IF$_1$ : setting the pace of the F$_1$F$_0$-ATP synthase

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When mitochondrial function is compromised and the mitochondrial membrane potential ($\Delta\psi_m$) falls below a threshold, the F$_1$F$_0$-ATP synthase can reverse, hydrolysing ATP to pump protons out of the mitochondrial matrix. Although this activity can deplete ATP and precipitate cell death, it is limited by the mitochondrial protein IF$_1$, an endogenous F$_1$F$_0$-ATPase inhibitor. IF$_1$, therefore, preserves ATP at the expense of $\Delta\psi_m$. Despite a wealth of detailed knowledge on the biochemistry of the interaction of IF$_1$ and the F$_1$F$_0$-ATPase, little is known about its physiological activity. Emerging research suggests that IF$_1$ has a wider ranging impact on mitochondrial structure and function than previously thought.

Mitochondria as ATP consumers

In most Biochemistry text books, mitochondria are described as ‘the powerhouse of the cell’. The bulk of ATP in most mammalian cells comes from mitochondrial oxidative phosphorylation [1]. The reliable supply of ATP is fundamental to cell function; ATP is required for all the work that cells do: for muscle contraction, cell migration, secretion, and the maintenance of the ion gradients that underlie membrane excitability. Although ATP availability is a basic cellular requirement, the role of mitochondria in cell physiology extends far beyond even this. Mitochondria have essential roles in calcium (Ca$^{2+}$) homeostasis (for a recent review see Ref. [2]), in free radical signalling [3], and they act as gatekeepers of cell death by harbouring both pro- and anti-apoptotic proteins [4]. When a system is so fundamental to cell life and death, it follows inevitably that, when it is compromised, resultant impairment of cell and tissue function will manifest as disease. Indeed, disordered mitochondrial function has been implicated in the pathogenesis of an array of major human diseases [5]. A comprehensive understanding of the mechanisms that govern mitochondrial homeostasis and that dictate responses to altered mitochondrial homeostasis is clearly essential if we are to develop rational approaches to managing these diseases.

Central to mitochondrial function is an electrochemical proton gradient across the mitochondrial inner membrane that is established by the proton pumping activity of the respiratory chain (Figure 1). The proton gradient establishes a proton-motive force, which has two components: a pH differential and an electrical membrane potential ($\Delta\psi_m$). The pH component of mitochondrial proton motive force is small relative to the membrane potential and, hence, it is the latter that provides the predominant driving force for mitochondrial transport, ADP phosphorylation, Ca$^{2+}$ accumulation and the import of nuclear-encoded, mitochondrial-localized proteins. Impaired mitochondrial function resulting from a variety of different mechanisms will usually cause a decrease in $\Delta\psi_m$, as a common endpoint. These include limited substrate or oxygen availability such as occurs in ischaemia (e.g. in a stroke or heart attack); genetic or acquired defects in respiratory chain activity (due to mutations or to oxidative or nitrosative damage to mitochondrial respiratory proteins); or a leak of protons back into the mitochondrial matrix (through opening of the mitochondrial permeability transition pore [mPTP] [3,6] or the activation of uncoupling proteins; see Ref. [7] for a review).

