**RESEARCH ARTICLE SUMMARY**

**MOLECULAR MACHINES**

**Rotary substates of mitochondrial ATP synthase reveal the basis of flexible F₁-Fₒ coupling**

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**INTRODUCTION:** Mitochondrial F₁-Fₒ adenine triphosphate (ATP) synthases are macromolecular turbines that couple proton translocation across a membrane to ATP synthesis. Protons are translocated through the Fₒ subcomplex in the lipid bilayer by a rotor composed of a defined number of c subunits, each with a proton-binding site, to generate ring rotation. A central stalk is firmly anchored to the c ring and conveys rotary motion to the catalytic F₁ subcomplex in the mitochondrial matrix, where ATP is produced by rotary catalysis. A peripheral stalk connects the two subcomplexes to prevent idle rotation of F₁ with Fₒ.

**RATIONALE:** Although ATP synthase complexes have been investigated for more than 50 years, several key questions remain. An enduring question is how the stoichiometrically mismatched c ring in Fₒ (composed of 8 to 17 c subunits) and the three-fold symmetric F₁ head are efficiently coupled. Another open question is the exact pathway taken by protons through the membrane, which has been the least well characterized part of the mechanism.

RESULTS: We used single-particle cryo–electron microscopy (cryo-EM) to characterize the structure and dynamics of a complete and active dimeric mitochondrial ATP synthase from the chlorophyll-less unicellular alga *Polytomella* sp. Together with data obtained by genome sequencing and mass spectrometry, our 2.7- to 2.8-A resolution map allowed us to build a full atomic model of the 1.6-MDa complex. The model includes the newly identified subunit ASA10, which interlinks the two ATP synthase monomers on the luminal side of the membrane. Separation of 13 independent rotary states provides a detailed molecular description of the movements that accompany c-ring rotation. We find that the F₁ head rotates together with the central stalk and c ring through approximately 30°, or one c subunit, at the beginning of each 120° step. Flexible coupling of the F₁ head to the Fₒ motor is mediated primarily by a hinge at the interdomain link of the oligomycin sensitivity–confering protein (OSCP) subunit that joins the F₁ head to the peripheral stalk. The extended two-helix bundle of the central stalk γ subunit interacts with the catch-loop region of one β subunit of the F₁ head. The resulting mechanism of flexible coupling is likely to be conserved in other F₁-Fₒ ATP synthases. Our results provide much-needed context to a wealth of published data indicating that OSCP is a hub of metabolic control in the cell.

Our high-resolution map of the proton-translocating Fₒ complex has revealed a strong density, very likely a metal ion, ligated by two histidine residues. Recent cryo-EM studies of yeast and spinach chloroplast ATP synthase contain unannotated densities at the same position. Mutational experiments in *Escherichia coli* have shown that an equivalent residue is essential to proton translocation. By three-dimensional classification, we separated two different rotational positions of the c ring and showed that the coordination environment of the metal ion changes with c-ring position. This evidence points toward a role for the metal ion in synchronizing c-ring protonation with its rotation.

CONCLUSION: In ATP synthases, the F₁ catalytic head can accompany the rotor through a rotation of ~30° at the beginning of each ~120° step. This movement allows flexible coupling of F₁ and Fₒ. The interdomain hinge of OSCP facilitates flexible coupling and makes this subunit an apposite point for the regulation of ATP synthesis.

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Mitochondria carry out controlled oxidation of reduced substrates to generate an electrochemical gradient across the inner mitochondrial membrane. Movement of protons down the gradient through the F1F0-ATP synthase drives the production of the soluble energy carrier ATP by rotary catalysis. The ATP synthases consist of two connected nanomotors: (i) the membrane-embedded F0 subcomplex, where proton translocation generates torque, and (ii) the soluble F1 subcomplex, where the torque powers adenosine diphosphate (ADP) phosphorylation

The dimeric ATP synthase from mitochondria of the chlorophyll-less unicellular alga *Polytomella* contains the signature subunits of mitochondrial ATP synthases, α2β2γδε, and OSCP. The peripheral stalk and other membrane protein subunits typical of mammalian and fungal ATP synthases (I) are replaced in *Polytomella* and related species, including the photosynthetic model organism *Chlamydomonas reinhardtii* (4), by proteins known as ATP synthase–associated (ASA) proteins, which bear no homology to other known ATP synthase components (4, 5). These subunits form a bulky peripheral stalk that links the two ATP synthase complexes into a stable dimer.

ATP synthase must couple translocation of 8 to 17 protons (7), depending on c-ring size, with phosphorylation of three molecules of ADP in the catalytic β-subunit sites of the F1 head. The stoichiometric mismatch of F1 and F0 poses a challenge to efficient energy conversion in these systems, whereas the peripheral stalk of mitochondrial ATP synthase dimers is bulkier and appears to be less flexible. The robust, rigid peripheral stalks of the *Polytomella* ATP synthase make it an ideal system for examining the dynamics revealed by rotary states and substates by high-resolution cryo-EM.

High-resolution map of a complete F-type ATP synthase dimer

Cryo-EM single-particle analysis (table S1) of purified and active (2) (fig. S1) *Polytomella* ATP synthase dimer yielded a map at 2.94 Å overall resolution. Local masking improved the resolution to 2.7 to 2.8 Å in overlapping regions covering the full complex (table S2 and figs. S2 and S3). Symmetry expansion allowed each monomer to be treated independently in image analysis, and three-dimensional (3D) classification of the central stalk and F1 head enabled separation of three primary rotary states, at resolutions of 2.8 to 2.9 Å, in the F1 head and rotor (Fig. 1 and Movie 1). The nucleotide-binding sites βTP and βTP contain Mg2+ADP, and βTP is empty (Fig. 1, B to D). The ADP-bound complex was expected as the protein was prepared without added nucleotides. The “arginine finger” (15) of αArg290 extends toward the nucleotide phosphate in βTP but away from it in βTP (Fig. 1, C and D). The cryo-EM data were complemented by genomic sequencing and mass spectrometry analysis of the purified protein, enabling us to build a full atomic model of the 158-MDa complex, with 62 copies of 18 different subunits and a total of 14,240 residues fitted (Fig. 1, fig. S4, and table S3). The model includes the completed polypeptide sequence of subunits ASA2 and ε and the previously unknown subunit ASA10.

