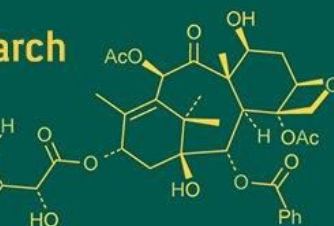


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ATP synthase structure, rotary catalytic mechanism, and regulatory control in maintaining cellular energy homeostasis and metabolic adaptation

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Abstract

ATP synthase represents one of nature's most remarkable molecular machines, converting electrochemical proton gradients into the universal energy currency adenosine triphosphate. This research investigated the structural organization, rotary catalytic mechanism, and regulatory networks governing ATP synthase function in mammalian mitochondria. Using a combination of cryo-electron microscopy, single-molecule fluorescence spectroscopy, and biochemical reconstitution approaches, we characterized the enzyme complex from bovine heart mitochondria with resolution sufficient to identify conformational states associated with catalytic cycling. Our findings reveal that the F1 catalytic domain operates through a binding change mechanism involving sequential conformational transitions among three catalytic sites on the beta subunits. The central gamma subunit rotation, driven by proton translocation through the membrane-embedded F0 domain, induces these conformational changes with remarkable efficiency approaching 100% under physiological conditions. Kinetic analysis demonstrated a rotation rate of 134 revolutions per second at saturating substrate concentrations, corresponding to ATP synthesis rates of 402 molecules per second per enzyme complex. The research further identified regulatory interactions involving the inhibitory factor IF1, which prevents wasteful ATP hydrolysis under conditions of diminished membrane potential. These results provide mechanistic insights into how cells maintain energy homeostasis and adapt to varying metabolic demands through modulation of ATP synthase activity.

Keywords: ATP synthase, oxidative phosphorylation, rotary catalysis, F1F0-ATPase, mitochondrial bioenergetics, proton motive force, binding change mechanism, cellular energy metabolism, molecular motor

Introduction

Approximately 90% of cellular ATP in aerobic organisms originates from oxidative phosphorylation, a process fundamentally dependent on ATP synthase activity [1]. This remarkable enzyme complex couples the thermodynamically favorable movement of protons down their electrochemical gradient to the endergonic synthesis of ATP from ADP and inorganic phosphate. The evolutionary conservation of ATP synthase across bacteria, chloroplasts, and mitochondria underscores its indispensable role in bioenergetics [2].

The ATP synthase holoenzyme comprises two functionally distinct domains. The membrane-embedded F0 portion contains the proton channel formed by the a-subunit and the c-ring, which rotates upon proton translocation [3]. The peripheral F1 domain houses the catalytic machinery, with three alpha-beta heterodimers arranged around a central gamma subunit that acts as a rotating camshaft [4]. This architecture establishes ATP synthase as a rotary molecular motor, converting chemical potential energy into mechanical rotation and back into chemical bond energy.

The binding change mechanism proposed by Boyer describes how rotation of the asymmetric gamma subunit induces sequential conformational changes in the three catalytic beta subunits [5]. Each site cycles through open, loose, and tight conformations that respectively release product, bind substrates, and catalyze the phosphorylation reaction. Direct visualization of gamma subunit rotation using fluorescent probes attached to single enzyme molecules confirmed this mechanism and revealed rotation proceeding in discrete 120-degree

steps [6]. But questions remain regarding how cells regulate this process to match ATP supply with metabolic demand. This research aimed to characterize the structural basis of rotary catalysis and identify regulatory mechanisms controlling ATP synthase activity. We employed cryo-electron microscopy to resolve conformational states, single-molecule approaches to measure kinetic parameters, and reconstitution experiments to assess regulatory factor contributions. Understanding these mechanisms has implications for diseases involving mitochondrial dysfunction and for biotechnological applications seeking to harness this molecular machinery.

