Cryo-EM structures define ubiquinone-10 binding to mitochondrial complex I and conformational transitions accompanying Q-site occupancy

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Mitochondrial complex I is a central metabolic enzyme that uses the reducing potential of NADH to reduce ubiquinone-10 (Q10) and drive four protons across the inner mitochondrial membrane, powering oxidative phosphorylation. Although many complex I structures are now available, the mechanisms of Q10 reduction and energy transduction remain controversial. Here, we reconstitute mammalian complex I into phospholipid nanodiscs with exogenous Q10. Using cryo-EM, we reveal a Q10 molecule occupying the full length of the Q-binding site in the ‘active’ (ready-to-go) resting state together with a matching substrate-free structure, and apply molecular dynamics simulations to propose how the charge states of key residues influence the Q10 binding pose. By comparing ligand-bound and ligand-free forms of the ‘deactive’ resting state (that require reactivating to catalyse), we begin to define how substrate binding restructures the deactive Q-binding site, providing insights into its physiological and mechanistic relevance.
Mitochondrial complex I (NADH:ubiquinone oxidoreductase) is an intricate ~1 MDa multimeric membrane-bound complex that is essential for mitochondrial metabolism\(^\text{1,2}\). It comprises 14 catalytic core subunits that are conserved across all kingdoms of life, and up to 31 supernumerary subunits that contribute to its stability, regulation, and/or biogenesis\(^\text{3,4}\). As an entry point into the electron transport chain (ETC), complex I is a key contributor to oxidative phosphorylation, mitochondrial homeostasis, and redox balance. Specifically, it couples electron transfer from NADH to ubiquinone (Q) to proton translocation across the inner mitochondrial membrane, building the proton motive force \((\Delta \rho)\) to drive ATP synthesis. Due to its central roles in metabolism, complex I is implicated in ischaemia-reperfusion (IR) injury\(^\text{5}\) and its dysfunctions lead to neuromuscular and metabolic diseases\(^\text{6}\).

Two biochemically characterised, physiologically relevant states, the ‘active’ and ‘deactive’ resting states described initially by Vinogradov and coworkers\(^\text{7}\), have previously been identified by electron cryomicroscopy (cryo-EM) on mammalian complex I\(^\text{3,9,10}\). They are distinguished by subtle domain movements and conformational changes at the Q-binding site and in adjacent membrane-domain subunits\(^\text{3,9-13}\). In the absence of substrates and at physiological temperatures, mammalian complex I relaxes gradually from the ready-to-catalyse active resting state into the profoundly active resting state, with a partially unstructured Q-binding site\(^\text{3,7-10,14,15}\). The deactive state forms spontaneously during ischaemia (when lack of O\(_2\) prevents ETC turnover). Then, when NADH and ubiquinone are added/replenished (for example, upon reperfusion), it slowly reactivates and returns to catalysis. During reperfusion the deactive state protects against IR injury by minimising complex I-mediated production of reactive oxygen species by reverse electron transfer\(^\text{13,16,17}\). Cryo-EM studies of mammalian complex I have also identified a third state, for which the density map lacks information in key regions of the enzyme, which we suggested previously to arise from complex I in the first stages of dissociation\(^\text{3,11}\).

The Q-binding site in complex I is an unusually long and heterogeneous channel. Advances in cryo-EM have allowed identification of several inhibitors\(^\text{12,18-22}\), adventitious detergents\(^\text{23}\), and substrate analogues\(^\text{19,20,24,25}\) bound in the site, whilst native Q species (Q\(_9\) and Q\(_{10}\) with nine and ten isoprenoid units, respectively) have predominantly been observed with their Q-headgroups close to the channel entrance\(^\text{25-28}\). There is currently only one report of complex I containing a fully-bound Q\(_{10}\) molecule, which is for porcine complex I in the mammalian respirasome\(^\text{22}\). As expected, the structure shows the fully-bound Q\(_{10}\) spanning the entirety of the channel, but, consistent with observations of the short-chain substrate analogues decylubiquinone (dQ)\(^\text{19,20,24,25}\) and piericidin A \(^\text{12,19}\), it does not exhibit the expected dual-ligation of the two redox-active carbonyls on the Q-headgroup. Biochemical data\(^\text{29}\) and molecular dynamics simulations\(^\text{30-32}\) both indicate that, for redox-coupled proton transfer, the headgroup must be ligated between two proton-donor ligating partners (His\(^{59}\)\(^\text{NDUF5}\) and Tyr\(^{108}\)\(^\text{NDUF5}\)) at the tip of the channel.

Here, we describe the structures of several states of bovine complex I reconstituted into phospholipid nanodiscs with exogenous Q\(_{10}\). The nanodiscs provide a native membrane-like environment and eliminate potential artefacts from the detergent micelle typically present in cryo-EM analyses. Using cryo-EM, we resolve five distinct structures at global resolutions up to 2.3 Å, including one with a Q\(_{10}\) molecule occupying the full length of the Q-binding channel. By comparing the structures of substrate/ligand-bound and apo (substrate/ligand-free) forms of both the active and deactive states, as well as a structure of the poorly characterised third state, we probe substrate/ligand-driven conformational changes in the Q-binding site and the physiological and catalytic relevance of each state.

### Results

**Reconstitution of bovine complex I into nanodiscs.** Complex I was purified from bovine heart mitochondria in the detergent \(n\)-dodecyl \(\beta\)-D-maltoside (DDM)\(^\text{10,33}\). Then, to exchange the detergent micelles for nanodiscs, it was reconstituted in a mixture of synthetic phospholipids, Q\(_{10}\) and the membrane scaffold protein MSP2N\(^\text{34}\), using a protocol derived from that for preparing complex I proteoliposomes\(^\text{21,33,35}\). The nanodisc-bound complex I (Cxi-ND) was then isolated by size-exclusion chromatography (from proteoliposomes, protein–free nanodiscs, free MSP2N\(_2\), and residual detergent) and shown to be monodisperse (Supplementary Fig. 1a). Following addition of CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate) and asolectin to dissociate the scaffold proteins and provide a larger hydrophobic phase, the sample used for cryo-EM exhibited 86.0 ± 0.1% (17.5 ± 0.4 µmol min\(^{-1}\) mg\(^{-1}\)) of the piericidin-sensitive NADH:dQ oxidoreductase activity of the DDM-solubilised enzyme before reconstitution (20.4 ± 0.6 µmol min\(^{-1}\) mg\(^{-1}\)), indicating that the complex I in Cxi-ND is highly catalytically competent. The intact Cxi-NDs (without CHAPS/asolectin) displayed very little piericidin-sensitive NADH:dQ activity (2.01 ± 0.10 µmol min\(^{-1}\) mg\(^{-1}\)) indicating that dQ does not exchange effectively in and out of the nanodisc and/or Q-binding site (see also Supplementary Fig. 1b).