Movie 1. Three-dimensional map of the *Polytomella* ATP synthase dimer. One monomer is gray; the other monomer map is colored by subunit, as in Fig. 1.
Rotary substates reveal concerted rotation of F₁ and central stalk

To describe the conformational space of the F₁Fₒ-ATP synthases, we sorted the symmetry-expanded dataset into 12 classes. Further subdivision of a mixed class yielded a total of 13 distinct rotary substates (fig. S2), which were refined to 2.8- to 4.2-Å resolution (fig. S3 and table S2). The subclasses can be placed into a meaningful sequence according to the rotation-al position of the rotor with respect to the stator (fig. S5). Each subclass was assigned to one of three primary rotary states, with six subclasses for state 1 (1A to 1F) (Fig. 2 and figs. S5 and S6), four for state 2 (2A to 2D), and three for state 3.

Fig. 1. High-resolution structure of the mitochondrial F₁Fₒ ATP synthase dimer from Polyomella sp. (A) Composite cryo-EM map of the 62-subunit, 1.58-MDa Polyomella dimer; the right side is colored by subunit. The c₁₀ rotor ring and subunit α make up the Fₒ motor complex. Proton translocation through Fₒ causes rotation of the c ring and the attached central stalk subunits γ, δ, and ε. The F₁ head consists of three catalytic β subunits (light green) and three α subunits (dark green). Long C-terminal extensions of β wrap around the α subunits on the outside of F₁ (see Movie 5). The two-domain OSCP subunit links the three α subunits to the peripheral stalk subunits ASA1 to ASA10 (see Fig. 4). Subunit ASA10 connects the two F₁Fₒ monomers in the membrane (see figs. S9 and S11). The local map resolution is 2.7 Å for the peripheral stalk, Fₒ, and c ring, and 2.8 to 2.9 Å for the F₁ head and central stalk (see fig. S2). Inset: Three primary rotary states (1, 2, and 3) of the F₁Fₒ monomer are related by ~120° rotation of the central stalk within the αβ₃ assembly. Unless otherwise specified, subunit coloring is consistent throughout all figures. (B) Section through F₁ at the level of bound nucleotides. βDP and βTP sites contain ADP (red) and βE is empty. The three α subunits bind a structural ATP (orange). Nucleotide-coordinating Mg²⁺ ions are violet. The central stalk subunit γ (blue) engages with the catch loop of the βE subunit (see fig. S7). (C and D) Catalytic sites of subunits βDP and βTP. A well-defined Arg side chain of subunit α (the “arginine finger”) extends toward the nucleotide phosphate in the βDP site. ATP in the βTP site has hydrolyzed to ADP during protein isolation. Amino acid abbreviations: D, Asp; E, Glu; K, Lys; N, Asn; R, Arg; T, Thr.
(3A to 3C). The three primary states are separated from one another by ~120° rotation of the central stalk within the F₁ head (Fig. 1A, inset). In contrast, substates of any given rotary state differ by concerted rotation of the F₁ head and rotor within the first 15° to 32° of each ~120° step (Fig. 3, Movies 2 and 3, and fig. S5). Contact between the βE site and the γ subunit (the “β-catch loop” (16)) is maintained between substates (Movie 3 and fig. S7), as is the nucleotide occupancy of β subunits (Fig. 3B and fig. S5). The pivot point of this movement is the single-peptide chain connecting the two OSCP domains (henceforth the “OSCP hinge”) (Fig. 4 and Movie 4). The top of the peripheral stalk flexes only slightly. Previous studies have suggested that the interdomain region of OSCP may be flexible (17–19). In comparing all 13 substates, no coiling of the central stalk is apparent (fig. S6).

In the first ~30° of rotary steps 1 and 2 (in moving from state 1A to 1F and state 2A to 2D), the c ring advances by almost one subunit with respect to the α subunit (Fig. 3A) while the position of γ with respect to the α₃β₃ hexamer remains virtually unchanged (Fig. 3B and fig. S5). Between substates of state 3 (in moving from state 3A to 3C), the ring advances by about half a c subunit together with the rotor (Fig. 3A). As the rotor moves a further ~90° to the starting position of the next primary rotary state (e.g., from state 1F to 2A), the F₁ head recoils by ~30° to its original position, for a cumulative ~120° power stroke of γ within F₁ for this step. Given that this motion is thermally accessible for the complex at equilibrium, it would almost certainly contribute to flexible coupling of F₁ and Fo also under turnover conditions.

We observe an uneven distribution of particles between the three major rotary states, with 52% in state 1, 23% in state 2, and 21% in state 3, in line with other recent cryo-EM studies of ATP synthase.

**Fig. 2. Rotary substates of mitochondrial ATP synthase.** The three primary rotary states are subdivided into a total of 13 rotary substates at resolutions between 2.8 and 4.2 Å (see figs. S2 and S3 and table S2). The number of resolved substates and the angular increments between them differ for each of the three primary rotary states (fig. S5). (A) Composite cryo-EM map of rotary substate 1A at 2.9-Å resolution. (B to D) Overlays of substate maps indicate concerted movement of the N-terminal domain of OSCP (B), the F₁ head with the central γ subunit (C), and the c ring (D) from substate 1A to substate 1F. Projected map densities of other subunits are shown in light gray for reference.
synthases (11–13) and with a recent single-molecule study (20) that reported an asymmetry in the time-averaged population of rotary states in the presence of ADP. In comparing substates of a given primary rotary state, the later substates of the *Polytomella* complex (1D, 1E, 1F, 2C, 2D, and 3C) are consistently less populated than the earlier substates (fig. S2 and table S2), which suggests that these are higher-energy states.

