Keilin Memorial Lecture

The ATP synthase: the understood, the uncertain and the unknown

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Abstract

The ATP synthases are multiprotein complexes found in the energy-transducing membranes of bacteria, chloroplasts and mitochondria. They employ a transmembrane protonmotive force, $\Delta p$, as a source of energy to drive a mechanical rotary mechanism that leads to the chemical synthesis of ATP from ADP and $P_i$. Their overall architecture, organization and mechanistic principles are mostly well established, but other features are less well understood. For example, ATP synthases from bacteria, mitochondria and chloroplasts differ in the mechanisms of regulation of their activity, and the molecular bases of these different mechanisms and their physiological roles are only just beginning to emerge. Another crucial feature lacking a molecular description is how rotation driven by $\Delta p$ is generated, and how rotation transmits energy into the catalytic sites of the enzyme to produce the stepping action during rotation. One surprising and incompletely explained deduction based on the symmetries of $c$-rings in the rotor of the enzyme is that the amount of energy required by the ATP synthase to make an ATP molecule does not have a universal value. ATP synthases from multicellular organisms require the least energy, whereas the energy required to make an ATP molecule in unicellular organisms and chloroplasts is higher, and a range of values has been calculated. Finally, evidence is growing for other roles of ATP synthases in the inner membranes of mitochondria. Here the enzymes form supramolecular complexes, possibly with specific lipids, and these complexes probably contribute to, or even determine, the formation of the cristae.

Introduction

"From the earliest times breathing and the warmth of the body were recognized as the chief manifestations of life". So wrote David Keilin at the beginning of the first chapter of his book *The History of Cell Respiration and Cytochrome* [1]. This famous work, completed posthumously by his daughter, Joan Keilin, traces the history of respiration from the Roman physician, surgeon and philosopher, Galen of Pergamon (AD 129–216) via Harvey, Lavoisier, Spallanzani, Berzelius, Hoppe-Seyler and others through to Keilin’s own great scientific work on the cytochromes and cytochrome $c$ oxidase. Keilin conducted his studies at the Molteno Institute for Parasitological Research in Cambridge from 1925 until his death in 1963. Since then, a deep understanding has emerged of how the cytochromes $a$, $a_3$, $b$, $c_1$ and $c$, which Keilin described, participate in electron-transfer processes in mitochondria and bacteria, and, in a development that Keilin could not have anticipated, how release of cytochrome $c$ from mitochondria triggers apoptosis [2]. This knowledge about electron transfer has been integrated into a wider comprehension of bioenergetics, stemming from chemiosmosis, developed in the 1960s and 1970s by Peter Mitchell [3], and Mitchell acknowledged his indebtedness to Keilin’s work in his Nobel Lecture [4]. In his chemiosmotic theory, Mitchell described how the redox energy derived from oxidative metabolism is used to generate a proton electrochemical gradient, $\Delta \mu_{H^+}$ across the inner membranes of mitochondria. He coined the phrase ‘protonmotive force’ (pmf or $\Delta p$), as a way of describing this gradient, and he proposed that $\Delta p$ drives the ATP synthase to make ATP, the energy currency of the biological world, from ADP and $P_i$. Since the 1980s, many of the details have emerged of the atomic structures and molecular mechanisms of the extraordinarily complicated multisubunit protein complexes that carry out the key steps of respiration in mitochondria and...
**Figure 1 | Possible mechanisms that were considered to explain the coupling of force to the synthesis of ATP**

On the left, in a direct coupling mechanism, protons are carried through the inner membranes of mitochondria into the catalytic region of ATP synthase, where they participate directly in the chemistry of the formation of ATP. On the right, in an indirect coupling mechanism, the passage of protons through the membrane domain of ATP synthase brings about intermediate effects that are then transmitted to the catalytic sites of the enzyme and lead to synthesis of ATP. Today, we know that the protonmotive force, \( \Delta p \), is coupled indirectly to ATP synthesis by a mechanical rotary mechanism.

bacteria [5–15]. Together, they provide a landmark in biology, and they represent the culmination of the pioneering efforts of Keilin and Mitchell.

The present article concerns the ATP synthase (also known as the F-ATPase or F\(_{1}\)F\(_{0}\)-ATPase), which, although not studied by Keilin himself, is one of the main beneficiaries of the electron-transfer processes in mitochondria, bacteria and chloroplasts, and is a key enzyme in energy conversion in biology. Mitchell wrote extensively about the ATP synthase, chloroplasts, and is a key enzyme in energy conversion in the electron-transfer processes in mitochondria, bacteria [5–15]. Together, they provide a landmark in biology, and they represent the culmination of the pioneering efforts of Keilin and Mitchell.

The present article concerns the ATP synthase (also known as the F-ATPase or F\(_{1}\)F\(_{0}\)-ATPase), which, although not studied by Keilin himself, is one of the main beneficiaries of the electron-transfer processes in mitochondria, bacteria and chloroplasts, and is a key enzyme in energy conversion in biology. Mitchell wrote extensively about the ATP synthase, chloroplasts, and is a key enzyme in energy conversion in the electron-transfer processes in mitochondria, bacteria and chloroplasts [19]. They are close relatives of the V-ATPases (vacuolar ATPases) found in secretory membranes that use the energy from ATP hydrolysis to generate ion gradients across those membranes, and of the A-ATPases (archaeal ATPases) that synthesize ATP in the archaea. Under aerobic conditions, the ATP synthases make ATP from ADP and Pi, using \( \Delta p \), generated by respiration or photosynthesis, as a source of energy. Instead of using \( \Delta p \) as an energy intermediate, some bacteria, such as \textit{Hylobacter tartaricus}, generate an Na\(^+\) ion motive force and couple it to ATP synthesis [20]. The structures and functions of these Na\(^+\)-dependent ATP synthases are broadly similar to the ATP synthases that are driven by \( \Delta p \). Many bacterial ATP synthases can operate also in reverse under anaerobic conditions, and hydrolyse ATP made by glycolysis, using the energy that is released to translocate protons out of the bacterial cytoplasm thereby generating \( \Delta p \), which then drives other essential cellular functions, such as chemotaxis, and membrane transport steps that depend on \( \Delta p \). In contrast, the ATP hydrolytic activities of the ATP synthases from mitochondria, chloroplasts and some bacteria are inhibited; \textit{in vivo}, these enzymes can only synthesize ATP although their ATP hydrolase activities can be activated artificially \textit{in vitro} [21].

