Essentials for ATP Synthesis by F₁F₀ ATP Synthases

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Abstract

The majority of cellular energy in the form of adenosine triphosphate (ATP) is synthesized by the ubiquitous F₁F₀ ATP synthase. Power for ATP synthesis derives from an electrochemical proton (or Na⁺) gradient, which drives rotation of membranous F₀ motor components. Efficient rotation not only requires a significant driving force (ΔμH⁺), consisting of membrane potential (Δψ) and proton concentration gradient (ΔpH), but also a high proton concentration at the source P side. In vivo this is maintained by dynamic proton movements across and along the surface of the membrane. The torque-generating unit consists of the interface of the rotating c ring and the stator a subunit. Ion translocation through this unit involves a sophisticated interplay between the c-ring binding sites, the stator arginine, and the coupling ions on both sides of the membrane. c-ring rotation is transmitted to the eccentric shaft γ-subunit to elicit conformational changes in the catalytic sites of F₁, leading to ATP synthesis.

Key Words

ATP synthase assembly, coupling ion coordination, driving forces, torque generation
INTRODUCTION

To support life, cells must be continuously supplied with external energy in the form of light or nutrients and must be equipped with chemical devices to convert these external energy sources into adenosine triphosphate (ATP). ATP is the universal energy currency of living cells and as such is used to drive numerous energy-consuming reactions, e.g., biosyntheses, mechanical motility, transport through membranes, regulatory networks, and nerve conduction. When performing work, ATP is usually converted to ADP and phosphate. It must therefore continuously be regenerated from these compounds to continue the cell energy cycle. The importance of this cycle can be best illustrated by the demand for a daily turnover of 50 kg of ATP in a human body on average. The majority of this ATP is synthesized by the ubiquitous F1F0 ATP synthase with energy that has been supplied by the external sources mentioned above.

ATP Synthase Structure and Function

The F1F0 ATP synthase is a miniature engine composed of two opposing rotary motors, which are connected in series as described separately below (Figure 2). The F1 motor is a water-soluble protein complex with the subunit composition α3β3γδε that catalyses ATP synthesis or hydrolysis by a rotary mechanism.
Figure 1

Ion cycling across biological membranes leading to ATP synthesis. In heterotrophic organisms, e.g., animals or bacteria, the metabolism of nutrients generates reducing equivalents (NADH, succinate). These are oxidized by the respiratory-chain enzymes, using oxygen as terminal electron acceptor. The free energy of the oxidation reactions is converted into an electrochemical gradient of protons (ΔμH+) across the membrane. Chloroplasts and phototrophic bacteria convert light energy into a ΔμH+ across the membrane. The anaerobic bacterium P. modestum couples the decarboxylation of methylmalonyl-CoA to electrogenic Na+ transport, which results in an electrochemical sodium gradient. The electrochemical H+ or Na+ gradients established by these membrane-bound complexes serve as energy sources for ATP synthesis from ADP and inorganic phosphate by an F1F0 ATP synthase. Abbreviations: N side, the negatively charged side of a membrane; P side, the positively charged side of a membrane.

The F0 motor is membrane embedded and in its simplest bacterial form has the subunit composition ab2c10−15. It catalyses ion translocation across the membrane that is coupled to rotation of the c ring versus the stator subunits a and b1. The two motors are physically connected by two stalks, a central one containing the γ- and ε-subunits and a peripheral one involving the δ- and b-subunits. Mammals contain additional subunits, which are mostly in the stalk region. Under mild dissociation conditions, the F1F0 complex disintegrates into the F1 and F0 complexes, which can be isolated and studied individually.

The crystal structure of F1 from bovine heart mitochondria revealed a hexameric assembly of alternating α- and β-subunits around an eccentric α-helical coiled-coil γ-subunit (4). The catalytic nucleotide-binding sites are located on each of the three β-subunits at the interface with the α-subunits. As these sites are in different nucleotide-bound states during catalysis, each β-subunit adopts a different conformation, causing a diverse interaction with the central γ-subunit. On rotation of the γ-subunit, the conformations of the β-subunits change sequentially such that each conformation of varying nucleotide-binding affinity is adopted by each site during one rotational cycle. Hence, the rotational model can explain the binding change mechanism that was predicted from numerous biochemical studies.
Figure 2
The structural organization of an F₁F₀ ATP synthase. The structures of individual subcomplexes were taken from RCSB Protein Data Bank (http://www.rcsb.org/pdb/static.do?p=general_information/web_links/databases.html) and assembled by eye according to biochemical data. The structures used were from the c ring of *Ilyobacter tartaricus* (1CYE), the F₁ organization of *Escherichia coli* (1JNV), the δ-subunit of *E. coli* (2A7U), the peripheral stalk from bovine mitochondria (2CLY), and the membranous part of subunit b of *E. coli* (1B9U). No high-resolution structural data are available for subunit a and the hinge region of subunit b. The illustration was created using PyMOL (DeLano Scientific LLC).

These studies are consistent with several cross-linking experiments, which have been important to define subunits γ, ε, and c₁₀₋₁₅ as the rotor and subunits α₃, β₁, δ, a, and b₂ as the stator (8–11). A physical connection between the c₁₀ ring and the γ- and ε-subunits is similarly seen in the electron density map of an F₁ complex from yeast with an attached c₁₀ ring (12).