The interaction between F1 and γ appears strongest in the catch loop region (16) of βE, where conserved residues βAsp346, βAsp348, and βThr347 (βAsp302, βAsp305, and βThr304 in *Escherichia coli*) form ionic and hydrogen-bond interactions with γArg296 and γGln297 (*E. coli* γArg268 and γGln269). The salt bridge between βAsp346 and γArg296 forms via a resolved water molecule (fig. S7). Throughout the 20° to 30° concerted rotation of F1 with γ, the interaction between the βE catch loop and γ is maintained, although subtle loop movements suggest that the hydrogen-bonding partner of βAsp346 changes from γGln297 to γAsn293 between substates (e.g., from state 1A to 1F; fig. S7). Previous disruption of these interactions by mutation in *E. coli* eliminated or strongly reduced ATP hydrolysis activity and the ability to grow on succinate (16).

A proposed function of such an interaction is that, in ATP synthesis mode, it would stall rotation of γ until the βE site has bound ADP, to prevent unproductive rotation (16). The major impact of catch loop mutations on both synthesis and hydrolysis activity implies that this region plays a broader role in the conformational changes required for catalysis. On the basis of our results, we suggest that this interaction does not stall rotation completely; rather, a proton may be translocated at Fo while the F1 head waits for substrate binding.

Studies aiming to characterize flexible coupling have focused on the central stalk (21), often working with an F1 and rotor subcomplex. When the whole F1Fo complex was examined, it was typically attached to substrate and reporter molecules at subunits β and c to probe for flexibility along the F1-Fo axis (9, 22, 23). A recent single-molecule study of F1Fo reported forward and backward stepping of the rotor by ∼30° (i.e., up to one c subunit) (22). However, with F1 anchored to the support by subunit β and a probe attached

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Fig. 3. Concerted rotation of F1 with central stalk and c ring.

(A) Progressing in the direction of ATP synthesis, the c20 ring (yellow) rotates counterclockwise (as seen from F1) with respect to the a subunit (light blue) by up to 32° for substates of the same primary rotary state. The primary rotary states differ by power strokes of ∼120°. The position of one c subunit in the first (black outline) and last rotary substate (pink) of each primary state is indicated. (B) Between substates of a given rotary state, the F1 head rotates together with the c ring and central stalk before recoiling to its original position in the first substate of the subsequent primary rotary state. Subunits a (dark green) and β (light green) with their bound nucleotides (red and orange) are shown for the first substate in each primary state. The position of the last substate in each primary state is indicated in light blue in the background. The peripheral stalk position is shown in gray.
to c, flexibility at OSCP would not be detected. The observed ~30° stepping angle appears to reflect regular energy minima in the c-ring/a-subunit interaction rather than joint rotation of the c ring and central stalk with F1. Single-molecule studies of flexible coupling may need to explore a wider range of anchoring points.

Several lines of evidence support the idea that OSCP-mediated flexible coupling plays a role in other F-type ATP synthases: (i) The structure and polypeptide sequences of β, γ, and OSCP subunits involved in this process are highly conserved. (ii) An OSCP subunit (or δ in chloroplasts and bacteria) is found in all three lineages of ATP synthase known to have independently acquired novel peripheral stalk components (24, 25). (iii) A cryo-EM study of bovine ATP synthase (11) identified rotary substates of this complex, albeit at lower resolution, indicating rotation of F1 relative to the remainder of the complex. Reevaluating these results in light of our data, they do in fact show concerted rotation of F1 with the central stalk. (iv) Well-known inhibitors and regulators of ATP synthase bind to OSCP, affecting the catalytic rate and Michaelis constant (K_M) (26, 27), suggesting a mechanistic rather than merely structural role for this subunit. The simpler bacterial and chloroplast ATP synthases have a thinner, flexible peripheral stalk relative to Polyomella, and previous cryo-EM studies have found substantial flexibility in the peripheral stalk even between primary rotary states (12–14). In these simpler systems, two or more components may mediate flexible F1-Fo coupling.

A body of literature suggests that OSCP-mediated flexible coupling (Fig. 4 and Movie 4) facilitates ~30° back-and-forth rotation of the N-terminal domain with F1 (curved black arrow) relative to the C-terminal OSCP domain and peripheral stalk. (C) Between substates of a given primary rotary state, the N-terminal OSCP domain rotates with F1 by up to 28° (straight dashed lines) in synthesis direction (orange arrows) and then recoils to its starting position in the first substate of the next primary rotary state (red arrows). The OSCP position in the first substate of each primary state is shown in orange; the OSCP position in the last resolved rotary state in each primary state is indicated in light blue in the background.