**Overall structure of ATP synthases**

Irrespective of their source, ATP synthases have related structures, as summarized in Figure 2. They consist of two major functional domains, a membrane-extrinsic F\(_{1}\) sector and a membrane-intrinsic F\(_{0}\) sector joined together by central and peripheral stalks. The F\(_{1}\) domain is the catalytic part of the enzyme where ATP is formed from ADP and Pi. The F\(_{0}\) domain contains a motor, which generates rotation using the potential energy stored in \( \Delta p \), as explained in Figure 3. The rotational energy of the motor is transmitted to the catalytic domain by the central stalk, which is attached directly to the rotary motor. The peripheral stalk links the \( \alpha_{3}\beta_{3}\gamma\delta\epsilon \) domain to subunit a in the F\(_{0}\) domain, so that together they form the integral stator of the enzyme.

The F\(_{1}\) domain of the ATP synthase is an assembly of five globular proteins, \( \alpha, \beta, \gamma, \delta \) and \( \epsilon \), with the stoichiometry \( \alpha_{3}\beta_{3}\gamma\delta\epsilon \) [22]. The combined molecular mass of these subunits is approximately 350 kDa. In the \( \alpha_{3}\beta_{3}\gamma\delta\epsilon \) subassembly, which is common to all ATP synthases, the three \( \alpha \)- and the three \( \beta \)-subunits form an approximately spherical \( \alpha_{3}\beta_{3} \) structure approximately 100 Å (1 Å = 0.1 nm) in diameter, with the six subunits arranged in alternation around an elongated \( \alpha \)-helical structure in the \( \gamma \)-subunit [18]. This part of the \( \gamma \)-subunit is completely enveloped in the \( \alpha_{3}\beta_{3} \) domain and occupies its central axis. Thus the \( \alpha_{3}\beta_{3} \) structure resembles an orange made of six segments arranged around the central pith stalk of the fruit. The rest of the \( \gamma \)-subunit protrudes approximately 30 Å beyond the \( \alpha_{3}\beta_{3} \) domain, and forms part of a ‘foot’ that attaches the \( \alpha_{3}\beta_{3}\gamma\delta \) complex firmly to the F\(_{0}\) domain. This attachment is augmented by another protein, known for historical reasons as the \( \epsilon \)-subunit in bacteria and chloroplasts, and as the
The bacterial and chloroplast ATP synthases depicted on the left, and the more complex mitochondrial enzyme is shown on the right. The upper part of each model contains the subunits in the F\textsubscript{1} catalytic domain. One of the three \(\alpha\)-subunits (red) has been removed to expose the elongated \(\alpha\)-helical structure in the \(\gamma\)-subunit (dark blue), which lies approximately along the central axis of the spherical \(\alpha_{3} \beta_{3}\) domain. The \(\gamma\)-subunit (and associated subunits) is in contact with the F\textsubscript{o} membrane domain, which contains the c-ring (brown) and the associated a-subunit (grey). The number of c-subunits in the c-ring differs between species. The rotor of the enzyme consists of the ensemble of the c-ring and the \(\gamma\)-subunit (and associated subunits). The pathway for protons through the F\textsubscript{o} domain is in the vicinity of the interface between the c-ring and a-subunit. The peripheral stalk is on the right of each model. In some bacterial enzymes, it consists of the \(\delta\)-subunit (light blue) and two identical b-subunits (pink). In other bacterial enzymes, and in the chloroplast enzyme, the two b-subunits are replaced by single copies of homologous, but non-identical, subunits b and b’. The N-terminal domain of the \(\delta\)-subunit binds to the N-terminal region of one of the three \(\alpha\)-subunits, and the b- and b’-subunits interact with the a-subunit via their N-terminal transmembrane \(\alpha\)-helices. In the mitochondrial enzyme, the peripheral stalk consists of single copies of subunits OSCP, b, d and F\textsubscript{6}. OSCP is the homologue of the bacterial \(\delta\)-subunit. The sequences of mitochondrial subunits b, d and F\textsubscript{6} are not evidently related to those of the bacterial \(b, b'\) subunits. Their structures also differ significantly, although they are both dominated by \(\alpha\)-helices, except for the C-terminal domain of OSCP (and presumably the bacterial and chloroplast \(\delta\)-subunit), which contain \(\beta\)-structures. The membrane domains of the mitochondrial enzyme contain a number of membrane subunits with single transmembrane \(\alpha\)-helices that are not found in bacteria and chloroplasts. These supernumerary subunits have no known roles in the generation of ATP; subunits e, f, g, A6L and DAPIT and the 8 kDa proteolipid, which are found in the complex when phospholipids are maintained during the purification of the complex, are shown.

\(\delta\)-subunit in mitochondria. Despite their different names, these \(\varepsilon\)- and \(\delta\)-subunits are folded and probably bind in a similar way, and their functions are probably related also, although they are not identical. In mitochondria, the attachment of the foot of the \(\gamma\)-subunit to the F\textsubscript{o} domain is augmented by a second subunit. Known, confusingly, as the \(\varepsilon\)-subunit, this mitochondrial protein has no counterpart in bacterial and chloroplast enzymes [23]. The \(\gamma\)-subunit, and the associated \(\varepsilon\)-subunits in the bacterial and chloroplast enzymes, and the associated \(\delta\)- and \(\varepsilon\)-subunits in the mitochondrial enzyme, are bound firmly to one end of a hydrophobic cylindrical structure made of a ring of c-subunits in the F\textsubscript{o} membrane domain of the enzyme [24,25]. This cylinder, together with the \(\gamma\)-subunit and its associated \(\varepsilon\)- and \(\delta\)-subunits, forms the rotor of the enzyme.