Single-molecule experiments with small magnetic or gold beads or with fluorescent dyes instead of the large actin filament provided insights into mechanistic details of the enzyme. Rotation in the fully coupled F₁F₀ Na⁺ ATP synthase was observed in both synthesis and hydrolysis mode (13). The rotor turns counterclockwise in the ATP hydrolysis direction and clockwise in the ATP synthesis direction, when viewed from the F₀ domain, and performs up to 700 revolutions per second (14, 15). In F₁, the γ-subunit rotates in steps of 120° for each ATP molecule hydrolyzed (Figure 3). Each 120° step can be further divided into four stages (Reference 16 and references therein). In the ATP-binding dwell, an ATP molecule binds to the empty β₁-site and elicits a rapid 80° substep of the γ-subunit rotation. In the following catalytic dwell, ATP is thought to be cleaved at the β₂-site, and ADP and phosphate are released from the β₃-site (17). This initiates a 40° substep of the γ-subunit, completing its 120° rotation. The rotational behavior of F₁F₀ resembles that of F₁, indicating that friction in the F₀ motor is negligible during ATP-driven rotation (18). Tributyltin chloride, a specific inhibitor of ion access through subunit a, inhibits rotation by 96% in accordance with strict coupling between mechanical and ion translocation events (15). When the performance of the F₁ motor was probed by sophisticated single-molecule experiments in femtoliter-sized chambers, the hydrolysis of three ATP molecules per revolution was directly observed and showed very high mechanochemical coupling (19). If the γ-subunit of F₁ was forced to rotate in the ATP synthesis direction, ATP was synthesized from ADP and phosphate. The mechanochemical coupling efficiency was low.
Figure 3

Model of the binding change mechanism during ATP hydrolysis (adapted from Reference 99). The F₁ part is seen from the membrane and is divided in β₁, β₂, and β₃, representing the α/β interface of each catalytic unit. The pink unit is thought to generate the power stroke responsible for γ-rotation, and the resting units are shown in blue. The rotating, central γ-subunit is shown in orange. In states I and V, when F₁ is waiting for ATP, it is assumed that the catalytic β₁, β₂, and β₃ sites are empty or contain ATP and ADP/Pi, respectively. The angular position of subunit γ in state I is set to be 0°. (I–II) ATP-binding dwell: ATP is bound to the empty catalytic β₁ site. (II–III) A 80° rotation: ATP binding initiates rotation of γ by 80°, accompanied by ADP release from β₃. (III–IV) ATP hydrolysis/Pi-release dwell: ATP is cleaved into ADP and Pi at β₂, and Pi is released from β₃. The sequence of events is not yet determined. (IV–V) A 40° rotation: The subsequent rotation completes one 120° step, yielding an empty β₃ site, which is ready for ATP binding (similar to β₁ in I). The catalytic turnover of a particular ATP molecule needs a 360° rotation of the γ-subunit, with ATP binding to β₁ at 0°, ATP cleavage at 200°, ADP-release at 240°–320°, and Pi release at 320°. Also shown is the azide-inhibited structure of bovine F₁ as seen from the membrane, with AMP-PNP (cyan), ADP (magenta), and Pi (green) in the respective catalytic-binding sites, which somewhat represent state III (100).

for an F₁ subcomplex lacking the ε-subunit but reached more than 70% after reconstitution with ε (18). The ε-subunit thus has an important role in the synthesis of ATP, but the mechanism for this function has not yet been elucidated.

THE F₀ MOTOR

The bacterial F₀ motor is a membrane-embedded protein complex consisting of an oligomeric ring of c subunits, a single a subunit, and a dimer of b subunits, which flank the c ring laterally. Each c subunit consists...
of two membrane-spanning α-helices that are connected by a hydrophilic loop at the cytoplasmic side of the membrane. In the c ring, the loop regions form an extensive area of contact with the foot of the central stalk subunits γ and ε (12). The α subunit is a very hydrophobic protein that in most models is composed of five transmembrane helices (20, 21). Ion translocation takes place through subunit a and its interface with subunit c. Torque generated during this process is conferred to the γ-subunit to drive ATP synthesis in F1. The b subunits are anchored within the membrane by an N-terminal α-helix and extend as the peripheral stalk all the way to the head of the F1 domain (22). According to cross-linking studies, the b subunits contact the C-terminal part of the c subunit and the loop between helices 4 and 5 of the α subunit at the periplasmic surface. In the solvent-exposed part of the b subunit, the tether, dimerization, and δ-subunit interaction domains have been defined (23). The δ-subunit forms a strong complex with the latter domain and the α-subunit (24, 25). The connection of F1 and F0 by the peripheral stalk is an important device to counter the tendency of the stator to follow the rotation of the rotor. In mitochondria, the peripheral stalk consists of more subunits (named OSCP, b, δ, and F0), and its high-resolution X-ray structure indicates that it forms a rigid complex in accord with the suggested function to counteract the torque created through the rotor (26).

Assembly of the Rotor Complex

To date, there is only little information on the assembly of the ATP synthase or the subcomplexes of this enzyme (27). It is remarkable, however, that a typical bacterial ATP synthase consists of eight different subunits, whereas nine open reading frames are present in the respective ATP (or unc) operon with the uncI gene preceding the structural genes for the eight subunits of the ATP synthase. The uncI gene of *Escherichia coli* encodes a membrane protein of 14 kDa that is not a component of the ATP synthase. The protein copurifies with the enzyme in stoichiometric amounts and might thus have a functional role in the assembly of the enzyme. Disruption of the uncI gene leads to diminished growth yields without significantly affecting the function of the ATP synthase and thus could not provide conclusive evidence for uncI gene function (28, 29). More recently, studies with the UncI protein from *P. modestum* showed that it plays a chaperone-like role in the assembly of the c11 ring of its ATP synthase (30). When expressed in *E. coli*, UncI of *P. modestum* was required for the formation of the fully assembled c11 rings, and in its absence, neither c11 nor c1 protein products are observed, indicating degradation of the c subunit under the experimental conditions employed. With another expression system, containing plasmids with the corresponding c subunit genes under control of the strong T7 promotor, however, small amounts of correctly assembled c11 rings from *P. modestum* or *Ilyobacter tartaricus* were synthesized in *E. coli* (31). As uncI from the respective organism was not present in these experiments, either the UncI protein from *E. coli* or another protein must have taken the role to direct the correct assembly of these c rings. These results are not unexpected given the formation of catalytically active *E. coli* ATP synthase in the uncI deletion background and argue for an overlapping function of another protein with UncI in promoting the assembly of the c ring. Indeed, YidC has been shown to assist in the formation of the c ring from monomers (32). The chaperone-like function of UncI of *P. modestum* was corroborated by an in vitro system, which showed the oligomerization of c1 into c11 upon UncI addition (33).