**Resolution of three major classes of Cxi-ND particles.** Single-particle cryo-EM analyses on a Titan Krios microscope with a K3 detector yielded a total of 343,213 particles (Table 1), which were separated into three major classes by 3D classification in RELION\(^\text{36}\), following subtraction\(^\text{37}\) of the nanodisc density (Supplementary Fig. 2). By map-to-map comparisons with biochemically characterised mouse and bovine structures\(^\text{3,9,10,12,13}\) (Supplementary Table 1a), two classes were assigned to complex I in the ‘active’ resting state (61,658 particles, 2.65 Å resolution) and in the ‘deactive’ resting state (259,540 particles, 2.28 Å). The third class (22,019 particles, 3.02 Å) matched best to a state proposed earlier to correspond to particles in the first stages of dissociation; here we name it ‘state 3’ to reassess it without the bias from early suggestions based on 5.60 Å low-resolution data\(^\text{3,11}\). Focused 3D classifications subsequently resolved two sub-states for each of the active and deactive states, providing a total of five distinct maps and models (Table 1 and Supplementary Figs. 2, 3 and 4).

All the established hallmarks for the active state\(^\text{3,10,12}\) are present in the active maps/models (Supplementary Fig. 5), including well-defined densities for the loops in NDUF52, ND3 and ND1 (residues 52–62, 24–55, and 194–217, respectively) that constitute the Q-binding site and for the region of NDUA9 closest to the membrane, an extended NDUF5/NDUA10 interface, and a fully α-helical ND6-transmembrane helix (TMH) 3. [Note we use the human subunit nomenclature throughout for simplicity]. The same is true for the deactive maps/models (Supplementary Fig. 5), where the hallmarks include disordered/alternate conformations of the above loops, disorder in a short stretch of a loop in NDUA9 (residues 324–331) adjacent to the disordered loop in ND3, a restricted NDUF5/NDUA10 interface, and a π-bulge in ND6-TMH3\(^\text{3,9,10,13}\). Furthermore, NDUF57 residues 47–51 form a loop in the active state and a β-strand in the deactive state, and the adjacent loop (residues 74–83) is ‘flipped over’ between the two states, reorientating the hydroxylated conserved Arg\(^{77}\)\(^\text{NDUF57}\). As reported previously\(^\text{3}\), the state 3 map (see Supplementary Fig. 6) lacks clear densities for...
Table 1 Cryo-EM data collection, refinement, and validation statistics.

| Data collection and processing | Grid I | Grid II |
|--------------------------------|--------|--------|
| Magnification                 | 81,000 | 81,000 |
| Voltage (kV)                  | 300    | 300    |
| Electron exposure (e/Å²)      | 40.5   | 40.5   |
| Defocus range (µm)            | −1.0 to −2.4 | −1.0 to −2.4 |
| Super-resolution pixel size (Å)| 0.535  | 0.535  |
| Final pixel size (Å)          | 0.750  | 0.750  |
| Symmetry imposed              | C1     | C1     |
| Initial particle images (no.) | 701,236| 377,697|
| Final particle images (no.)   | 212,841| 130,372|
| Total final particle images (no.) | 343,213 |        |

| Classes                        | Active-Q<sub>10</sub> | Active-apo | Deactive-apt<sup>a</sup> | Deactive-apo | State 3 (Slack) |
|--------------------------------|------------------------|------------|--------------------------|--------------|---------------|
|                                 | EMD-14132              | EMD-14133  | EMD-14134                 | EMD-14139    | EMD-14140      |
| Final particle images (no.)    | 23,449                 | 38,205     | 235,957                  | 23,583       | 22,019         |
| Map resolution (Å) [FSC threshold: 0.143] | 2.84                   | 2.76       | 2.30                     | 2.81         | 3.02           |
| Map resolution range (Å)       | 2.61-7.02              | 2.49-6.70  | 2.05-3.99                | 2.48-7.16    | 2.61-6.49      |
| Map sharpening B-factor (Å²)   | −34                    | −42        | −(49-53)                 | −44          | −54            |
| Model statistics               |                        |            |                          |              |               |
| Initial model (PDB ID)         | 7QSD                   | 7QSD       | 7QSD                     | 7QSD         | 7QSD          |
| Model resolution (Å) [FSC threshold: 0.5] | 2.85                   | 2.73       | 2.23                     | 2.73         | 2.91           |
| Model composition              |                        |            |                          |              |               |
| Non-hydrogen atoms             | 69,987                 | 69,743     | 71,642                   | 69,322       | 66,114         |
| Protein residues               | 8,287                  | 8,283      | 8,299                    | 8,198        | 8,015          |
| Ligands                        | 45                     | 42         | 48                       | 45           | 32             |
| Waters                         | 1,060                  | 1,024      | 2,774                    | 1,096        | 0              |
| Average B-factors (Å²)         | 49.62                  | 43.88      | 40.03                    | 42.27        | 51.36          |
| Protein                        | 50.65                  | 44.98      | 42.75                    | 43.24        | 50.30          |
| Ligand                         | 44.47                  | 37.52      | 37.10                    | 36.74        | N/A            |
| Root Mean Square deviations    |                        |            |                          |              |               |
| Bond lengths (Å)               | 0.007                  | 0.009      | 0.006                    | 0.006        | 0.006          |
| Bond angles (*)                | 0.641                  | 0.732      | 0.647                    | 0.648        | 0.618          |
| MolProbity score               | 1.30                   | 1.36       | 1.13                     | 1.37         | 1.44           |
| All-atom clash score           | 3.18                   | 3.76       | 2.69                     | 3.68         | 4.03           |
| EMRinger score                 | 4.82                   | 5.35       | 6.42                     | 4.68         | 4.07           |
| Ramachandran plot              | 0.15                   | 0.04       | 0.58                     | 0.01         | 0.00           |
| Favored (%)                    | 96.87                  | 96.83      | 97.64                    | 96.69        | 96.21          |
| Allowed (%)                    | 3.07                   | 3.13       | 2.34                     | 3.28         | 3.75           |
| Outliers (%)                   | 0.06                   | 0.04       | 0.01                     | 0.02         | 0.04           |

*Data refers to the composite map, except for the map resolution (FSC = 0.143) which comes from the consensus map. For the consensus map data, see Supplementary Fig. 3.
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around the enzyme, and 2.66 Q 10. Although this equates to 295 phospholipids, suf cient for a layer only one or two deep — protein voids in others (Fig. 1 and Supplementary Fig. 7a).

The overlapping densities for the 14 core (coloured) and 31 supernumerary (grey) subunits support quinone diffusion, consistent with the low dQ/Q 10 (~0.26–0.31 Å) for the membrane-bound core subunits, 0.32–0.35 Å overall). For the deactive-state structures, we were able to model substantially more of subunit NDUFA9 than has previously been possible in detergent-solubilised deactive/open structures. Only residues 324–331, adjacent to the disordered loop in ND3, were not modelled, suggesting that the nanodisc environment is able to contain the disorder that further propagates to residues ca. 186–195, 253–278, and 323–334 in the detergent-solubilised enzyme. It is thus possible that structural changes to NDUFA9 in the membrane-bound deactive state are less extensive than previously supposed. As expected, none of the three DDMs modelled in the active-state bovine reference structure were observed in CxI-NDs, while the total number of phospholipids observed has increased to 47, including all 22 that were modelled in the reference structure (Supplementary Fig. 7b). There is no evidence that lateral pressure ‘tightens’ the subunit interfaces in the CxI-ND membrane domain, and further comparisons with detergent-solubilised mammalian complex I structures revealed only three very minor structural differences (see Supplementary Note 2).

