLLMGVFPGYFARGLHP LAEAFKLLGGENLYFQQQFSKGEELFTGVVPILVELDGDVNGHKFSVSG EGEGDATYGKLTCLKFICTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKRH DFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKE DGNILGHKLEYNYN SHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHYQ QNTPIGDGPVLLPDNHYLSTQSALS KDPNEKR DHMV LLEFVTAAGITHGM DELYKTSHHHHHHHHHH*</p>
Nqo13 K235M	<p>MVVLA VLLPVVFGALLLGLPRALGVLGAGLSFLLNLYLFLTHPGGVAHAF QAPLLPGAGVYWAFGLDGLSALFFLTIALTVFLGALVARVEGRFLGLALLM EGLLLGLFAARDLLVFYVFFEAALIPALLMLYLYGGEGRTRALYTFVLFTLV GSLPMLAAVLGARLLSGSPTFLLDLLAHPLQEEAAFWVFLGFALAFAIKT PLFPLHAWLPPFHQENHPSGLADALGTLYMVGVF AFFRFAIPLAPEGFAQ AQGLLLFLAALSALYGAWVAFAAKDFKTLLAYAGLSHMGVAALGVFSGTP EGAMGGLYLLAASGVYTGGLFLLAGRLYERTGTLEIGRGRGLAQSAAPGLA ALALILFLAMVGLPGLSGFPGEFTLLGAYKASPWLAALAFLSVIASAAAYAL TAFQKTFWEEGGSGVKDLAAGWGFALLSVLALLLMGVFPGYFARGLHP LAEAFKLLGGENLYFQQQFSKGEELFTGVVPILVELDGDVNGHKFSVSG EGEGDATYGKLTCLKFICTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMKRH DFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKE DGNILGHKLEYNYN SHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHYQ QNTPIGDGPVLLPDNHYLSTQSALS KDPNEKR DHMV LLEFVTAAGITHGM DELYKTSHHHHHHHHHH*</p>
Nqo13 E377Q	<p>MVVLA VLLPVVFGALLLGLPRALGVLGAGLSFLLNLYLFLTHPGGVAHAF QAPLLPGAGVYWAFGLDGLSALFFLTIALTVFLGALVARVEGRFLGLALLM EGLLLGLFAARDLLVFYVFFEAALIPALLMLYLYGGEGRTRALYTFVLFTLV GSLPMLAAVLGARLLSGSPTFLLDLLAHPLQEEAAFWVFLGFALAFAIKT PLFPLHAWLPPFHQENHPSGLADALGTLYKVGVF AFFRFAIPLAPEGFAQ AQGLLLFLAALSALYGAWVAFAAKDFKTLLAYAGLSHMGVAALGVFSGTP EGAMGGLYLLAASGVYTGGLFLLAGRLYERTGTLEIGRGRGLAQSAAPGLA ALALILFLAMVGLPGLSGFPGEFTLLGAYKASPWLAALAFLSVIASAAAYAL TAFQKTFWEEGGSGVKDLAAGWGFALLSVLALLLMGVFPGYFARGLHP PLAEAFKLLGGENLYFQQQFSKGEELFTGVVPILVELDGDVNGHKFSVS GEGEGDATYGKLTCLKFICTTGKLPVPWPPTLVTTTLTYGVQCFSRYPDHMK RHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTLVNRIELKGI DFKEDGNILGHKLEYNYN SHNVYITADKQKNGIKANFKIRHNIEDGSVQL ADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKR DHMV LLEFVTA AGITHGMDELYKTSHHHHHHHHHH*</p>

Supplementary Table 7 | Expression strains and tested plasmids.

Expression strain	Plasmid	Construct	Properties
BL21 (DE3), C41 (DE3), C43 (DE3)	pET21a	His-TEV-Nqo13	No expression
BL21 (DE3), C41 (DE3), C43 (DE3)	pBAD	His-TEV-Nqo13	No expression
BL21 (DE3), C41 (DE3), C43 (DE3)	pWALDO	Nqo13-TEV-GFP-His	Low expression
BL21 (DE3)	pWALDO	Nqo12-TEV-sfGFP-His	Low expression, no binding to Ni-NTA
LEMO21 (DE3)	pWALDO	Nqo12-linker-TEV-sfGFP-His	Successful
LEMO21 (DE3)	pWALDO	Nqo13-TEV-His	Successful

Supplementary Table 8 | Benchmarking the proton transfer energetics. The model system contains $N=137$ atoms (see also Supplementary Fig. 9). Electronic energies were evaluated at def2-TZVP/ $\epsilon=4$ (DFT)¹⁷⁻²³ and def2-TZVPPD/ $\epsilon=4$ (RPA)²⁴ levels.

