Conformational changes in mitochondrial complex I of the thermophilic eukaryote
Chaetomium thermophilum

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Mitochondrial complex I is a redox-driven proton pump that generates proton-motive force across the inner mitochondrial membrane, powering oxidative phosphorylation and ATP synthesis in eukaryotes. We report the structure of complex I from the thermophilic fungus Chaetomium thermophilum, determined by cryoEM up to 2.4-Å resolution. We show that the complex undergoes a transition between two conformations, which we refer to as state 1 and state 2. The conformational switch is manifest in a twisting movement of the peripheral arm relative to the membrane arm, but most notably in substantial rearrangements of the Q-binding cavity and the E-channel, resulting in a continuous aqueous passage from the E-channel to subunit ND5 at the far end of the membrane arm. The conformational changes in the complex interior resemble those reported for mammalian complex I, suggesting a highly conserved, universal mechanism of coupling electron transport to proton pumping.

INTRODUCTION
Mitochondrial NADH:ubiquinone oxidoreductase (complex I) is the major entry point of electrons into the respiratory chain (1, 2). Complex I is a multi-subunit membrane protein complex consisting of around 45 different protein subunits. It couples the transfer of two electrons from reduced nicotinamide dinucleotide (NADH) to ubiquinone (Q) to the transfer of four protons across the inner mitochondrial membrane (3–5). Electron and proton transfer accounts for a large part of the proton-motive force that drives adenosine triphosphate (ATP) synthesis by the F-type ATP synthase in the same membrane (6).

A minimal version of mitochondrial complex I is found in bacteria. The bacterial complex consists of 14 strictly conserved core subunits that are sufficient for catalytic activity. In the course of eukaryotic evolution, ~30 accessory subunits have been incorporated into complex I, increasing its molecular mass from ~550 kDa to ~1 MDa. Although the exact roles of most accessory subunits are unknown, some have been shown to regulate complex I assembly, stability, and activity (7).

Mitochondrial complex I has a characteristic L-shape, with a membrane arm embedded in the lipid bilayer of the inner mitochondrial membrane, joined at one end to the peripheral arm that extends into the mitochondrial matrix. The peripheral arm transfers electrons from NADH to Q via a bound flavin mononucleotide (FMN) along a chain of eight iron-sulfur (Fe-S) clusters (8–11). The membrane arm pumps protons across the inner mitochondrial membrane. It contains three antiporter-like subunits that form an elongated hydrophilic passage with numerous internal water molecules. Of these, only the antiporter-like subunit ND5 at the distal end of the membrane arm appears to have an aqueous connection to the intermembrane space (IMS) (12, 13). The Q substrate binds in a cavity above the level of the membrane surface near the junction of the two arms, where it is reduced to ubiquinol (QH2) by Fe-S cluster N2 (14). An aqueous tunnel referred to as the E-channel is lined by strictly conserved glutamate residues of core subunits ND1, ND3, and ND4L. The E-channel connects the Q-binding cavity to the internal hydrophilic passage within the membrane arm.

Recent cryo–electron microscopy (cryoEM) structures of complex I show that the Q headgroup binds at two primary sites within the cavity (13, 15, 16). The deeper binding site (referred to as Qd) is located next to N2. The Q site is located in the shallower part of the cavity close to its entrance. Presumably, binding of Q, QH2, or Q intermediates at different binding sites within the Q-binding cavity during the catalytic cycle is linked to proton translocation (12, 13, 15), but the exact molecular mechanism is not understood.

Mammalian mitochondrial complex I incubated at elevated temperatures (>30°C) without NADH and Q substrates undergoes a reversible transition from an active to a deactive state, characterized by a substantial decrease in turnover rate (17, 18). The deactive state is thought to prevent the production of reactive oxygen species (ROS), generated by reverse electron transfer in complex I during reperfusion in ischemic tissues (19, 20). When fresh substrate is added, complex I reverts to the active state. The transition between the active and deactive state is characterized by a lag phase in NADH:Q oxidoreductase activity that lasts up to 2 min (21), before NADH is again oxidized at a linear rate (17).

CryoEM of mammalian (13, 15, 22–24) and plant complex I (25) has resolved two different main conformations, characterized by a tilt of the peripheral arm relative to the membrane arm. In the mammalian complex, internal rearrangements have been described in the Q-binding cavity and at transmembrane helix (TMH) 3 of core subunit ND6 (13, 22, 24). At present, it is not clear how these two conformations or the associated internal rearrangements relate to the mechanism of electron transport and proton translocation, whether they represent the deactive and active state of complex I (22, 24) or are substates of the active state and thus represent different catalytic intermediates (13).

In the present study, we investigate mitochondrial complex I from the filamentous fungus Chaetomium thermophilum, one of the few well-characterized thermophilic eukaryotes. C. thermophilum has an optimal growth temperature of 50° to 55°C but tolerates temperatures up to 60°C (26). It grows naturally in dung or compost heaps but is easily cultivated in standard media. Proteins and macromolecular

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complexes from thermophiles are good targets for structural biology, as they are usually more robust than their mesophilic counterparts (27, 28), and hence more easily purified and crystallized. The properties of thermotable proteins, in particular their higher resistance to denaturation, are equally beneficial for cryoEM. We determined the cryoEM structure of mitochondrial complex I from *C. thermophilum* at 2.4-Å resolution. Although we expected this thermotolerant complex to be more rigid than complex I from mammals or plants, we found that it undergoes major conformational changes. Structures of three different conformations were resolved, which we refer to as state 1 and state 2, plus an inhibited conformation.

**RESULTS**

**Subunit composition and overall structure of *C. thermophilum* complex I**

Complex I from *C. thermophilum* (*Ct*-complex I) was solubilized either in the detergent *n*-dodecyl β-β-maltoside (DDM) or in lauryl maltose neopentyl glycol (LMNG). The 2.4-Å map of *Ct*-complex I in LMNG allowed us to model the 14 core subunits and 29 accessory subunits with confidence (Fig. 1). For subunit assignment and atomic model building, we initially used the cryoEM structure of complex I from the aerobic yeast *Yarrowia lipolytica* (*Yl*-complex I; Protein Data Bank 6RFR) (16). For consistency, we adopted the subunit nomenclature of human complex I, except for the accessory subunit NUXM of the membrane arm, which is absent in mammals.

The overall structure of *Ct*-complex I is similar to that of *Y. lipolytica* (29). The subunit composition is well-conserved, except for an accessory subunit at the top of the peripheral arm, binding at the same site as subunit NDUFV3, which shares a weak sequence similarity. We assume that its role is analogous to that of mammalian NDUFV3 and refer it to as Ct-NDUFV3 (fig. S1).

Subunits NDUFB2 and NDUF4A1 were not annotated in the genome of *C. thermophilum* (30). To determine their sequences as well as that of Ct-NDUFV3, we initially modeled these three subunits as polyalanine. Subsequently, map features indicating particular side chains were used to place matching residues. The resulting peptide fragments served as query sequences for BLAST searches in a six-frame-translation database from the *C. thermophilum* genome. Missing residues were then modeled according to the amino acid sequence of the returned hits. In this way, all subunits of *Ct*-complex I were modeled and finally confirmed by mass spectrometry (MS).