The interfaces of the rotor subunits can be inferred from the medium-resolution crystal structure of the F1–c10–ring complex from yeast (12). In this structure, the cytoplasmic loop of the c ring forms the contact site with the foot of the γ- and ε-subunits. In the in vitro assembly of the rotor complex has been demonstrated with the isolated c11 ring and a preformed complex of subunits γ and ε from *I. tartaricus* (34). Using surface plasmon resonance
spectroscopy, association and dissociation kinetics were recorded. The data yielded a $K_D$ of about 7 nM, indicating the formation of a very stable complex. The value is similar to the binding affinity determined for the $ab_2F_1$ stator complex of *E. coli* (35). The high intrinsic stability of the rotor and the stator complexes appears to be necessary to maintain the enzyme's integrity under operation conditions. Structurally, the assembly of the rotor is based primarily on attachment of the $\gamma$-subunit to the c ring because a separate complex between the c ring and the $\epsilon$-subunit is not observed. The assembly is severely affected by mutagenesis of conserved amino acids in the contact region of all three subunits (34). Interaction kinetics between the $\gamma\epsilon$-complex and the c-ring mutants suggest that at least two steps are involved in the assembly of the complex. Following low-affinity binding between the polar loops of the c ring and the bottom part of the $\gamma$-subunit, the complex is stabilized by a high-affinity interaction involving the c ring and residues $\gamma$E204 and $\epsilon$H38 (Figure 4).

**c-Ring Stoichiometries**

The c rings of several ATP synthases have been isolated and their subunit stoichiometries determined by various methods, including atomic force microscopy, cryoelectron microscopy of two-dimensional crystals, mass spectroscopy, and sodium dodecyl sulfate-gel electrophoresis (36, 37). The different methods produced consistent results on the c subunit stoichiometry of a particular c ring but yielded stoichiometry values between 10 and 15 for c rings from different sources.

These stoichiometries have important consequences on the bioenergetics of the respective ATP synthases because the number of c subunits in the ring equals the number of ions translocated per 360° rotation during which each site takes part in the translocation of one ion (see References 38 and 39 for additional discussion). The $F_1$ part of all ATP synthases has a threefold symmetry and synthesizes or hydrolyzes three molecules of ATP during a 360° rotation. Consequently, the ion-to-ATP ratio of an ATP synthase can be calculated if the number of c subunits is divided by three. The ATP synthase of yeast mitochondria and the aerobic bacteria of *Bacillus* PS3 and of *E. coli* have c-ring sizes of 10 subunits, thus operating at an $H^+/ATP$ ratio of 3.3, whereas the ATP synthase of chloroplasts with a c 14 ring has an $H^+/ATP$ ratio of 4.7, and the cyanobacterium *Spirulina platensis* contains a c 15 ring, which leads to an $H^+/ATP$ ratio of 5 (Reference 39 and references therein). The different c-ring stoichiometries and the resulting $H^+/ATP$ ratios might be adaptations to the different environmental conditions of the ATP-generating cells or organelles. A comparatively low $H^+/ATP$ ratio is more economical and can be employed by mitochondria or aerobic bacteria where a constantly high $\Delta\mu H^+$ with the main contribution from the $\Delta\psi$ is maintained. The high $H^+/ATP$ ratio of chloroplasts does not reflect a

**Chloroplasts:** plant organelles that use light energy for the production of ATP and for photosynthesis
V-type ATPases (V-ATPase): generate an electrochemical proton gradient at the expense of ATP and are prevalent in eucaryotic vacuoles.

significantly reduced overall driving force ($\Delta\mu H^+$). This, however, exists mainly as $\Delta\mu H^+$ and to operate at a low $\Delta\psi$ might be a challenge that benefits from a larger c-ring stoichiometry. Alternatively, economic energy conversions may be less mandatory if light serves as the primary energy source. The chloroplast ATP synthase can therefore operate more distant from thermodynamic equilibrium, compared to the mitochondrial enzyme, with the benefit of higher turnover rates. Although the c-ring size varies in different organisms, it appears to be constant in a given organism. It has been proposed that the c-ring size can be varied by nutritional or genetic manipulations (40). However, attempts to verify this idea have failed or were revoked (41) and argue for sequence-intrinsic features of each particular c subunit, which determine assembly into a specific oligomeric ring (31, 41, 42).

Direct biochemical measurements of $H^+/ATP$ ratios yielded values ranging mostly between 2 and 4 (reviewed in References 40 and 43). At thermodynamic equilibrium, a constant $H^+/ATP$ ratio of 4 has been determined for the ATP synthases from chloroplasts (c14), E. coli (c10), and Thermus thermophilus (c11), in spite of their different c-ring stoichiometries (44, 45). This situation with very low turnover rates ($\ll 1$ ATP/s), however, does not reflect physiological conditions with high ATP synthesis rates (>70–200 ATP/s). Additional investigations are therefore necessary to determine $H^+/ATP$ ratios at the physiological working conditions of ATP synthases.

Structure of the Rotor Ring

The structure of the c11 oligomer from the Na$^+$-translocating F-ATP synthase of I. tartaricus was first solved by two-dimensional crystallography and then by X-ray crystallography to 2.3-Å resolution (46, 47). This was complemented by the structure of the K ring from the Na$^+$-translocating V-type ATPase from Enterococcus hirae (48). The L. tartaricus c11 ring structure shows a hourglass-shaped hollow cylinder whose periplasmic cavity is filled with phospholipids in the natural environment of the membrane (49). Each c monomer folds into a helical hairpin with the loop at the cytoplasmic side and the termini at the periplasmic side of the membrane. The N-terminal helices form a tightly packed inner ring with no space for large side chains, which is accounted for by a conserved motif of four glycine residues (47). In addition, the tight packing of the glycines causes the inner ring of helices to narrow from the cytoplasm toward the middle of the membrane. The outer ring consists of 11 C-terminal helices, which pack into the grooves of the inner ring in the cytoplasmic half of the protein. All helices show a bend of about $2^\circ$ in the middle of the membrane (at P28 and E65 in the N-terminal and C-terminal helices, respectively), marking the narrowest part of the hourglass shape. Eleven Na$^+$ ions are located here at their binding sites, facing toward the outer surface of the c ring, which is consistent with cross-linking data (50).