State	B3LYP-D3	CAM-B3LYP-D3	CAMh-B3LYP-D3	TPSSh-D3	ω B97X-D	RPA
I	0.0	0.0	0.0	0.0	0.0	0.0
II	6.3	6.1	6.4	6.1	6.9	8.1
III	4.6	4.5	4.4	4.7	4.7	4.8
IV	17.8	17.6	17.8	16.4	19.1	18.8
V	8.3	8.7	8.4	8.4	8.0	6.5

Supplementary Table 9 | List of QM/MM simulations

Simulation	System	QM region	Description	Length (ps)	Method
1	Nqo13 WT	K235, H292, E377, T232, S291, S318, Y321, S402, Y405, 10 H ₂ O ($N=137$)	Lateral proton transfer pathway from K235 to E377	75 x 2.3 ps = 173 ps	US/WHAM
2	Nqo13 E123Q	K235, H292, E377, T232, S291, S318, Y321, S402, Y405, 10 H ₂ O ($N=137$)	Lateral proton transfer pathway from K235 to E377	75 x 2.3 ps = 173 ps	US/WHAM
3	Nqo12 WT	K329, K385, H321, H325, S298, Q302, Y305, F328, S384, T413, Y416, 4 H ₂ O ($N=149$)	Lateral proton transfer pathway from K329 to K385	52 x 2.3 ps = 120 ps	US/WHAM
4	Nqo12 E132Q	K329, K385, H321, H325, S298, Q302, Y305, F328, S384, T413, Y416, 4 H ₂ O ($N=149$)	Lateral proton transfer pathway from K329 to K385	52 x 2.3 ps = 120 ps	US/WHAM
5	Nqo13 WT	K235, H211, T232, T322, F217, H218, D228, F326, N221, K282, 9 H ₂ O ($N=148$)	N-side medial proton transfer from K282 to K235	10 x 22.5 ps = 225 ps	MWE/MBAR

Supplementary Methods

Derivation of an analytical model for ion-pair induced barrier tuning

An analytical model of the ion-pair induced barrier reduction was derived based on an electrostatic consideration. To this end, the influence of the ion-pair opening on the transition state energy was modelled by considering the electrical tuning effect of moving the proton from the middle lysine residue (Lys235 in Nqo13) to a nearby protonated water species (H_3O^+ or H_5O_2^+ at the transition state E^\ddagger), located at a distance a from the latter (Supplementary Fig. 7). The effect of the electrostatic tuning was modelled by summing over all charged interactions,

$$V_{ij}(r) = \frac{e^2}{4\pi\epsilon\epsilon_0} \sum_{i>j} \frac{q_i q_j}{r_{ij}} \quad (1)$$

leading to the barrier tuning based on ion-pair distance x ,

$$\Delta E^\ddagger(x) = \alpha \left[\frac{1}{R+a-(x-d)} - \frac{1}{R+d+a} - \frac{1}{R-(x-d)} + \frac{1}{R+d} \right] \quad (2)$$

where $\alpha = q_i q_j e^2 / 4\pi\epsilon\epsilon_0 = 332 \text{ kcal mol}^{-1} \text{ \AA}^{-1}/\epsilon$, R is the distance between the middle lysine and the ion pair, d and x are the distances between the ion-paired residues in the respective closed and open conformations, and, a is the distance between the protonated water species and Lys235. The dielectric constant of the medium was modelled with $\epsilon=10$. The geometry of the model is shown in Supplementary Fig. 7, with $R=15.9 \text{ \AA}$, $a=9.2 \text{ \AA}$, $d=3.2 \text{ \AA}$.

Poisson-Boltzmann Electrostatic with Monte Carlo sampling

pK_a values for titratable residues in Nqo12 and Nqo13 were estimated based on Poisson-Boltzmann (PB) electrostatic calculations together with Monte Carlo (MC) sampling. To this end, the protein residues were treated as explicit atoms using the CHARMM36m force field¹. The protein surroundings were modelled as an inhomogeneous polarisable low-dielectric ($\epsilon=10$) medium, with explicit lipid molecules included from the MD simulations. The bulk water was modelled as a homogenous high dielectric medium ($\epsilon=80$). The PB equations were solved numerically using the Adaptive Poisson-Boltzmann Solver (APBS)². The 2^N protonation states in the protein were sampled by allowing for single, double, and triple moves in the MC routine, as implemented in Karlsberg⁺³⁻⁵. The sidechain atoms were optimised upon protonation state changes using the ABNR minimiser in CHARMM²⁵. The pK_a s were estimated based on their shifts from experimental solvent (water) pK_a values,