Apart from the 43 protein subunits, the structure of *Ct*-complex I resolves 13 bound cofactors (Fig. 2A). The peripheral arm contains eight Fe-S clusters (six Fe₄S₄ and two Fe₂S₂), a coordinated Zn²⁺ ion, one FMN, one NADPH (reduced form of nicotinamide adenine dinucleotide phosphate), and a phosphopantetheine group, covalently attached to subunit NDUFAB1α. NDUFAB1 belongs to the group of mitochondrial acyl carrier proteins, which are involved in mitochondrial fatty acid synthesis (31, 32) and the assembly and activation of respiratory chain complexes (33). *Yl*-complex I contains two isoforms of subunit NDUFAB1 (ACPM in *Y. lipolytica*), one in the
peripheral arm (ACPM1) and one at the tip of the membrane arm (ACPM2). In *C. thermophilum*, both positions are occupied by identical copies, referred to as NDUFAB1α and NDUFAB1β.

FMN binds to subunit NDUFV1 at the top of the peripheral arm, where NADH is oxidized and the electrons are fed into the chain of Fe-S clusters that runs vertically through the peripheral arm, with one off-pathway Fe₄S₄ cluster (N1a). The Fe-S cluster chain ends at cluster N2, which transfers electrons to the Q substrate in the Q-binding cavity near the junction between the peripheral and membrane arms. Arg154 of the NDUF87 subunit, which is close to N2, is modified to

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**Fig. 2. Cofactors and lipids in *C. thermophilum* complex I.**

(A) Cofactors, lipids, and detergent molecules identified in the map of DDM-solubilized Ct-complex I. Top inset: Distances (in angstrom) along the redox chain between FMN and the Fe-S clusters (N). Bottom inset: Fatty acid tails of lipids near subunit ND1 run almost parallel to the membrane plane. (B) View from the mitochondrial matrix side on the membrane arm of complex I in DDM (peripheral arm omitted for clarity). Inset: Top view of tilted lipids near subunit ND1. (C) Lipids and detergent molecules in the membrane arm of LMNG-solubilized Ct-complex I. (D) View of the membrane arm in LMNG from the matrix side (peripheral arm omitted for clarity). CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PP, phosphopantetheine.
a dimethyl arginine (fig. S2), as also observed in the cryoEM structures of ovine (13), mouse (22), and Y. lipolytica complex I (12). Most likely the dimethyl arginine modulates the redox potential of the N2 cluster (34).

Subunit NDUFAB9 binds one molecule of NADPH. A noncovalently attached NADPH in this position has been found in mammalian, yeast, and plant complex I, but its function, if any, is unclear. Most likely it does not participate in electron transfer because of its long distance of ~37 Å to the nearest Fe-S cluster (N2) (1). Studies in which NADPH binding is blocked indicate that it is required for complex I assembly and stability, rather than for catalytic activity (35–37).

The membrane arm of Ct-complex I contains one phosphopentetehine group as a cofactor bound to the second copy of subunit NDUFAB1 (NDUFAB1β) at the tip of the membrane arm. In addition to the cofactors, we identified 17 phospholipids and 14 detergent molecules in the cryoEM map of the DDM-solubilized complex I (Fig. 2, A and B). In the LMNG-solubilized complex, we identified 32 phospholipids and 3 detergent molecules (Fig. 2, C and D). No densities for NADH or Q were visible, as both samples were prepared and plunge-frozen without added substrates.

### Two different conformations of *C. thermophilum* complex I

To inspect the cryoEM structures for conformational variability and heterogeneity, we performed 3D Variability Analysis (3DVA) in cryoSPARC (see Materials and Methods) (38). 3DVA of the DDM-solubilized complex indicated a flexing and slight twisting motion of the peripheral arm relative to the membrane arm (fig. S3 and movie S1), suggesting a flexible hinge between the two arms. 3DVA of the LMNG-solubilized complex likewise indicated flexibility in the same region. However, the twisting motion of the peripheral arm was much more pronounced than in DDM and accompanied by a major reorganization of the map density in the hinge region (fig. S4 and movie S2). 3DVA separated the Ct-complex I particles into two different populations, suggesting distinct conformations. Repeated 3DVA of the LMNG dataset with a mask around the hinge region improved the separation of the two particle populations and finally resolved two clusters of 153,568 (87.5%) and 21,989 (12.5%) particles (fig. S4 and movie S3). Both clusters were three-dimensional (3D)–refined separately, yielding maps of 2.4- and 2.8-Å resolution for the major and minor cluster, respectively. Model building then produced structures of Ct-complex I in two different conformations. We refer to these two conformations as state 1 (major cluster) and state 2 (minor cluster) rather than as open and closed, because no significant opening or closing of the angle between two arms was evident. The transition between the two states of Ct-complex I is characterized by a twisting rather than a tilting motion of the peripheral arm relative to the membrane arm (movie S4). The transition concurs with substantial changes of the complex I structure in the hinge region, the Q-binding cavity, and the E-channel.

Loops of the Q-binding cavity subunits ND1, NDUF2, and NDUF7 (referred to as ND1, PSST, and 49 kDa in the bovine complex) rearrange in the transition from state 1 to state 2 (Fig. 3A). The loop linking TMH5 and TMH6 of subunit ND1 between the Qa and Qd site includes a number of highly conserved residues, in particular Glu211 and Glu223 (see table S2). In state 2, Glu211ND1 and Glu223ND1 form salt bridges with Arg128 and Arg132 of subunit NDUF7. In state 1, the TMH5–6 loop of ND1 moves out of the Q-binding cavity, and both salt bridges break. The rupture of these two salt bridges is also observed in ovine complex I (fig. S5, A and B) (13) and is likely to be a key element in the complex I mechanism. Site-directed mutagenesis indicated a significant decrease in Q reductase activity when residues in the loop region containing Arg128 and Arg132 of NDUF7 were targeted (39).

The loop between the first and second β-strand of subunit NDUF52 (known as the β1–β2 loop) is part of the Qd site and includes two conserved histidines (His124 and His128) that are critical for ubiquinone reductase activity of complex I (40, 41). The β1–β2 loop reorganizes in the 1-to-2 transition. In state 1, His128NDUF52 extends into the Q-binding cavity but retracts from it in state 2 (fig. S3A and figs. S5C and S6A). In state 2, loop residues 99 to 102 of subunit NDUF7 are in an extended conformation and protrude into the Q-binding cavity, but in state 1, they arrange into a β-strand and retract from the Q-binding cavity (fig. 3A and figs. S5B and S6B).

The loop connecting TMH1 and TMH2 of subunit ND3 in the membrane arm interacts with peripheral arm subunits that contribute to the Q-binding cavity. In state 1, the TMH1–2 loop is disordered, but in state 2, it is well ordered and appears to be locked near the NDUF52 core subunit and accessory subunit NDUF6 (Fig. 3B and fig. S6). In this ordered formation, the conserved Cys40 in the loop comes close to the Q-binding cavity (fig. S7). The exact role of the TMH1–2 loop of ND3 is under debate, but it clearly is important. Modification of the conserved cysteine has a major impact on complex I activity (21, 42, 43). In the human complex, mutations in this loop result in Leigh syndrome and progressive mitochondrial disease (44, 45). In the 1-to-2 transition, the adjacent ND1 loop connecting transverse helix α1 and TMH2 rearranges and appears to keep the TMH1–2 loop of subunit ND3 in the ordered and defined position seen in state 2 (Fig. 3B and fig. S5, D and E). In addition, site-directed mutagenesis of residues in accessory subunit NDUF6 close to the TMH1–2 loop of ND3 causes a substantial decrease in complex I activity (Fig. 3B) (46). We propose that locking the TMH1–2 loop of ND3 in the position observed in state 2 is a critical part of the mechanism and is supported by the adjacent subunits ND1 and, in mitochondria, NDUF6. Locking of the TMH1–2 loop by subunit NDUF6 appears to be triggered by the twisting motion of the peripheral arm during the 1-to-2 transition.