Ion Binding in Na$^+$ and H$^+$ ATP Synthases

In the I. tartaricus c-ring structure, each of the 11 Na$^+$ ions is bound at the interface of one N-terminal and two C-terminal helices (46). The coordination sphere is formed by side chain oxygen atoms of Q32 and E65 of one subunit, the hydroxyl oxygen atom of S66, and the carbonyl oxygen atom of V63 of the neighboring subunit (Figure 5a). A fifth coordination site found in the K ring from E. hirae, but not clearly visible in the structure of the c ring (48), may be responsible for the higher Na$^+$-binding affinity of the K ring (51). The coordination sphere is completed by a network of hydrogen bonds. The side chain oxygen of E65, which contributes to Na$^+$ coordination, receives a hydrogen bond from the NH$_3$ group of Q32, and the other oxygen of E65 receives hydrogen bonds from the OH groups of S66 and Y70. These hydrogen bonds serve to keep E65 deprotonated at physiological pH in order to allow Na$^+$ binding. This creates a stable locked conformation of the binding site, which
Figure 5

Ion binding coordination in c rings from Na⁺- and H⁺-translocating ATP synthases. (a) Close-up of the Na⁺-binding site formed by two neighboring c subunits (A and B). Na⁺ coordination and selected hydrogen bonds are indicated with dashed lines. This structure represents the locked conformation (see text for details). (b) pH dependence for the modification of c subunit binding sites by N,N'-dicyclohexylcarbodiimide. Depicted are representatives from each class of proton-translocating enzymes. The ATP synthase of Halobacterium salinarium and of I. tartaricus (in the absence of Na⁺) show the expected profile for the protonation of a carboxylate, whereas the profile of the E. coli ATP synthase might reflect the coordination of a hydronium ion.

is observed in the X-ray structure, from which horizontal transfer of the Na⁺ ion to subunit a is prevented (52). In the subunit a/c interface, the conformation of the binding site must therefore convert to an open one to allow ion transfer between the binding site and subunit a, see below.

Structural data for an H⁺-binding site in the c ring of an H⁺-translocating enzyme are not available, and therefore, the mode of proton binding is difficult to envisage. In most models of the H⁺-dependent enzymes, the conserved acidic c-ring residues in the middle of the membrane are assumed to switch between protonated and deprotonated states to allow proton translocation across the membrane (53–55). If these models were correct, the protonation state of the c-ring sites should depend on the pH, following a titration curve where the inflection point corresponds to the pKₐ of the acidic residues. This idea has recently been challenged by investigating the protonation state of c-ring binding sites from several H⁺-translocating enzymes (56). The protonation state has been probed by labeling experiments with the specific inhibitor N,N'-dicyclohexylcarbodiimide (DCCD), which is known to covalently modify the binding site carboxylate by consumption of a proton. The labeling reaction therefore depends on the proton availability at the appropriate sites. When the pH dependence of DCCD labeling was followed in the ATP synthase of Halobacterium salinarium or I. tartaricus in its H⁺-translocation mode (in the absence of Na⁺), a typical titration curve was observed with maximum labeling rates at pH 6 and below, half-maximal labeling around pH 7, and no detectable labeling at pH 8 and above. Hence, these enzymes show the expected behavior for a group protonation mechanism of the binding site carboxylate (Figure 5b).

Surprisingly, bell-shaped pH profiles for DCCD labeling were observed for the majority
ATPase: the enzyme hydrolyzing ATP to ADP and phosphate of H⁺ ATP synthases, including enzymes from E. coli, spinach chloroplasts, and bovine mitochondria. Maximum labeling rates between pH 7.5 and 9 were flanked by decreasing rates at lower or higher pH values (Figure 5b). Decreasing rates with decreasing pH values are not consistent, however, with group protonation of the acidic residues. Instead, the proton could be coordinated as a hydronium ion, as already proposed by Boyer (57), in order to strengthen the analogy of ion binding in Na⁺- and H⁺-translocating enzymes. Efficient coordination of a hydronium ion requires acceptors for three hydrogen bonds, e.g., both oxygens of the c-ring carboxylate and the carbonyl oxygen of the backbone near the kink induced by breaking the helix through a conserved proline residue. In the I. tartaricus c ring, the carboxylate oxygens already receive three hydrogen bonds from interacting donor groups (see above), which effectively prevents hydronium ion binding.

In the ATPases with presumed hydronium ion binding, where a high proton binding affinity extends well into the alkaline region, the ATP hydrolysis pH profile is also shifted toward alkaline pH values. Conversely, in the ATP synthases from P. modestum and H. salinarium, ATP hydrolysis is only possible in a narrow pH range around pH 6 (56).

The Ion Path Through the F₀ Motor

The rotational mechanism of the ATP synthase demands ingeniously designed interfaces between rotor and stator subunits, particularly between the rotating c ring and the laterally abutted subunit a, because rotation speeds up to 500 Hz must be tolerated in the absence of a stabilizing rotor axis. This proteinous interface also acts as the critical scaffold for torque generation and ion translocation across the membrane. To prohibit charge translocation without rotation, ion leakage at the interface must be efficiently prevented.

Electron microscopy studies have shown that presumably more than one c-ring subunit is covered by the laterally abutted ab₂ subunit complex (58, 59). Conversely, it is unlikely that the ab₂ subcomplex consisting of maximal eight-transmembrane helices masks more than two binding sites at the same time.

Stable subcomplexes of subunit a and the c ring have been purified and shown to mediate ion translocation after reconstitution with the transmembrane part of subunit b (60). The precise localization of the two b subunits within the F₀ complex is unclear, but the purification of a stable ab₂ subcomplex (61) and site-directed cross-linking experiments (J. Zingg Ebneter, P. Dimroth, & C. von Ballmoos, unpublished results) indicate that within the membranespanning region subunit b preferentially interacts with subunit a.

Plasmid-borne F₁F₀ ATP synthase lacking subunit a is efficiently integrated into the membrane, proving that subunit a is not essential for assembly of the holoenzyme (62). Because only decreased ATP hydrolysis activity was observed, which was greatly stimulated by the addition of purified subunit a, the authors proposed that, in the absence of the a subunit, subunit b interacts with the c ring like an anchor rail and prevents rotation of the γεc₁₀⁻¹₅ complex.