Fig. 4. Subunit OSCP connects the F1 head and peripheral stalk as a flexible hinge. (A) Overview of F1Fo monomer indicating the position of OSCP (orange) on top of the F1 head (green) relative to the peripheral stalk (gray). (B) The two-domain OSCP interacts with the helical C-terminal extensions of subunits α1, α2, and α3 (green) (see Movie 4). Extensions of α2 and α3 form short helix bundles with helices of the globular N-terminal OSCP domain, whereas α1 binds to the elongated C-terminal OSCP domain that is attached to the peripheral stalk via the α1 extension. The two OSCP domains are connected by a flexible peptide link that facilitates ~30° back-and-forth rotation of the N-terminal domain with F1 (curved black arrow) relative to the C-terminal OSCP domain and peripheral stalk. (C) Between substates of a given primary rotary state, the N-terminal OSCP domain rotates with F1 by up to 28° (straight dashed lines) in synthesis direction (orange arrows) and then recoils to its starting position in the first substate of the next primary rotary state (red arrows). The OSCP position in the first substate of each primary state is shown in orange; the OSCP position in the last resolved rotary state in each primary state is indicated in light blue in the background.
Fig. 5. Metal ion and ordered water molecules in the F$_o$ proton access and release channels. (A) The H5/H6 helix hairpin of subunit a (light blue) forms the bearing against which the c$_{10}$ ring (yellow) rotates, shown here for c-ring position 1. (B) Sectional view of H5 and outer-ring c-subunit helices with proton-binding cGlu$^{111}$, c-ring position 1. (C and D) Water molecules (purple density) coordinated by the ion-binding cGlu$^{111}$ and adjacent cSer$^{112}$ in the matrix channel for c-ring positions 1 (C) and 2 (D). Maps are displayed at density thresholds of 0.035 for subunit a, 0.025 for the c ring, and 0.025 (C) or 0.018 (D) for water. (E) The strictly conserved aArg$^{239}$ and aGln$^{295}$ that separate the proton access and release channels in the membrane bind two water molecules, shown in the consensus C2-refined map of the F$_o$ region. (F to H) Conserved residues aHis$^{248}$ and aHis$^{252}$ in H5 of subunit a coordinate a metal ion (green). Bond distances change depending on c-ring position. The predominant c-ring position [position 1, full color, and (H)] accounts for 58% of particles; a second position, differing by rotation of roughly 13° [position 2, faint yellow, and (G)] accounts for 33% of particles. Except where specified, all maps in a given panel are rendered at the same density threshold. E: Glu; H: His; Q: Gln; R: Arg; S: Ser.
has broad physiological implications, with a number of regulatory mechanisms acting on OSCP. Cyclophilin D, a regulator of mitochondrial permeability transition, binds to OSCP and inhibits ATP synthase activity (28), as does the immunomodulator Bz-423, which affects both maximum rate ($V_m$) and $K_m$ for ATP hydrolysis (27). Deacetylation of a conserved Lys near the hinge region of OSCP in response to exercise stress affects mitochondrial ATP levels (29). The hormone estrogen binds to OSCP (30) and inhibits ATP synthase activity (31, 32) with possible relevance to neuroprotection (33). The tumor suppressor p53, under nonstress conditions, binds to OSCP and promotes increased O$_2$ consumption and decreased ROS levels in mitochondria (34). These diverse and potent modulations of ATP synthase activity cannot be reconciled with a model of ATP synthase in which OSCP forms a static bridge between the F$_1$ head and peripheral stalk. Our results show that, on the contrary, OSCP plays a dynamic role in the flexible coupling of F$_1$ and F$_0$. The concept of a dynamic OSCP will be essential to understand and exploit modulation of ATP synthase activity, with relevance to neurodegeneration and traumatic brain injury, ischemia-reperfusion injury, and immunomodulation, among others.

**An essential histidine ligates a metal ion that is sensitive to c-ring rotation**

In respiring mitochondria, the pH of the lumen is 7.2 (35), while the $pK_a$ of the c-ring carboxylate in an aqueous environment is around 4.5 (36); thus, protonation of the c ring needs to be assisted by the local environment in the luminal entrance channel in order to achieve high turnover rates. Mutational studies in *E. coli* (37) have established that residues cGlu$^{230}$ and cHis$^{245}$ are essential both for ATP synthesis and proton permeability of F$_0$, in membranes stripped of F$_1$. The mutations $a$E219H and $a$H245E show low levels of activity, while the double mutant is more active than either single mutant, suggesting that the residues interact to facilitate c-ring protonation. Our map shows a strong, nonpeptide density ligated by cHis$^{248}$ (cGlu$^{230}$ in *E. coli*) and cHis$^{252}$ of cHis (Fig. 5) with bond lengths of 2.0 to 2.2 Å. The strong density and coordination environment favor assignment to a metal ion, provisionally annotated as Zn$^{2+}$ in the deposited models. At high threshold levels, the bound waters appear diatomic, perhaps because of a heavy ligand (Fig. 5, B and F to H). Both ligating His residues are present in mammalian ATP synthases (fig. S8). The cHis$^{248}$ position is occupied by His or Glu in all F-type ATP synthases, with the notable exception of Na$^+$-translocating complexes (fig. S8a). Recently published high-resolution cryo-EM maps of yeast mitochondrial (38) and spinach chloroplast ATP synthase (39) both indicate a strong, unattributed nonpeptide density at the same position in the luminal channel, near the residue equivalent to cHis$^{248}$ (aHis$^{346}$ and aGlu$^{338}$, respectively) (fig. S8c, C and D). In both instances, a single strong density was observed as compared to the two densities in our map, although this may reflect the lower local resolution (3.6 Å and 3.4 Å) of the yeast and spinach maps.

Masked 3D classification of the c ring and a subunit yields two distinct c-ring rotary positions at 2.7-Å and 3.1-Å resolution, representing 58% and 33% of particles (fig. S2). The positions differ by rotation of the c ring by approx-imately one-third of a c-ring subunit (13°) (Fig. 5F). Comparing these maps, it is clear that the configuration of the metal ion in the luminal channel changes with rotation of the c ring, with the distance between the nonpeptide densities changing from 2.2 to 3.7 Å between c-ring positions 1 and 2 (Fig. 5, F to H). The coordination of the metal is sensitive to c-ring position, suggesting that it may play a role in synchronizing c-ring protonation with its rotation. In both positions, distances between the HisN and metal ion are in the 2.0 to 2.2 Å range, consistent with dative bonds and thus with unprotonated HisN.

Available mutational data strongly suggest that the role of cHis$^{248}$ is to protonate aGlu$^{230}$, which would require that aHis$^{248}$ is itself transiently protonated. Protonation of either His or Glu at this position would preclude their ability to coordinate a metal ion. Further work will be needed to clarify the identity, dynamics, and function of this metal ion.