We used NADH:Q1 oxidoreductase measurements to ascertain that our Ct-complex I preparations were active. At 30°C, the LMNG-solubilized complex showed an activity of ~10 μmol min⁻¹ mg⁻¹, which increased to ~33 μmol min⁻¹ mg⁻¹, if the measurements were performed at 50°C (fig. S8A), in line with the thermostability and thermophilic adaptation of Ct-complex I. Notably, the activity traces showed no measurable lag phase (fig. S8, C and D), which is characteristic of the reactive state as it reverts to the active state. The activity traces closely resemble those for the active ovine complex I (13). Thus, we conclude that Ct-complex I in our preparations is predominantly in the active state.

### Internal water molecules rearrange during the conformational transition

Density modification of the cryoEM maps (47) reduced noise and improved the overall resolution (table S3) to the point where water molecules could be built into the density with confidence. We modeled 2649 water molecules in state 1 of Ct-complex I, 1593 in state 2, and 1219 in the DDM-solubilized complex.

In the center of the membrane arm, hydrophilic residues coordinate numerous internal water molecules, creating an internal aqueous
The hydrophilic residues are positioned at discontinuous helix regions of the membrane core subunits. The Q-binding cavity and the aqueous passage through core subunits ND2, ND4, and ND5 are joined via the E-channel, which also contains numerous coordinated water molecules. In state 1, a nonhydrated region in the E-channel at TMH3 of subunit ND6 breaks the aqueous passage, separating the Q-binding cavity from the chain of water molecules in the membrane arm (Fig. 4). The peptide geometry of TMH3 ND6 deviates from that of a regular $\alpha$-helix and forms a $\pi$-bulge at the conserved residue Tyr77 ND6 roughly in the middle of the helix. In state 2, the C-terminal half of TMH3 ND6 on the matrix side that includes the conserved residue Phe85 ND6 rotates around its axis by almost 180°, and the $\pi$-bulge reverts to a regular $\alpha$-helix (Fig. 4A and fig. S6D).

Focused 3D refinement improved the resolution of the membrane arm and enabled us to model a total of 136 water molecules in the E-channel.

Laube et al., Sci. Adv. 8, eadc9952 (2022) 25 November 2022
along the entire hydrophilic passage (fig. S9). Of the three antiporter-like core subunits ND5, ND4, and ND2, only ND5 at the tip of the membrane arm seems to form an aqueous connection to the IMS, as also observed in Y. lipolytica (12) and the ovine complex I (13). The transition between the two states of complex I did not appear to be associated with a rearrangement of water molecules or conformational changes beyond the π-gate in the membrane arm.

Most of the water molecules in Ct-complex I were modeled in the peripheral arm. Apart from the hinge region and the Q-binding cavity, these water molecules did not rearrange in the 1-to-2 transition. As in the ovine complex (13), none of the ~1700 water molecules in the hydrophilic peripheral arm were found directly between neighboring Fe-S clusters.

**A bridge between the peripheral and membrane arm**

Accessory subunit NDUFA5 is part of the peripheral arm. In C. thermophilum, this subunit is larger than in any other known complex I structure. It interacts with subunit ND2 in the membrane arm, forming a protein bridge (here referred to as NDUFA5 bridge) between the peripheral and membrane arms that has not been observed before (fig. S10). Remarkably, C- and N-terminal sequence extensions of core subunits ND3 and NDUFS2 tether these subunits to the NDUFA5 bridge (Fig. 6). The N-terminal extension of NDUFS2 (here referred to as tether 1) is bound to the surface of the NDUFA5 bridge. It directly precedes the β1-β2 loop, which is, as described above, well known to be involved in Q binding and reduction (40, 41). Tether 1 therefore establishes a direct connection between the β1-β2 loop and the NDUFA5 bridge. In the transition between the two states, tether 1 reorganizes (Fig. 6). In state 1, tether 1 is exposed on the surface of the membrane arm, extends across the tip of the NDUFA9 latch, and interacts with the latch via a salt bridge between Arg114NDUF2 and Glu368NDUF9 close to the β1-β2 loop (Fig. 6, inset a). In state 2, tether 1 relocates, enabling a rearrangement of Arg114NDUF9. Also, the NDUFA9 latch retracts from its position at the hinge between the two arms and the salt bridge between Glu368NDUF9 and Arg114NDUF2 breaks (Fig. 6).

The C-terminal extension of subunit ND3 is enveloped by subunit NDUFA5 and remarkably contributes a β-strand to the β-sheet of the NDUFA5 bridge (Fig. 6, orange arrow). This β-strand and TMH3 of subunit ND3 are directly linked by another peptide tether (tether 2), which rearranges and attaches to subunit NDUFS2 in the 1-to-2 transition (Fig. 6).

**Detergent effects in C. thermophilum complex I**

In the structure of DDM-solubilized complex I at 2.8-Å resolution, the ND1 subunit is seen to have a 40-residue extension joining TMH6 and TMH7 that is absent in other complex I structures. In the DDM structure, the ND1 extension is ordered and forms a hook (the ND1 hook) extending to the detergent belt (Fig. 7; see fig. S11 for a sequence alignment), which is notably thinner (~25-Å) in this region than around the remainder of the membrane arm. Lipids in this region of the matrix leaflet are clearly tilted, with their acyl chains oriented almost parallel to the membrane plane (Fig. 2, A and B). Several amphipathic helices from core and accessory subunits, including NDUFA9, NDUFA12, NDUFS8, or NDUFS7, are located near the matrix leaflet at subunit ND1 and are likely involved in...
orienting the lipids in this region (16). The distortion of the lipid bilayer reduces the local membrane thickness, which may facilitate access to the Q-entrance tunnel, framed by TMH1, TMH6, and the transverse helix $\alpha_1$ of subunit ND1 (16). The ND1 hook in Ct-complex I may maintain a thinner lipid bilayer near the Q-entrance tunnel, perhaps to improve accessibility for the Q substrate. Presumably, this loop is unstable in the LMNG-solubilized complex, due to some difference in shape, size, or surface properties of the detergent micelle. Unexpectedly, we did not observe the two conformations state 1 and state 2 of Ct-complex I in the DDM-solubilized complex. In this detergent, all particles assumed a single conformation, which closely resembles state 1 in LMNG, except that the ND1 loop connecting TMH5 and TMH6 forms a short $\alpha$-helix and is shifted toward the N2 cluster (Fig. 8). We identified two molecules of DDM in the Q-binding cavity (Fig. 8A and fig. S12). One of these (DDM-1) binds at the Q-entrance tunnel, as also observed in the DDM-solubilized Yl-complex I (48). The hydrophilic headgroup of DDM-1 sits near the Q site, coordinated by Asp121 of subunit NDUFS7, and its hydrophobic acyl tail extends into the Q-entrance tunnel. DDM-2 is bound in the E-channel, with its headgroup coordinated by the conserved residues Glu211 and Glu236 of subunit ND1, and pointing into the Q-binding cavity. The movement of the ND1 loop toward N2 appears to trigger a reorganization of the $\beta_1$-$\beta_2$ loop of NDUFS2. The $\beta_1$-$\beta_2$ loop protrudes into the Q$_d$ site, where it would interfere with the coordination of the Q head group by the conserved Tyr177 in subunit NDUFS2 (Fig. 8, inset b) (49).