$$\text{pK}_a(\text{protein}) = \text{pK}_a(\text{water}) + \Delta \text{pK}_a(\text{water} \rightarrow \text{protein})$$

with solvent pK_a s of Glu (4.1), Asp (3.9), Tyr (10.1), His (6.04), and Lys (10.5). The ΔpK_a term was calculated based on changes in the electrostatic interactions of the protonated (AH) and deprotonated (A) species with surrounding protein background charges (ΔG_{back}), the Born desolvation energy (ΔG_{Born}) for moving AH and A from water ($\epsilon=80$) to the protein medium ($\epsilon=10$), and from interaction of the 2^N possible protonation states in the protein medium ($\Delta G_{ij}^{\text{protein}}$),

$$\Delta pK_a (\text{water} \rightarrow \text{protein}) = \Delta \Delta G^{\text{water} \rightarrow \text{protein}} / 2.303 RT \quad (3)$$

with

$$\Delta \Delta G^{\text{water} \rightarrow \text{protein}} = \Delta G_{\text{Born}} + \Delta G_{\text{back}} + \sum_{i \neq j} \Delta G_{ij}^{\text{protein}} \quad (4)$$

The calculations were performed for the isolated Nqo12 and Nqo13 models based on the X-ray structure of *Thermus thermophilus* Complex I (PDB ID: 6i0d⁶), and based on MD simulations of the WT-Nqo13, E132Q-Nqo13, WT-Nqo12, E123Q-Nqo12 models (see *Methods*, main text). The pK_a shifts were also compared to MD simulations of the intact Complex I from *T. thermophilus*⁷.

Analysis of hydration profiles

The analysis and visualisation of hydration profiles from MD simulations were carried out using CAVER v.3.0⁸ and Visual Molecular Dynamics (VMD)⁹. Proton pathways were first characterised by starting the tunnel search from Lys329 in Nqo12^{ΔTH} or Lys235 in Nqo13, using a probe radius set to 0.9 Å. Sidechains of histidine residues, Phe378^{Nqo13}, and the headgroups of charged residues (Glu, Asp, Lys, Arg) were excluded from the initial tunnel search. The hydration profiles were computed based on the identified tunnels, by probing the water occupancy within a 2 Å radius along the tunnel, based on the last 100 ns of a given trajectory. The hydration percentage was calculated as the ratio between occupied water molecules at a given tunnel coordinate and the total number of analysed frames.

Constant-pH MD simulations

Constant-pH MD (cphmd) simulations were performed based on the 1 μs MD simulations of Nqo13 embedded in a POPC membrane and solvated in a water box (see main text *Methods*). The cphmd simulations were performed using 15 independent simulations, with 20 replicas each, sampling a pH range from 0.5 to 13.5, and with initial protonation states assigned from the PBE/MC calculations (see above). Each simulation included 1000 minimisation steps, followed by 500 cycles of 1000-10000 production steps and 7500 non-equilibrium switching steps, leading to a total simulation length of *ca.* 50 ns. Final pK_a values were computed based on all sampled conformations using the PBE/MC methodology (see above) by re-weighting each conformation by its protonation probability and electrostatic energy to derive average protonation states. The cphmd simulations were performed, as implemented in NAMD¹⁰⁻¹², and pK_a calculations using an in-house APBS/Karlsberg+ implementation²⁻⁵.

Supplementary Discussion

Conformational changes coupled to proton transport

To test how the proton transfer reactions coupled to conformational changes in the antiporter-like subunits, we performed additional MD simulations in multiple states. In the WT-Nqo13, we find that upon transfer of the proton from Lys235 to Glu377, mimicking a proton transfer step along the central pathway, the sidechain of His292 flips to a different rotameric state, suggesting that the conformation of His292 could affect the back transfer of protons in the reverse direction (*cf.* also Refs.^{13, 14}, Supplementary Fig. 1g). Moreover, we note that the deprotonation of the Lys235 leads conformational changes in conserved residues in TM7a/b and TM8 (Supplementary Fig. 1h) that could regulate the proton uptake from the N-side bulk. The constant pH-MD simulations (see *Supplementary Methods*) further support conformational changes around the broken TM7a/b helix, suggesting that the effective pK_a of Lys235 is around 9.3 in the WT-Nqo13, and shifts to 6.5 for E132Q-Nqo13 (Supplementary Fig. 16).

Supplementary References

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