Q10 bound in the active state. Following focused 3D classiﬁcation to resolve heterogeneous density features in the Q-binding site (see Methods and Supplementary Fig. 3), the active class was split into a ligand-bound sub-class with a continuous density matching a Q10 molecule spanning the Q-binding channel (henceforth active-Q10; 23,449 particles, 2.84 Å resolution), and a substrate-free sub-class with a presumably water-ﬁlled cavity (henceforth active-apo; 38,209 particles, 2.76 Å) (Table 1 and Supplementary Fig. 4). The Q-binding site is fully structured in both cases.

In active-Q10, the Q10 density occupies the entirety of the channel, starting between subunits NDUFS2 and NDUFS7 (with the Q-headgroup adjacent to Tyr108NDUFS2 and His59NDUFS2) then extending, as the isoprenoid chain, along the NDUFS2–NDUFS7 then ND1–NDUFS7 interfaces to exit from ND1. The His sidechain forms a hydrogen bond (H-bond) with the 3-methoxy of the Q-headgroup (3.2 Å), rather than with either of the reactive carbonyls, which are in geometrically unfavourable positions (Fig. 2a). The Tyr sidechain is too distant (>4.3 Å) from the headgroup for a H-bond, but interacts via two mediating water molecules and a water is also H-bonded between the headgroup and Asp160NDUFS2. In an alternative lower-occupancy orientation of the Q10, represented by weaker density, the headgroup is ﬂipped by 180° and the ﬁrst isoprenoid is in a different position (Fig. 2a inset). While the His now interacts with the 2-methoxy the H-bonding pattern is similar. In both cases, attempts to reposition the headgroup to create reactive H-bonds to the Tyr and His without moving the isoprenoid chain out of its density were unsuccessful. A number of waters were also observed adjacent to the isoprenoid chain, stabilised by H-bonding to nearby sidechains, clustered particularly in the more charged protein section around the middle of the isoprenoid chain in both cases.

To probe further why the Q10 appears to have ‘paused’ in this pre-reactive conformation, we applied molecular dynamics simulations with enhanced sampling to explore the charge-state and flexibility of reactive groups in the Q-binding site (see Methods for Charaterisation of nanodisc-bound complex I. In all three states, two belt-like densities representing two MSP2N2 monomers are visible, enveloping the membrane domain in place of the usual detergent-phospholipid micelle (Fig. 1). Two 391 residue-long polyalanine models fit into them well, overlapping as four parallel helices below NDUFA9 (Fig. 1b), a subunit just above the expected membrane surface that has been observed to bind phospholipids and reported as an important feature of the active to deactive transition. The overlapping contrasts with the ‘dangling’ termini observed for shorter MSPs. Quantitative biochemical analyses showed the CxI-NDs contain an average of 295 phospholipids, sufficient for a layer only one or two deep around the enzyme, and 2.66 Q 10. Although this equates to ~12 mM Q10 in the phospholipid phase, substantially above the Kam value in proteoliposomes, there is no ‘bulk’ bilayer to support quinone diffusion, consistent with the low dQ/Q10 reductase activity (Supplementary Fig. 1b). Furthermore, the MSP2N2s wrap tightly around the enzyme like stretched rubber bands, creating direct enzyme-MSP2N2 contacts in some regions and phospholipid-ﬁlled crevices and cavities where there are protein voids in others (Fig. 1 and Supplementary Fig. 7a).
Our active-Q$_{10}$ model was built with the His59$^{\text{NDUFS2}}$ dihedral angle $\chi_2 = -95.5^\circ$ (Fig. 2a), but the conformer with a ‘flipped’ sidechain also fits well. Interestingly, simulations for the [AspH+His] charge-state (Fig. 3e) revealed a flexible His59$^{\text{NDUFS2}}$ sidechain with a barrier for the ring to flip of <15 kJ mol$^{-1}$ at the stable binding position (CV = 23.8 Å), consistent with $\chi_1 = -100^\circ$ or +80$^\circ$ being equally populated at the physiological temperature simulated. When the Q-headgroup approaches Tyr108$^{\text{NDUFS2}}$ (CV = 22.5 Å; Fig. 3b), the flexibility of His59$^{\text{NDUFS2}}$ $\chi_2$ is slightly decreased (Fig. 3e) and a H-bond, which is absent at the stable binding position (Fig. 3c), forms between His59$^{\text{NDUFS2}}$-$\text{N}_2$ and the protonated Asp160$^{\text{NDUFS2}}$ sidechain (Fig. 3f). It may also re-form when the Q-headgroup dissociates (Fig. 3d), in line with previous simulations on complex I from *T. thermophilus*.[31,41] The H-bond between His59$^{\text{NDUFS2}}$-$\text{N}_8$1 and the Q-headgroup 3-methoxy is easily broken (Fig. 3g). In fact, when the Q-headgroup occupies its stable binding position it has sufficient conformational freedom to twist and flip during the physiological-temperature simulations, also visiting conformations compatible with the flipped subpopulation observed in the cryo-EM data (Fig. 2a inset).
The reactive site, the His59NDUFS2 sidechain is rotated by ∼90° relative to in the active-Q_{10} structure (Fig. 2c) and stabilised by H-bonding to the carbonyl backbone of Ile423NDUFS2; the water coordinate and coloured by free energy are shown in coloured shadows. The collective variable (CV) describes the distance between the Q-headgroup 3-methoxy-O and His59NDUFS2-NHz atoms. Symbols correspond to the structural properties observed in cryo-EM models active-Q_{10} with the primary (star) and flipped (bullet) Q-headgroups.

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In the absence of contiguous density attributable to bound Q_{10} (active-apo structure), we suggest that the discrete densities scattered throughout the Q-binding channel, including in isoprenoid-binding regions, are dynamic water molecules in H-bonding networks (Fig. 2c). Intriguingly, there is also a clear but unidentified density observed at the channel entrance (it may be a Q_{10} or phospholipid inserted tail-first, but is also consistent with a molecule of MOPS buffer) that appears to separate the water-filled cavity from the hydrophobic membrane (Fig. 2c). In the reactive site, the His59NDUFS2 sidechain is rotated by ∼90° relative to in the active-Q_{10} structure (Fig. 2c) and stabilised by H-bonding to the carbonyl backbone of Ile423NDUFS2; the water molecule between the Q-headgroup and Asp160NDUFS2 shifts by -1 Å in response (Fig. 2c). The two waters between Tyr108NDUFS2 and the Q-headgroup are not resolved in the active-apo sub-state, but we suggest a poorly-resolved density extending from Tyr108NDUFS2 represents a network of dynamic water molecules, in place of the headgroup. Apart from His59NDUFS2, the only difference in residue conformation between the Q-binding sites in the active-Q_{10} and active-apo structures is at Glu202ND1. Although, as may be expected for carboxylates, the sidechains are not well-resolved, the models suggest that in active-Q_{10} Glu202ND1 forms a water-mediated H-bond to Glu227ND1 whereas in active-apo, Glu202ND1 has undergone a rotameric shift that reconfigures the H-bonding interactions between the two glutamates. These observations hint that Glu202ND1 responds to the channel occupancy, and may function as a ‘control point’ for proton transfer.