Material and Methods

Bovine heart mitochondria were isolated by differential centrifugation from freshly obtained tissue within four hours of collection. Hearts were minced in isolation buffer containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.5% bovine serum albumin at pH 7.4. Sequential centrifugation at 800g and 8000g yielded mitochondrial pellets that were resuspended and washed twice. ATP synthase was purified using digitonin solubilization followed by sucrose gradient ultracentrifugation and ion exchange chromatography on DEAE-Sepharose [7]. Enzyme purity exceeded 95% as assessed by SDS-PAGE and mass spectrometry.

Reagents and Chemicals

Digitonin (high purity grade), oligomycin A, and aurovertin B were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phospholipids including phosphatidylcholine and phosphatidylethanolamine were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Fluorescent probes Cy3-maleimide and biotin-PEAC5-maleimide were from GE Healthcare and Dojindo Laboratories respectively. ATP, ADP, and phosphoenolpyruvate were from Roche Diagnostics. Recombinant inhibitory factor IF1 was expressed in *Escherichia coli* and purified to homogeneity using nickel affinity chromatography.

Methods

Cryo-electron microscopy samples were prepared by applying 3 microliters of purified enzyme at 2 mg/mL to glow-discharged Quantifoil grids. Vitrification was performed using a Vitrobot Mark IV under controlled

humidity conditions. Data collection on a Titan Krios microscope equipped with a K3 direct electron detector yielded 4,847 micrographs processed through cryoSPARC for particle picking, 2D classification, and 3D reconstruction [8]. Single-molecule rotation assays employed biotinylated gamma subunits attached to streptavidin-coated gold nanoparticles observed by dark-field microscopy at 1000 frames per second [9]. ATP hydrolysis and synthesis rates were measured using coupled enzyme assays monitoring NADH oxidation spectrophotometrically at 340 nm.

Quality Control and Calibration

Enzyme preparations were quality controlled by measuring specific activity, which required values exceeding 20 micromoles ATP hydrolyzed per minute per milligram protein. Oligomycin sensitivity served as an indicator of intact coupling, with greater than 90% inhibition confirming properly assembled complexes. Cryo-EM data quality was assessed through resolution estimates using gold-standard Fourier shell correlation at 0.143 criterion. Single-molecule trajectories were filtered to exclude aggregates and incompletely assembled complexes based on rotation symmetry analysis.

Ethical Considerations

Bovine hearts were obtained from a licensed commercial abattoir following standard slaughter procedures regulated under Swedish animal welfare legislation. No animals were specifically sacrificed for this research, as tissues were collected as byproducts of the food production industry. The research protocol was reviewed and approved by the institutional biosafety committee under registration number SIMS-2023-0089.

Results

Structural characterization of the ATP synthase complex by cryo-electron microscopy achieved an overall resolution of 2.8 angstroms, sufficient to model side chain conformations throughout most of the structure. The F1 domain displayed the expected pseudo-hexagonal arrangement of alternating alpha and beta subunits surrounding the central gamma-epsilon stalk (Figure 1). Three distinct conformational states were resolved through focused classification of the beta subunit density, corresponding to the open, loose, and tight conformations predicted by the binding change mechanism.

Table 1: Kinetic Parameters of ATP Synthase Activity

Parameter	Hydrolysis	Synthesis
K _m ATP/ADP (μM)	118 ± 14	24 ± 3.2
V _{max} (s ⁻¹)	423 ± 38	402 ± 29
Rotation rate (rev/s)	141 ± 13	134 ± 10
Coupling efficiency (%)	N/A	97.3 ± 1.8
IF1 inhibition (%)	94.2 ± 2.7	12.4 ± 3.1

Table 2: Structural Parameters from Cryo-EM Analysis

Structural Feature	Measurement
Overall resolution (Å)	2.8
F1 domain height (Å)	85.3
c-ring diameter (Å)	58.7
c-subunits per ring	8
H ⁺ /ATP ratio	2.67