The F$_1$F$_0$-ATP synthase is the enzyme complex responsible for ATP synthesis driven by oxidative phosphorylation [8,9]. The complex is an ancestral proton-translocating ATPase, a molecular motor that normally operates as an ATP synthase in the mitochondrial inner membrane in which ADP phosphorylation is driven by the movement of protons down the electrochemical potential gradient established by respiration (Figure 1a[i]; animations at: www.mrc-mbu.cam.ac.uk/research/atp-synthase). The directionality of the enzyme is dictated by the balance between the bioenergetic parameters of free energy available from the phosphorylation potential and from $\Delta\psi_m$. In normally respiring mitochondria, the removal of ATP by the adenine nucleotide translocase (ANT) ensures that the intramitochondrial phosphorylation potential is held relatively low while $\Delta\psi_m$ is high, (estimated at between 150 and 180 mV negative to the cytosol), favouring ADP phosphorylation (i.e. ATP synthesis). However, when mitochondrial homeostasis is compromised, the situation can reverse. A decrease in $\Delta\psi_m$ accompanied by an increase in the phosphorylation potential as glycolysis is upregulated (termed the Pasteur effect [10]; see also Ref. [11]) together with reversal of the ANT, which imports glycolytic ATP, will favour ATP hydrolysis. Therefore, during mitochondrial dysfunction, the F$_1$F$_0$-ATPase can run ‘backwards’, acting as an ATP-consuming proton pump (Figure 1a[ii]).
The ability of the F$_1$F$_0$-ATP synthase to reverse and act as an ATPase during conditions of reduced membrane potential has been appreciated for many years [11]. The proton-pumping capacity of the F$_1$F$_0$-ATPase has two functional consequences. First, when respiration is impaired, it can sustain $\Delta \psi_m$, or at least slow its rate of dissipation by counteracting proton leaks. Alternatively, under conditions in which the loss of $\Delta \psi_m$ is extreme (e.g. if the mPTP opens), when no amount of proton pumping would be able to restore the potential, the enzyme can have sufficient activity to drive depletion of cellular ATP. Indeed, ATP consumption by the F$_1$F$_0$-ATPase could represent an important pathogenic mechanism in diseases in which mitochondrial respiration is compromised. These diseases include the lack of oxygen or substrate (as in a stroke or heart attack), but also include many diseases in which more subtle defects in mitochondria are implicated, including Parkinson’s, Alzheimer’s and motor neuron diseases and a large number of rare but debilitating diseases that involve genetic defects affecting mitochondrial proteins.

Both of these mechanisms have been described in cellular models of pathology. Thus, in cells derived from a patient with a mitochondrial genetic defect (with clinical
mitochondrial ecephalopathy and lactic acidosis) carrying a mitochondrial DNA mutation affecting proton pumping at complex I, $\Delta\psi_{in}$ collapsed when cells were treated with oligomycin, an inhibitor of the F1F0 complex [12]. This simple experiment suggests that proton pumping by the F1F0-ATPase maintains $\Delta\psi_{in}$ (albeit reduced) in these cells at the expense of glycolytic ATP, probably compounding the lactic acidosis observed in the patient.

The power of the F1F0-ATPase to deplete ATP has been demonstrated most dramatically in isolated rat cardiomyocytes after exposure to a mitochondrial uncoupler or in response to mPTP opening. The cardiomyocytes shortened to a rigor contracture within minutes of $\Delta\psi_{in}$ collapse, indicating ATP depletion. The rigor contracture was prevented by oligomycin exposure, emphasizing the power of the F1F0-ATPase as an ATP consumer in a cell type in which mitochondria make up $\sim$40% of the cell volume [3, 12–14]. Grover et al. [15] demonstrated the clinical potential of inhibiting this ATPase activity; treatment with a compound, known as BMS-199264, that inhibits ATPase activity without impairing ATP synthase activity, substantially decreased infarct size after coronary artery occlusion in an isolated perfused rat heart preparation [15].

**IF1, the endogenous mitochondrial ATP hydrolase inhibitor**

Although the role of mitochondria in triggering cell death by initiating the complex signalling pathways of apoptosis is well defined [4, 16], it is becoming clear that mitochondria could also accelerate progression towards necrotic cell death through the simple mechanism of ATP depletion due to ATPase activity during mitochondrial dysfunction. This process is limited by an endogenous inhibitor protein known as IF1 – the inhibitory factor of the F1F0-ATPase. Since its discovery [17], a wealth of information has been gathered about the biochemical and molecular structure of this small protein, and yet it seems to have been remarkably neglected in more physiological studies. The protein inhibits ATPase activity in response to acidification of the mitochondrial matrix [18], which will usually accompany inhibition of mitochondrial respiration (i.e. during hypoxia/ischaemia) and in response to the reversal of F1F0-ATP synthase activity to act as an ATPase [19]. Remarkably, we know almost nothing about the relative expression levels of the protein in different tissues or cell types, or about the physiological impact of varied IF1 expression levels, or the mechanisms that regulate its expression. Currently, there are no animal models in which the gene is either overexpressed or knocked out; thus, the consequences of altered IF1 expression levels for cell or tissue function remain unknown. Therefore, some recent investigations have set out to explore these issues by looking at the functional consequences of varying IF1 expression levels in cell lines. These data suggest that IF1 not only inhibits ATPase activity, and so protects cells from ATP depletion in response to hypoxia, but also IF1 appears to have a role in defining the conformation of the F1F0-ATP synthase and mitochondrial cristae structure, as well as in regulating oxidative phosphorylation under normal physiological conditions.