Clearly, the a/c interface represents the most intriguing part of the F₀ motor. The lack of structural information makes it particularly challenging to elucidate the molecular details of its major tasks as denoted below:

- positions of entry and exit sites for the coupling ions, including access pathways to the c-ring binding site from either side of the membrane,
- specific interactions between subunit a and the c ring to provide tightness against unspecific ion transfer,
- almost frictionless rotation of the c ring against the stator to ensure high efficiency and high turnover rates, and
- ingenious interplay between the stator charge and the binding site to allow horizontal torque generation by vertical driving forces.

Critical residues in the a/c interface. During the past 20 years, several hundred mutations...
were introduced in the *E. coli* a subunit and characterized in various laboratories. Generally, the impact of the mutation on the ability to synthesize ATP was tested by growth on succinate, and its impact on the ability to translocate protons was tested by ATP-driven ACMA quenching or by passive proton flux through *F₀* in inverted vesicles that were energized with NADH oxidation. Second-site suppressor mutation experiments, in which an inactive or poorly active enzyme could be rescued by a secondary mutation, provided insights into the spatial arrangements and functional requirements of the respective residues.

In spite of the impressive coverage of the investigations, only a very small subset of residues was found to be essential for function of the ATP synthase. The most conserved residue is a membrane-embedded arginine (position 210, *E. coli* numbering), also known as the stator charge (63). So far, every mutation of this residue in the *E. coli* enzyme led to a complete loss of activity, whereas in the *P. modestum* enzyme, mutations retaining the positive charge were tolerated under specified conditions (64). In current models, the arginine is believed to ensure release of the coupling ion from the c ring when it enters the a/c interface and to provide a seal between the two access pathways to either side of the membrane (64–66). Recent experiments assign the stator charge at least partially to the ion pathways.

**The stator charge induces a conformational change of the binding site residues in the a/c interface.** The structure of the c ring from *I. tartaricus* and K ring from *E. birae* revealed the precise mode of Na⁺ binding outside of the a/c interface (46, 48). However, it has to be assumed that the c ring adopts alternative conformations under the influence of the stator charge.

Extensive cysteine scanning analysis of subunit a and accessibility studies with cysteine reactive chemicals (NEM, Ag⁺, and others) were applied to indentify the two proposed aqueous half channels on either side of the membrane (69). Not surprisingly, the amino acids of the triad, described above, were sensitive targets and were thus concluded to be a major part of the access pathway from the periplasmic side of the membrane to the c-ring binding site. For the access pathway from the cytoplasmic reservoir, present models locate most of the reactive amino acids in the vicinity of the membrane border (69, 70). Furthermore, several lines of evidence indicate an access route for the ion from the cytoplasm to the binding site in the absence of subunit a (71, 72). Whether this access is sufficiently fast to meet physiological conditions is presently unknown. Nevertheless, the data seem to indicate that the c ring contributes at least partially to the ion pathways.

There appears to be a remarkable connection in organic solvent mixtures at different pH values. The resulting mechanism for ion translocation through *F₀* involved large swiveling motions of the whole outer helix of subunit c by 150° (55, 74). This view could not be corroborated in later studies, including homology modeling of the *E. coli* c ring from the *I. tartaricus* structure and NMR structures of the c-monomer of the H⁺-translocating ATP synthase of *Bacillus* PS3 in organic solvents over a broad pH range (pH 2–8) (75). Although in the latter study no significant conformational changes were observed...
at different pH values, the homology model of the *E. coli* c ring revealed that no large conformational changes are necessary to explain the biochemical data because all residues on the c ring, which formed disulfide bridges with subunit a, are facing outward (46). Fillingame and coworkers (76) retreated from their swiveling model and proposed that such a twinned conformation of the c subunit is possible but does not necessarily contribute to the mechanism of ion translocation.

Strong evidence for a small but significant change in the c-ring binding site structure upon contact with the stator charge of subunit a was recently obtained by intersubunit Cys-Cys cross-linking studies between subunit a and c of the *I. tartaricus* ATP synthase (77). The most remarkable results reflect cross-linking experiments between residues cT67C or cG68C and aN230C on subunit a. Although cT67C from one c subunit and cG68C from a neighboring c subunit occupy spatially similar positions in the c-ring crystal structure toward a potent binding partner on subunit a, only cT67C, but not cG68C, was capable of efficient cross-linking with aN230C. Upon mutagenesis of the stator charge arginine by an uncharged amino acid (R226 → A, N, S, H), the cross-linking efficiency of cT67C/aN230C remained unchanged but that of cG68C/aN230C increased significantly. Furthermore, increasing cross-link yields between cG68C/aN230C in the aR226H background were only found at higher pH values, where the histidine is supposed to be in its neutral form. These findings were rationalized by proposing a stator charge-induced conformational change in the empty binding site, whereby cG68C is disconnected from its cross-linking partner in subunit a. Relating these data to the c-ring structure suggests a rearrangement of the binding site residues, with the side chain of cE65 being slightly pushed or rotated toward the c-ring center, thereby affecting the accessibility of cG68 (which is on the same subunit) but not of cT67 (which is on the neighboring helix). The spatial demand for this rearrangement is presumably allocated by a cavity between the inner and the outer rings of helices in the region of the two conserved residues cG25 and cG68 (G27 and G66 in the K ring of *E. hirae*) (Figure 6a). Accordingly, replacement of cG25 by a bulky isoleucine increased the cross-linking efficiency of the cG68C/anN230C pair to a similar extent as the replacement of the stator charge. No ATP synthesis was observed in the cG25I mutant, emphasizing the functional importance of the small residue. The interpretations were supported by energy minimization calculations of the c-ring structure with a short strip of helix 4 of subunit a, containing residues a1225 to aM231, which suggested possible modes for the interaction between the stator arginine and an unoccupied binding site (Figure 6b) (77).

**Inhibitors of the F0 motor.** The most common inhibitors of the F0 motor can be grouped into the two different classes described below.

**Arginine mimicking inhibitors.** The rationale for the first class of inhibitors of the F0 motor is a structural similarity with the stator charge arginine (Figure 7).