**How ATP is hydrolysed in the \(F_1\) domain**

The understanding of the mechanism of how ATP is hydrolysed in the catalytic \(F_1\) domain of the enzyme is based on a series of high-resolution structures determined by X-ray crystallography with the bovine [18,25–38] and yeast [24,39–41] enzymes. Many of them represent either the ground state in the catalytic cycle of ATP hydrolysis or synthesis [24,25,30,33,35,37,39,42], or of the closely related ADP-inhibited state [18,26–29,32,34,36,38,43]. One such structure is summarized in Figure 4. In these structures, the three
The α-subunit (green) is shown in contact with the cylindrical ring of c-subunits (blue). The C-terminal α-helix of each c-subunit contains a carboxy group (in the side chain of an aspartate or glutamate residue; dark blue circles) exposed on the external circumference of the cylinder. From the bottom of the Figure, protons from the side of the membrane distal from the F\textsubscript{1} domain of the ATP synthase are shown entering a half-channel and neutralizing a negatively charged carboxy group in the interface between the α-subunit and the c-ring [119]. Once neutralized, this residue moves by Brownian motion to the more hydrophobic environment of the lipid bilayer, generating a rotation in the direction indicated. This rotational step brings another negatively charged carboxy group into the lower half-channel allowing another proton to be consumed, generating another rotational substep. Thus, as more negative charge is neutralized by protons, the protons are carried around on the external surface the ring by the neutralized carboxy group, until they reach a second site in contact with the α-subunit, where the local environment reionizes the carboxy group, releasing the proton through a second half-channel on the opposite of the membrane from which it entered. The direction and magnitude of Δψ (−180 mV inwards in mitochondria) ensures that the rotation is unidirectional, and there is no requirement for a ratchet mechanism. The number of protons required to generate each 360° rotation of the c-ring corresponds to the number of c-subunits that form the ring. The γ-subunit (and associated subunits) are attached firmly to the ring, and each 360° rotation of this part of the rotor provides the energy to generate three molecules of ATP from the F\textsubscript{1} domain.

Non-catalytic α-subunits and the three catalytic β-subunits of the enzyme are arranged in alternation around an asymmetric α-helical structure in the single γ-subunit. The α- and β-subunits are similar, each consisting of an N-terminal domain with six β-strands, a central nucleotide-binding domain made of both α-helices and β-strands, and an α-helical C-terminal domain containing six α-helices in β-subunits and seven in α-subunits. Because the γ-subunit is asymmetrical, F\textsubscript{1}-ATPase is an inherently asymmetrical enzyme, and a structure of yeast F\textsubscript{1}-ATPase without bound nucleotides demonstrated that the asymmetry is derived from the protein assembly itself. The asymmetry of the single γ-subunit obliges each of the three catalytic β-subunits to adopt a different conformation with different nucleotide-binding and catalytic properties. Two of them have similar conformations, but in structures, now recognized as the ADP-inhibited state of the enzyme (see the section on Regulatory mechanisms), one subunit, designated β\textsubscript{DP}, contains bound MgADP, and the second subunit, β\textsubscript{TP}, has bound MgAMP-PNP (adenosine 5'-[βγ-imido]triphosphate) (AMP-PNP is a non-hydrolysable analogue of ATP). However, in other structures, which represent the ground state of the active catalytic cycle, two molecules of MgAMP-PNP [24,25,35,39], MgADP-BeF [33] or MgADP [30,33,37,42,43] are bound to both β\textsubscript{TP}- and β\textsubscript{DP}-subunits in the same structure. Hence the β\textsubscript{DP}- and β\textsubscript{TP}-subunits can bind either ADP or ATP. In all of these structures, the third subunit, the β\textsubscript{E}-subunit, adopts a radically different conformation, in which part of the nucleotide-binding domain and the attached C-terminal domain have hinged outwards together, in response to the curvature of the γ-subunit. In these structures, this subunit has no bound nucleotide, and so it is known as the ‘empty’ or ‘open’ state, designated β\textsubscript{E}. None of the α-subunits opens in a similar way. All three of them remain closed, each binding an Mg\textsuperscript{2+} ion and a nucleotide, and they remain bound throughout the catalytic cycle. The nucleotides bound to α-subunits have no known direct role in the formation of ATP. Other intermediate states in the catalytic cycle have been resolved by X-ray crystallography, most notably a transition state analogue structure [31], where the catalytic cycle has been arrested before the release of MgADP from the β\textsubscript{E}-subunit, and a pre-nucleotide-release state that demonstrates that, following the hydrolysis of an ATP molecule, the Mg\textsuperscript{2+} ion and P\textsubscript{i} are released from the enzyme in an unknown (possibly random) order before the nucleotide ADP [44]. The same state has been captured in a structure of yeast F\textsubscript{1}-ATPase inhibited with the yeast inhibitor protein F1\textsuperscript{I} (G.C. Robinson, J.V. Bason, M.G. Montgomery, I.M. Fearnley, D.M. Mueller, A.G.W. Leslie and J.E. Walker, unpublished work).

