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REVIEW

ATP in Mitochondria: Quantitative Measurement, Regulation, and Physiological Role

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Abstract—Oxidative phosphorylation in mitochondria is the main source of ATP in most eukaryotic cells. Concentrations of ATP, ADP, and AMP affect numerous cellular processes, including macromolecule biosynthesis, cell division, motor protein activity, ion homeostasis, and metabolic regulation. Variations in ATP levels also influence concentration of free Mg^{2+}, thereby extending the range of affected reactions. In the cytosol, adenine nucleotide concentrations are relatively constant and typically are around 5 mM ATP, 0.5 mM ADP, and 0.05 mM AMP. These concentrations are mutually constrained by adenylate kinases operating in the cytosol and intermembrane space and are further linked to mitochondrial ATP and ADP pools via the adenine nucleotide translocator. Quantitative data on absolute adenine nucleotide concentrations in the mitochondrial matrix are limited. Total adenine nucleotide concentration lies in the millimolar range, but the matrix ATP/ADP ratio is consistently lower than the cytosolic ratio. Estimates of nucleotide fractions show substantial variability (ATP 20-75%, ADP 20-70%, AMP 3-60%), depending on the organism and experimental conditions. These observations suggest that the 'state 4' – inhibition of oxidative phosphorylation in the resting cells due to the low matrix ADP and elevated proton motive force that impedes respiratory chain activity – is highly unlikely *in vivo*. In this review, we discuss proteins regulating ATP levels in mitochondria and cytosol, consider experimental estimates of adenine nucleotide concentrations across a range of biological systems, and examine the methods used for their quantification, with particular emphasis on the genetically encoded fluorescent ATP sensors such as ATeam, QUEEN, and MaLion.

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INTRODUCTION

Mitochondria are traditionally called the powerhouses of eukaryotic cells, as one of their main functions is ATP production through oxidative phosphorylation. Most biochemistry textbooks explain that the majority of ATP is synthesized in the mitochondrial matrix from ADP and inorganic phosphate by the H^+-transporting ATP synthase (also known as F_OF_1, Complex V, F-ATPase, or F_1F_O-ATPase) located in the inner mitochondrial membrane. ATP synthesis is driven by the proton motive force (*pmf*) across the inner mitochondrial membrane. The *pmf* is generated by the respiratory-chain enzymes that oxidize NADH and succinate produced in the tricarboxylic acid (TCA) cycle, and, depending on the organism or tissue, by the oxidation of additional substrates such as glycerol-3-phosphate. The adenine nucleotide translocator (ANT; also known as the ATP/ADP carrier, ATP/ADP antiporter, AAC, or ATP/ADP translocase) exports ATP from the matrix into the intermembrane space in exchange for ADP, whereas inorganic phosphate enters the matrix via the phosphate carrier (PiC, encoded by the SLC25A3 gene in humans and MIR1

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in baker's yeast). Nucleotide and phosphate exchange between the cytosol and the intermembrane space occurs through the mitochondrial porins – voltage-dependent anion channels (VDAC) – located in the outer mitochondrial membrane.

This simplified outline captures the basic principles of oxidative phosphorylation, but it is insufficient for understanding the regulatory mechanisms governing the process or clarifying how its dysfunction is related to various pathologies.

Decades of *in vitro* research have generated an extensive body of data on the functions of individual proteins involved in oxidative phosphorylation and the mechanisms that regulate them. Recent advances in microscopy have further accelerated structural studies of mitochondria. Notably, cryo-electron microscopy has recently enabled high-resolution (2.5 Å) visualization of mammalian respiratory-chain supercomplexes in their native cellular environment, revealing previously unknown aspects of their organization and interactions [1]. This is only one of many studies that have revealed the structure of mitochondrial proteins, their relative positions, dynamics, and roles in the formation of cristae and contacts with the endoplasmic reticulum. The body of experimental data on the structure and localization of mitochondrial proteins *in vivo* is also growing rapidly.

Despite the considerable progress in mitochondrial biology, surprisingly little is known about the precise conditions under which oxidative phosphorylation proceeds *in vivo*. We have very little quantitative data on the absolute concentrations of ATP, ADP, and AMP in the mitochondrial matrix, on how these concentrations vary under different physiological and pathological states, and on how such changes relate to the *pmf* or to reactive oxygen species (ROS) production. Because ATP and ADP are exchanged rapidly between the matrix and the cytosol, mitochondrial nucleotide concentrations modulate cytosolic pools and are themselves shaped by them. Thus, shifts in the intramitochondrial ATP/ADP/AMP ratio influence both mitochondrial enzyme function and metabolic processes in the cytosol.

Moreover, changes in ATP concentration in both the cytosol and the mitochondrial matrix affect cellular physiology by altering free magnesium levels. ATP binds Mg^{2+} roughly an order of magnitude more strongly than ADP. In solutions with an ionic strength of 0.1-0.15 M, the logarithms of the magnesium binding constants are approximately 4.0-4.6 for ATP, 3.1-3.5 for ADP, and 1.9-2.1 for AMP [2]. Therefore, ATP hydrolysis to ADP increases the concentration of free magnesium, which in turn influences numerous cellular processes, including those that are not directly sensitive to the changes in nucleotide levels. A notable example is chromatin condensation during mitosis, which is triggered by the rise in cytosolic free magnesium from ~0.5 to 1 mM. This increase occurs in parallel with the drop in ATP concentration, and it has been proposed that the ATP decline results from the elevated ATP hydrolysis rate [3].