Consistently, our activity measurements of Ct-complex I solubilized in DDM show only weak Q$_1$ reduction activity, compared to complex I in LMNG (fig. S8, A and B; $P < 0.01$ between complex I in LMNG and DDM at 30° and 50°C, without rotenone). Rotenone inhibits the oxidoreductase activity of Ct-complex I significantly. However, at 30°C, the activity of DDM-solubilized Ct-complex I is so low that rotenone does not inhibit the reaction further (fig. S8B; DDM buffer, 30°C). In agreement with the observed occupation of the Q-binding cavity, this indicates a clear inhibitory effect of DDM on the activity of Ct-complex I. Presumably, at 50°C, the inhibitory effect of DDM compared to rotenone is diminished by the decreased binding affinity of the detergent in the Q-binding cavity at elevated temperatures (fig. S8B; DDM buffer, 50°C). Notably, the oxidoreductase activity increases significantly when DDM-solubilized Ct-complex I is transferred to a buffer containing LMNG (fig. S8B; LMNG buffer). The quick recovery indicates that inhibition by DDM is reversible.
The DDM molecule is bound in the Q-entrance tunnel (DDM-1) with its head group Cₜ-complex I. In DDM-solubilized Cₜ-complex I, one DDM molecule is bound in the Q-entrance tunnel (DDM-1) with its head group near the Qsite, coordinated by Asp121NDUF5. A second DDM molecule binds in the E-channel (DDM-2) with its head group coordinated by Glu211ND1 and Glu236ND1. DDM molecules are shown with EM density (transparent green). Figure S12 shows their position in the Q-entrance tunnel and E-channel. Inset b: Superposition of the ND1 hook formed in DDM-solubilized Cₜ-complex I in DDM (colored by subunit) and in LMNG in state 1 (gray). Overall, the two structures are very similar, but in the DDM structure, the TMH5-6 loop of subunit ND1 shifts toward N2 and forms a partial α-helix. The TMH5-6 loop reorganization is accompanied by a rearrangement of the β1-β2 loop of subunit NDUF52, which protrudes into the Qsite in DDM-solubilized Cₜ-complex I. Red arrow: Movement of Phe220ND1 by ~16 Å.

Fig. 7. The ND1 hook. The core subunit ND1 (blue) of Cₜ-complex I forms a hook (inset a) that extends onto the DDM detergent belt (red) near the Q-entrance tunnel. In the vicinity of the ND1 hook, the thickness of the detergent micelle is reduced from ~36 to ~25 Å. Inset b: CryoEM map density of the ND1 hook with fitted side chains.

DISCUSSION
CryoEM of mitochondrial complex I from the thermophilic eukaryote C. thermophilum revealed that Cₜ-complex I undergoes a transition between two different conformations, which we refer to as state 1 and state 2. The transition is characterized by a twisting motion of the peripheral arm relative to the membrane arm and by a substantial restructuring of the hinge region, the Q-binding cavity, and the E-channel (Fig. 9). We are aware that two different conformations have been described also of the mammalian (13, 22–24) and plant complex I (25). In mammals, the two conformations are referred to either as open and closed (13) or as the active and inactive state of complex I (22, 24).

NADH:O₂ activity measurements with the Cₜ-complex I did not indicate a lag phase that would be expected of the inactive state. As the sample used for cryoEM grid preparation was treated identically, we conclude that our structures show the active state of Cₜ-complex I. The ovine complex I was driven into the reactive state by incubating it at 37°C in the absence of substrates (13). The structure of the complex deactivated in this way closely resembled the open conformation (our state 1) but indicated a ~40° tilt of TMH4 of subunit ND6. The tilt of this helix is thought to arrest complex I in the reactive state, which presumably represents an off-pathway state of the open complex (13). No tilt of TMH4ND6 was apparent in any of our Cₜ-complex I structures, consistent with our conclusion that the two states of Cₜ-complex I both show the active complex.

The open/closed terminology derives from the slight movement, "open" (around 112°) or more "closed" angle (around 105°) between the two arms in the mammalian complex (13, 23). Because this angle is not noticeably different in the two states of the Chaetomium complex, the term open or closed does not apply. The cryoEM structure of plant complex I from Arabidopsis thaliana mitochondria (25) likewise revealed two conformations, one with a narrower angle (106°) and the other with a slightly wider angle (112°) between the two arms, which, in analogy to the mammalian complex, were also referred to as open and closed. However, neither of them showed the rearrangements in the E-channel or the Q-binding cavity observed in the Chaetomium and ovine complex I (13). In bacterial complex I from Escherichia coli (50), three different conformations with different angles between the membrane and peripheral arms were resolved. None of these conformations indicated rearrangements in the Q-binding cavity or E-channel, similar to those we see in the Chaetomium complex. The authors proposed that these conformations of the E. coli complex I represent uncoupled states, in which redox reaction is decoupled from proton pumping (50). Evidently, both the mitochondrial and bacterial complex I can adopt more "open" or more "closed" conformations, which are, however, not necessarily linked to the internal rearrangements we observe for the Chaetomium complex. Thus, the open/closed terminology can be misleading as it might apply to many different complex I conformations that are not related to our states 1 and 2. We suspect that the hinge region of complex I is intrinsically flexible, and that this flexibility can result in an apparent opening and closing of the angle, which may, however, not reflect functionally relevant structural rearrangements in the Q-binding cavity and E-channel.

Restructuring of the E-channel in the 1-to-2 transition results in the formation of a continuous water chain through the π-gate formed by TMH3 of subunit ND6. Similar water and residue reorganizations at the π-gate were also observed in ovine complex I (13). However, the π-gate is "closed" in the open state (our state 1) and "open" in
the closed state (our state 2) (Fig. 9 and fig. S13), which is potentially confusing. On this basis, we propose that the two conformations should be referred to simply as state 1 and state 2, until a consensus on their functional relevance has been reached.

The opening of the \( \pi \)-gate in the 1-to-2 transition creates a continuous aqueous passage from the Q-binding site all the way to the antiporter-like subunit ND5, where protons can be expelled into the IMS. The higher resolution of our \( C_t \)-complex I structure revealed additional water molecules at the open \( \pi \)-gate that contribute to an uninterrupted water chain in state 2 (fig. S13) and suggests that protons can flow easily from the Q-binding sites to the interior of the membrane arm. An essential role of TMH3\( \beta^6 \), which contains the \( \pi \)-gate, is consistent with its high degree of sequence conservation (51) and with an impaired complex I activity as a result of substantial changes. The \( \pi \)-gate connects the E-channel and Q-binding cavity to the aqueous passage inside the membrane arm (dashed red arrow). In state 1, the \( \pi \)-gate formed by TMH3\( \beta^6 \) is closed, breaking the aqueous passage. In state 2, the \( \pi \)-gate opens as TMH3\( \beta^6 \) regularizes into an \( \alpha \)-helix, resulting in a continuous aqueous passage from the Q-binding cavity to the tip of the membrane arm.