In order to explain the interconversion of catalytic sites through ‘tight’, ‘loose’ and ‘open’ states required by a binding change mechanism of catalysis of ATP hydrolysis by F\textsubscript{1}-ATPase, it was proposed that the interconversion of sites was effected by a mechanical rotation of the γ-subunit, each 360° rotation taking each β-subunit through the three states represented by the β\textsubscript{TP}-, β\textsubscript{DP}- and β\textsubscript{E}-subunits, thereby hydrolysing three ATP molecules [18]. It was shown subsequently by biophysical experiments conducted with single enzyme complexes that, during ATP hydrolysis, in either a bacterial α\textsubscript{3}β\textsubscript{3}γ complex or the intact F\textsubscript{1}F\textsubscript{0}-ATPase, the direction of rotation is counterclockwise (as viewed from the membrane domain of the enzyme) [45,46], and that the rotation proceeds in 120° steps which were resolved subsequently into 90° and 30° substeps (or 80° and 40° substeps in other experiments) [47,48]. The ground-state structure [35] probably represents the state of the enzyme at the end of the complete 120° rotary step. During ATP synthesis, the direction of rotation is reversed,
The subunits of the protein are depicted in ribbon representation. The $\alpha$, $\beta$, $\gamma$, $\delta$ and $\varepsilon$-subunits are red, yellow, blue, green and purple respectively. Top left: the complete $F_1$ domain with three $\alpha$- and three $\beta$-subunits and single copies of $\gamma$, $\delta$- and $\varepsilon$-subunits [30]. In the intact ATP synthase, the rotor of the enzyme consists of the central stalk interacting via its foot with loop regions between the N- and C-terminal $\alpha$-helices of the eight c-subunits in the $F_0$ membrane domain [25]. In the top right, bottom left and bottom right diagrams, the three different conformations of the catalytic $\beta$-subunits that are present in the structure in the top left panel are shown, together with the central stalk ($\gamma$, $\delta$- and $\varepsilon$-subunits) and an $\alpha$-subunit, for reference. Each $\beta$-subunit has three domains: the N-terminal domain (top) is made of $\beta$-sheets, the nucleotide-binding domain (middle) is a mixed $\alpha$-helix/$\beta$-sheet structure, and the C-terminal domain (bottom) is a bundle of six $\alpha$-helices. Each of the three $\beta$-subunits has been obliged by the asymmetry of the $\alpha$-helical coiled-coil region of the $\gamma$-subunit to adopt a different conformation, denoted $\beta_{TP}$, $\beta_{DP}$ and $\beta_{E}$, with different affinities for nucleotides, the $\beta_{TP}$- and $\beta_{DP}$-subunits can each bind either ADP or ATP, and the $\beta_{E}$-subunit is unable to bind any nucleotide. During the catalytic cycle of ATP synthesis or hydrolysis, the rotation of the central stalk takes each $\beta$-subunit through each of the three conformations. As the rotor turns, this action leads to the binding and entrapment of ADP and phosphate at the $\beta_{E}$-site as it closes, ATP formation at the $\beta_{DP}$-site and ATP release from the $\beta_{TP}$-site as it opens and converts back into the $\beta_{E}$-site. Each 360° rotation of the central stalk (clockwise as viewed from beneath) leads to the formation of an ATP molecule from each of the three $\beta$-subunits.

and it is assumed that the order of the structural changes accompanying ATP hydrolysis is reversed also.

The combined high-resolution structural information from the ground state [35], transition-state analogue [31] and pre-nucleotide-release state [44] of the bovine $F_1$-ATPase provides a detailed account of the mechanism of ATP binding and hydrolysis, and release of the products of hydrolysis (summarized in Figure 5). The upper part of the Figure shows the interconversion of the $\beta_{TP}$-, $\beta_{DP}$- and $\beta_{E}$-subunits with the accompanying 120° rotary steps described above. The
Figure 5 | Structural description of the binding of substrates and the release of products during the catalytic cycle of ATP hydrolysis by bovine F₁-ATPase

The structures are arranged to depict the events that occur cyclically in one of the three catalytic sites of the enzyme. In the upper row, the conformations of the βE-, βTP- and βDP-subunits are taken from the 'ground-state' (GS) structure of bovine F₁-ATPase [35], and are placed in the order described in [35], each conversion requiring a 120° step of the central stalk of the enzyme. In the βDP-subunit, ATP is about to undergo hydrolysis. βTP-TS (transition state) depicts the active site containing the transition analogue of ATP, ADP-AlF₄ [31]. βE-TS represents the state immediately following the scission of the γ-phosphate of ATP, before release of any of the products [44]. Its formation is postulated to require a 90° rotary step [47]. F₁-PH represents the state of the catalytic site in βE-subunit of the structure of bovine F₁-ATPase determined with crystals grown in the presence of phosphonate, a chelator of Mg²⁺ ions. The formation of this state from the transition-state conformation is postulated to require a 30° rotary substep [47]. The Mg²⁺ ion, its hydrating water molecules and bound nucleotide are shown in green, red and grey respectively. In βTP-GS, the Mg²⁺ ion is occluded.

lower part of the Figure shows intermediates formed during a 120° step. They are the transition-state intermediate, the state following the scission of the γ-phosphate bond, but before product release, and the state following the release of Pᵢ and the Mg²⁺ ion, where ADP is still bound to the enzyme. The release of the Mg²⁺ ion involves the disruption of its six coordinating interactions (with four ordered water molecules, the β-hydroxy group of the threonine residue βThr¹⁶³ and phosphate groups in the nucleotide) [44]. In the final step (lower left), the ADP is released, and the βE conformation is regenerated.

The main features of the mechanism of ATP synthase and the synthesis of ATP are summarized in a series of movies at http://www.mrc-mbu.cam.ac.uk/research/atp-synthase/molecular-animations-atp-synthase. These movies demonstrate the rotary action of the enzyme driven by proton translocation through F₁. Substrates are shown entering the catalytic interfaces in the F₁ domain, and after its formation, ATP is shown leaving those interfaces.

The uncertain

Regulatory mechanisms

The different types of ATP synthases have various mechanisms of inactivation and reactivation of ATP synthesis under conditions of low and increasing available energy. In mitochondria and chloroplasts, when Δp is low, MgADP (without Pᵢ) remains bound to one of the three catalytic sites of the enzyme forming an inactive complex. In chloroplasts, it is thought that this inactive ADP-inhibited state is stabilized during the hours of darkness by formation of an
intramolecular disulfide linkage in the γ-subunit of the ATP synthase. When daylight is restored, the enzyme is reactivated by thioredoxin-regulated reduction of the disulfide and energy-dependent dissociation of the MgADP [49,50].

Mitochondria contain an inhibitor protein known as IF1 [51]. *In vitro*, at a pH of ~6.7 or below, this protein forms a 1:1 complex with either F1-ATPase or with the intact enzyme complex, the formation of this inhibited complex requiring the hydrolysis of two ATP molecules. In the presence of Δp, the direction of rotation of the rotor reverses, the bound IF1 is released and ATP synthesis resumes. Hence it has been assumed from these properties that the physiological role of IF1 is to prevent the wasteful hydrolysis of ATP in mitochondria under anaerobic conditions, although there remains doubt that this is its true role *in vivo*.