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**Ligand binding to the deactive state.** Q-binding site loops in subunits NDUFS2, ND1 and NDUFS7 are characteristically disordered in the deactive state^{4,10,13} and so further classification of the deactive particles was focused on the whole Q-binding region (Supplementary Fig. 3). Two sub-classes were resolved: an occupied, ligand-bound sub-class (active-ligand; 235,957 particles, 2.30 Å resolution) and an apparently unoccupied apo sub-class (active-apo; 23,583 particles, 2.81 Å) (Table 1 and Supplementary Fig. 4). In the deactive-apo structure, the Q-binding site loops in NDUFS2, ND3 and ND1 are largely disordered, whereas in the deactive-ligand structure the NDUFS2 and ND1 loops are in ordered conformations different from those in the active state (Fig. 4 and Supplementary Fig. 5). The overall structure remains deactive (with a restricted NDUFA5/NDUA10 interface, disordered ND3 loop, π-bulge in ND6-TMH3, and deactive NDUFS7 conformation). The restructured NDUFS2-B1-B2 loop (which carries His59) has moved into the space occupied by the Q-headgroup in the active-Q_{10} structure (Fig. 4c, f), and together with the restructured ND1-TMH5-6 loop, it constrains the Q-binding channel (Fig. 4c). In contrast, in the deactive-apo structure, the disordered loops fail to enclose the channel, which appears as a gaping crevice open to the matrix (Fig. 4d).

The ligand density observed in the deactive-ligand structure displays features consistent with both Q_{10} and DDM, suggesting a mixed population that could not be separated by focused classification. The DDM used for complex I preparation may have been retained in the channel when the external DDM molecules were removed during reconstitution. The shape of the headgroup density suggests Q_{10} at low map thresholds but matches two six-membered maltoside rings at higher thresholds, while a long, zigzagged protrusion resembles an isoprenoid chain (Supplementary Fig. 9). Fitting a Q10 molecule into the density reveals just one polar interaction, a H-bond from Arg274ND1 to the Q-headgroup, while the fitted DDM molecule additionally interacts with His55NDUFS2 and Glu202ND1, and an intervening water molecule bridges it to Glu24ND1 (Fig. 5a). DDM may thus stabilise the deactive state, but whether it also promotes deactivation remains unclear. His55NDUFS2 and Glu202ND1 are on the displaced NDUFS2-B1-B2 and ND1-TMH5-6 loops, respectively, consistent with their restructuring in the deactive-ligand structure and with continued disorder in the ND3-TMH1-2 loop, which is consistent with the active state by interaction between His55NDUFS2 and Cys39ND3. Notably, the DDM molecule modelled here differs from the one observed in Y. lipolytica complex I, which is slightly further into the Q-binding channel with its maltoside rings in different positions (Supplementary Fig. 10g-i). Similarly, the modelled positions of Q_{10} in plant complex I and porcine complex I in Y. lipolytica complex I, and dQ in closed and open ovine complex I (Supplementary Fig. 10m-p) overlap with the Q_{10}/DDM modelled here, but do not align well. Clearly, the lower section...
of the Q-binding channel can accommodate a variety of extended hydrophobic and amphipathic molecules, including substrates, inhibitors and detergents, and their binding may modify surrounding protein structures.

**Ligand binding to state 3.** Following focused classification procedures, all the state 3 particles were retained in a single homogenous class that contains densities for two bound ligands. First, a clear density at the entrance to the Q-binding channel (Fig. 4e, f) is consistent with a cholate molecule (added for the reconstitution) in two orientations (Fig. 5b), in close proximity to either Arg25ND1, Asp51ND1, and Trp46NDUFS7, or Arg274ND1, Thr21ND1, and Tyr228ND1 for polar interactions. As in the deactive-ligand structure, the NDUFS2-β1-β2 loop is translated up the channel relative to both its active and deactive-ligand conformations, bringing the backbone carbonyls of Ala58NDUFS2 and His59NDUFS2 to within H-bonding distance (2.6–2.8 Å) of the Tyr108NDUFS2 hydroxyl and restricting the cavity. The ND1-TMH5-6 loop does not run across the Q-binding channel as in the deactive-ligand state, but is retracted downwards, beyond its conformation in the active state, so the Q-binding site again appears open to the matrix. It is not possible to tell if these changes result from cholate binding or are intrinsic to state 3. Regardless of the physiological and mechanistic relevance of state 3, our structure affirms the flexible nature of the Q-binding site and its ability to accommodate ligands. Second, a
1.4 Å diameter probe (Figs. 2b, c, 4a, ND3, and ND6, were visualised using the CASTp software with a like subunit, ND2. To probe whether the different Q-site structures – bulge in ND4-TMH6 not present in the active or deactive states. However, the Q10 headgroup is ~100 Å away from cluster N2 and the Q-binding channel, which is clearly too far for catalytic electron transfer. Similarly, a non-catalytic Q10 has also been proposed to bind in subunit NDUFA9 (outside of the canonical Q-binding site), in the active state of porcine complex I also. In contrast, the Q10 headgroup in a crystal structure of T. thermophilus complex I9,19 is flipped and rotated out-of-plane, with Tyr108NDUFS2 (Tyr87Nqo4) H-bonding with both the 4-carbonyl and 3-methoxy but His59NDUFS2 (His38Nqo4) not interacting. A similarly flipped binding pose was captured in another set of simulations on T. thermophilus complex I4, where the Q10 headgroup was stabilised by a H-bond to (protonated) His59NDUFS2 but did not engage Tyr108NDUFS2. The binding poses observed in two piericidin-bound structures12,19 match the pose for dQ in the T. thermophilus structure9, although neither recapitulates the currently unique NDUS2-β1-β2 loop conformation that increases the separation of Tyr108NDUFS2 and His59NDUFS2 in this dQ-bound bacterial structure.

Here, we showed excellent agreement between our experimentally determined Q10 binding pose and predictions from simulations with cluster N2 oxidised and a defined [AspH + His] charge-state for Asp160NDUS2 and His59NDUS2. Our results imply that the protonation and charge states of key active-site residues are intimately connected to the binding pose. Furthermore, in both our structure and simulations, cluster N2 is oxidised. Reduction of cluster N2 is likely a key driver for the changes required to bring the Q-headgroup from the pose we observe into the reactive state, including ‘flipping’ the Q-headgroup to orientate the 4-carbonyl toward Tyr108NDUS2, shifting ionisation equilibria to protonate His59NDUS2, and changing the conformation of the NDUS2-β1-β2 loop to bring it into a coordinating position. We suggest that our observed state is structurally equivalent to a ‘pre-reactive’ state that occurs.