Fig. 9. Conformational changes in \( C. \) thermophilum complex I. The conformational transition between state 1 and state 2 entails a twisting motion of the peripheral arm relative to the membrane arm, as well as rearrangements in the Q-binding cavity and the E-channel. The Q-binding cavity is located close to the interface between the two arms at the end of the chain of iron-sulfur clusters (orange and yellow spheres). Loops of subunits ND1, ND3, NDUFS2, and NDUFS7 in and around the Q-binding cavity undergo substantial changes. The \( \pi \)-gate connects the E-channel and Q-binding cavity to the aqueous passage inside the membrane arm (dashed red arrow). In state 1, the \( \pi \)-gate formed by TMH3\( \beta^6 \) is closed, breaking the aqueous passage. In state 2, the \( \pi \)-gate opens as TMH3\( \beta^6 \) regularizes into an \( \alpha \)-helix, resulting in a continuous aqueous passage from the Q-binding cavity to the tip of the membrane arm.

State 1

- ND6 \( \pi \)-gate: closed
- NDUSF2 \( \beta^1-\beta^2 \)-loop: extended
- NDUSF7 \( \beta \)-strand: retracted
- NDUSF7 \& ND1 TMH5-6 loop: loose
- NDUSF7 \& ND1 TMH5-6 loop: no salt bridges

State 2

- ND6 \( \pi \)-gate: open
- NDUSF2 \( \beta^1-\beta^2 \)-loop: retracted
- NDUSF7 \( \beta \)-strand: locked
- NDUSF7 \& ND1 TMH5-6 loop: extended
- NDUSF7 \& ND1 TMH5-6 loop: 2 salt bridges

Aqueous passage

Matrix

IMS

The conformational transition between state 1 and state 2 entails a twisting motion of the peripheral arm relative to the membrane arm, as well as rearrangements in the Q-binding cavity and the E-channel. The Q-binding cavity is located close to the interface between the two arms at the end of the chain of iron-sulfur clusters (orange and yellow spheres). Loops of subunits ND1, ND3, NDUFS2, and NDUFS7 in and around the Q-binding cavity undergo substantial changes. The \( \pi \)-gate connects the E-channel and Q-binding cavity to the aqueous passage inside the membrane arm (dashed red arrow). In state 1, the \( \pi \)-gate formed by TMH3\( \beta^6 \) is closed, breaking the aqueous passage. In state 2, the \( \pi \)-gate opens as TMH3\( \beta^6 \) regularizes into an \( \alpha \)-helix, resulting in a continuous aqueous passage from the Q-binding cavity to the tip of the membrane arm.

The opening of the \( \pi \)-gate in the 1-to-2 transition creates a continuous aqueous passage from the Q-binding site all the way to the antiporter-like subunit ND5, where protons can be expelled into the IMS. The higher resolution of our \( C_t \)-complex I structure revealed additional water molecules at the open \( \pi \)-gate that contribute to an uninterrupted water chain in state 2 (fig. S13) and suggests that protons can flow easily from the Q-binding sites to the interior of the membrane arm. An essential role of TMH3\( \beta^6 \), which contains the \( \pi \)-gate, is consistent with its high degree of sequence conservation (51) and with an impaired complex I activity as a result of mutations in this region (52). The formation of a continuous aqueous passage from the Q-binding site to subunit ND5 through the \( \pi \)-gate appears to be a key element of the complex I mechanism.

Substantial rearrangements during the transition between the two states of complex I also affect the Q-binding cavity. Reorganization of the ND1 TMH5-6 loop, the NDUFS2 (49-kDa) \( \beta^1-\beta^2 \)-loop, and the NDUFS7 (PSST) loop within the Q-binding cavity is implicated in regulating the diffusion of Q intermediates between the binding sites and the ejection or uptake of QH\(_2\) and Q (13, 39) and appears to be well-conserved between mammalian (13) and \( C_t \)-complex I (fig. S5).

The structures of these loops are strikingly similar in the closed and open states of complex I. In state 2 of \( C_t \)-complex I, presumably, the better-defined conformation of these loops in state 2 is important for locking the Q substrate near N2 for reduction. The ND3 TMH1-2 loop and its essential cysteine adopt a defined and conserved position near the Q\(_2\) site in the transition to state 2 (figs. S5E and S7). Whether this is critical for Q binding, reduction, and protonation remains to be investigated.

The structural rearrangements of the Q-binding cavity and E-channel in the open-to-closed transition of ovine complex I are very similar to the rearrangements we observe in the 1-to-2 transition of the \( C_t \)-complex I and thus appear to be well-conserved (figs. S5 and S13). Conformations of complex I from the bacterium \( T. \) thermophilus show movements of the peripheral arm that are linked to rearrangements in the Q-binding cavity and in TMH3 of subunit Nqo10, which is homologous to subunit ND6 of the eukaryotic complex. These rearrangements appeared to be similar, although much less pronounced than in the \( C. \) thermophilum or ovine complex. The authors nevertheless assumed that they might correspond to the open and closed conformation of the ovine complex I (54).

\( T. \) thermophilus complex I does not undergo an active-to-deactive transition (55), therefore, it can be excluded that any of its conformations represents the deactive state. The fact that complex I from bacteria, mammals, and now also fungi undergoes a transition that is linked to very similar rearrangements in the Q-binding cavity and E-channel, in particular at TMH3\( \beta^6 \), leads us to the assumption that such a transition represents a principal and conserved feature of the complex I mechanism.

\( C_t \)-complex I solubilized in the detergent DDM did not undergo the 1-to-2 transition or any of the structural rearrangements observed in LMNG. NADH:Q\(_1\) activity measurements with \( C_t \)-complex I indicated strong inhibition by DDM. A similar effect has been reported for the detergent Triton X-100 (56). We discovered two DDM molecules bound in the Q-entrance tunnel and the E-channel. In line with our activity measurements, we conclude that DDM bound to this site prevents substrate binding and inhibits enzymatic activity.
As the 1-to-2 transition was not apparent in this detergent, we propose that DDM inhibits the transition. Ovine complex I retains its ability to undergo the transition with the inhibitor rotenone bound in the Q-binding cavity (13). However, rotenone does not have a flexible hydrophobic tail, whereas the acyl chain of one DDM molecule extends into the E-channel and obstructs it. Because the E-channel is a site of major reorganization in the 1-to-2 transition, its obstruction by a bound inhibitor is likely to prevent this transition.