The mode of inhibition of F1-ATPase by IF1 has been studied in detail (Figure 6). The active bovine protein is a homodimer, where the C-terminal regions of the largely α-helical proteins form an antiparallel coiled coil [52] (Figure 6A). The protruding N-terminal regions provide the inhibitory part of the protein, and each active dimer can bind to two F1-ATPase moieties [53]. At pH values of ~8.0 and higher, the dimers of bovine IF1 form dimers of dimers and higher aggregates, occluding the inhibitory portion of the protein and rendering the inhibitor inactive. Disaggregation of these oligomers into dimers appears to be controlled by the ionic state of a histidine residue, His49, which is thought to provide a pH-sensitive switch between inactive and active states [54]. Removal of the capacity to dimerize by deletion of the C-terminal region from residues 61–84 produces a potent monomeric inhibitor that binds to a single F1-ATPase moiety [37]. In some species, for example *Saccharomyces cerevisiae*, IF1 lacks the C-terminal dimerization region, and the protein is naturally monomeric (G.C. Robinson, J.V. Bason, M.G.
The structures of the monomeric inhibited bovine [37] and yeast F1–IF1 [G.C. Robinson, J.V. Bason, M.G. Montgomery, I.M. Fearnley, D.M. Mueller, A.G.W. Leslie and J.E. Walker, unpublished work]) complexes show that IF1 is bound in a complex binding site at a catalytic interface between the αDP- and βDP-subunits (Figure 6B). In the bovine complex, the inhibitor protein occupies a deep groove lined with α-helices in the C-terminal domains of the αDP- and βDP-subunits (Figure 6C), and its N-terminal region interacts with the coiled-coil region of the γ-subunit via a short α-helix (Figure 6D), and extends into the central aqueous cavity of F1-ATPase. Yeast IF1 binds to yeast F1-ATPase in a similar manner. However, the yeast inhibitor arrests the catalytic cycle at an earlier stage than in the bovine enzyme, and ATP hydrolysis activity has been shown to be associated with plasma membranes, and ATP hydrolysis activity has been detected by immunological means in these enzymes.

Another potential use of the IF1 active fragment with a C-terminal affinity tag is in the isolation of the so-called ecto-ATPase, a version of the F-ATP synthase that has been proposed to be associated with plasma membranes (see, for example, [56]). Although individual subunits of the enzyme have been detected by immunological means in these membranes, and ATP hydrolysis activity has been shown to be associated with the membranes, the presence of a fully assembled enzyme complex capable of hydrolysing ATP has not been demonstrated, and so the existence of such an ectopic complex remains in doubt. The isolation and characterization of the subunit composition and enzyme properties of the complex would help to resolve this issue.

The bacterial ATP synthase from Paracoccus denitrificans and other Alphaproteobacteria have their own inhibitor protein, known as the ξ-subunit, that is thought to prevent ATP hydrolysis [57]. However, the protein is not obviously closely related to mitochondrial IF1, and its mode of action is not known. Other bacterial ATP synthases, exemplified by the enzymes from Escherichia coli and from the moderate thermophile Bacillus PS3 appear to be able both to synthesize and to hydrolyse ATP, depending on whether they are operating under aerobic or anaerobic conditions. An inhibitory mechanism has been demonstrated in vitro for the enzymes from E. coli [58] and Bacillus PS3 [59] involving the ζ-subunit which is bound to both to the γ-subunit in the central stalk of the F1-domain and to the c-ring in the membrane domain of the enzyme (Figure 2). In a structure of the isolated ζ-subunit [60], the N-terminal domain of the protein is folded into a ten-stranded β-sandwich, and the C-terminal region consists of two side-by-side α-helices. In the intact enzyme, the β-sandwich domain is involved in binding the subunit to the γ-subunit and the c-ring, and the α-helical C-terminal region has been proposed to adopt two different conformations, ‘down’ and ‘up’ [61]. In the ‘down’ conformation, the two α-helices are held associated closely with the β-sandwich by an ATP molecule bound (evidently without an Mg2+ ion) between the two domains. In the absence of bound ATP, the two α-helices are thought to assume the ‘up’ position, where it has been proposed that they could interact with the αβγ-complex and inhibit the enzyme in the ATP hydrolysis direction. The ‘up’ position has been captured in a structure of the intact F1-domain of the E. coli enzyme [62], but a coherent explanation of the physiological context for such an inhibitory mechanism is lacking. Other bacterial ATP synthases, exemplified by the enzymes from Caldalkalibacillus thermarum [21] and Mycobacterium tuberculosis [63], synthesize ATP in the usual way under aerobic conditions, but they are not able to hydrolyse ATP under physiological conditions. Their latent ATP hydrolyase activities can be activated only under non-physiological conditions. Currently, there is no adequate molecular explanation for these observations.

Generation of rotation

The cylinder of the rotor of ATP synthases is made of c-subunits [24,25], simple membrane proteins folded into two transmembrane α-helices, with the loop regions joining the α-helices exposed on the same side of the membrane as the F1 domain, and with some of the loops at least in contact with, and binding to, the foot of the central stalk. The N-terminal α-helices of c-subunits form an inner ring in the core of the cylinder, and the C-terminal α-helices provide its external annulus. Most importantly for the generation of rotation of the rotor by Δp, each c-subunit contains an essential acidic amino acid residue (aspartate or glutamate depending on the
species) found around the midpoint of the C-terminal α-helix in the membrane domain of the enzyme [64]. During ATP synthesis, the rotation of the c-ring is driven by $\Delta p$ in a clockwise direction (as viewed from the membrane domain towards the F$_1$ domain), with an estimated rotary speed of approximately 100–150 rev/s, depending on the species. In the ATP synthases that hydrolyse ATP under anaerobic conditions, the energy to drive rotation is now provided by the hydrolysis of ATP, which drives the rotor in a counterclockwise direction, and pumps protons across the energy transducing membrane in the outward direction (away from the F$_1$ domain of the enzyme), thereby generating the transmembrane $\Delta p$. The c-ring is in contact with, and rotates against, the surface of another hydrophobic membrane protein, known variously as subunit a (in bacteria), ATPase-6 or A6 (in mitochondria) and subunit IV (in chloroplasts). For simplicity, it will be referred to in the present paper as subunit a. A model of its transmembrane topography has been proposed [65]. Otherwise, little is known about the detailed structure of this protein. It contains a basic amino acid (Arg$^{110}$ in the protein from E. coli) that participates in the translocation of protons through the F$_{\alpha}$ membrane domain of the enzyme [66]. This residue is strictly conserved in all species that have been examined. It is likely that the a protein provides a pathway (a channel or two half-channels) to allow protons to access the negatively charged carboxy residues on the C-terminal α-helices of c-subunits on the external surface of the c-ring in the rotor, and two polar residues (an aspartate and a glutamine residue) have been proposed to lie on this pathway [67–69]. Following neutralization of the successive carboxy groups on the surface of the c-ring, rotation of the ring, and successive reionization of the carboxy groups, the protons are released on the opposite side of the membrane domain of the enzyme. One significant question that arises is how is the stepping action of the γ-subunit generated from the turning of the rotor? The most likely explanation is that, before a 120° step occurs, energy is stored from the turning rotor by an elastic element, and then released in a quantum or packet (or two subpackets corresponding to the substeps) in order to generate the stepping action. However, the site of transient energy storage is not known, although it could reside in the central stalk, or in the peripheral stalk, or both.