An example of a magnesium-dependent process in mitochondria is the A/D transition of complex I in the mammalian respiratory chain. Under hypoxic conditions, complex I shifts from the active (A) to the de-activated (D) state, and upon restoration of respiration, the reverse transition normally occurs within minutes. However, in the presence of 1-5 mM Mg^{2+}, this reactivation is slowed by several orders of magnitude [4]. Given that the free magnesium concentration in the matrix exceeds that in the cytosol, this phenomenon is likely to contribute to the regulation of mitochondrial recovery from ischemia *in vivo*.

In this review, we have aimed to summarize the available literature on adenine nucleotide concentrations in the cytosol and mitochondrial matrix of eukaryotic cells, as well as the methods used to measure ATP levels within mitochondria.

PROTEINS AFFECTING ATP CONCENTRATION IN MITOCHONDRIA

The two main 'players' controlling the dynamics of ATP concentration in the mitochondrial matrix are ATP synthase [5] and adenine nucleotide translocator ANT [6], both located in the inner mitochondrial membrane. Each of these proteins accounts for a significant proportion of the total mitochondrial membrane protein – up to 12% for ANT [6] and up to 20% for ATP synthase [7, 8]. Analysis of the mitochondrial proteome of the HEK293T cell line derived from human embryonic kidneys showed that, in terms of the number of molecules per cell, ANT (all isoforms) is the most abundant mitochondrial protein, with more than 8 million molecules per cell, while the number of ATP synthase molecules reaches nearly 2 million per cell [9]. Only about 20 mitochondrial proteins exceed ATP synthase in copy number. These include, besides ANT, chaperones HSP60, HSP10, and mt-HSP70, peroxiredoxin PRDX3; membrane channels such as the outer membrane porins VDAC1 and VDAC3; several enzymes of the central carbon metabolism such as malate dehydrogenase (MDH2) and citrate synthase (CS) from the TCA cycle; phosphate transporter SLC25A3; and several proteins involved in mitochondrial translation and protein import [9].

ATP synthase can catalyze not only ATP synthesis but also ATP hydrolysis. It thus links the activity of the respiratory chain enzymes, membrane proton conductivity, and the ATP/ADP ratio in the matrix. When *pmf* decreases due to increased proton

conductivity or reduced activity of respiratory chain enzymes, ATP synthase switches from synthesizing to hydrolyzing ATP, pumping protons from the mitochondrial matrix into the intermembrane space and preventing depolarization of the inner mitochondrial membrane. If *pmf* is absent, the enzyme may exhibit high ATPase activity: it can hydrolyze from several hundred to thousands of ATP molecules per second [10].

Under the low-*pmf* conditions, the ATPase activity of mitochondrial ATP synthase is suppressed via non-competitive inhibition by the MgADP complex (the so-called ADP inhibition [10]) and by the regulatory protein IF1, which binds to the enzyme and blocks its ATP hydrolysis activity without impeding ATP synthesis [11]. In mammals, IF1 is believed to prevent ATP depletion in the cell under the energy-deprived conditions, such as ischemia [11]. Studies conducted by our group on baker's yeast have shown that IF1 helps to resume division more quickly after starvation [12]. Along with that, attenuated ADP inhibition increased the growth rate in yeast lacking mitochondrial DNA (*rho*^{0} strain), which are incapable of oxidative phosphorylation and instead synthesize ATP in the cytosol via glycolysis [13]. Such cells lack a fully functional ATP synthase; however, their mitochondria contain the hydrophilic F_1 subcomplex, which is encoded by the nuclear genes and can hydrolyze but not synthesize ATP. For the *rho*^{0} cells, intensive ATP hydrolysis in the mitochondrial matrix proves advantageous, as ANT exchanges ADP produced in the matrix for cytoplasmic ATP, transferring one negative charge from the cytosol to the matrix and thereby maintaining *pmf* necessary for protein import and several other mitochondrial functions. Thus, activities of ATP synthase and its F_1 subcomplex, along with regulation of these activities, are important factors influencing mitochondrial ATP concentration and, consequently, cell physiology.

ANT is highly specific for ATP and ADP. It can transport some other nucleotides and small molecules, but only at more than 100 times lower rates, rendering such fluxes negligible under physiological conditions. It is ANT that determines the specificity of oxidative phosphorylation for ADP; ATP synthase can, although with lower efficiency, phosphorylate other nucleoside diphosphates, primarily GDP [14]. ANT is an electrogenic carrier, and under normal conditions, high *pmf* across the inner mitochondrial membrane shifts the equilibrium toward the export of ATP from mitochondria to the cytosol.

The ATP/ADP exchange is inhibited in the presence of magnesium because the nucleotides are transferred in their free form rather than as magnesium complexes [15, 16] – a rare case for proteins that bind nucleoside triphosphates.