**Discussion**

The active-Q10 structure described here shows an exogenous native ubiquinone substrate inserted fully into the complex I Q-binding channel. However, the Q-headgroup is not bound in the expected reactive state with its 1,4-carbonyls H-bonding to Tyr108NDUS2 and His59NDUS2, ready to receive their protons following electron transfer from cluster N2. Simulations of the reactive state in T. thermophilus complex I proposed that the 4-carbonyl always H-bonds to Tyr108NDUS2, but the 1-carbonyl may either H-bond or π-stack with His59NDUS2. While H-bonding between the 4-carbonyl and Tyr108NDUS2, but not between the 1-carbonyl and His59NDUS2, was described in a recently published active Q10-bound pore structure25, our active-Q10 structure shows neither of these interactions. Furthermore, previous structures with the Q10 analogue dQ and the inhibitor piericidin A1 bound are also not modelled in the expected reactive state (Supplementary Fig. 10a–f). In the ovine ‘closed’ state supplemented with dQ and NADH the dQ-headgroup is rotated ~35° (in-plane) relative to our primary binding pose so that His59NDUS2 H-bonds to the 4-carbonyl instead of the 3-methoxy, but the 1-carbonyl is ~4.5 Å from Tyr108NDUS2. The dQ-headgroup is 5-6 Å away from Tyr108NDUS2 (Tyr144NDUS2) in Y. lipolytica complex I also.24

In our active states, this unorthodox Q10-binding site is well ordered and occupied by a phospholipid molecule.

State-dependent structural features in the ND1 cavity and E-channel. To probe whether the different Q-site structures propagate changes to the membrane domain, we investigated the structures of the E-channel, which leads from the Q-binding site to ND4L in the (Fig. 6a, b). Two water molecules, on each side of the ~12.5 Å-long Asp66ND3-to-Glu34ND4L H-bond network that is continuous in the active state, disrupting it and releasing Glu34ND4L to flip away towards ND2 (Fig. 6c, d). In this region, state 3 matches the deactive state (Fig. 6e). Beyond this region, we did not observe any substantial changes in the membrane domain between the active-Q10-apo and deactive-ligand/-apo states.

**Discussion**

The active-Q10 structure described here shows an exogenous native ubiquinone substrate inserted fully into the complex I Q-binding channel. However, the Q-headgroup is not bound in the expected reactive state with its 1,4-carbonyls H-bonding to Tyr108NDUS2 and His59NDUS2, ready to receive their protons following electron transfer from cluster N2. Simulations of the reactive state in T. thermophilus complex I proposed that the 4-carbonyl always H-bonds to Tyr108NDUS2, but the 1-carbonyl may either H-bond or π-stack with His59NDUS2. While H-bonding between the 4-carbonyl and Tyr108NDUS2, but not between the 1-carbonyl and His59NDUS2, was described in a recently published active Q10-bound pore structure,25 our active-Q10 structure shows neither of these interactions. Furthermore, previous structures with the Q10 analogue dQ and the inhibitor piericidin A1 bound are also not modelled in the expected reactive state (Supplementary Fig. 10a–f). In the ovine ‘closed’ state supplemented with dQ and NADH the dQ-headgroup is rotated ~35° (in-plane) relative to our primary binding pose so that His59NDUS2 H-bonds to the 4-carbonyl instead of the 3-methoxy, but the 1-carbonyl is ~4.5 Å from Tyr108NDUS2. The dQ-headgroup is 5-6 Å away from Tyr108NDUS2 (Tyr144NDUS2) in Y. lipolytica complex I also.24

In contrast, the dQ-headgroup in a crystal structure of T. thermophilus complex I9,19 is flipped and rotated out-of-plane, with Tyr108NDUS2 (Tyr87Nqo4) H-bonding with both the 4-carbonyl and 3-methoxy but His59NDUS2 (His38Nqo4) not interacting. A similarly flipped binding pose was captured in another set of simulations on T. thermophilus complex I4, where the Q10 headgroup was stabilised by a H-bond to (protonated) His59NDUS2 but did not engage Tyr108NDUS2. The binding poses observed in two piericidin-bound structures12,19 match the pose for dQ in the T. thermophilus structure9, although neither recapitulates the currently unique NDUS2-β1-β2 loop conformation that increases the separation of Tyr108NDUS2 and His59NDUS2 in this dQ-bound bacterial structure.

Here, we showed excellent agreement between our experimentally determined Q10 binding pose and predictions from simulations with cluster N2 oxidised and a defined [AspH + His] charge-state for Asp160NDUS2 and His59NDUS2. Our results imply that the protonation and charge states of key active-site residues are intimately connected to the binding pose. Furthermore, in both our structure and simulations, cluster N2 is oxidised. Reduction of cluster N2 is likely a key driver for the changes required to bring the Q-headgroup from the pose we observe into the reactive state, including ‘flipping’ the Q-headgroup to orientate the 4-carbonyl toward Tyr108NDUS2, shifting ionisation equilibria to protonate His59NDUS2, and changing the conformation of the NDUS2-β1-β2 loop to bring it into a coordinating position. We suggest that our observed state is structurally equivalent to a ‘pre-reactive’ state that occurs.
naturally on the catalytic cycle, similar to that proposed previously by Teixeira and Arantes\cite{31}, in which the Q₁₀ pauses before dehydrogenation and ligation of the 1,4-carbonyls finally brings it into the reactive conformation. The active Q₁₀-bound porcine structure described recently\cite{25} differs from our structure because there the Q₁₀ headgroup is flipped and the 4-carbonyl is H-bonded to Tyr108NDufs2. This structure was further suggested to be in a different charge-state, with His59NDufs2 protonated and a much shorter distance to Asp160NDufs2 than in our structure (1.7 Å vs. 5.0 Å) suggesting an ion-pair interaction that places it in the [Asp⁺ + HisH⁺] charge-state. Nevertheless, His59NDufs2 remains unable to coordinate to the 1-carbonyl. Complex I is expected to encounter several different charge-states, including for Asp160NDufs2 and His59NDufs2, during its turnover cycle and therefore the two different Q₁₀ binding poses observed may illustrate the interplay of charge-state and Q-headgroup ligation that the system explores prior to entering the reactive conformation.

On the surface of it, our results may alternatively be taken to suggest the possibility of ubiquinone reduction in improperly ligated states, consistent with the considerable (inhibitor-sensitive) NADH:Q₁₀/Q₈ activities of the Tyr108NDufs2-equivalent Y144F/W/H mutants in Y. lipolytica\cite{29}. However, ubiquinone reduction potentials depend on the ligation environment and binding mode\cite{46}, and engaging water molecules around the headgroup as non-specific proton donors appears unlikely in the context of an efficient energy-conserving mechanism. Bound Q₁₀/Q₈ have much greater conformational freedom without the long isoprenoid tail to constrain them\cite{33,34}, so they may exhibit alternative hydration patterns and conformational distribution\cite{31}, and become artefactually activated for reduction.