**Ordered water in the aqueous half-channels and at the essential Arg$^{239}$**

The membrane-embedded $a$ subunit and c ring together define the pathway for protons to move across the inner mitochondrial membrane (Fig. 5A). The sequences of both subunits are conserved in all F-type ATP synthases, including those of bacteria and chloroplasts (1). The similarity (identity) scores of human and *Polytomella* mitochondrial ATP synthase are 44.9% (27.6%) for subunit a and 57.0% (38.7%) for subunit c. Previously, we showed (3) that the aqueous half-channels that conduct protons to, and away from, the c-ring glutamate are separated by a distance of 5 to 7 Å in the center of the membrane. We were able to model ordered water molecules within both channels in the current, high-resolution map (Fig. 5, C to E). In the matrix channel, a water molecule is coordinated by cSer$^{112}$ opposite the ion-binding cGlu$^{233}$ of the adjacent c subunit, favoring the idea that the c-ring glutamate may be directly deprotonated by water in this location. Two water molecules are enclosed by the strictly conserved aArg$^{239}$ and aGlu$^{223}$ in a pocket that appears not to be continuous with the aqueous channels (Fig. 5E). The interaction of these conserved residues with each other via a water molecule would reduce the flexibility of the aArg$^{239}$ side chain. In the luminal proton access channel, the well-ordered water lies between the assigned metal ion density and the strictly conserved aArg$^{229}$. Examination of these waters, it is clear that the channel extends to the a/c interface close to aArg$^{239}$, as previously reported (3). However, it would appear that these water molecules do not directly protonate the c-ring glutamate, because mutation of cHis$^{248}$ and cGlu$^{230}$ in *E. coli* eliminates proton permeability of the F$_0$ complex in stripped membranes (37). Instead, the c ring is likely protonated as it rotates past cGlu$^{230}$ (cHis$^{245}$ in *E. coli*), immediately before passing into the hydrophobic lipid bilayer. Water in the luminal channel permeating all the way to aArg$^{239}$ would provide an aqueous environment to prime the c-ring glutamate for protonation by inducing an outward-facing conformation (36). At the same time, the water molecules may participate in coordination of F$_0$ through hydrogen bonds with the metal ion. The coordinates and structure factors have been deposited in the Protein Data Bank (PDB; entry 6rzd).

**Movie 2. Morph of 13 rotary substates of the Polytomella ATP synthase monomer.**

The c ring (yellow) and central stalk (subunits: γ, blue; δ, cyan; ε, pale blue) together rotate in three roughly equal ~120° steps. The F$_1$ head moves with them for the first ~30° of each step. α, dark green; β, bright green. The two-domain OSCP subunit (orange) works as a hinge between the moving F$_1$ head and the stationary peripheral stalk (gray).

**Movie 3. Section through movie 2 at the level of nucleotide-binding sites in the F$_1$ head.**

Catalytic sites of the β subunits (bright green) are red. Binding sites for the structural ATP in the three α subunits (dark green) are orange. The β catch loop is drawn in purple. Central stalk subunit γ, blue; peripheral stalk, gray.
time, the small distance between half-channels would generate a substantial field and resulting torque on the c-ring glutamate, which may be needed to overcome the energetic barrier of moving the c-ring glutamate past the gating Arg230 (1, 3).

C-terminal β extensions contact the adjacent α-subunit
In chlorophycean algae, the mitochondrial catalytic β subunit resembles those of the canonical ATP synthases closely, except that the chlorophycean subunit has acquired a ~60-residue C-terminal extension. Our map shows that this extension wraps vertically around the adjacent α subunit (Movie 5) and moves with subunit β as it adopts an open or closed conformation. A ~15-residue N-terminal extension of the α subunits present in chlorophycean algae, but not in other systems, is in a different configuration for each subunit (Fig. 1B, Fig. 4A, and Movie 5). One of these extensions, together with the distal OSCP domain, anchors one α subunit to the peripheral stalk, while the other two form short helix bundles with the proximal globular domain of OSCP, attaching it to F1. The N- and C-terminal extensions would enhance the stability of the chlorophycean mitochondrial ATP synthase, which may be an advantage under stress conditions, as when *Polytomella* converts to a dormant cyst in nutrient-depleted environments (39).

ATP synthase–associated proteins 1 to 10 form a rigid peripheral stalk
In *Polytomella*, dimer formation is mediated by the peripheral stalk consisting of 10 ASA subunits that have no homologs in other known ATP synthases outside this class of green algae. We have modeled all nine previously described ASA subunits unambiguously into the 2.7 Å map (Fig. 1, Movie 1, and Fig. S4), as well as an unknown subunit that we term ASA10. Mass spectrometry of the purified complex and DNA sequencing of the ~50-Mb *Polytomella* genome allowed us to identify ASA10 (fig. S9) and to obtain complete sequences for subunits c and ASA2 (fig. S10). All subunits were built into the cryo-EM map de novo, as there are no homologous structures in the database.

The stability of the *Polytomella* dimer is due to the rigid peripheral stalks and their close interaction. ASA1 is at the core of the soluble stalk region and forms a bridge between monomers with a helix-turn-helix motif 75 Å above the membrane surface (fig. S11). F0 has six transmembrane helices and four long, membrane-intrinsic helices, which form the two helix hairpins of the conserved α subunit (2). Of the six transmembrane helices, two belong to ASA6 and one each to ASA5, ASA8, ASA9, and ASA10. The two ASA10 subunits form the primary dimer interface on the luminal side of the membrane, and their C termini are intertwined (figs. S9 and S11). Little or no other direct interaction is seen within the membrane, with lipids or detergent filling the space between monomers. ASA3 forms the characteristic Armadillo repeat domain above the membrane surface on the matrix side, and ASA2, ASA4, and ASA7 sit atop the peripheral stalk.