Adenylate kinases also influence the concentrations of ATP and other nucleotides in the cytosol and mitochondria. These enzymes catalyze the reversible transfer of a phosphate group between nucleoside triphosphates (usually ATP or GTP) and AMP, producing two nucleoside diphosphates. Currently, nine isoforms of adenylate kinase have been characterized in human cells ([17], see also a recent review [18] on the physiological role of adenylate kinases in health and disease). Here, we will consider only two mitochondrial enzymes: AK2 of the intermembrane space and AK3 (GTP:AMP phosphotransferase) of the mitochondrial matrix. Another member of the adenylate kinase family, AK4, is also localized in the mitochondrial matrix in some tissues but apparently lacks enzymatic activity [19, 20]; therefore, it is not discussed in detail here. (It should be noted, however, that the recombinant human AK4 with intact mitochondrial localization peptide expressed in *Escherichia coli* cells demonstrated the ability to phosphorylate AMP in the presence of ATP or GTP [21]). Despite its probable lack of enzymatic activity *in vivo*, AK4 is an important regulator of cellular processes that plays a role in the oxidative stress response, participates in hypoxic regulation of mitochondria, and is associated with drug resistance, aggressive tumor progression, and metastasis in several cancers, making it a promising diagnostic and therapeutic target [18].

Adenylate kinase AK2 interconverts ATP, ADP, and AMP in the mitochondrial intermembrane space and catalyzes the reaction (1):

$$\mathrm{NTP} + \mathrm{AMP} \leftrightarrows \mathrm{ADP} + \mathrm{NDP}. \quad (1)$$

Both purine and pyrimidine nucleoside triphosphates (NTPs) can act as phosphate donors, but only AMP can be phosphorylated by AK2 [22]. Like the vast majority of enzymes whose substrates are nucleoside triphosphates, AK2 and all other adenylate kinases work only with their magnesium complexes. AMP, which has about two orders of magnitude lower affinity for magnesium ions than ATP, binds and is phosphorylated without magnesium [23]. The apparent equilibrium constant of the adenylate kinase reaction varies between 0.4 and 1.3 depending on the magnesium concentration, reaching a maximum at approximately 0.5 mM of free magnesium [24]. AK2, which is localized in the intermembrane space, and cytosolic adenylate kinases establish a strict relationship between the concentrations of cytoplasmic adenine nucleotides (see the section 'Adenine nucleotides in the cytosol' below). AK2 also plays an important role in the dynamic disequilibrium of nucleotide composition between the cytosol and the intermembrane space, converting AMP from the cytosol into ADP using ATP exported from the mitochondria by ANT [25].