Here, reconstituting complex I into phospholipid nanodiscs with Q₁₀ allowed us to resolve substrate/ligand-bound and substrate/ligand-free forms of both the active and deactive states of mammalian complex I, plus a ligand-bound form of state 3. Whether deactive-like states (in which the NDufs2-β₁-β₂, ND1-TMH5-6 loops and a loop in subunit NDufs7 are altered/disordered\cite{10,22}) are intermediates in catalysis\cite{20} or pronounced off-cycle resting states\cite{7,14,15,25} is currently a major controversy. First, we note the high similarity of our bovine/murine active and deactive states with the closed and open conformations, respectively, of ovine complex I, supported by high-map-map correlations, small overall RMSD values (Supplementary Table 1b) and matching structural hallmarks\cite{9,10,5}. Whereas we attribute the deactive state to an off-cycle pronounced resting state, Kampjut and Sazanov\cite{20} proposed that ‘open’ deactive-like states must form during catalysis for Q₁₀ to enter the Q-binding channel, which would then ‘close’ as it moves to the reactive site. Their model excludes unoccupied active-like/closed states as catalytic intermediates. However, the simplest explanation for our observation of both the active-Q₁₀ and active-apo states, with near-identically structured Q-binding sites (as also observed in several inhibitor-bound structures\cite{12,20–22}), is that Q-binding may occur within active-like/closed conformations. In this case, individual structural elements involved in the deactive transition may move during catalysis, but would not be coordinated to produce the extensive domain-level conformational changes required to generate a full deactive-like state. Simulations of Q-binding/dissociation differ in the extent to which they predict conformational changes are required\cite{31,41,48,49}, and the controversy will probably only be resolved when the intermediates generated by turnover of a sample in which deactive-like states are not already present are observed. The presence of deactive-like/open states in both the pre-turnover and turnover samples characterised by Kampjut and Sazanov\cite{20} is consistent with both models, as they may represent off-cycle resting states that are not actively catalysing. A very different mechanism, involving only active-like states, was recently proposed by Gu et al.\cite{25}, with an invariably bound Q₁₀ molecule shutting up and down within the Q-binding channel, collecting electrons from N₂ and transferring them to a secondary Q₁₀ bound in the membrane outside the channel\cite{25}. However, our data suggest the existence of active-like enzyme molecules without Q₁₀ bound (active-apo state), and we have not identified any densities from Q₁₀ bound outside the channel. Therefore, our data do not align with the proposed ‘two-Q’ model\cite{25}. Nevertheless, it should be noted that our complex I was not frozen during turnover, and so further evidence is required to confirm the relevance of ‘empty’ Q-site species to the catalytic cycle.

Common to both interpretations of deactive/open states is the concept that substrate binding triggers restructuring of the Q-binding channel, either during catalysis or reactivation. Here, comparison of our deactive-ligand and deactive-apo structures shows how Q₁₀/DDM-binding in the lower section of the channel restructures the NDufs2-β₁-β₂ and ND1-TMH5-6 loops in the oxidised deactive state. These restructured loop conformations match the conformations observed in the NADH-reduced open states of ovine complex I, with ubiquinone/ol modelled in a position overlapping with that occupied here by Q₁₀/DDM\cite{20}. On the contrary, loop reordering was not observed in porcine complex I in the deactive state\cite{25}, in which ubiquinone/ol is modelled in the same position as we have modelled cholate in the Q-binding site in state 3. It is possible that a heterogeneous population of the Q-binding site in the porcine enzyme has led to discontinuous or weaker densities that were not successfully classified into ligand/substrate-bound and -apo states. Finally, in our partially restructured deactive-ligand state the NDufs2-β₁-β₂ loop blocks substrate access to the reactive centre, suggesting that the deactive state is resistant to reactivation under the conditions for reverse electron transport (ubiquinol and NAD⁺)\cite{7} because ubiquinol is unable to react with the oxidised enzyme. As a result, the deactive resting state of complex I protects against the damage caused by reverse electron transfer and the coupled generation of reactive oxygen species in ischaemia-reperfusion injury\cite{13,16,17}.

Further modifications of our CxI-ND reconstitution strategy now provide new opportunities to capture and characterise hitherto-unknown catalytic intermediates that are formed as the native Q₁₀ substrate binds and is reduced, triggering energy transfer to the proton-pumping membrane domain.

**Methods**

**Transformation and recombinant expression of membrane scaffold protein, MSP2N2.** Bacterial strain Escherichia coli NiCo2(1-DE3) was kindly provided by Dr Ali Ryan, Northumbria University, UK. Chemically competent E. coli NiCo2(1-DE3) cells were prepared and transformed with pMS2P2N2 (Addgene) using a standard New England BioLabs (NEB) heat shock protocol. For heterologous overexpression of MSP2N2, a starter culture of E. coli NiCo2(1-DE3) containing pMSP2P2N2 was grown overnight at 37 °C in LB broth with 50 µg mL⁻¹ kanamycin. 4 × 2 L Fernbach flasks containing 500 mL of fresh LB media supplemented with antibiotic were inoculated with 1% v/v starter culture and grown at 37 °C, 250 rpm until the culture reached an OD₆₀₀ of 0.6. Protein expression was induced using 0.1 mM IPTG and the cells were cultured for a further 4 h at 37 °C with 250 rpm shaking. Cells were harvested by centrifugation at 6,000 x g for 20 min at 4 °C, resuspended in 40 mL lysis buffer (50 mM Tris-HCl pH 8, 500 mM NaCl, 5% glycerol, 1% v/v Triton X-100, 0.002% w/v PMSF, and 1 EDTA-free protease inhibitor tablet (Roche)) and stored at −80 °C.

**Purification of MSP2N2.** The method for purification of MSP2P2N2 was adapted from a published protocol\cite{34}. Cell suspensions were thawed and lysed by sonication on ice using a Q700 sonicator (Qsonica) (50% output amplitude, 30 cycles of 10 s on, 20 s off). The cell lysate was clarified by centrifugation using a S34 rotor at 30,000 x g for 1 h at 4 °C. The supernatant was collected, syringe filtered through a 0.22 μm membrane (Merck Millipore Ltd.) and applied to a 1 mL Ni-NTA column (His-Trap™ HP, Cytiva) equilibrated with 50 mM Tris-HCl (pH 8), 500 mM NaCl (=buffer A) + 1% v/v Triton X-100. The column was washed with 10 column...
Preparation of bovine mitochondria, membranes, and complex I. Bovine heart mitochondria were prepared as described previously33, and mitochondrial membranes were prepared using a method modified from that used previously for Mus musculus10. Brieﬂy, mitochondria were thawed and diluted to 5 mg mL−1 with 20 mM Tris-HCl (pH 7.5 at 20 °C), 1 mM EDTA, 10% glycerol, but then ripurified by three 5 s bursts of sonication with 30 s intervals on ice using a Q700 micro-tip Sonicator (Qsonica) at 65% output amplitude setting. The membranes were pelleted at 75,000 g using an MLA80 rotor (Beckman Coulter) for 1 h, then resuspended in the same buffer. Bovine complex I was prepared as described previously33 with a minor modiﬁcation to match the mouse complex I preparation10 (solubilised membranes were centrifuged at 48,000 × g for 12 min and kept on ice until reconstitution into nanodiscs.