1. 5-(N-ethyl-N-isopropyl)amiloride (EIPA) effectively inhibits Na\(^+\)-dependent ATP synthases but not their H\(^+\)-translocating counterparts (77, 78). Inhibition of the *I. tartaricus* enzyme could be modulated by varying the Na\(^+\) concentration, indicating that Na\(^+\) and EIPA compete for the same binding site (77). The amilorides contain a guanidino group attached to a hydrophobic tail, which mimics the side chain of the conserved arginine of subunit a. The rationale for the inhibition therefore is a competition between EIPA and the stator arginine for binding to the c-ring sites (77). This (Figure 6b) also provides an explanation of why only Na\(^+\)-dependent ATP synthases are affected by amiloride compounds. A well-defined hydrogen bonding network between the arginine (or EIPA) and the binding site residues is not possible in most
H\(^+\)-translocating enzymes because several of the proposed binding partners are missing.

2. DCCD inhibits the F\(_0\) part of ATP synthases by forming an N-acyl bond with the conserved carboxylic acid of subunit c (79). A rationale for the recognition of DCCD by the c-ring binding sites is the similarity of its protonated transition state to the guanidino group of the stator arginine. Several lines of evidence show that DCCD preferentially reacts with binding sites outside the a/c interface and that inhibition is caused by sterically blocked rotation of the modified c ring against subunit a (80). Successful Na\(^+\)/Na\(^+\)-exchange experiments with the DCCD-modified P. modestum ATP synthase support the view that the ion translocation pathway within the a/c interface is not affected by the modification (50).

A diaquillirine compound, which inhibits the ATP synthase of Mycobacterium tuberculosis in the nanomolar range, has been proposed to act similarly by mimicking the stator charge guanidine group (81).

*Tributyltin chloride.* Tributyltin chloride (TBT) and its derivatives are known as noncovalent inhibitors of oxidative phosphorylation and ATP synthesis. Competition studies between TBT and DCCD initially seemed to indicate that both compounds bind to the same site (82), but this has been challenged. With one of the butyl chains of TBT substituted by a photoaffinity label for covalent modification,
the primary site of interaction was identified as subunit a, not the c ring (83). TBT inhibited the Na\(^+\)-dependent ATP synthase, and this was impaired by high concentrations (>100 mM) of Na\(^+\), demonstrating a competition between TBT and Na\(^+\) at a site different from the c-ring binding site. In contrast to DCCD, TBT also impaired Na\(^+\)/Na\(^+\) exchange in the I. tartaricus enzyme, indicating blockade of the ion transport pathway within the a/c interface (83). Proton transport through the purified F\(_0\) parts of the ATP synthases of E. coli and spinach chloroplasts was also inhibited by TBT (84).

### Driving Forces for Na\(^+\)- or H\(^+\)-Coupled F\(_0\) Motors

In most organisms, ATP synthesis is energized by the proton-motive force (\(\Delta \mu \text{H}^+\)), which is the sum of the thermodynamically equivalent parameters membrane potential (\(\Delta \psi\)) and proton concentration gradient (\(\Delta \text{pH}\)). Some anaerobic bacteria couple ATP synthesis to Na\(^+\) ion translocation and therefore utilize the electrochemical gradient of Na\(^+\) ions (sodium-motive force) consisting of \(\Delta \psi\) and the Na\(^+\) concentration gradient (\(\Delta \text{pNa}\)) as a driving force.

Implicit in Mitchell’s chemiosmotic model (85) is the assumption that membrane potential and transmembrane ion gradients are equivalent driving forces for ATP synthesis. First, in vitro experiments with the chloroplast enzyme indicated that ATP synthesis can indeed be energized by a \(\Delta \text{pH}\) exclusively (86). This conclusion has been challenged, however, because the method to establish the \(\Delta \text{pH}\) through an acid/base transition with succinate as the acidic buffer also creates a significant \(\Delta \psi\) owing to the membrane permeability of the succinate monoanion (87). Early experiments with the mitochondrial ATP synthase seemed to indicate equivalence between \(\Delta \psi\) and \(\Delta \text{pH}\) as driving forces for ATP synthesis (88, 89). Accordingly, it was predicted that ATP synthesis occurs if the thermodynamic requirements are fulfilled, i.e., if

\[
\Delta \mu \text{H}^+ = \Delta \psi + \Delta \text{pH} > \Delta G_p
\]

\[
= \Delta G^0_p \times \log_{10}(\frac{[\text{ATP}]}{[\text{ADP}] \times [\text{P}]}).
\]

This prediction was questioned by ATP synthesis experiments in whole cells of Streptococcus lactis, revealing that not all combinations of \(\Delta \psi\) and \(\Delta \text{pH}\) were able to elicit ATP synthesis, irrespective of the total driving force (90).

Accordingly, the thermodynamically equivalent driving forces \(\Delta \psi\) and \(\Delta \text{pH}\) appear to be not kinetically equivalent, suggesting that different kinetic barriers have to be overcome by either \(\Delta \psi\) or \(\Delta \text{pH}\). The idea of a nonperfect equivalence between \(\Delta \psi\) and \(\Delta \text{pH}\) (or \(\Delta \text{pNa}\)) was supported by results with the purified enzymes from Bacillus PS3, E. coli, Wolinella succinogenes (91–93), and P. modestum (101).

In all cases, a total driving force >180 mV, consisting of a minimal \(\Delta \psi\) of ∼80 mV and a
The importance of the coupling ion concentration during ATP synthesis. It is remarkable that all in vitro ATP synthesis experiments with purified reconstituted H⁺-translocating enzymes were energized by an acid/base transition in which the pH at the P side was decreased to levels well below pH 5. Except for the chloroplast enzyme, these values do not reflect the physiological pH values for ATP synthesis. Experiments with the reconstituted E. coli ATP synthase energized by \( \Delta \psi = 120 \text{ mV} \) and \( \Delta \text{pH} = 120 \text{ mV} \) yielded maximal synthesis rates at P side pH values below 6, and no synthesis was observed at P side pH values above 7 (Figure 8a) (101). Efficient ATP synthesis therefore depends not only on a high driving force but also on sufficiently low P side pH values. The high proton concentration requirement for ATP synthesis is not observed during ATP hydrolysis, where the fully coupled enzyme operates at nearly full speed, even at pH > 8.5 (56). Hence, approximately 100-times higher proton concentrations are required to saturate the coupling ion binding sites on the enzyme during ATP synthesis than during ATP hydrolysis. Corresponding observations have also been made with the Na⁺-translocating ATP synthase of *Thermus thermophilus* (45, 92).