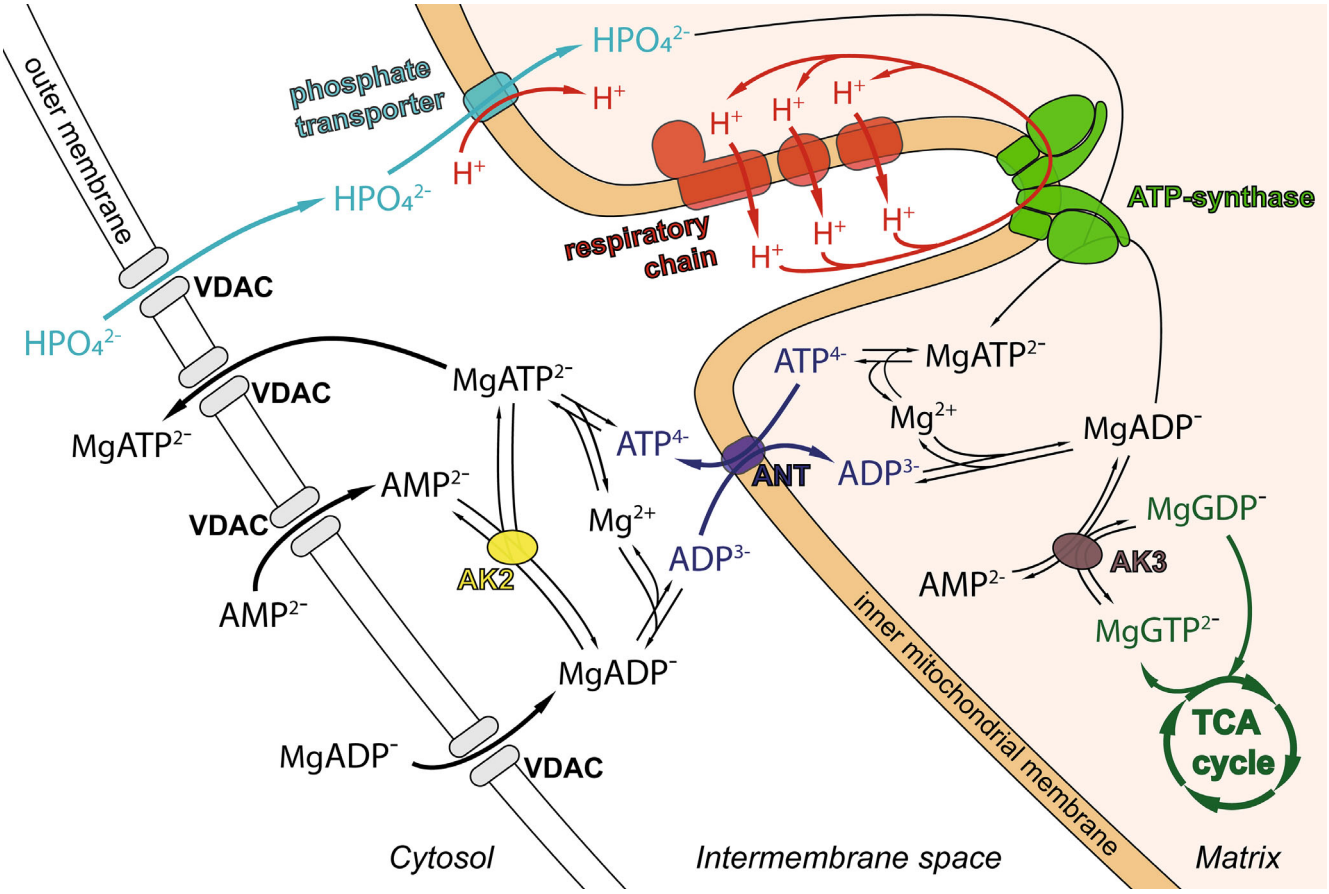

Fig. 1. Oxidative phosphorylation in human mitochondria. Proteins are labeled in bold: green – ATP synthase, cyan – phosphate transporter, red – respiratory chain enzymes, purple – adenine nucleotide translocator (ANT), yellow – adenylate kinase AK2, brown – adenylate kinase AK3, gray – porin VDAC. Bold arrows indicate transport of compounds; thin arrows indicate chemical transformations. The diagram depicts the situation when the respiratory chain generates a sufficiently high proton motive force (*pmf*) for ATP synthesis; pH is 8.0 in the matrix and 7.4 in both the cytosol and intermembrane space. TCA cycle stands for tricarboxylic acid cycle.

Adenylate kinase AK3 (GTP:AMP phosphotransferase) is localized in the mitochondrial matrix [26] and catalyzes the reaction (2):

$$GTP + AMP \leftrightarrows ADP + GDP. \quad (2)$$

This enzyme is present in mitochondria in a significant amount: a proteomic analysis of the human HEK293T cell line has shown that the number of AK3 molecules per cell is approximately 700,000 [9].

Functions of the proteins described above, as well as the phosphate transporter SLC25A3 [27], are shown schematically in Fig. 1.

Experimental studies from the 1970s and 1980s indicate that the rate of oxidative phosphorylation is determined by the ATP/ADP ratio in the intermembrane space [28, 29], and that the rate-limiting process is ATP/ADP translocation through ANT [30, 31]. However, a more detailed analysis has revealed that ANT is only one of several key factors constraining the rate of oxidative phosphorylation, with its role being most significant when the respiration rate is approximately 80% of the maximum possible value [32].

Moreover, the ATP-Mg/P_i carrier located in the inner mitochondrial membrane also influences nucleotide concentration dynamics in the mitochondrial matrix. This protein facilitates electroneutral transport of the $MgATP^{2-}$ complex and inorganic phosphate through the inner mitochondrial membrane [33] (not shown in Fig. 1). According to the proteomic study mentioned above, the total number of all isoforms of this membrane protein approaches 300,000 molecules per cell [9].

Outside mitochondria, the phosphocreatine shuttle (and, in many invertebrates, the phosphoarginine shuttle) significantly influences ATP concentration. Mitochondrial creatine kinase (or arginine kinase), located in the intermembrane space, uses ATP to phosphorylate creatine (or arginine). Cytosolic isoforms of the same enzymes catalyze the reverse reaction, regenerating ATP from phosphocreatine and ADP,

and are associated with the sites of high energy consumption, such as muscle fiber myosin, sarco/endoplasmic reticulum Ca^{2+}-ATPase, plasma membrane Na^+/K^+-ATPase, and other ATP-utilizing proteins. However, a detailed examination of how these enzymes regulate cytosolic ATP concentrations lies beyond the scope of this review.

ADENINE NUCLEOTIDES IN THE CYTOSOL

As shown in Fig. 1, oxidative phosphorylation in mitochondria results in the conversion of cytosolic AMP, ADP, and inorganic phosphate to ATP. It should be noted that, with high adenylate kinase activity, the concentrations of ATP, ADP, and AMP will remain close to the thermodynamic equilibrium. Thus, if the rate of ATP consumption in the cell exceeds the rate of its synthesis, ATP concentration will decrease while ADP and AMP concentrations will increase. Importantly, in this situation, the AMP concentration relative to ATP will increase much more than the ADP concentration [34]. Indeed, for the adenylate kinase reaction (3):

$$\frac{[ATP] \times [AMP]}{[ADP]^2} = K, \quad (3)$$

where [ATP], [ADP], and [AMP] are the equilibrium concentrations of the corresponding nucleotides, and K is the equilibrium constant, we have (4):

$$[ATP] \times [AMP] = K \times [ADP]^2, \quad (4)$$

and dividing both sides of the equation by $[ATP]^2$, we obtain (5):

$$\frac{[AMP]}{[ATP]} \propto \left(\frac{[ADP]}{[ATP]}\right)^2, \quad (5)$$

meaning the AMP/ATP ratio is proportional to the square of the ADP/ATP ratio. Therefore, it is not surprising that an increase in the AMP concentration signals energy deficiency in the cell. The hypothesis that the AMP/ATP ratio regulates the activity of enzymes involved in energy metabolism was first proposed by Atkinson in 1964 during his study of yeast phosphofructokinase [35]. Atkinson later expanded this hypothesis by introducing a variable reflecting the number of high-energy phosphoanhydride bonds per adenine nucleotide in the cell. Since an ATP molecule contains two such bonds, and ADP contains one, this variable is calculated by the formula (6):