The peripheral stalk

The peripheral stalk is an elongated and largely α-helical structure that extends from the F$_{\alpha}$ membrane domain to the topmost extremity of the F$_1$-domain, where it binds to the N-terminal region of an α-subunit [38,70–72]. The structure of the membrane-extrinsic region of the peripheral stalk of the enzyme from bovine mitochondria has been studied in detail. It is a complex of single copies of subunits OSCP (oligomycin-sensitivity-conferral protein), b’ (subunit b lacking its two transmembrane α-helices), d and F$_{6}$ [73]. OSCP has two domains. Its α-helical N-terminal domain provides the binding site for the N-terminal region of one of three α-subunits, which extends above the $\alpha_6\beta_3$ domain [38,71,72,74], and its C-terminal domain interacts with the C-terminal region of the b-subunit and with F$_{6}$ [38,75,76]. The region between the two domains appears to be flexible and could provide an ‘elbow’ in an otherwise seemingly rather rigid structure. The rest of the peripheral stalk is made of α-helices in the b, d and F$_{6}$ subunits, which lie roughly parallel to each other, giving the impression of a stiff structure, rather than resembling a flexible rope [77]. The bacterial δ-subunit is homologous with OSCP, and the structures of their N-terminal domains are very similar [78,79]. The sequences of mitochondrial and bacterial b-subunits are not evidently related [80], although the two bacterial b-subunits (or single copies of related b and b’-subunits) are thought to form an α-helical coiled coil [81,82]. However, the detailed structures of the C-terminal domain of the bacterial δ-subunit and of the b- and b’-subunits with the exception of their membrane-bound N-terminal α-helices [83], and how they interact, remain to be established.

The coupling of $\Delta p$ to ATP synthesis depends upon an integral peripheral stalk, and the peripheral stalk has been proposed to perform a number of roles. They include the clamping of subunit a to the c-ring [84], transient storage of elastic energy during the 120° rotary steps of the enzyme [85] and helping the $\alpha_6\beta_3$ domain to resist the rotational torque of the rotor [38]. The possible presence of a flexible elbow in the peripheral stalk argues against the first role, and the only biophysical experiments that have addressed the issue of where energy is stored transiently during the 120° steps suggest that only the central stalk participates in this part of the enzyme’s mechanism [86]. Therefore the only remaining role appears to be that of resisting the rotational torque of the rotor.

Although the location of the elastic element responsible for transient energy storage is currently not known for certain, the suggestion that it is most likely to reside in the α-helical coiled-coil region of the γ-subunit is supported by a molecular dynamics simulation based on the structure of bovine F$_1$-ATPase [87]. This simulation suggested that the coiled-coil region of the γ-subunit undergoes structural changes during a 120° step. However, in possible contradiction of this observation, pairwise comparisons of all of the known structures of F$_1$-ATPase have shown that the α-helical coiled-coil region of the γ-subunit behaves as a rigid body in all of these structures, but there are points of flexion above and below the coiled coil (M.G. Montgomery, A.G.W. Leslie and J.E. Walker, unpublished work). Thus the issue of how and exactly where transient energy is stored remains unresolved.

Bioenergetic cost of making an ATP molecule

Knowledge of the main architectural features of the ATP synthases and their functions allows a fundamental deduction to be made about energy transduction in the organisms in which these enzymes are found, namely the number of protons that are required to be translocated through the energy-transducing membrane for each ATP molecule that is synthesized by the ATP synthase from ADP and P$_i$. This
parameter is the energetic cost to the organism of making each ATP molecule. Since each 360° rotation of the rotor of the ATP synthase produces three ATP molecules from the F₁ domain of the enzyme, and each 360° rotation of the c-ring requires the translocation of the same number of protons as there are c-subunits in the ring, the bioenergetic cost to the ATP synthase is the number of c-subunits in the c-ring divided by 3 [24]. The c₈-rings in the bovine enzyme [25] are the smallest c-rings that have been hitherto observed, and it is likely that they occur in all vertebrate ATP synthases, where, in examples from each class, the sequence of the c-subunit is conserved almost completely. They are probably found in the enzymes from invertebrates also, where key residues that allow c₅-rings to form and to function are conserved in all known sequences of c-subunits. It has been estimated that there are approximately 50,000 species of vertebrates on Earth today and approximately 2 million species of invertebrates. The associated bioenergetic cost to the ATP synthases in these multicellular organisms is 8/3 or 2.7 protons/ATP. By the same logic, in unicellular organisms including fungi and bacteria (and also chloroplasts from green plants), where ring sizes of 10, 11, 13, 14 and 15 have been observed [24,88–91] (Figure 7), the bioenergetic cost to the ATP synthase will be 3.3, 3.7, 4.3 and 5.0 protons/ATP respectively. In mitochondria, ATP is produced in the matrix of the organelle, and it is made available as a source of cellular energy by exchanging it for external ADP. It is also transported actively back into the matrix of mitochondria. The combination of the electrogenic exchange of an integral ATP for an external ADP and the non-electrogenic symport of P, and a proton adds one proton to the total required to provide ATP to the cellular cytoplasm, and so the cost to mitochondria containing an ATP synthase with a c₅-ring will be 3.7 protons. In mammalian mitochondria, for each two electrons transferred to oxygen or succinate, ten or six protons respectively are translocated out of the matrix. Therefore the number of moles of ADP phosphorylated to ATP per two electrons transferred to oxygen, known as the P/O ratio [92,93], will be 10:3.7 and 6:3.7 or 2.7 and 1.6 respectively for NADH and succinate, similar to the experimental values of 2.5 and 1.5. P/O ratios have been less studied in other species, and there is uncertainty about the proton stoichiometries of their electron-transfer complexes. Nonetheless, similar considerations apply to their ATP synthases, except that in bacteria there is no requirement to translocate ATP, and ADP and P, across the energy-transducing membrane.