In terms of its overall structure, Ct-complex I resembles that from other eukaryotes closely, but it also has some interesting aspects that so far appear to be unique. The enlarged subunit NDUFA5 forms a protein bridge between the peripheral and membrane arms that has not been seen in any other complex I structure. The NDUFA5 bridge seems to stabilize the connection between the two arms and might therefore help to maintain structural integrity at elevated temperatures. Recent structures of plant complex I revealed a protein bridge between the two arms formed by the subunits NDUFA1α, NDUFA6, and the plant-specific ferredoxin-like subunit C1-FDX. This bridge is on the other side of the membrane arm, as seen from the peripheral arm, and may have a regulatory function (25). The NDUFA5 bridge in Ct-complex I is connected to the core subunits NDUF52 and ND3 by three polypeptide tethers. Tether 1 is formed by subunit NDUF52 and connects the NDUFA5 bridge directly with the β1-β2 loop of NDUF52, which rearranges in the 1-to-2 transition and might be a key element in the Q reductase activity (26, 40). We assume that tether 1 imposes a dragging force on the β1-β2 loop and might be an element in regulating its function. Tether 2 of subunit ND3 forms a β-strand within NDUFA5, connecting the NDUFA5 bridge directly to TMH3 of subunit ND3. Tether 2 is likely to stabilize the NDUFA5 bridge by forming a β-sheet that is incorporated into a β-sheet in the NDUFA5 bridge. Thus, we speculate that the NDUFA5 bridge combines stabilizing and regulatory functions in Ct-complex I.

The structural features described above have not been found in complex I from other species, including that from Y. lipolytica, the only other structure of a fungal complex I. Sequence alignments suggest that nonthermophilic relatives of C. thermophilum, such as Neurospora crassa or Podospora anserina, have similar extensions in subunit NDUFA5 and ND1 (figs. S10C and S11). In the absence of complex I structures from these organisms, it is difficult to say whether these particular features represent adaptations of Ct-complex I to higher growth temperatures or have developed at some point in fungal evolution.

In Ct-complex I, the C-terminus of subunit NDUFA9 extends into the hinge between the peripheral and membrane arms. It forms a latch that rearranges in the 1-to-2 transition and interacts closely with subunits NDUF52 and ND6. In state 1, the latch establishes a salt bridge with tether 1 of subunit NDUF52 close to the β1-β2 loop. As this salt bridge breaks in state 2, we assume that the latch participate in the rearrangement of the critical β1-β2 loop. In state 1, the NDUFA9 latch extends across the TMH3-4 loop of subunit ND6. In the transition to state 2, the latch retracts from this position, while the Ω-bulge in TMH3N586 reverts to a regular α-helix. The Ω-bulge is likely to form or disappear in response to some compression or stretching force on TMH3S586, which in Ct-complex I appears to be exerted by the NDUFA9 latch via the TMH3-4 loop. Structures of mammalian and plant complex I do not show a NDUFA9 latch. In these organisms, its role must be taken over by some other element of the convoluted complex I structure. In Y. lipolytica, the NDUFA9 latch is present and notably disordered under turnover conditions (12), consistent with a regulatory role in fungal complex I.

In the transition between state 1 and state 2 of complex I, the peripheral arm twists, and this movement triggers conserved rearrangements in the Q-binding cavity and the formation of a water channel through the π-gate, enabling proton and charge transfer from the E-channel to the aqueous passage of the membrane arm. The cryoEM structures of Ct-complex I in two different conformations provide strong support for a common mechanism of coupling electron transfer in the peripheral arm to proton translocation in the membrane arm.

**MATERIALS AND METHODS**

**Isolation of mitochondria from C. thermophilum**

Wild-type *C. thermophilum* (La Touche) var. *thermophilum* was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany (no. 1495) and grown as described (26) with slight modifications. Mycelium was grown on LB agar plates at 52°C for 2 days. CCM medium (500 ml) was inoculated with freshly scraped off and chopped mycelium in a 1-liter Erlenmeyer flask. After 1 day at 52°C and 100 rpm in a rotary shaker, the submerging cultures were shredded in a blender for 10 s and used to inoculate 1 liter of CCM in 5-liter Erlenmeyer flasks. Cultures were incubated at 52°C for ~18 hours at 100 rpm. For harvest, cultures were strained through a metal sieve (180 μm pore size) and excessive liquid was squeezed out with a silicone spatula. Cell pellets were stored at −20°C until use.

Mitochondria were isolated from ~100 g of mycelium. All subsequent steps were performed at 4°C or on ice. Cells were resuspended in isolation buffer [50 mM Hepes/NaOH (pH 7.8), 350 mM sorbitol, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication with a Branson Model 250 device at 25% amplitude, in two cycles of 2 min each, with 3-s pulses and 2-s pause times. The cell lysate was centrifuged at 150g for 5 min and 4000g for 5 min to remove cell debris. Mitochondria were pelleted by centrifugation at 12,000g for 15 min. To improve purity, mitochondria were resuspended in isolation buffer, and the previous two centrifugation steps were repeated. Mitochondria were finally resuspended in resuspension buffer [10 mM Hepes/NaOH (pH 7.8), 350 mM sorbitol, and 1 mM EGTA], and the concentration of mitochondrial protein was measured by a Bradford assay. The concentration of the resuspension was adjusted to ~10 mg/ml with resuspension buffer. Mitochondria were flash-frozen in liquid nitrogen and stored at −80°C until further use.

**Purification of complex I**

Purified mitochondria from *C. thermophilum* containing ~50 mg of mitochondrial protein were thawed on ice and centrifuged at 12,000g for 10 min at 4°C. Supernatant was discarded, and the membrane pellet was resuspended either in DDM solubilization buffer [100 mM Hepes/NaOH (pH 7.8), 2 mM MgCl2, 50 mM NaCl, and 3% (w/v) DDM] or in LMNG solubilization buffer [100 mM Hepes/NaOH (pH 7.8), 2 mM MgCl2, 50 mM NaCl, and 2.5% (w/v) LMNG] to a final detergent:protein weight ratio of 2:1. The resuspension was rotated for 30 min at 4°C. Insoluble material was removed by centrifugation at 21,000g for 15 min at 4°C. The supernatant was filtered (0.22 μm pore size) and loaded onto a POROS GoPure HQ column (Thermo Fisher Scientific) connected to an Äkta Purifier system (GE Healthcare). The column was equilibrated with IEX buffer 50 [30 mM Hepes/NaOH (pH 7.8), 2 mM MgCl2, and 50 mM NaCl] supplemented with 0.015% (w/v) DDM, if mitochondria were...
solubilized with DDM, or 0.0015% (w/v) LMNG, if mitochondria were solubilized with LMNG. After loading, the column was washed with IEX buffer 50 supplemented with detergent, until a constant baseline was reached. Complex I was eluted with a linear gradient to 70% of the high-salt buffer IEX 1000 [30 mM Hepes/NaOH (pH 7.8), 2 mM MgCl₂, and 1000 mM NaCl] over 60 min with a flow rate of 1 ml/min. IEX buffer 1000 was supplemented with detergent in the same way as IEX buffer 50. Fractions containing complex I were concentrated using Amicon Ultra 4 columns with 100,000 molecular weight cutoff and loaded onto a Superose 6 Increase 3.2/300 size exclusion column (GE Healthcare) connected to an Äkta Ettan system. Complex I was eluted in SEC buffer [20 mM Hepes/NaOH (pH 7.4) and 100 mM NaCl] supplemented with the same type and amount of detergent as IEX buffer 50. Eluted complex I was used directly for cryoEM specimen preparation.