$$\frac{2[ATP] + [ADP]}{[ATP] + [ADP] + [AMP]}, \quad (6)$$

ranging from zero (pure AMP; no ADP or ATP) to two (pure ATP; no ADP or AMP). To normalize the range to unity, Atkinson divided the expression by 2 and called the result the 'adenylate energy charge' (AEC) of the cell, which reflects the energy available in the phosphoanhydride bonds of adenine nucleotides for metabolic reactions [36]. Thus, cellular AEC is calculated from intracellular concentrations of ATP, ADP, and AMP using the formula (7):

$$AEC = \frac{[ATP] + ½[ADP]}{[ATP] + [ADP] + [AMP]} \quad (7)$$

and ranges from zero to one.

Soon after its initial success, the AEC hypothesis became less popular because the number of enzymes directly regulated by adenine nucleotides was found to be quite small. A revival occurred in the late 1980s with the discovery of the AMP-activated protein kinase (AMPK) cascade [37], which in response to an increase in AMP initiates a series of reactions that stimulate catabolism and suppress ATP-consuming processes both on a short-term (via enzyme phosphorylation) and long-term (via gene expression) time scale [38]. The subsequent extensive body of data on AMPK activation during hypoxia, ischemia, muscle activity, and other states of cellular energy depletion provided a solid experimental basis for the role of AEC in regulating cellular bioenergetics and broadened its relevance beyond the strictly metabolic regulation [34].

As previously noted, due to the activity of adenylate kinases, concentrations of adenine nucleotides in the cytosol are tightly interconnected, governed by thermodynamics of the adenylate kinase reaction. Blair [24] calculated these concentrations and the free magnesium levels equilibrated by adenylate kinase at different AEC values under conditions close to those in the cytosol (pH 7.5, 100 mM K^+; Fig. 2).

The data shown in Fig. 2 illustrate the connection between the ATP hydrolysis and the increase in free magnesium concentration, as well as the relative stability of ADP concentration across a wide range of AEC values.

To date, a significant amount of experimental data on nucleotide concentrations in the cytosol has been accumulated. Most of these data are obtained from measurements using whole cells and, strictly speaking, reflect not the cytosolic concentrations but the total, averaged concentrations in both cytosol and mitochondria. However, since the volume of mitochondria is generally much smaller than that of the cytosol, the contribution of mitochondrial nucleotides to the total pool is also small and, as a first approximation, can be neglected, except for cardiomyocytes and other cells with very high mitochondrial content. In a large-scale metabolomic study quantifying the most abundant low-molecular-weight metabolites in human cells and baker's yeast, concentrations of

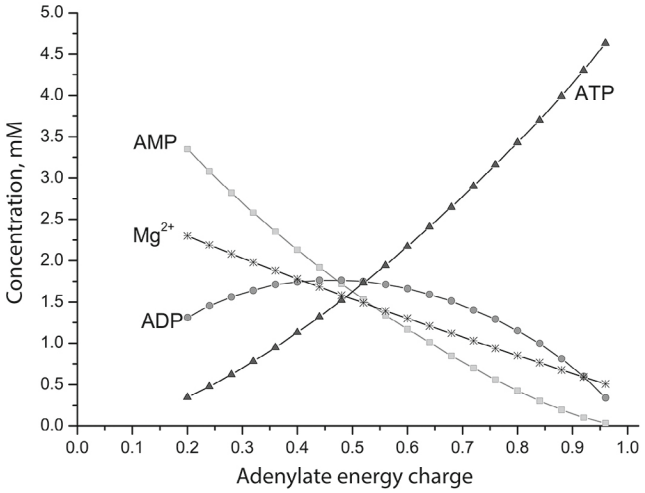

Fig. 2. Relationship between adenylate energy charge (AEC) and concentrations of free magnesium and adenine nucleotides in the presence of adenylate kinase. Data were taken from [24] for the following conditions: total nucleotide concentration 5 mM, total magnesium concentration 5 mM, potassium concentration 100 mM, pH 7.5. The graph shows total nucleotide concentrations (magnesium-bound plus free) for ATP, ADP, and AMP, and concentration of free Mg^{2+}.

adenine nucleotides in eukaryotic cells were reported to fall within the following ranges: ATP, 1.4-7.0 mM; ADP, 0.43-0.57 mM; and AMP, 0.036-0.103 mM [39]. These values are in good agreement with the majority of previously obtained data [40, 41] and correspond to an AEC value of about 0.95 (see Fig. 2).

ADENINE NUCLEOTIDES IN MITOCHONDRIA

The interconversion of adenine nucleotides in mitochondria is less understood than that in the cytosol. As noted above, in mammals, the mitochondrial matrix apparently lacks adenylate kinase, which could regenerate ADP from ATP and AMP. However, AK3 is capable of converting AMP to ADP using GTP. Mammalian mitochondria lack a carrier protein that transports GTP across the inner membrane, so the mitochondrial GTP and GDP pools can be considered isolated from the cytosolic pool on a short timescale. GTP in the matrix is synthesized by the succinyl-CoA synthetase, an enzyme of the TCA cycle. Notably, animal mitochondria have two isoforms of this enzyme: one synthesizes GTP and the other ATP [42]. The relative abundance of each isoform varies among different tissues.