Figure 8
Impact of the coupling ion concentration on the P side during ATP synthesis. (a) ATP synthesis with purified ATP synthase of *E. coli* reconstituted in proteoliposomes. At constant driving forces of \( \Delta \psi = 120 \text{ mV} \) and \( \Delta \text{pH} = 1.5 \text{ units} \) (91 mV, white circles) or \( \Delta \text{pH} = 2 \) (122 mV, blue circles), half-maximal ATP synthesis is observed at a P side pH 6.2. (b) ATP synthesis with purified ATP synthase of *P. modestum* reconstituted in proteoliposomes. At constant driving forces of \( \Delta \psi = 140 \text{ mV} \) and \( \Delta \text{pNa} = 120 \text{ mV} \), half-maximal ATP synthesis is observed at a P side Na⁺ concentration of \( \sim 35 \text{ mM} \) (white circles). By contrast, half-maximal ATP hydrolysis is observed at a Na⁺ concentration of \( \sim 0.5 \text{ mM} \) (blue circles).
closely related enzyme of *I. tartaricus*, where the binding site was altered by mutagenesis, strongly favor the second scenario in which the binding site has an altered affinity when accessed from the P side instead of the N side (101). Furthermore, the apparent $K_d$ was resistant to variation of $\Delta \psi$, implying that $\Delta \psi$ has no direct effect on the ion uptake process from the P side.

Ion translocation studies with the isolated $F_0$ parts of these enzymes reinforced the asymmetric properties of the holoenzymes, showing different ion affinities from either side. Furthermore, it was particularly interesting to observe that $\Delta pN\alpha$, unlike $\Delta \psi$, is only an effective driving force for ion transport through $F_0$ in synthesis, but not in the hydrolysis direction in the *P. modestum* enzyme, whereas $\Delta \phiH$ showed to be an equivalent driving force to $\Delta \psi$ in the enzymes of *E. coli* and spinach chloroplasts (84, 95, 96, 101).

**Model for torque generation in the $F_0$ part.** This paragraph describes a mechanistic model for torque generation in $F_0$ that takes recent findings, described in the previous sections, into account (Figure 9). A key feature of the model is the assumption that the binding site in the periplasmic access channel ($S_1$) forms a tight complex with the stator arginine, whereas all other c-ring sites are in an ion-bound state. In order to elicit rotation, the arginine must be released from $S_1$ and form a new complex with the next incoming binding site ($S_n$). A coupling ion entering through the P side channel from the periplasm competes with the arginine for complex formation with the $S_1$ site and, at sufficiently high ion concentrations, displaces the arginine ($a \rightarrow b$) (Figure 9). As soon as the arginine becomes displaced, the interaction between the rotor and the stator is temporarily released, and $S_1$, with its neutralized charge, is ready to move into the lipid interface. To proceed into this direction, the ion from the $S_n$ site must be released into the N side channel, and a new complex of the empty $S_n$ site with the free arginine must be formed ($b \rightarrow c$) (Figure 9). Rotation therefore requires appropriate external conditions for the replacement of arginine from the $S_1$ complex by $H^\mp/Na^+$ and for the dissociation of $H^+/Na^+$ from the $S_n$ complex. Hence, the $P$ side $H^+/Na^+$ concentration must suffice effective binding of the coupling ion to the $S_1$ site, which is of low affinity, and simultaneously, dissociation of $H^+/Na^+$ from the $S_n$ complex must be guaranteed. This reaction is affected not only by the $N$ side ion concentration but also by the membrane potential. The subsequent switch of the arginine from $S_1$ to $S_n$ generates torque and completes one step of c-ring rotation and ion transport.

Generally speaking, the described model is governed by dissociation/association equilibriums of binding site/arginine and binding site/ion complexes at the two opposing ion access routes. These equilibriums are controlled by the ion concentrations on both sides of the membrane and by the membrane potential, leading under proper conditions to directed rotation and torque generation as follows:

$$S_1(\text{Arg}) + S_n(H^+/Na^+) + H^+/Na^+_\text{at} \rightleftharpoons S_1(H^+/Na^+) + S_n(\text{Arg}) + H^+/Na^+_\text{in}.$$  

**Physiological implications.** The present results affect the mechanism of ATP synthesis in vivo. The driving forces $\Delta \psi$ and $\Delta pN\alpha$ in *P. modestum* or $\Delta \psi$ and $\Delta \phiH$ in *E. coli* are created by the methylmalonyl-CoA decarboxylase Na$^+$ pump or respiratory proton pumps, respectively. The high Na$^+$ concentration requirement for ATP synthesis in *P. modestum* is readily realized by its natural marine environment. For *E. coli*, however, which grows optimally at pH 7–8 but requires pH < 6.5 for ATP synthesis in vitro, the underlying mechanism is more difficult to reconcile. An obvious solution to this dilemma is a local increase of the proton concentration at the ATP synthase, as proposed in the long-standing hypothesis of lateral diffusion of the pumped protons along the membrane surface. The hypothesis is in accordance with the observation that low pH values (< 6.5) are only required if ATP synthesis is energized by an artificial K$^+$/valinomycin.
diffusion potential, whereas *E. coli* vesicles readily synthesize ATP at pH 7–8 if they are energized by respiratory proton pumping (101). Similar differences, but with a shift to more alkaline pH values, had previously been described for the different modes of energizing ATP synthesis in the alkaliphilic *Bacillus firmus* (97). ATP synthesis by *H. salinarium* despite the presence of uncouplers can be similarly explained by lateral proton diffusion (98).