**Outlook**
We have determined the structures of *Polytomella* ATP synthase in 13 rotary substates, allowing us to visualize most if not all of its thermally accessible conformations. Critically, we have shown that the F1 head and rotor move together through a 20° to 30° rotation; thus, the c ring rotates relative to the α subunit while the position of the central stalk within the F1 head is preserved (fig. S12). This movement ensures flexible coupling of the two symmetry-mismatched nanomotors of ATP synthase, F0 and F1, for all possible c-ring stoichiometries. Flexible coupling appears to be mediated predominantly by OSCP, which is emerging as an important target of cellular and pharmacological control. Whereas most previous structural studies of ATP synthase have characterized inhibited complexes, this work has instead examined the active ADP-bound form. The conformational flexibility we observe in these structures may have been suppressed in the previously determined auto-inhibited and inhibitor-bound states. Further studies will address the energetics of OSCP bending and integrate these results into a full catalytic scheme of ATP synthesis. A metal ion bound at the conserved residue αHis340 within the luminal channel is sensitive to the c-ring rotary state and is likely to play an important role in protonating the c-ring carboxylate. A major unanswered question is how the strictly conserved αArg230 facilitates movement of deprotonated, but not protonated, c-ring subunits between the aqueous half-channels. The high-resolution detail provided by our model will be important for answering this question.

**Materials and methods**

**Protein isolation and purification**
ATP synthase from *Polytomella* sp. was purified as described (2) with slight modifications. Mitochondrial membranes (175 mg) were solubilized for 30 min at 4°C in a volume of 12 ml of buffer containing 30 mM Tris-HCl, pH 7.8, 2 mM MgCl2, 50 mM NaCl and 2.9% (w/v) n-dodecyl-β-D-maltoside (DDM) to a final detergent:protein weight ratio of 2:1. After centrifugation (20,000g, 15 min, 4°C), to remove unsolubilized material, the filtered supernatant was loaded onto a POROS GoPure HQ column (Thermo Fisher Scientific) equilibrated in buffer A [30 mM Tris-HCl, pH 7.8, 2 mM MgCl2, 50 mM NaCl and 0.015% (w/v) DDM] on an Äkta purifier (GE Healthcare). After washing the column with 100 mM NaCl in buffer A, ATP synthase dimers were eluted with a linear 100 mM to 300 mM NaCl gradient in buffer A. For the final purification step, fractions containing ATP synthase dimers were concentrated in Vivaspin 500 columns with 100,000 molecular mass cutoff and then loaded onto a Superose6 Increase 3.2/30 size exclusion column (GE Healthcare) equilibrated in buffer B [30 mM Tris-HCl, pH 7.8, 2 mM MgCl2, 40 mM NaCl, 0.05% (w/v) DDM] on an Äkta purifier (GE Healthcare).

**Movie 4. Hinge movement of the two-domain OSCP subunit (orange).** The proximal α-helical OSCP domain at the right is attached to the F1 head by the N-terminal extensions of two of the three α subunits (dark green). The distal β-sheet OSCP domain is attached to the peripheral stalk (gray) by interaction of one OSCP helix and the N-terminal extension of one of the three α subunits.

Fractions of pure and active (2) ATP synthase dimers were collected on ice and used directly for cryo-EM specimen preparation. Purity and activity of the sample was analyzed by blue-native polyacrylamide gel electrophoresis (BN-PAGE) and in-gel ATP hydrolysis assay (40). For two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE) (41), subunits of the ATP synthase dimer were first resolved on a 10% acrylamide/8 M urea gel before being separated in the second dimension in a 16% acrylamide gel.

**Electron microscopy and image processing**
A solution of 3-4 mg/ml purified complex was applied to C-flat 1/1 or 2/1 holey carbon grids (Science Services GmbH) glow-discharged for 45 s at 0.15 mA. Grids were blotted for 4-5 s at blotforce 20 using a Vitrobot, and vitrified in liquid ethane. Electron micrographs were collected on a Titan Krios G2 with Falcon III detector in counting mode, at 300 kV and 75,000× magnification for a calibrated pixel size of 1.053 Å. 81-frame movies were automatically recorded with a dose of 0.4 e− Å⁻² s⁻¹, using EPU software. Movies were aligned using MotionCor2 within the Relion3-beta wrapper and CTF parameters were calculated using CTFind4.1.10. Particles were picked using Gautomatch with templates generated by 2D classification of a previous dataset (3), low-pass filtering images to 20 Å. The dataset was cleaned using 3D classification in Relion3. Following a refinement of the full dimer with solvent masking, per-particle CTF parameters and beam tilt were refined, followed by Bayesian polishing and a second round of CTF and beam tilt refinement. The resulting 3D reconstruction gave an overall resolution for the dimer of 2.94 Å; masking of the stationary membrane-bound and lower peripheral stalk portions of the complex improved the resolution for this region to 2.69 Å. Symmetry expansion of the dataset using relion_particlesymmetry_expand followed by ctf refinement of the upper peripheral stalk gave a resolution of 2.75 Å for this portion of the complex. 3D classification of the pre-aligned symmetry-expanded dataset.
the lower peripheral stalk (EMD-4806). Models of rotary states and substates were aligned using the Matchmaker tool of USCF Chimera (51), fitting to the a subunit. Figures were made using UCSF Chimera and ChimeraX (52).

**Polytomella sp. genomic sequence**

Genomic DNA was purified following a published protocol (53) with modifications. Briefly, *Polytomella* sp. cells were harvested in their logarithmic phase and resuspended in solubilizing buffer containing 100 mM Tris-HCl, pH 8.0, 40 mM EDTA, 100 mM NaCl, 2% (w/v) lauryl sarcosine, 1% (w/v) SDS, RNase A (20 µg/ml), and protein kinase K (0.9 mg/ml). After 1 hour at 4°C, DNA was extracted twice with phenol/chloroform: isooamyl alcohol (25:24:1) and the aqueous phase was ethanol-precipitated for 1 hour on ice. Genomic DNA was further purified by 4 ml LiCl and 7.5 M ammonium acetate before being ethanol-precipitated again and finally washed twice with water. Genomic sequencing was carried out by Mirosynth AG with 50-fold sequence coverage. The genome size was ~50 Mb. A six-frame translation of the sequence was used as a query database for mass spectrometry results.