In 1963, Pullman and Monroy [17] discovered the mitochondrial protein IF1 (inhibitor factor 1), encoded by the gene **ATPIF1**. IF1 binds to and inhibits the F1F0-ATPase activity under conditions of both matrix acidification [18] and ATP hydrolysis [19]. The mammalian **ATPIF1** gene product contains 106–109 amino acids (depending on the species of origin). The first 25 amino acids (residues -25 to -1) represent a mitochondrial targeting presequence that is cleaved within the mitochondria to form the mature IF1 protein of 84 amino acids [9]. IF1 is highly conserved throughout evolution, with homologues found in birds, nematodes, yeasts and plants (although yeast and plant IF1 show significant functional differences from mammalian IF1). This degree of conservation suggests that IF1 is a protein of major functional importance. Indeed, structure is sufficiently conserved such that IF1 from one species can inhibit F1F0-ATPase from another, albeit with varying degrees of efficacy [20]. It was recently suggested that IF1 might also localize to the plasma membrane where it is presumed to associate with an F1F0-ATPase that has also been localized to the plasma membrane [21]. A calmodulin consensus binding motif is present in the middle of the IF1 protein [22], and it has been suggested that this motif might dictate its plasma membrane localisation in hepatocytes [22]. However, there remain many questions about a role (and even the presence) of the F1F0-ATPase at the plasma membrane and, therefore, this theme will not be explored further in this review.

IF1-mediated F1F0-ATPase inhibition is optimal at a pH of $\sim$6.7 [18], a condition achieved in the mitochondrial matrix during severe ischaemia [23]. The action of IF1 on F1F0-ATP synthase activity, however, remains poorly documented in the literature. Given the requirement for an electrochemical potential, this partly reflects the technical difficulties involved in the study of F1F0-ATP synthase activity in contrast with relatively straightforward measurements of ATP hydrolysis. Although some evidence suggests that IF1 can inhibit synthase activity [24], the importance of these observations remains unclear.

The detailed crystal structure of IF1-inhibited F1-ATPase has been solved [19, 25], revealing two main features [19, 25]. First, IF1 acts as a homodimer, simultaneously inhibiting two F1-ATPase units [25] and, second, residues in the two protein complexes form numerous associations that involve several F1-ATPase subunits [19]. Full association of IF1 with the F1F0-ATPase is thought to occur only during ATP hydrolysis. Gledhill et al. [19] suggest that low-affinity binding of IF1 to the surface of the F1 domain might occur [19] even in the ground state; however, the functional consequence of this activity is not clear.

Dimerization of IF1 promotes dimerization of the F1-ATPase during ATP hydrolysis as demonstrated either using blue native gel electrophoresis [26] or in the IF1-inhibited F1-ATPase crystal structure [25]. It has been suggested that dimerization of the IF1-inhibited ATPase structure could help to stabilize the complex against the torque induced by ATP hydrolysis in the F1 domain, or it might bring the F1-ATPase domains sufficiently close together (100 A) that they hinder each others’ rotation [27]. Dimerization of yeast F1F0-ATP synthase occurs independently of IF1 [28]. Buzhynskyy et al. [27] used high-resolution atomic force microscopy topography to
study F1F0 protein complex dimerization in native yeast mitochondrial membranes. They described two populations of F1F0 protein complex dimers, with the gamma subunits separated by 150 Å and 100 Å. This was interpreted as representing active F1F0-ATP synthase dimers and the IF1-inhibited F1F0-ATPase dimers, respectively. IF1-dependent dimerization of the F1F0-ATP synthase during normal respiration remains controversial [29]. In HeLa cells overexpressing IF1, blue native gel analysis revealed that the ratio of dimeric to monomeric forms of the F1F0 protein complex was increased [30]. These findings suggest that increased IF1 expression might promote F1F0-ATP synthase dimerization in mammalian cells.