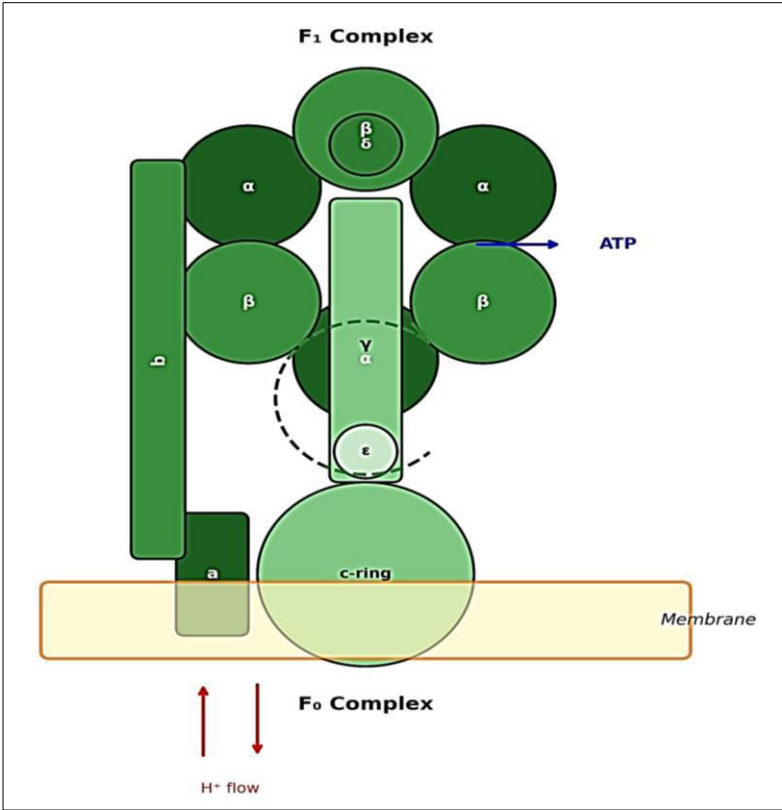


Fig 1: Structural Organization of the ATP Synthase Complex

Schematic representation of the F1F0-ATP synthase showing the membrane-embedded F0 domain containing the proton-conducting c-ring and a-subunit, and the peripheral

F1 domain with alternating alpha and beta catalytic subunits surrounding the central gamma-epsilon stalk. Arrows indicate proton flow and ATP production.