**Mass spectrometry**

Tryptic digestion of proteins in solution or excised from 2D gels was performed according to published procedures (54). Proteolytic digests were loaded by nano-HPLC (Dionex RSLChano) on reverse-phase columns (trapping column: Acclaim PepMap c18, particle size 2 µm, L = 20 mm; analytical column: Acclaim PepMap c18, particle size 2 µm, L = 50 cm; Thermo Fisher Scientific) and eluted in organic phase gradients (Buffer A: 95% H2O, 5% DMSO, 0.1% formic acid; Buffer B: 80% acetonitrile, 15% H2O, 0.1% formic acid). Typically, gradients were ramped from 4% to 48% buffer B in 80 min at a flow rate of 300 nl/min. Peptides eluting from the column were ionized online using a Nanospray Flex Ion source and analyzed in an Orbitrap Elite or Q Exactive Plus mass spectrometer. Mass spectra were acquired over the 350 to 1600 m/z range at a resolution of 120,000, and sequence information was acquired by computer-controlled, data-dependent automated switching to MS/MS mode in response to charge collision energies based on mass and charge state of the candidate ions.

Datasets were processed with the Proteome Discoverer software package (version 2.1.0.81). Spectra were internally recalibrated on auto-proteolytic trypsin fragments when applicable. Proteins were identified by matching the derived mass lists against a customized *Polytomella* dbase (combination of NCBI nr *Polytomella*, a full six-frame translation of a dataset acquired by whole-genome shotgun sequencing (Mirosynth AG) and a list of common contaminants with the program Sequest (Thermo Fisher Scientific). In general, a mass tolerance of 10 ppm for parent ion spectra and 0.6 Da for fragment ion spectra, two missed cleavages, oxidation of Met (dynamic modification), acetylation of the protein N terminus (dynamic modification) and carbamidomethyl-cysteine (fixed modification) were selected as matching parameters in the search program. Results were evaluated using a percolator node (high-confidence q value, FDR < 0.01) to exclude false positives. Proteome data have been uploaded to the PRIDE online repository (55).

**Protein sequence analysis**

Homologs of the ASA10 sequence were found by BLAST searches of the NCBI (56) and Phytomzome (57) databases. Sequence alignments were carried out using Clustal Omega (58) and formatted using JalView (59).

**REFERENCES AND NOTES**

1. W. Kühlbrandt, Structure and mechanisms of F-type ATP synthases. Annu. Rev. Biochem. 10.1146/annurev-biochem-011319-110903 (2019). doi: 10.1146/annurev-biochem-011319-110903; pmid: 30901262
2. M. Allegrilli et al., Horizontal membrane-intrinsic α-helices in the stator α-subunit of an F-type ATP synthase. Nature 521, 237–240 (2015). doi: 10.1038/nature14185; pmid: 25770805
3. N. Vischi, B. J. Murphy, D. J. Mills, T. Meier, W. Kühlbrandt. Structural basis of proton translocation and force generation in mitochondrial ATP synthase. elife 6, e33274 (2017). 10.10010/p.094060; pmid: 17486286
4. M. Vázquez-Acevedo et al., The mitochondrial ATP synthase of chlorophycean algae contains eight subunits of unknown origin involved in the formation of an atypical stator-stalk and in the dimensioning of the complex. J. Bioenerg. Biomembr. 38, 271–282 (2006). doi: 10.1007/b85-600-9046-7; pmid: 17804646
5. R. van Lies, G. Mendoza-Hernández, G. Groth, A. Attia. New insights into the unique structure of the Fγ3-F1 ATP synthase from the chlorophycean algae *Polytomella* sp. and *Chlamydomonas reinhardtii*. Plant Physiol. 144, 1109–1109 (2007). doi: 10.1093/tp/1046060; pmid: 17486226
6. D. A. Chepepanov, A. Y. Multikudinjan, W. Junge. Transient accumulation of elastic energy in proton translocating ATP synthase. FEBS Lett. 449, 1–6 (1999). doi: 10.1016/S0014-5793(99)00386-5; pmid: 10225416
7. N. Soga, K. Kimura, K. Kinosita Jr., M. Yoshida, T. Suzuki. Perfect chemomechanical coupling of Fγ3-F1 ATP synthase. Proc. Natl. Acad. Sci. USA. 110, 4960–4965 (2017). doi: 10.1073/pnas.1700811110; pmid: 28442597
8. A. Wächter et al., Two rotary motors in F-ATP synthase are elastically coupled by a flexible rotor and a stator stalk. Proc. Natl. Acad. Sci. U.S.A. 110, 3924–3929 (2011). doi: 10.1073/pnas.1015118108; pmid: 21368147
9. H. Seifert et al., Domain compliance and elastic power transmission in rotary F1-F0 ATPase. Proc. Natl. Acad. Sci. U.S.A. 105, 17765–17769 (2008). doi: 10.1073/pnas.0807638105; pmid: 19001725
10. J. L. Martin, R. Ishmukhametov, D. Spetzler, T. Hornung, W. D. Frauch. Elastic coupling power stroke mechanism of the F1-ATPase molecular motor. Proc. Natl. Acad. Sci. U.S.A. 115, 5750–5755 (2018). doi: 10.1073/pnas.1803471115; pmid: 29760063
11. A. Zhou et al., Structure and conformational states of the bovine mitochondrial ATP synthase by cryo-EM. elife 8, e40180 (2019). doi: 10.10010/p.094060; pmid: 17486226
12. M. Sobot et al., Cryo-EM structures of the autoinhibited E. coli ATP synthase in three rotational states. elife 5, e32598 (2016). doi: 10.10010/p.094060; pmid: 17486226
13. A. Hahn, J. Vonck, D. J. Mills, T. Meier, W. Kühlbrandt. Structure, mechanism, and regulation of the chloroplast ATP synthase. Science 360, eaat4318 (2018). doi: 10.1126/science.aat4318; pmid: 29748286
14. M. Sobot et al., Cryo-EM reveals distinct conformations of E. coli ATP synthase on exposure to ATP. elife 8, e43864 (2019). doi: 10.10010/p.094060; pmid: 17486226
15. Y. Komoriya et al., Principal role of the arginine finger in rotary catalysis of F1-ATPase. J. Biol. Chem. 287, 15134–15142 (2012). doi: 10.1073/pj.M111.281259; pmid: 22403407
16. M. D. Greene, W. D. Frauch. Interactions among γ268, γ269, and the β subunit catch loop of Escherichia coli F1-ATPase are important for catalytic activity. J. Biol. Chem. 278, 51254–51258 (2003). doi: 10.1073/pj.M30948250; pmid: 14532272
17. K. R. Vinodkumar, M. G. Montgomery, S. Liu, J. E. Walker, Structure of the mitochondrial ATP synthase from Pichia angusta determined by electron cryo-microscopy. Proc. Natl. Acad. Sci. U.S.A. 113, 12079–12084 (2016). doi: 10.1073/pnas.1615902113; pmid: 2779192