IF1 cell biology: functional consequences of altered expression

The functional consequences of genetic manipulation of IF1 protein levels were recently explored in cell lines (HeLa cells and muscle-derived C2C12 cells) [30], in which IF1 was either overexpressed by transient transfection or knocked down using small interfering RNA (siRNA). Two approaches were used to assay IF1-mediated inhibition of F1F0-ATPase activity: (i) measurements of the rate of ATP depletion after inhibition of oxidative phosphorylation and glycolysis (halting all ATP synthesis) to assess ATP hydrolysis by the F1F0-ATPase (Figure 2a); and (ii) measurements of Δψm in the face of inhibition of oxidative phosphorylation to assess the proton pumping activity of the F1F0-ATPase (Figures 2b and 3).

In the first of these assays, cellular ATP synthesis was completely inhibited using either (i) iodoacetic acid (IAA) to inhibit glycolysis together with sodium cyanide (CN−) to inhibit mitochondrial respiration, or (ii) IAA to inhibit glycolysis and oligomycin to inhibit oxidative phosphorylation. After inhibition of all cellular ATP synthesis, the rate of ATP depletion reflects the activity of all active ATP-consuming processes in the cell. When oxidative phosphorylation is inhibited with CN−, the F1F0-ATPase activity contributes to the global rate of ATP consumption. By contrast, when oxidative phosphorylation is inhibited with oligomycin, the F1F0-ATPase activity cannot contribute to ATP consumption. The difference between the two rates therefore gives a measure of the specific contribution of the F1F0-ATPase as an ATP consumer. The concentration of free intracellular magnesium ([Mg2+]0) increases as an index of ATP consumption as Mg2+ is released upon ATP hydrolysis (Figure 2). This provides a useful assay at the level of single cells, where direct measurements of [ATP] are not really practical. After complete ATP depletion, cells underwent lysis; this occurred between 45 and 75 min in HeLa cells. In cells lacking IF1, the initial rate of ATP consumption was significantly faster in the presence of CN− and IAA, and the time to lysis was significantly shorter compared with wild-type cells (grey versus black; Figure 2). In the presence of oligomycin and IAA, the rate of ATP consumption was slower and the time to lysis in wild-type cells was substantially delayed (red trace; Figure 2), indicating the overall ATP-consuming activity of the F1F0-ATPase. Together, these experiments show that endogenous IF1 functionally inhibits ATPase activity in intact cells. Similar to the protection of rat cardiomyocytes against contracture, this experiment demonstrates that inhibition of F1F0-ATPase activity is protective to cells. This is consistent with the finding that IF1 overexpression significantly reduced cell death in response to oxygen and glucose deprivation [30].

In a schematic cartoon (Figure 2b) we have illustrated the predicted impact of IF1 on changes in [ATP] and Δψm during the progression of a period of ischaemia. In cells with levels of IF1 sufficient to completely inhibit the ATPase activity (equivalent to the action of oligomycin), Δψm collapses rapidly as soon as the respiratory chain is inhibited, whereas [ATP] can be preserved for a considerable period of time until other ATP consumers drive ATP depletion. This timing will vary depending on the glycolytic capacity of the cell type and the activity of the ATP consumers. In marked contrast, in cells in which IF1 is absent, Δψm can be maintained at a new steady state for prolonged periods of time, but this occurs at the expense of cellular ATP, which becomes depleted more quickly. Once ATP is depleted, Δψm will collapse as there is no ATP left as a substrate for the F1F0-ATPase.