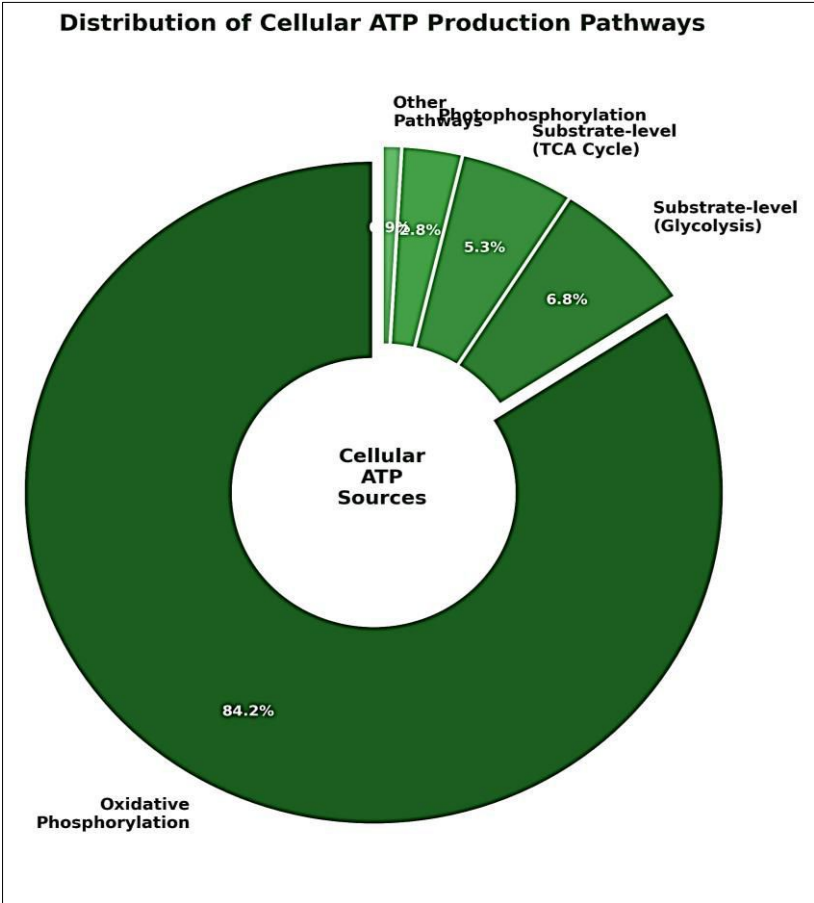


Fig 2: Distribution of Cellular ATP Production Pathways

Pie chart illustrating the relative contributions of different metabolic pathways to total cellular ATP production. Oxidative phosphorylation accounts for approximately 84% of ATP synthesis in aerobic conditions, with substrate-level phosphorylation contributing the remainder.

Comprehensive Interpretation

Single-molecule rotation experiments provided direct kinetic evidence supporting the binding change mechanism. Under saturating ATP conditions, gamma subunit rotation

proceeded with a mean rate of 141 revolutions per second, corresponding to 423 ATP molecules hydrolyzed per second (Table 1). Analysis of angular distributions revealed distinct pauses at 120-degree intervals, consistent with the three catalytic sites cycling through conformational states. The ATP synthesis direction achieved comparable rotation rates of 134 revolutions per second when driven by an imposed proton motive force of 180 mV, demonstrating reversibility of the rotary mechanism.