**Sample vitrification and cryoEM data acquisition**

Three microliters of purified complex I at a final concentration of 3.6 mg/ml (DDM-solubilized) or 1.5 mg/ml (LMNG-solubilized) was applied onto freshly glow-discharged (15 mA for 45 s in a PELCO easiGlow system) C-flat 1.2/1.3 300 mesh copper grids (Science Services GmbH). Samples were blotted for 4 s with blot force 20 (595 Whatman paper) at 4°C in 100% humidity and plunge-frozen in liquid ethane with a FEI Vitrobot Mark IV. CryoEM data were collected automatically using EPU software (Thermo Fisher Scientific) on a Titan Krios G3i microscope at 300 kV, equipped with a K3 detector (Gatan) operating in electron counting mode. Movies were acquired at a nominal magnification of ×105,000, resulting in a pixel size of 0.837 Å. Each movie was recorded for 2.1 s and subdivided into 45 frames. The electron flux rate was set to 15 e⁻ per pixel per second at the detector, resulting in an accumulated exposure of 45 e⁻/Å² at the specimen. An energy filter with a slit width of 30 eV was used, and a 70 μm C2 and a 100 μm objective aperture were inserted during acquisition. A total of 1996 movies at a defocus range of −1 μm to −3.5 μm were collected for complex I solubilized in DDM and 6570 movies at a defocus range of −0.5 to −3.0 μm for complex I solubilized in LMNG. An overview of the data collection statistics is shown in table S3.

**Image processing**

Image processing was performed in RELION3 (57, 58), unless otherwise specified. For complex I in DDM, dose-fractionated movies were motion-corrected using the RELION implementation of the MotionCor2 algorithm (59) and contrast transfer function (CTF) parameters were estimated with CTFFIND4 (60). A total of 126,638 particles were picked in cryoYLO 1.7.3 with a model trained on 20 micrographs (61). Particle coordinates were imported into RELION and extracted with down-sampling to a pixel size of 3.348 Å. Particles were then subjected to 2D classification, resulting in 24,016 particles, which were selected to reconstruct an initial model. The initial model was used as a reference for 3D classification with 102,840 particles, selected from classes of the previous 2D classification. After 3D classification, broken and low-resolution particles were sorted out and the remaining 37,815 particles were re-extracted with a pixel size of 1.0299 Å and a box size of 512 pixels. An additional round of 3D classification was performed. The remaining particles were subjected to 3D refinement, followed by two rounds of per-particle CTF refinement with an intermediate Bayesian polishing step. After removal of particle duplicates, the refined particles were transferred to cryoSPARC v3.0.1 (38) to perform a nonuniform refinement in combination with per-particle-defocus and per-group CTF parameter refinement (62), which resulted in a map with an overall resolution of 2.77 Å.

For complex I in LMNG, motion correction and CTF estimation were again performed in RELION with the RELION implementation of the MotionCor2 algorithm and CTFFIND4, respectively. Micrographs with an estimated resolution below 7 Å were excluded, and 1,087,651 particles were picked from the remaining 6503 micrographs in cryoYLO. Particles were extracted with a down-sampled pixel size of 3.348 Å. After 2D classification, 177,728 particles were selected and subjected to an ab initio reconstruction in cryoSPARC. The ab initio reconstruction was used as a reference for 3D classification with 996,588 particles, selected from classes of the previous 2D classification. 3D classes with broken particles were removed, and the remaining 199,973 particles were re-extracted with a box size of 540 pixels, giving a pixel size of 0.9765 Å. The re-extracted particles were subjected to 3D refinement, followed by two rounds of per-particle CTF refinement with an intermediate Bayesian polishing step. A 3D classification yielded a class of 175,581 particles that indicated high-resolution features. As 3D refinement yielded a map of 2.55 Å, close to Nyquist frequency, the particles were re-extracted into 588-pixel boxes with a pixel size of 0.837 Å. Particles were again subjected to two rounds of per-particle CTF refinement with an intermediate Bayesian polishing step and 3D refinement, resulting in a map at 2.50 Å resolution. After removal of particle duplicates, the dataset was further processed in cryoSPARC by performing nonuniform refinement in combination with per-particle-defocus and per-group CTF parameter refinement, which improved the global resolution to 2.39 Å.

3DVA was performed in cryoSPARC (63) with the nonuniform refined particles and a real-space mask excluding solvent and the detergent micelle. 3DVA was run using three variability components and a low-pass filter resolution of 6 Å. 3DVA with the DDM dataset revealed flexibility at the hinge between the membrane arm and peripheral arm, but no discrete cluster of particles with a different conformation of complex I. 3DVA of the LMNG dataset indicated a heterogeneous distribution of the particles along the latent coordinate for component 2 and resolved a subset of particles with a strongly twisted peripheral arm in relation to the membrane arm. Repeating 3DVA with the LMNG dataset by masking the hinge region between the peripheral and membrane arms of complex I (where the variability was greatest) and decreasing the low-pass filter resolution to 4 Å resulted in a better separation of particle subsets along component 2. By using the cluster mode function in cryoSPARC, the particle subsets were separated into two clusters of 21,989 and 153,568 particles each. Both particle sets were again subjected to nonuniform refinement in cryoSPARC, which resulted in a map of 2.44 Å (153,568 particles) and 2.78 Å resolution (21,989 particles), showing complex I in two distinct conformations (states 1 and 2). 3D classifications of the refined particles in RELION (without image alignment and increased T values) confirmed the presence of the two conformations in the LMNG-solubilized complex I dataset resolved by 3DVA (with similar particle distribution) and delivered only one conformation for the DDM-solubilized complex I.

To further improve the resolution of the complex I membrane arm in the tip region, the nonuniformly refined particles of states 1 and 2 (in LMNG) and the DDM-solubilized complex were subjected to a local refinement in cryoSPARC by masking the membrane arm.
The locally refined particles of the membrane arm of complex I in DDM yielded a map of 2.76-Å resolution. The membrane arm of states 1 and 2 in LMNG resulted in maps of 2.47- and 2.83-Å resolution, respectively. All resolutions were estimated according to the Fourier shell correlation (FSC) 0.143 cutoff criterion of two independently refined half maps (64). As a final step, one cycle of density modification with phenix.resolve_cryo_em (47) was carried out with all six reconstructed maps, using in each case the unsharpened map, two unfiltered half-maps, and an estimate of the protein mass as inputs. This yielded a resolution of 2.75-Å at FSCref = 0.5 for both the map of the entire DDM-solubilized complex I and for the membrane arm. FSCref gave a resolution of 2.68-Å for state 2 of the entire complex and 2.75-Å for the membrane arm. For state 1, the FSCref = 0.5 resolution was 2.32-Å for the entire complex and 2.39-Å for the membrane arm. An overview of the processing workflow and the results is given in figs. S14 to S16.