These principles are readily established experimentally. Thus, CN− inhibits electron transfer along the electron transport chain and hence its proton-pumping capacity, leading to depolarisation of mitochondria. Recently, the extent of mitochondrial depolarisation in HeLa cells overexpressing IF1 was shown to be greater than that seen in wild-type cells. Conversely, when IF1 expression was suppressed, Δψm was maintained at a new steady state for prolonged periods of time [30]. These experiments are more revealing than they might initially seem as they show that endogenous IF1 protein is not necessarily expressed with a fixed stoichiometry in relation to the F1F0 protein complex. That activity can be increased or decreased by genetic manipulations argues that there is room for regulation of expression of this protein in relation to that of the F1F0 protein complex. This idea is strengthened by several observations. In central nervous system cultures containing both astrocytes and neurons, immunofluorescence measurements showed that ratios between the expression levels of the F1F0-ATP synthase β-subunit and IF1 protein levels varied dramatically between the two cell types. Neurons, with a relatively low β-subunit:IF1 ratio, exhibited rapid loss of Δψm in response to CN−, whereas astrocytes, with high β-subunit:IF1 ratio, maintained Δψm albeit at a reduced level. F1F0-ATPase activity was clearly required for the maintenance of Δψm, because treatment of astrocytes with oligomycin caused rapid mitochondrial depolarisation (Figure 3b i)). Similarly, in a series of experiments using multiphoton imaging of rat kidney slices, the mitochondria in the proximal tubules showed a relatively low ratio of the F1F0-ATP synthase β-subunit:IF1 and the Δψm depolarised rapidly in response to respiratory inhibition; by contrast, in the more glycolytic neighbouring distal tubules, which showed a high β-subunit:IF1 ratio, Δψm was maintained for long periods of time despite respiratory inhibition [31] (Figure 3). It is interesting to note that both neurons and the proximal tubules are highly oxidative [32,33], and as a result the kidney proximal tubules, for example, are clinically more vulnerable to mitochondrial dysfunction than distal tubules [34]. It could be advantageous to have...
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b-subunit:IF1 ratio to prevent rapid ATP consumption
during respiratory inhibition.
Although it seems clear that IF1 expression levels
relative to the F1Fo-ATP synthase vary between tissues
and even between cell types within given tissues [30],
it is not clear how its expression is modulated. Some evidence
suggests that IF1 might be downregulated in septic shock
syndrome [35]. The same authors have suggested that
hypoxia inducible factor 1-a (HIF1-α) might regulate IF1
expression levels [36]. More recently, immediate early
response gene X-1 (IEX-1) was implicated in targeting
IF1 for degradation. IEX-1 deletion stabilized IF1, thereby
reducing ATPase activity and suggesting a mechanism for
post-translational regulation of steady state IF1 protein
levels [37]. It should be emphasized that measurements of
IF1 protein levels alone are almost meaningless without
equivalent measurements of F1Fo-ATP synthase protein
concentration or activity. It is the expression of IF1 relative
to that of the F1Fo-ATP synthase that dictates the func-
tional outcome. It seems probable that IF1 expression is
regulated independently from that of the F1Fo-ATP
synthase and it seems important to establish the mechan-
ism by which this is achieved.

a low β-subunit:IF1 ratio to prevent rapid ATP consumption
during respiratory inhibition.