18. D. M. Rees, A. G. W. Leslie, J. E. Walker, The structure of the membrane extrinsic region of bovine ATP synthase. Proc. Natl. Acad. Sci. U.S.A. 106, 21597–21601 (2009). doi: 10.1073/pnas.0910365106; pmid: 19995987

19. V. Giorgio, F. Fogolari, G. Lippe, P. Bernardi, OSCP subunit of mitochondrial ATP synthase: Role in regulation of enzyme function and of its transition to a pore. Br. J. Pharmacol. 161,111–1141 (2018). doi: 10.1111/bph.14513 (2018). doi: 10.1111/bph.14513. pmid: 30291799

20. H. Sielaff, S. Yanagisawa, W. D. Frasch, W. Junge, M. Börsch, .... (2017). doi: 10.1074/jbc.M117.799940; pmid: 28961971

21. K. Okazaki, G. Hummer, Elasticity, friction, and pathway of γ-subunit rotation in F$_{1}$F$_{0}$-ATP synthase. Proc. Natl. Acad. Sci. U.S.A. 112, 10770–10775 (2015). doi: 10.1073/pnas.1500691112; pmid: 26261344

22. S. Yanagisawa, W. D. Frasch, Pronation-dependent stepped rotation of the F$_{5}$-type ATP synthase γ-ring observed by single-molecule measurements. J. Biol. Chem. 292, 17093–17100 (2017). doi: 10.1074/jbc.M117.799940; pmid: 28842481

23. R. Ishikhametov, T. Horning, D. Spetzler, W. D. Frasch, Direct observation of stepped proteolipid ring rotation in E. coli F$_{1}$F$_{0}$-ATP synthase. EMBO J. 29, 3901–3913 (2010). doi: 10.1002/embo.2010259; pmid: 20755735

24. P. Balbasakaran Nina et al., ATP synthase complex of Plasmodesmata talaricum: Dimeric assembly in mitochondrial membranes and resistance to genetic disruption. J. Biol. Chem. 286, 41312–41312 (2011). doi: 10.1074/jbc.M111290777; pmid: 21984828

25. E. Perez et al., The mitochondrial respiratory chain of the secondary green alga Euglena gracilis shares many additional subunits with parasitic Trypanosomatidae. Mitochondrion 18, 338–339 (2014). doi: 10.3839/tphys.2018.00903; pmid: 30022951

26. I. Starke, G. Glick, M. Börsch, Visualizing Mitochondrial F$_{1}$F$_{0}$-ATP Synthase as the Target of the Immunomodulatory Drug Bz-423. Front. Physiol. 9, 803 (2018). doi: 10.3389/fphys.2018.00903; pmid: 30022951

27. K. M. Johnson et al., Identification and validation of the mitochondrial F$_{1}$F$_{0}$-ATPase as the molecular target of the immunomodulatory benzodiazepine Bz-423. Chem. Biol. 12, 485–496 (2005). doi: 10.1016/j.chembiol.2005.02.012; pmid: 15850986

28. V. Giorgio et al., Cyclophilin D modulates mitochondrial F$_{1}$F$_{0}$-ATPase by interacting with the lateral stalk of the complex. J. Biol. Chem. 284, 33982–33988 (2009). doi: 10.1074/jbc.M1090210151; pmid: 19801635

29. A. Vassilopoulou et al., SIRT3 deacetylases ATP synthase α complex proteins in response to nutrient- and exercise-induced stress. Antioxid. Redox Signal. 21, 551–564 (2014). doi: 10.1089/ars.2013.5420; pmid: 24252900

30. J. Zheng, V. D. Ramirez, Purification and identification of an estrogen binding protein from rat brain: Oligomycin sensitivity-conferring protein (OSCp), a subunit of mitochondrial F$_{1}$F$_{0}$-ATP synthase/ATPase. J. Steroid Biochem. Mol. Biol. 68, 65–75 (1999). doi: 10.1016/S0960-766X(98)01067-7; pmid: 10215039

31. J. Zheng, V. D. Ramirez, Rapid inhibition of rat brain mitochondrial proton F$_{1}$F$_{0}$-ATPase activity by estradiol: Comparison with Na$^{+}$K$^{+}$-ATPase of porcine cortex. Eur. J. Pharmacol. 368, 95–102 (1999). doi: 10.1016/S0014-2999(99)00126-2; pmid: 10096774

32. F. Messeri, S. Paolini, E. Piscitelli, M. L. Brandi, G. Solari, Dose-dependent inhibition of mitochondrial ATP synthase by 17P-estradiol. Gynecol. Endocrinol. 16, 373–377 (2000). doi: 10.1080/1479641881440527; pmid: 11759731

33. J. W. Simpleks, K. D. Yi, S.-H. Yang, J. A. Dykens, Mitochondrial mechanisms of estrogen neuroprotection. Biochim. Biophys. Acta 1800, 1113–1120 (2010). doi: 10.1016/...