Complex I reconstitution into nanodiscs. Complex I was reconstituted into nanodiscs using a protocol based on the reconstitution of complex I into proteoliposomes25,33. Two batches of 0.5 mg of chloroform-dissolved synthetic lipids (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 18:1 cardiolipin, Avanti Polar Lipids; stock at a mass ratio of 8:1:1 (DOPC:DOPE:cardiolipin) and total concentration of 25 mg mL−1) were each mixed with 200 µl of chloroform-dissolved ubiquinone-10 (Q10; i.e. 40 mM Q10 per mg lipid) in a test tube. The solvent was evaporated off under a stream of N2 and any residual chloroform removed in a desiccator under vacuum for at least 2 h. The dried lipid-Q10 mixtures were each resuspended in 45.75 µL 10 mM MOPS (pH 7.5 at 4 °C), 50 mM KCl by vigorous vortexing, and sonication in an ultrasonic bath (Grant Instruments (Cambridge) Ltd.) for 10 min together with 42.5 µL of 20% w/v lipid (KCl) (i.e. ﬁnal concentration of 40 mM). Each sample was transferred into a 1.5 mL Eppendorf tube, centrifuged at 7,000 × g for 10 min in a bench-top centrifuge, then each supernatant was transferred to a new tube and incubated on ice for 10 mins. MSP2N2 and bovine complex I (prepared as described above) were pooled gently with the lipid-Q10 mixture at a molar ratio of 400:1:1 (lipid:MSP2N2:complex I). Each sample was then diluted 2 fold with 0.5 ml 10 mM MOPS (pH 7.5 at 4 °C), 50 mM KCl and 1 µM Q10, and incubated on ice for 20 min. Then each sample was run over a separate PD10 desalting column (Cytiva) at 4 °C to remove the peripheral detergents. The eluates were pooled together, concentrated using a 100 kDa MWCO Amicon® Ultra concentrator (Merck Millipore Ltd.) to ~100 µL, and ﬁltered using a 0.22 µm Corning® Contour® Spin® 0.45 uL membrane (Cytiva) equilibrated with 10 mM MOPS (pH 7.5 at 4 °C), 50 mM KCl, and the most concentrated fractions from the monodisperse Cx1-Nd peak were used for grid preparation.

Characterisation of complex I-reconstituted nanodiscs. The complex I concentration in the nanodisc preparation was quantiﬁed relative to a detergent solubilised sample of known concentration using the NADH:APAD concentration in the nanodisc preparation was quantiﬁed relative to a detergent solubilised sample of known concentration using the NADH:APAD ratio from each measured rate was subtracted from each measured rate where noted. Isolated complex I used for reconstitution and Cx1-ND samples used for cryo-EM analyses were diluted to 200 µM decylubiquinone (dQ), 0.15% asolectin/CHAPS, and/or 10 µg mL−1 alternative oxidase (AOX), prepared as described previously33.

Cryo-EM grid preparation and image acquisition. UltraThin gold grids (0.1/6, Quantifoil Micro Tools Ltd.) were prepared as described previously21. Brieﬂy, grids were glow discharged (20 mA, 90 s), incubated in a solution of 5 mM [11-met]mountzcyt dend xhexaethylenglycol (TH 001—m11n6-0.01, ProChima Surfaces) in ethanol for two days in an ananaric glovebox (Belle), then washed with ethanol and dried just before use. Using a Vitrobot Mark IV (FEI), 2.5 µl of 4.8 mg mL−1 Cx1-ND solution (from the same solution) were applied to the grid after blotting for 10 s at force setting –10, at 100% relative humidity and 4 °C, and then plunge frozen into liquid ethane. Twelve grids were screened for particle number and distribution and two grids were selected for two 2-D data collections. They were imaged using a Gatan K3 detector and a post-column imaging energy filter (0.5% of maximum) operating at a zero-energy mode (50% of 20 eV) mounted on an FEI 300 kV Titan Microscope (Thermo Fisher Scientiﬁc) with a 100 µm and 70 µm objective and C2 apertures, respectively, and EPU v. 2.7.0.5806REL at the Department of Biochemistry, University of Cambridge. Data were collected in super-resolution electron counting mode at a pixel size of 0.535 Å pixel−1, with a nominal magniﬁcation of 180,000× with a dose rate of 4.4 e−·Å−2·s−1 in 0.2 µm intervals, and the autofocus routine run every 10 µm. Aberration-free image shift (AFIS) was used for data acquisition on day 1 of the 2-D data collection for the ﬁrst grid but was abandoned due to frequent occurrences of erratic image beam shifts observed in collected movies. The dose rates for the two datasets were 16.9 electronsÅ−2·s−1, with 2.4 s exposures captured in 40 frames. The total dose was thus 40.5 electrons Å−2 in both cases. Data were retrieved as non-gain-corrected LZW-compressed tiff movie stacks.

Cryo-EM data processing. The two datasets were processed separately until stated otherwise, using RELION 3.1.00 (Supplementary Figs. 2 and 3). The micrographs were motion-corrected using RELION’s implementation of motion correction with 5 × 5 patches, and contrast transfer function (CTF) estimated using CTFFIND-4.123. All catalytic activity assays were conducted at 32 °C in 96-well plates using a Titertek Multiflo Microplate Reader (Titertek) with 10 column volumes of buffer A (10 mM MOPS (pH 7.5 at 20 °C), 50 mM KCl, 1 µM piericidin and 100 µM NADH, as described previously21,33,35, except that 0.15% soy bean asolectin (Avantor Polari Lipids) and 0.15% CHAPS (Merck Chemicals Ltd.) were present. Cx1-Nd concentrations (i.e. combined protein concentrations of complex I and MSP2N2) were determined using the Pierce® bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientiﬁc) as described previously21. Each sample was subjected to centrifugation (10,000 × g for 12 min) and the protein concentration of the supernatant was determined by SDS-PAGE, and the protein flash frozen and stored at –80 °C.

All catalytic activity assays were conducted at 32 °C in 96-well plates using a Molecular Devices Spectramax 340 plus plate reader and Softmax Pro. Catalysis was initiated by addition of 200 µM NADH and monitored at 340 and 380 nm (εNADH = 4.81 mM−1 cm−1). Linear rates were used for activity calculations, and inhibitor-insensitive rates (determined by the addition of 1 µM piericidin A) subtracted from each measured rate were used for activity calculations. Reconstitution and Cx1-Nd samples used for cryo-EM analyses were diluted to 0.5 µg mL−1 in 20 mM Tris-HCl (pH 7.5 at 32 °C), and activity assays performed with 200 µM decylubiquinone (dQ), 0.15% asolectin/CHAPS, and/or 10 µg mL−1 alternative oxidase (AOX), prepared as described previously33.
performed. The identified sub-states outlined in the main text were then signal rerouted to give the global map, and the global resolution estimated from the FSC between 0.143 and 0.133 (Table 1) following half of the relevant maps to the resolution limit using Phenix MaskCreate. Mollweide projections were plotted using Python and Matplotlib, and the degree of directional resolution anisotropy calculated using the 3DFSC program suite52 (Supplementary Fig. 4).

**Model building, refinement and validation.** Working models derived from a model for bovine complex I in the active state (PDB ID: 7QOD)49 were rigid-body fitted into maps using the Fit in Map tool in UCSF ChimeraX52, rigid-body real space refinement against the respective locally sharpened consensus (active-Q10, active-apo, active-apo, and state 3) for working model building (Supplementary Table 1). The active-apo, active-apo, and state 3 models were then used to generate the final model (PDB 6YJ4)23 and adjusted accordingly.

**Comparisons of cryo-EM maps and models.** Map-to-map real-space correlations were performed using the Fit in Map function in UCSF ChimeraX2 (Supplementary Table 2) for the final model. Map-to-map real-space correlations were performed using the Align command in PyMOL.