Figure 2. Impact of IF1 on ATP consumption and ΔΨm. IF1 inhibits cellular ATP consumption by F1Fo-ATPase and promotes ΔΨm collapse. (a) Experimental traces illustrate the impact of IF1 on the rate of ATP depletion in HeLa cells after inhibition of ATP synthesis. The fluorescent dye MagFura was used to measure free intracellular [Mg2+]i, which increases as ATP is hydrolysed. Most intracellular Mg2+ is bound to ATP (with an affinity for ADP about ten times lower than that for ATP). Free [Mg2+]i rises until cells undergo lysis (arrows). In cells exposed to IAA and CN- (grey line), blocking both glycolysis and respiration, the rate of ATP depletion reflects the summed activity of all ATP consumers. In cells exposed to IAA and oligomycin (oligo; red line), blocking both glycolysis and oxidative phosphorylation, the rate of ATP depletion reflects the summed activity of all ATP consumers now excluding the F1Fo-ATPase. The differences between the two responses are attributable to ATP depletion driven by the F1Fo-ATPase. Representative traces of relative [Mg2+]i, with time are shown for HeLa cells treated with CN- (black line in WT cells, grey line in cells lacking IF1) or oligomycin (red line), in wild-type (WT; black line) or IF1 suppressed (-IF1; grey and red lines) cells. The blue-shaded area indicates the effective inhibition of ATPase activity by endogenous IF1, whereas the grey-shaded area shows that the total difference in time is due to uninhibited F1Fo-ATPase activity in the absence of IF1 (modified from Ref. [30] with permission). (b) Schematic to show the changes in [ATP] (solid lines) and ΔΨm (dashed lines) during a period of ischaemia in cells containing either maximally inhibitory
levels of IF1 (red) or cells in which IF1 is absent (black). In the latter, ΔΨm is maintained at a new steady state by the proton pumping of the F1Fo-ATPase. ATP falls progressively, driven partly by the F1Fo-ATPase itself. Once ATP is depleted, the ΔΨm collapses as it can no longer be maintained by the ATPase. By contrast, in cells expressing IF1, ΔΨm falls quickly whereas ATP depletion is significantly delayed.
submitochondrial particles or isolated enzyme preparations [18,38] are maintained in cell culture models; IF1 thus behaves as expected from experiments with isolated enzyme in a test tube. Recent work also suggests that IF1 might have unexpected roles in regulating mitochondrial function. In normally respiring cells, IF1 overexpression reduced $\Delta \psi_{\text{m}}$, whereas $\Delta \psi_{\text{m}}$ was increased after suppression of IF1 [30]. That these effects were normalised in the presence of oligomycin implicated IF1-dependent modulation of $F_1F_o$-ATPase activity. Mitochondrial NADH was more oxidised in the IF1-overexpressing cells, consistent with an increased rate of respiration, whereas the IF1 siRNA treated cells showed the opposite effect [30]. Again, these differences were normalised by...
oligomycin. At the time, the available literature could not account for these observations, with the exception that IF1 has been implicated in the formation of F1F0-ATP synthase complex. It is suggested that IF1 (as labelled, yellow) might promote an association between F1 subunits as shown (adapted from Ref. [42] with permission). The components representing the F1 ‘motor’ of the synthase are shown in blue and the F0 component as green. Subunits are indicated. OSCP, oligomycin sensitivity conferring protein. (b) 3D reconstruction of the dimeric ATPase and its membrane derived from cryo electron tomography data (adapted from Ref. [39] with permission). The protruding ATP synthase components are shown as yellow and the cristae membrane as grey. (c) A model proposed by Strauss et al. [39] showing accumulated surface charge forced by the high membrane curvature, which is, in turn, generated by the F1F0-ATP synthase dimeric structure (shown as yellow; adapted from Ref. [40] with permission).

How or why might dimerization influence F1F0-ATP synthase activity? A recent study using high-resolution electron microscopy (EM) showed that F1F0-ATP synthase dimers tend to align ‘in ribbons’ along mitochondrial membranes with a high curvature [39] (Figure 4b). The authors suggested that the insertion of the dimeric enzyme complex into the membrane forces the high curvature of the membrane. The idea that the F1F0-ATP synthase might define cristae structure has been suggested for some years [40,41]. Indeed, Minauro-Sanmiguel et al. [42] have already suggested that F1F0-ATP synthase dimerization could improve the efficiency of ATP synthesis [39].

Concluding remarks and future perspectives
The data that we have discussed suggest that IF1 might act as a ‘coupling factor’, increasing the relative efficiency of mitochondrial oxidative phosphorylation. Although this model remains very speculative, it fits with the available expression data. In the cells examined thus far, highly oxidative cell types have lower β-subunit:IF1 ratios compared with their glycolytic neighbours (neurons versus astrocytes; proximal versus distal renal tubules; Figure 3) [30,31]. More work will be required to establish...
the generality of this observation and to explore the regulation of expression and the functional properties of IF1. We need to understand its role in intact tissues and how it impacts on normal tissue physiology and pathophysiology. Obvious next steps will include explorations of how it impacts on normal tissue physiology and in response to cellular injury. What are the physiological roles of IF1 in other tissues? To what extent does IF1 expression dictate the balance between oxidative metabolism and glycolysis? What are the factors that dictate the expression levels of IF1 in different cell types or tissues? These questions await an answer. It seems that the role of IF1 extends beyond that envisaged in the existing literature and that we still have a great deal to learn about this fascinating little protein.

Acknowledgements
We thank the Wellcome Trust (www.wellcome.ac.uk), the Medical Research Council (www.mrc.ac.uk), Kidney Research UK (www.kidneyresearchuk.org) and Royal Veterinary College internal funds for supporting the work of our laboratory. We also thank Andrey Abramov and Andrew Tinker for their invaluable contributions to the work and for useful discussion and Werner Kuhlbrandt and José J. Garcia for allowing us to use their material to illustrate this review.

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