The unknown

The issues

Just as the boundaries are blurred between what is understood (we may ask ‘at what level?’) and what is uncertain, so are the boundaries between the uncertain and the unknown indistinct. Thus, in this final section of the present paper, some of the outstanding features of the ATP synthase that remain to be defined and explained will have been raised already in foregoing sections. In this section, the emphasis is on what remains to be established and how those goals might be reached.

Completion of the structure of ATP synthase

The current mosaic high-resolution structural model of mitochondrial ATP synthase lacks the subunits in the membrane domain, especially subunit α (Figure 9), which is required for a molecular understanding of proton translocation and the generation of rotation, but also of the membrane domain of subunit β and the so-called supernumery subunits e, f, g [94,95], A6L [96], DAPIT (diabetes-associated protein in insulin-sensitive tissue) and 6.8 proteolipid [97]. The way that the two transmembrane α-helices of subunit b contact subunit α, and possibly the c-ring, could also be important factors in understanding the enzyme’s mechanism, and the possible roles of the supernumery subunits are discussed below. At least two approaches are possible for deriving a complete structure of the ATP synthase. One is to continue to determine structures of individual subunits, or of subcomplexes that contain them, and thereby to add to, and to complete, the mosaic structure. The subcomplex approach is preferable to pursuing structures of individual subunits, since some of the information about how subunits interact with each other in the intact ATP synthase is retained, and, for example, knowing exactly how the α-protein and the c-ring interact could be crucial for understanding proton translocation. Another approach is to solve structures of complete complexes. Clearly, this approach would be advantageous as information about intersubunit contacts would be retained, but, so far, intact ATP synthase complexes have resisted attempts to induce them to form well-ordered three-dimensional crystals that are suitable for X-ray analysis. Some of the possible reasons for this are becoming apparent. One possible reason is that the intact ATP synthase is inherently unstable in the contact region between the α-subunit and the c-ring, and the removal of native lipids from the membrane domain and their replacement by detergents during purification adds to this instability. In other words, in purified ATP synthase complexes, the ATP synthetic and hydrolytic activities of the enzyme tend to become uncoupled from Δp. We have therefore put considerable effort into defining conditions of purification where this contact remains intact and the enzyme remains fully coupled to Δp. This objective has been achieved, and it remains to be seen whether appropriate crystals of the intact enzyme can be formed. However, a second possible reason for the formation of well-ordered crystals of the intact complex being difficult is that the isolated ATP synthase complex may be a mixture of unresolved structural isomers, differing in the mode of association of the peripheral stalk with the asymmetrical α₃β₄-domain of the enzyme. This issue has been discussed previously in the context of the structure of the F₁-domain with the peripheral stalk attached, where the peripheral stalk was aligned with one specific non-catalytic interface between the εDP- and βTP-subunits.
Another possible approach is to solve the structure of the intact complex by electron cryomicroscopy of single particles (single intact ATP synthase complexes) embedded in ice. This approach was used to produce models of the mitochondrial ATP synthase complex at 30, 24 and 17 Å resolution [98–100], and recently to produce a structure of the A-type ATP synthase complex from Thermus thermophilus at 9.7 Å resolution [101]. This model of the A-type enzyme indicates that the rotor ring of 12 L-subunits and the I-protein (equivalent to the c- and a-subunits respectively in the F-type ATP synthases) have a surprisingly small contact area. The A-type I-subunit and the F-type a-subunit are rather distantly related, and only the region around the essential arginine residue (Arg in the E. coli a-subunit) in the C-terminal regions of the protein is obviously conserved. Currently, one of the technical issues that limits the resolution of structures determined by electron cryomicroscopy is the ability to align the images of the single particles accurately. In this respect, the A-type ATP synthase is more favourable than the F-type enzyme as the two peripheral stalks of the A-type enzyme facilitate the alignment process. However, other technical issues originating from the electron microscope itself contribute to the current limitations of the resolution of this technique.

Reconciliation of structural and rotational experiments

It is unlikely that a detailed structure of intact ATP synthase on its own will be sufficient to explain the generation of rotation and the transmission of energy into the catalytic sites of the enzyme, including providing a description of the generation of the stepping action of the central stalk in the F1-domain of the integral enzyme complex. It is likely that a number of structures with the enzyme halted at various points in the catalytic cycle as well as biophysical single-‘molecule’ experiments will be required, and also biochemical and mutational experiments conducted in the light of the structural and biophysical information. One of the current difficulties in the ATP synthase field is that it is apparently difficult to reconcile structural data relating to the catalytic sites of the enzyme with the observations of dwells in the rotational cycle. This problem applies especially to the positions adopted by the foot of the central stalk in various structures of F1-ATPase, where it has been noted several times that the position could be influenced by crystal lattice contacts, and therefore that the observed positions should be interpreted cautiously [25,33,36]. It has also been proposed that the conformations of catalytic subunits in these structures are likewise influenced by crystal lattice contacts, and therefore that the observed conformations and nucleotide occupancies are ‘irrelevant’ in understanding catalysis by the enzyme [48,102–104]. Therefore we have investigated these issues systematically by comparing in a pairwise manner all of the 50 or more available crystal structures with each other (M.G. Montgomery, A.G.W. Leslie and J.E. Walker, unpublished work). The main conclusions from this study are that, with the possible exception of yeast F1-ATPase inhibited with the yeast inhibitor protein, IF1, the structures of the catalytic sites are not influenced by lattice contacts, and therefore that the observed structures describe the cycle...
Figure 8 | Arrangement of ATP synthase complexes in the inner membranes of mitochondria