Model building

Initial models for the C. thermophilum complex I subunits were built using the SWISS-MODEL server (65) on templates 6RFR and 6RFQ (16). Homology models were rigid body–fitted into the cryoEM map of complex I (state 1) using UCSF Chimera (66), followed by manual building in Coot (67). The structure model was rigid body–fitted into all other maps and corrected in Coot (67). Subunit NDUF2 and NDUF2A1 were not annotated in the genome of C. thermophilum (30, 68) and were initially modeled as poly-alanine chains. Characteristic residue-specific map features were subsequently used to place matching amino acids. The resulting short sequence fragments were used as query sequences for protein BLAST (69) searches in a six-frame-translation database from the genome of C. thermophilum. Missing residues were replaced by the amino acid sequence of the returned hits. Sequences of some subunits did not match map features, which can be attributed to false exon–intron boundaries in the annotated genome. These errors were corrected manually by extending or shortening the exons in the genomic sequences. Models were iteratively refined by using phenix.real_space_refine in PHENIX (70, 71) and manual refinement in Coot. Ligand restraint files were created by PHENIX el.BOW (72). Water molecules were built automatically using segmentation-guided water and ion modeling (SWIM) (73), implemented in the Segger tool of UCSF Chimera. Water molecules were checked and corrected with the “check/delete waters” function in Coot and also placed manually, if densities had nearly spherical shapes, did not clash with other atoms, and showed at least one hydrophilic interaction with neighboring residues or waters up to a distance of 3.5-Å. Validation checks for model stereochemistry were performed in MolProbity (74). An overview of the modeling statistics is shown in table S4. For structural comparison, models were aligned using the Matchmaker tool in UCSF ChimeraX (75). Cavities were calculated and drawn using Hollow (76) with a 1.4-Å probe radius. Finalized models were visualized using UCSF ChimeraX (75). Sequence alignments were performed with Clustal Omega (77).

Protein analysis by MS

Purified complex I samples were reduced with tris(2-carboxyethyl)phosphine (TCEP), and cysteines were alkylated with indole-3-acetic acid. Subsequent proteolytic digests were performed using S-TRAPs (Protifit) according to the manufacturer’s instructions. Peptides were further desalted and purified on C18 SPE cartridges and dried in an Eppendorf concentrator. After solubilization in 0.1% formic acid (FA) in acetonitrile/water [95/5 (v/v)], samples were subjected to liquid chromatography–MS/MS analysis on an Ultimate 3000 nanoRSLC (Thermo Fisher Scientific) system, equipped with an Acclaim Pepmap C18 trap column (2 cm by 75 μm, particle size: 3 μm; Thermo Fisher Scientific) and a C18 analytical column (50 cm by 75 μm, particle size: 1.7 μm; CoAnn Technologies) with an integrated liquid junction and fused silica emitter coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). Trapping was performed for 6 min with a flow rate of 6 μl/min using loading buffer [98/2 (v/v) water/acetonitrile with 0.05% trifluoroacetic acid], and peptides were separated on the analytical column at a flow rate of 250 nl/min with the following gradient: 4 to 48% B in 45 min, 48 to 90% B in 1 min, and constant 90% B for 8 min followed by 20-min column re-equilibration at 4% B with buffer A (0.1% FA in water) and buffer B [0.1% FA in 80:20 (v/v) acetonitrile/water]. Peptides eluting from the column were ionized online using a Nano Flex ESI source and analyzed in data-dependent mode. Survey scans were acquired over the 350 to 1400 mass/charge ratio (m/z) mass range in the Orbitrap [maximum injection time: 50 ms, AGC (automatic gain control) fixed at 2 × 10^6 with 120,000 mass resolution], and sequence information was acquired by a top–speed method with a fixed cycle time of 2 s for the survey and following MS/MS scans. MS/MS scans were acquired for the most abundant precursors with a charge state from 2 to 5 and an intensity minimum of 5 × 10^3. Picked precursors were isolated in the quadrupole with a 1.4 m/z isolation window and fragmented using HCD [NCE (normalized collision energy) = 30%]. For MS/MS spectra, an AGC of 10^4 and a maximum injection time of 54 ms were used and detection was carried out in the Orbitrap using 30,000 mass resolution. The dynamic exclusion was set to 30 s with a mass tolerance of 10 parts per million (ppm). Data analysis was performed in Proteome Discoverer (version 2.5) using Sequest HT as database search algorithm for peptide identification. Raw files were recalibrated and searched against the protein database obtained from structure modeling as well as the UniProt proteome for C. thermophilum (UP000008066; obtained 2020-10-09) and common contaminants. The search space was restricted to tryptic peptides with a length of 7 to 30 amino acids, allowing for up to two missed cleavages and with a minimum of one unique peptide per protein group as well as precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was set as a variable modification. Inference rescoring and Percolator nodes were used to estimate the number of false-positive identifications, and results were filtered for a strict target false discovery rate of <0.01.

Activity measurements

Complex I NADH:Q1 oxidoreductase activity was measured at 30° or 50°C by monitoring the oxidation of NADH at 340 nm (e = 6220 M⁻¹ cm⁻¹) using a Varian Cary 50 ultraviolet-visible spectrophotometer. Measurements were carried out with purified complex I protein (1 to 5 μg/ml) in 20 mM Heps/NaOH (pH 7.4), 50 mM NaCl, 1 mM EDTA, 2 mM Na3S, and either 0.015% (w/v) DDM or 0.015% (w/v) LMNG. The reaction was started by addition of complex I protein, after 100 μM 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butynyl)-1,4-benzoquinone (Q1) and 100 μM NADH were already added to the buffer. For complex I inhibition, 2 μM rotenone was added to the reaction mix. Values represent means ± SEM. Statistical analysis was performed using OriginPro 2020b (OriginLab).
Statistical significance was determined at the $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)) levels using independent two-tailed two-sample Student’s t test analysis.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.adc9952

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14 of 14

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Acknowledgments: We thank V. Zickermann and A.-N. A. Agip for critical comments on the manuscript and O. Yildiz for valuable assistance with model building. We thank N. Kellner and E. Hurt for invaluable help in establishing the culture of C. thermophilum in our laboratory.

Funding: This work was funded by the Max Planck Society. Author contributions: E.L. purified and characterized complex I, prepared cryoEM grids, acquired and processed cryoEM data, built and analyzed the atomic models, and drew the figures. J.M.-C. and J.D.L. acquired and analyzed MS data. E.L. and W.K. conceived the study and wrote the manuscript. Competing interests: The authors declare that they have no competing interests.

Data and materials availability: The cryoEM maps have been deposited in the Electron Microscopy Data Bank with accession codes EMD-14797 (complex I, state 1), EMD-14798 (complex I, state 1, only membrane arm), EMD-14794 (complex I, state 2), EMD-14796 (complex I, state 2, only membrane arm), EMD-14791 (complex I in DDM), and EMD-14792 (complex I in DDM, only membrane arm). The atomic models have been deposited in the Protein Data Bank under accession codes 7ZMG (complex I, state 1), 7ZME (complex I, state 2, only membrane arm). The atomic models have been deposited in the Protein Data Bank under accession codes 7ZMG (complex I, state 1), 7ZME (complex I, state 2, only membrane arm), 7ZMF (complex I in DDM), and 7ZMB (complex I in DDM, only membrane arm). The mass spectrometry proteomics data are available via ProteomeXchange with identifier PXD033234. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 13 May 2022
Accepted 7 October 2022
Published 25 November 2022
10.1126/sciadv.abe9952

Laube et al., Sci. Adv. 8, eadc9952 (2022) 25 November 2022 14 of 14