GTP in mitochondria is mainly required for RNA and protein synthesis, AMP rephosphorylation by adenylate kinase AK3, and for conversion of oxaloacetate to phosphoenolpyruvate by mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) [43].

Nucleoside diphosphate kinases (NDPKs) constitute another important class of enzymes influencing the relationship between nucleotide concentrations. They catalyze the exchange of phosphate groups between various nucleoside di- and triphosphates, maintaining thermodynamic equilibrium among ATP, ADP, and other nucleotides in the cytosol. However, in human cells, only one nucleoside diphosphate kinase, namely NDPK D, has a mitochondrial targeting sequence [44, 45]. This enzyme is present in the cell in significant amounts; proteomic studies of the HEK293T cells report up to 200,000 NDPK D molecules per cell [9]. Most experimental data suggest that it is localized in the intermembrane space and is anchored to the inner mitochondrial membrane [46]. (Some studies, however, report nucleoside diphosphate kinase activity in the mitochondrial matrix of vertebrate cells [47, 48]). Additional support for the absence of NDPK in the matrix comes from the experiments on baker's yeast. Yeast normally possesses the mitochondrial GTP/GDP transporter, which is absent in the vertebrate mitochondria. After genetic deletion of this transporter, it was necessary to express human NDPK with an added matrix localization signal to compensate for the deletion [49]. This observation implies that yeast mitochondria cannot produce GTP from ATP without NDPK artificially introduced into the matrix.

We have not found studies that address the relationship between guanine and adenine nucleotide concentrations in the mitochondrial matrix, or that report whether the adenylate kinase-driven thermodynamic equilibrium observed in the cytosol is likewise sustained in the matrix.

Accurate quantification of nucleotide concentrations in the mitochondrial matrix is crucial for elucidating the mitochondrial contribution to the cellular energy metabolism and its regulation. However, such measurements are rather difficult, primarily because of the substantial methodological difficulties. HPLC and other analytical approaches provide highly accurate measurements of total nucleotide content in the sample. Yet, when applied to intact cells, they cannot resolve relative contributions of the cytosolic and mitochondrial pools. Since the mitochondrial matrix usually occupies only a minor fraction of the cellular volume, the resulting values predominantly reflect cytosolic ATP, offering minimal information about its concentration in the matrix.

Measuring nucleotide levels in the isolated mitochondria provides only limited insight into *in vivo* concentrations of these nucleotides, as the isolation process subjects mitochondria to substantial stress and non-physiological conditions. The ATP/ADP/AMP ratios measured in the isolated mitochondria vary depending on biological source and experimental

Table 1. Relative proportions of adenine nucleotides in the isolated mitochondrial preparations from bovine heart and rat liver

Source	ATP, %	ADP, %	AMP, %	AEC	Reference
Bovine heart mitochondria	12	30	58	0.27	[50]
	39	33	28	0.56	[51]
Rat liver mitochondria	46	29	25	0.61	[52]
	35	50	15	0.6	[16]
	from 10 to 46	from 47 to 75	from 8 to 16	–	[53]
	20	69	11	0.55	[31]

Note. Absolute nucleotide concentrations were not determined in these studies, with the exception of reference [31]. The AEC was calculated as (ATP + ½ADP)/(ATP + ADP + AMP).

conditions, yet they consistently diverge from the cytosolic values and typically correspond to an AEC level of approximately 0.6 or less (Table 1).

Furthermore, when isolated mitochondria are incubated in the media containing phosphate and magnesium, adenine nucleotides leak out rapidly. This loss depends on the ATP/ADP/AMP ratio and could reach as much as 75% of the total nucleotide pool [52], implying that a substantial and variable fraction of adenine nucleotides could be lost during mitochondrial isolation. In light of these observations, the relative nucleotide contents measured in the isolated mitochondria seem to provide an unreliable basis for evaluating their matrix concentrations *in vivo*. Nevertheless, as discussed below, available *in vivo* estimates align reasonably well with the values shown in Table 1.

One of the few studies that attempted to quantify the *in vivo* ratios of ATP, ADP, and their magnesium complexes in the cytosol and mitochondria examined cambial cells of white maple (*Acer pseudoplatanus* L.) [54]. Using the ^{31}P-NMR spectra of living cells, cell extracts, and isolated mitochondria, the authors estimated nucleotide levels from the areas under the peaks corresponding to the γ-phosphate of ATP and the β-phosphate of ADP. The proportions of free nucleotides and their Mg^{2+} complexes were determined from the chemical shifts (δ), which depend on pH and Mg^{2+} concentration. ATP concentrations in the cytosol and mitochondrial matrix were reported to be similar – approximately 450 μM and 530 μM, respectively – whereas ADP concentrations were about 30 μM in the cytosol and 220 μM in the matrix. Thus, the cytosolic ATP/ADP ratio was several-fold higher than that in the matrix. It should be noted, however, that the cytosolic ATP and ADP concentrations reported in this study are far lower than those characteristic of animal cells and yeast, in which ATP and ADP levels are roughly 5 mM and 0.5 mM, respectively [39]. Moreover, the nucleotide concentrations reported in the study were not validated by the independent methods such as HPLC or luciferase assays, raising concerns about absolute accuracy of these measurements.