Left: molecular structure of the ATP synthase from bovine mitochondria. This overall structure is a mosaic of high-resolution molecular structures of F$_1$-ATPase [30], F$_1$-ATPase with the stator attached [38], the stator alone [77] and the F$_1$-c$_8$-ring subcomplex [25], all determined by X-ray crystallography. They have been assembled within a mask of the low-resolution structure of the intact ATP synthase complex determined by cryoelectron microscopy [98]. The green region is the residual region of the membrane domain of the enzyme where no high-resolution structures have been determined to date. This region contains subunit a, the membrane domain of subunit b (two transmembrane $\alpha$-helices), and subunits ATPase-8, e, f, g, DAPIT and 6.8 kDa proteolipid (each with a single transmembrane $\alpha$-helix). Right (A–D): dimeric ATP synthase complexes [109].

of catalytic events in the active sites of the enzyme. The second conclusion is that in many structures of F$_1$-ATPase, the ‘foot’ regions of the central stalk are displaced by lattice contacts. A wide range of positions has been observed with rotations in the range $-15^\circ$ to $+32^\circ$ relative to a specific ground-state structure. These rotations cannot be correlated with observations of rotation made in biophysical experiments. This conclusion is not surprising. In the intact ATPase or in F$_1$-ATPase actively hydrolysing ATP, or in a biophysical rotation experiment, the central stalk experiences a rotational torque, and energy is stored transiently, possibly in the coiled-coil region of the $\gamma$-subunit in the central stalk. Subsequently, this energy is released in a quantized manner to give the observed stepping action of the rotary motor [47,86]. In crystallization experiments involving F$_1$-ATPase, the enzyme is inactive, the elastic element is relaxed, and the foot can adopt any one of a wide range of positions, as observed, that are strongly influenced by crystal lattice contacts.

Thus it appears that, in order to understand fully the mechanism of ATP synthase, it may be necessary to obtain structural information about the enzyme in the presence of an imposed $\Delta p$. It is not immediately obvious how this requirement could be fulfilled either by X-ray crystallography or by electron cryomicroscopy of single-enzyme complexes. In principle, one possible way would be to obtain the structural information in situ in mitochondrial membrane vesicles, or in vesicles containing the reconstituted enzyme complexes, but this approach will await the improvement of current imaging techniques.

In the meantime, much can be done to improve the correlation of structural data of F$_1$-ATPases with rotational experiments. At present, rotational experiments have been carried out almost entirely on bacterial enzymes, where the mutations required for building the appropriate experimental constructs can be introduced easily. In contrast, almost all of the structural data on F$_1$-ATPases with which correlation with rotational observations is being attempted, are derived from bovine and, increasingly, yeast mitochondrial complexes. Therefore it would probably be beneficial to obtain more structural data with higher resolution than at present on bacterial complexes, and also to carry out
rotational experiments on yeast complexes. We are attempting to contribute to both approaches.

Roles of ATP synthase beyond oxidative phosphorylation

Over the years, evidence has accumulated in bacteria and mitochondria that respiratory complexes, including the ATP synthase complex, are organized into super- and supramolecular complexes [105,106]. Similar observations have been made about photosynthetic complexes in the thylakoid membranes of chloroplasts [106,107]. In the inner membranes of mitochondria, dimers of the ATP synthase are formed by the association of their e-subunits in their membrane domains [108]. These dimers form ribbons, or linear rows [109–111] (Figure 8), which are localized at the region of highest curvature along the edges of the cristae [112]. It is well known that cardiolipin (diphosphatidylglycerol) is essential for the activity of a coupled ATP synthase complex [113]. Cardiolipin molecules have been proposed to bind in the vicinity of a fully trimethylated lysine residue at position 43 [114] of the c-subunit [25]. This trimethylated lysine residue is located in the headgroup region of the bilayer (Figure 7B), and its presence would hamper the binding of the headgroups of phospholipids, providing sites for cardiolipin to bind preferentially. The ADP–ATP translocase, another abundant component of the inner membranes of mitochondria, also contains a trimethyl-lysine residue, which is also in the headgroup region of the membrane, and would be likely to have a similar preference for binding cardiolipin molecules. The proposal that these trimethyl-lysine residues mark cardiolipin-binding sites is borne out by molecular dynamics simulations (A. Duncan, A.J. Robinson and J.E. Walker, unpublished work), and the trimethylated lysine residue in subunit c is conserved in all vertebrate and invertebrate ATP synthase complexes that have been examined, but not in fungi or bacteria (T. Walpole, D. Palmer, S. Ding, K. Jayawardena, I.M. Fearnley and J.E. Walker, unpublished work). The cardiolipin molecules bound to the c₄-rings of mitochondrial ATP synthase may stabilize the c₄-rings to help them survive the rotational torque to which they are subjected. They may also help to lubricate the rotation of the ring in the membrane environment. Cardiolipin has been detected also in association with subunit a [115]. It is known that approximately 75% of cardiolipin in bovine heart mitochondria is associated with the matrix leaflet of the inner membrane [116,117], and the association of cardiolipin with subunit c and the ADP–ATP translocase would contribute to this local concentration. If those cardiolipin molecules associated with ATP synthase complexes were approximately cone-shaped [118], they would tend to give the cristae a negative curvature, the opposite to what is observed. Therefore the cardiolipin molecules associated with ATP synthase are more likely to be flattened to the surfaces of the proteins in the membrane sector of the enzyme, and their role is likely to be specific to the action of the ATP synthase itself.

Concluding remarks

The present article follows closely the contents of the Keilin Memorial Lecture delivered in Cambridge on 10 January 2012. It is intended to be an account of the contributions of my colleagues and I towards understanding the ATP synthase. Therefore emphasis has been placed especially on structural studies and, to some extent, on earlier biochemical work, where we identified the subunits of ATP synthase complexes, determined their sequences and the organization especially of the ‘Fₒ’ subunits, some of which we recognized by reconstitution as constituting the hitherto unknown peripheral stalk of the enzyme. In the present paper, these contributions have been related to biophysical studies on the rotational mechanism of the enzyme carried out largely by Japanese colleagues. Notwithstanding the current difficulties of their interpretation in a structural context mentioned above, these experiments have complemented our work exquisitely. The present paper is not intended to be a comprehensive review of the ATP synthase field. Thus I have not attempted to describe and acknowledge the many other important biochemical and genetic contributions that have come from many other colleagues around the world. I hope to have the opportunity to do so elsewhere.

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