**Identification of hydrogen bonds.** H-bonding contacts within individual CxI-ND models (with hydrogens added using phenix.ready_set and/or phenix.reduce53) were identified using the bHBonds command in UCSF ChimeraX52, for which the geometric criteria are based on a survey of small-molecule crystal structures53, and atom types adapted and extended from the program iDAT35.

**Quinone cavity determination.** The interior surface of the Q-binding channel was predicted using CASTp45, which computes a protein surface topology from a PDB model. The default 1.4 Å radius probe was used and the results were visualized in PyMOL using the CASTpyMOL 3.1 plugin and by UCSF ChimeraX52.

**Molecular dynamics.** The cryo-EM structure of active-state complex I from *M. musculus* at 3.1 Å resolution (PDB ID: 6ZJR)56 was used to build the initial simulation model. Protonation states of sidechains were adjusted to neutral pH, except that His59[ND2/DS2] and Asp160[ND2] were modelled initially as diprotonated (His+H+) and neutral (AspH), Glu68[ND3], Glu36[ND2/DS2], Glu262[ND1] and Glu114[ND4] as neutral (GluH), and His549[ND1] and His42[ND2] as di-protonated. The N-termini of NDUF7 (Ser34) and the truncated NDUF68 (Ser66) were modelled as neutral. These alternative protonation states were suggested by the chemical environment of the group and PropKa calculations54 (with nearby Fe cluster N2 in the oxidized state). All cofactors and post-translational modifications present in PDB 6ZJR were included. High confidence phospholipids were retained and built with linoleoyl (18:2) acyl chains. The polar headgroups were preserved, thereby they were modelled as 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine (DLPE) and 1,2,3-bis(1,2-dilinoleoyl-sn-glycero-3-phospho)-sn-glycero, as the cardiolipin dianion (CDL). Missing hydrogen atoms were built with the GROMACS suite52. Neutral His tautomers were chosen based on optimal H-bonding.

The modelled protein complex was embedded in a solvated bilayer with a composition mimicking that of the mitochondrial inner membrane. The final solvated and inserted system contains 368 DLPC (179 in the matrix leaflet), 294 DLPE (143 in the matrix leaflet), 96 CDL (half in each leaflet) and 22 oxidized Q(10) molecules, all initially in the membrane phase. The asymmetry in DLPC and DLPE composition between the two leaflets is due to chain NDUF19 occupying an area of the matrix leaflit only. The water phase contains 205,387 molecules plus 553 Na+ and 348 Cl− ions to neutralise the total system charge and maintain −0.1 M salt concentration. The resulting simulation model contains a total of 861,975 atoms. Water solvation of the apo Q-binding channel and regions near the oxidised N2 Fe centre was compared to the active-Q10 and previous cryo-EM models (PDB ID: 6YJ4)23 and adjusted accordingly. The simulation model was relaxed and equilibrated during molecular dynamics (MD) simulations of 740 ns in total. Initially all protein heavy atoms were tethered to their initial position by harmonic restraints, then the force constants were decreased progressively from 1000 to 10 kJ mol−1 nm−2, and all atoms were free to move (MD) simulations of 740 ns in total. Initially all protein heavy atoms were free to move (MD) simulations of 740 ns in total. Initially all protein heavy atoms were free to move (MD) simulations of 740 ns in total. Initially all protein heavy atoms were free to move (MD) simulations of 740 ns in total. Initially all protein heavy atoms were free to move (MD) simulations of 740 ns in total.
These configurations are provided here as Supplementary Data 1 to allow reproduction of our calculations. Finally, well-tempered metadynamics simulations were performed for each charge-state, starting from a configuration taken at 75 ns of each canonical MD trajectory described above. Metadynamics were activated in the CV coordinate (position on the path, p1.sss, and distance from the path, p1.zzz) and in the Hix(37,42) δ(ε, χ) dihedral (Cα-Cδ bond torsion) with Gaussians deposited every 500 time steps (1 ps), at initial height of 0.6 kJ mol⁻¹ widths of 0.4 and 0.02 units for the CV and dihedral, respectively, and a bias factor of 15.0. Wells were included to restrict sampling for the CV at 21 ± p1.sss < 2 Å and −2.45 < p1.zzz < −2.20 Å, with force constant of 1000 kJ mol⁻¹ nm⁻¹. Productive metadynamics simulations lasted 140 ns for each charge-state. Due to the enhanced sampling nature of this method, simulation times significantly shorter than for canonical MD are sufficient for an appropriate conformational sampling of confined regions, such as the reactive position of the complex I Q-binding site. Convergence within ±1 kJ mol⁻¹ of free energy differences in the CV profile (Fig. 3a) was reached after 70 ns. The effects of metadynamics and of restraints were removed by re-weighting the distribution of structural properties (distances, dihedral, CV) and the resulting free energies are shown in Fig. 3a, e–g and Supplementary Fig. 8. The statistical uncertainty was estimated as 95% confidence intervals by bootstrap analysis. In all MD simulations the interactions of protein, lipids and ions were described with the all-atom CHARMM36 model.26 Water was represented by the standard TIP3P model.25b FeS centres were described using the Chang and Kim25 parameters with corrections by McCullagh and Voth27. Qα interactions were represented by our calibrated force-field.28 The remaining cofactors were described by available CHARMM and CGenFF parameters (charmm36-mar2019.ff).34 All simulations were conducted with GROMACS (version 2020.3)61 at constant temperature (310 K) and pressure (1 atm) and a time step of 2 fs. Long-range electrostatics were treated with the Particle Mesh Ewald method.29 Metadynamics simulations were performed with the PLUMED plugin (version 2.6.1)30.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request. Structural data have been deposited in the EMDB and PDB databases under the following accession codes: EMD-14132 and 7QSK (active-Qα), EMD-14133 and 7QSL (active-apo), EMD-14134 and 7QSM (inactive-ligand; composite), EMD-14135, EMD-14136, EMD-14137, and EMD-14138 (active-ligand; consensual, hydrophilic domain, proximal and distal membrane domains, respectively), EMD-14139 and 7QSN (inactive-apo), and EMD-14140 and PDB 7QSO (state 3). Related data accession codes: EMD-14142 and 7QSD (DDM-solubilised bovine complex I).

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### Author contributions

I.C. performed Cxt-ND reconstitution, cryo-EM grid preparation, collected and processed cryo-EM data, carried out structure model building, analyses, and interpretations, and prepared figures. I.C. and J.I.W. carried out biochemical and kinetic characterisations. J.I.W. prepared complex I for reconstitution, assisted by I.C. H.R.B. advised on cryo-EM data collection and processing and built initial models. J.I.W. and H.R.B. contributed to data interpretation. B.S.I. prepared MSP2N2 for reconstitution, with contributions from J.I.W. O.B. established and optimised the initial reconstitution protocol. C.S.P. and G.M.A. performed and analysed molecular simulations. J.H. initiated and supervised the project and contributed to interpretation of the data. I.C. and J.H. wrote the paper with input from all authors.

### Competing interests

The authors declare no competing interests.

### Additional information

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