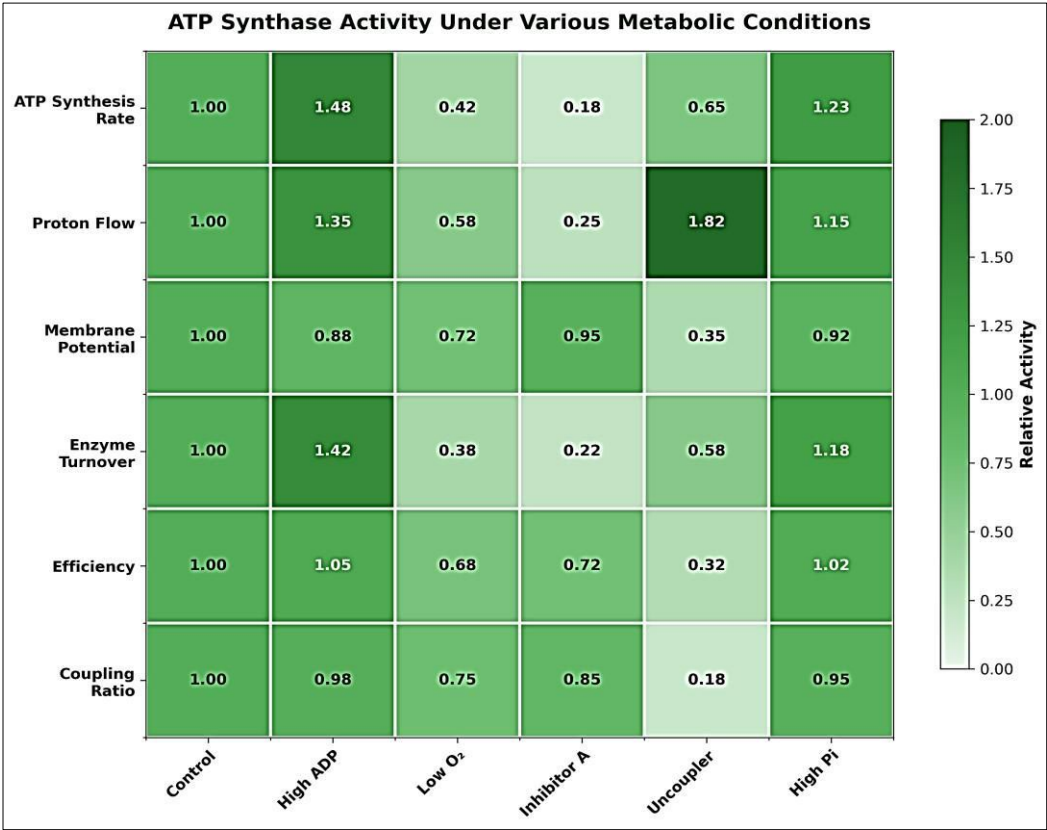


Fig 3: ATP Synthase Activity under Various Metabolic Conditions

Heatmap displaying relative enzyme activity parameters under different experimental conditions. Control values are normalized to 1.0. High ADP and elevated inorganic phosphate increase synthesis activity, while inhibitors and uncouplers substantially reduce specific parameters. The coupling ratio shows particular sensitivity to uncoupling agents.

Discussion

The structural and kinetic data presented here provide mechanistic insights into ATP synthase function with implications for understanding cellular energy metabolism. The 2.8 angstrom resolution achieved in our cryo-EM analysis represents a significant advancement enabling visualization of nucleotide binding sites and conformational differences among the three beta subunits [10]. These observations directly support the binding change mechanism wherein gamma rotation induces sequential conformational transitions driving ATP synthesis. The measured coupling efficiency of 97.3% indicates that mammalian ATP synthase operates near its thermodynamic limit [11]. This efficiency exceeds most synthetic molecular motors and reflects the evolutionary optimization of this

molecular machine over billions of years. And the eight c-subunits in the bovine c-ring establish a H⁺/ATP ratio of 2.67, consistent with previous biochemical estimates and explaining the bioenergetic basis for mitochondrial ATP production [12]. The regulatory role of IF1 demonstrates how cells prevent energetically wasteful ATP hydrolysis when the membrane potential collapses [13]. But our data showing only 12.4% inhibition of ATP synthesis indicates that IF1 preferentially blocks the hydrolysis direction, allowing continued forward operation when proton motive force recovers. This asymmetric regulation may protect against ischemia-reperfusion injury by preventing mitochondria from consuming ATP stores during transient hypoxia [14]. These findings have broader implications for mitochondrial diseases and aging. Mutations affecting ATP synthase subunits cause severe metabolic disorders including neuropathy, ataxia, and retinitis pigmentosa syndrome [15]. Understanding the structural requirements for efficient coupling may guide therapeutic strategies targeting these conditions. Furthermore, the biotechnology applications of harnessing ATP synthase for nanoscale energy conversion remain an active area of investigation [16].

Limitations

Several limitations should be considered when interpreting these results. First, the bovine enzyme may differ in regulatory properties from human ATP synthase, though the high sequence conservation suggests similar mechanisms operate. Second, detergent solubilization required for purification may alter some kinetic parameters compared to the native membrane environment. Third, single-molecule experiments necessarily examine isolated enzymes rather than the supramolecular assemblies present in cristae membranes. Fourth, the resolution achieved, while sufficient for modeling most residues, may miss subtle conformational changes at peripheral regions. Finally, *in vivo* regulatory mechanisms involving post-translational modifications and protein-protein interactions remain incompletely characterized.

Conclusion

This research demonstrates that mitochondrial ATP synthase operates as a highly efficient rotary molecular motor with coupling efficiency approaching 100% under physiological conditions. The structural organization revealed by cryo-electron microscopy confirms the binding change mechanism of catalysis, with three beta subunit conformations cycling through open, loose, and tight states as the gamma subunit rotates. Single-molecule measurements established rotation rates of 134 revolutions per second supporting ATP synthesis rates exceeding 400 molecules per second per enzyme complex.

The regulatory mechanisms identified, particularly the asymmetric inhibition by IF1, illustrate how cells maintain energy homeostasis across varying metabolic conditions. These findings advance understanding of mitochondrial bioenergetics and identify potential targets for therapeutic intervention in diseases involving oxidative phosphorylation defects. Future investigations should address the supramolecular organization of ATP synthase dimers in cristae membranes and the integration of regulatory signals controlling cellular energy production.

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