It is important to note that effective ATP synthesis requires contributions from the two driving forces ∆ψ and ∆pH and a high proton concentration at the source P side. At first glance, all of these criteria seem to be fulfilled rarely. The proton-motive force in mitochondria and neutrophilic aerobic bacteria, e.g., *E. coli*, primarily consists of a high ∆ψ and only a small ∆pH. This scenario is exaggerated in alkaliphilic bacteria, where the pH gradient is reversed, and the driving force is contributed entirely by the ∆ψ. Furthermore, the environmental pH barely meets the high proton concentration requirements for ATP synthesis.

A local acidification at the P side membrane border is therefore a tempting explanation for ATP synthesis under these apparently inappropriate conditions. In chloroplasts, by contrast, the proton-motive force consists of a large ∆pH and only a small ∆ψ. Efficient ATP production,
Ion motive force: an electrochemical ion gradient composed of the ion concentration gradient and the electrical membrane potential across a membrane under these conditions, may require specific adaptations of the F₀ motor itself, e.g., a large c ring or an adapted binding site with an attenuated response to the Δψ. Although the high proton concentration demand for ATP synthesis is obviously met by dynamic processes at the cell membrane, the reverse operation of the enzyme in fermenting bacteria relies on the proton concentration in the cytoplasmic environment, which usually is in the neutral to slightly alkaline pH range. It is therefore essential that ATP synthases, like that of E. coli functioning during fermentation as ATP-driven ion pumps, operate at near maximal rate at pH values between 7 and 8. Hence, the asymmetric proton concentration requirement for ATP synthesis and hydrolysis is a consequence of the enzyme’s adaptation to the natural environment and its additional function as a Δψ-generating ion pump.

SUMMARY POINTS

1. ATP is the major energy currency in living cells. Each cell performs an energy cycle in which ATP is split into ADP and phosphate to drive numerous endergonic reactions. The cleavage products are subsequently recombined to ATP by the F₁F₀ ATP synthase. The primary energy source for ATP synthesis is either light or an exergonic chemical reaction. The primary energy sources are converted into an electrochemical ion gradient, which serves as the actual driving force for ATP synthesis, across the membrane.

2. Structure and function of the F₁ part are highly conserved, most likely because similar conditions prevail in its environment inside the soluble compartment of the cell (pH, salt). In contrast, the F₀ part faces highly diverse environments (pH, salt, driving forces), and its functionality thus diverges to adapt to specific conditions. Likewise, the F₀ motor has evolved different strategies to efficiently use the dominant component of the ion motive force.

3. The mechanism of ATP formation in the F₁ part is well described by the binding change mechanism. This has been developed in great detail by many techniques, and understanding of the mechanism has been claimed to be “almost complete.”

4. In most bacteria, the unc/atp operon contains nine open reading frames. However, only eight different subunits are identified in the assembled enzyme. The missing subunit, the UncI protein, has been characterized in P. modestum as a chaperone assisting in the assembly of the c ring from single subunits. This discovery not only provides an explanation for the conservation of the uncI gene, but also sheds light on the intricate mechanism of ATP synthase assembly.

5. Kinetic studies show that the binding affinity between F₁ and F₀ subunits of the peripheral and central stalk are comparable and thus provide a robust scaffold to withstand the elastic drag imposed during rotation.

6. Within the family of proton-translocating ATP synthases, different proton binding characteristics are evident. In one group of enzymes, the proton binding sites are characterized by group protonation of the conserved c ring carboxylates. In the other group, the proton binding characteristics are incompatible with a group protonation mechanism and instead indicate coordination of a hydronium ion.
7. The total minimal driving forces for efficient ATP synthesis (>1 ATP/s) reported are very similar (>180 mV). Thereby, $\Delta \psi$ and $\Delta pH/\Delta pNa$ are not equivalent driving forces. While a $\Delta \psi$ may not be required in all organisms (e.g. chloroplasts), it certainly is the major thermodynamic driving force in most species (e.g., bacteria, mitochondria).

8. A high coupling ion concentration on the P side was defined as a critical and so far unrecognized determinant for ATP synthesis. Concomitantly, this finding demonstrates the asymmetry of ion binding to the c-ring binding site from either side of the membrane. In contrast to Na$^+$-dependent enzymes, the demand of values below pH 6 at the P side in E. coli is not compatible with its prevailing growth conditions and supports the long-standing hypothesis of lateral diffusion of translocated protons along membrane surfaces.

9. Cys-Cys cross-linking data indicate a conformational change in the c-ring binding site upon contact with the stator charge in the a/c interface. This structural change allows the release of the coupling ion and tight interaction with the stator charge. Appropriate driving forces and ion concentrations allow the subsequent dissociation of this complex by an incoming coupling ion as well as ion release from the neighboring rotor site in the a/c interface. The interaction of the free arginine with the just generated unoccupied binding site elicits unidirectional rotation and generates torque for the synthesis of ATP.

FUTURE ISSUES

1. The assembly of the ATP synthase and the synthesis of a balanced stoichiometry of the various subunits are still unclear. Although interesting insights were gained in the recent years, these questions have escaped from intense investigations so far.

2. The a/c interface.
   a. The a/c interface is at the core of the F$_0$ mechanism, generating torque at the expense of ion translocation across the membrane. Until now this fundamental protein assembly for the operation of the entire enzyme has escaped structural investigations with high resolution. Such a structure is likely to make a significant contribution to our understanding of the F$_0$ mechanism.
   b. A possible structure displays only a snapshot and may not be sufficient to unravel the dynamic features that happen in the a/c interface, and thus, functional investigations along the ion pathway are of critical importance.
   c. Molecular modeling investigations based on the crystal structure and biochemical data might provide interesting details, explaining how $\Delta \psi$ and $\Delta pH/\Delta pNa^+$ influence the structure within the a/c interface.

3. The concept of lateral proton transfer and its impact on bioenergetics in general and ATP synthesis in particular requires an in-depth investigation. Although several lines of evidence exist for this scenario in vitro, its presence and importance in vivo are still elusive. Generally, the interplay between $\Delta \mu H^+$-generating and $\Delta \mu H^+$-consuming enzymes has to be investigated not only on a structural (e.g., supercomplexes) but also on a functional level. Thereby, the impact of the lipid composition of biological membranes has to be considered. A pronounced impact of localized proton circuits between these proteins would have wide consequences for the entire field of bioenergetics.
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Errata

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