The authors also reported that most ATP exists as a magnesium complex in both compartments – 88% in the cytosol and 98% in the mitochondrial matrix [54]. In contrast, ADP was found to be predominantly free in the cytosol (71%) but largely magnesium-bound in the matrix (80%), implying that Mg^{2+} concentration is roughly an order of magnitude higher in the matrix than in the cytosol (2.4 mM vs. 250 μM).

Cytosolic ATP levels in the white maple cambial cells proved to be sensitive to metabolic context. They rose severalfold after exposure to excess adenine and decreased when cells were transferred into a phosphate-depleted medium or incubated with glycerol or choline, which are rapidly phosphorylated. In contrast, the cytosolic ADP levels remained comparatively stable. From this, the authors suggested that free ADP in the cytosol acts as a central regulator of respiration and cellular energy metabolism [54]. However, considering the limited dynamic range of cytosolic ADP permitted by adenylate kinase (Fig. 2) and the well-established role of AMP as a principal metabolic regulator, further experimental support might be necessary to confirm this interpretation.

Finally, the authors of [54] argued that the comparatively low Mg^{2+} concentration in the cytosol, relative to that of the mitochondrial matrix, favors the presence of ADP in its free form, thereby promoting its uptake by ANT and subsequent transport into the matrix, where it participates in oxidative phosphorylation as MgADP.

The principal advantage of the NMR approach used in this study is its ability to quantify adenine

nucleotides in living cells. However, the method has significant limitations, including technical complexity, requirement for large amounts of biological material, and low sensitivity to ADP and AMP.

Alternative approaches for assessing adenine nucleotide levels in animal cells rely on rapid fractionation to obtain a mitochondrial-enriched fraction within seconds. One method, reported effective for rat hepatocytes, uses brief exposure (20-40 s) to digitonin, followed by rapid centrifugation (20 s) [55]. Digitonin selectively disrupts the sterol-rich plasma membrane while leaving the sterol-poor inner mitochondrial membrane intact, which allows, after centrifugation, to obtain a supernatant containing cytosolic nucleotides and a pellet containing intact mitochondria. Using this technique, ATP, ADP, and AMP proportions of 45-75%, 28-31%, and 3-21% were reported in the mitochondria from rat hepatocytes [55, 56].

A different rapid-fractionation technique subjected rat liver cell suspensions to shear forces by passing them through a narrow metal needle, rupturing the plasma membrane while leaving most mitochondria intact [57]. The complete workflow – including disruption and centrifugation through silicone oil into fixative – required less than one minute. Using this method, the ATP/ADP ratio was found to be ~7 in the cytosolic fraction and close to 1 in the mitochondrial fraction.

Another study from the mid-1970s reported an intriguing and unconventional method for determining adenine nucleotide composition in rat liver mitochondria [58]. The technique involved rapid freezing of the tissue, its homogenization in the frozen state, lyophilization, and fractionation using a carbon tetrachloride–heptane solvent system. Despite appearing at first unsuitable for separating membrane-bound organelles, the procedure proved effective: electron microscopy and enzyme assays confirmed good separation of cytosolic and mitochondrial fractions. The authors found ATP, ADP, and AMP proportions of roughly 85%, 14%, and 1% in the cytosol and 27%, 47%, and 26% in the mitochondria, respectively. Notably, these ratios correspond well to the matrix values observed in the isolated mitochondria (Table 1) and to the cytosolic concentrations determined by other methods.

The reliability of this new method was further verified on isolated rat hepatocytes by direct comparison between the digitonin permeabilization protocol and the freezing-lyophilization-organic solvent fractionation. When both methods were applied to the same hepatocyte sample, they yielded the same values for mitochondrial adenine nucleotide content within experimental error [59]. A related study analyzing pre- and postnatal rat liver mitochondria with the organic solvent fractionation method reported the following values for the matrix adenine nucleotides: 25%, 32%, and 43% for ATP, ADP, and AMP in adult animals, and 21%, 19%, and 60% in the three-day-old pups [60].

A combination of the rapid freezing, lyophilization, and organic solvent fractionation method with an ultra-fast (3-second) procedure for extracting rat liver allowed estimation of the ATP/ADP ratio in mitochondria *in vivo*. In the control group of rats, this ratio was 0.92 ± 0.18; in the rats fasted for 48 h before liver extraction, it increased to 1.04 ± 0.03, and in the rats subjected to anesthesia, it decreased to 0.55 ± 0.06 (pentobarbital) or 0.86 ± 0.04 (ketamine) [61]. These data also agree with the experimental estimates of the ATP, ADP, and AMP ratios in the isolated rat liver mitochondria shown in Table 1.

Estimates of the ratio of ATP, ADP, and AMP concentrations in the mitochondrial matrix appear to be reliable, since many experiments using different objects and different methods yield similar results. At the same time, the absolute values of these concentrations remain difficult to assess. The range of absolute values reported in the literature is quite broad (Table 2).

In the study [62], an attempt was made to quantitatively evaluate the content of a wide range of metabolites in the mitochondrial matrix of HeLa cells. To quickly and selectively separate mitochondria from other cellular components, the authors used an immunoaffinity method, capturing mitochondria by an epitope of a recombinant protein localized in the outer mitochondrial membrane. To quantify metabolites in the mitochondrial preparations, the authors employed liquid chromatography-mass spectrometry (LC/MS) and estimated total mitochondrial matrix volume per cell – required for calculating absolute concentrations – using confocal microscopy. The adenine nucleotide concentrations reported in this work (Table 2, bottom row), however, raise substantial concerns. Most experimental data, including measurements obtained with fluorescent protein sensors [66-69], consistently show that the ATP levels in the matrix are in the millimolar range, nearly two orders of magnitude higher than the 50 μM value reported in [62]. A likely explanation is an error in the estimation of mitochondrial volume, a parameter that is difficult to determine precisely.

Taken together, the experimental evidence indicates that in the cytosol of mammalian cells, ATP, ADP, and AMP concentrations are constrained by the adenylate kinase equilibrium and typically are estimated as approximately 5 mM, 0.5 mM, and 0.05 mM, respectively, yielding an AEC of about 0.95. In the mitochondrial matrix, the total adenine nucleotide concentration is likewise in the millimolar range,

Table 2. Experimental estimates of absolute adenine nucleotide concentrations in the mitochondrial matrix

Source	ATP, mM	ADP, mM	AMP, mM	AEC	Reference
Guinea pig heart	5.6-7.2	1.4-2.0	ND	–	[63]
Rat liver	3.5	12.4	1.9	0.54	[31]
Rat liver (fasted rats)	5.2	7.8	1.5	0.63	[64]
Rat liver (fed rats)	1.4	7.8	3.8	0.41	[64]
Rat liver	12.5-16.4	12.0-14.1	ND	–	[57]
Rat liver	10.4	5.9	4.3	0.65	[56]
Rat; renal proximal tubular cells	2.62	ND	ND	–	[65]
HeLa cells	0.05	0.07	0.02	0.61	[62]

Note. In the studies cited, concentrations were calculated using approximate estimates of matrix volume per milligram of protein or per milligram of dry weight, except in the study [62], where the mitochondrial volume was derived from the microscopy data. AEC denotes the adenylate energy charge; ND indicates cases where no data were available.

but the ATP/ADP/AMP ratios are more variable and the AEC is lower – generally between 0.4 and 0.65.

It is widely assumed that in the resting state, when cellular ATP demand is low, the adenylate energy charge in the mitochondrial matrix rises and ADP becomes nearly fully phosphorylated to ATP. In this scenario, ATP synthase is unable either to continue ATP production or to consume *pmf* through proton translocation coupled to ATP synthesis. As a result, *pmf* increases and suppresses the activity of respiratory chain enzymes, producing the so-called 'state 4' [70], extensively characterized *in vitro* in isolated mitochondria. Inhibition of respiration elevates the reduction state of the respiratory electron carriers and increases the intracellular O_2 levels, thereby promoting non-enzymatic formation of reactive oxygen species (ROS) and increasing the risk of oxidative damage [71]. Vladimir Skulachev proposed that, in order to limit ROS production under these conditions, cells increase proton conductivity of the inner mitochondrial membrane ('mild uncoupling'), thereby lowering *pmf*, stimulating respiration, and reducing O_2 concentration [72].

The evidence summarized in this review, however, indicates that such complete depletion of matrix ADP – and thus a true state 4 – is unlikely to occur *in vivo*. All available data show that the matrix ADP concentration remains appreciable and is consistently higher than the cytosolic one, which itself remains remarkably stable at ~0.5 mM – more than sufficient to support continuous ATP synthesis by ATP synthase. Accordingly, the processes grouped under the term 'mild uncoupling' likely serve a physiological role distinct from the suppression of mitochondrial ROS production in the resting state.

APPLICATION OF FLUORESCENT PROTEIN SENSORS TO MEASURING ATP CONCENTRATION IN MITOCHONDRIA

A powerful strategy for quantifying ATP levels within both mitochondria and cytosol is the use of fluorescent protein-based sensors whose spectral characteristics shift upon ATP binding. The first such sensor, ATeam, was developed by Hiromi Imamura and colleagues [69]. ATeam comprises yellow and cyan fluorescent proteins joined by a small protein that undergoes a reversible conformational transition in response to ATP binding. This structural rearrangement decreases the distance between the fluorophores and enhances Förster resonance energy transfer (FRET). Consequently, fluorescence spectral measurements permit accurate monitoring of relative abundance of the free versus ATP-bound forms of the probe. A major advantage of this method is that its readout is independent of probe concentration. Application of ATeam in HeLa cells revealed mitochondrial ATP concentrations in the millimolar range, although consistently lower than those observed in the cytosol [69].

Further development of this approach substantiated the initial observation and enabled real-time assessment of ATP concentration changes within the mitochondrial matrix in response to various stimuli [66-68]. Parallel observations of a mitochondria-targeted ATeam probe and its cytosolic counterpart in cardiomyocytes demonstrated that hypoxia produces a substantial decline in the matrix ATP over several hours, whereas cytosolic ATP levels remain nearly constant. A similar pattern was observed under normoxia following the addition of oligomycin,

an ATP synthase inhibitor, although in this case the signal changes were developing within minutes [67].

In another study, ATeam probes were employed to analyze ATP concentration dynamics in the cytosol and mitochondria of HeLa cells following glucose depletion [73]. Under these conditions, cytosolic ATP levels remained largely stable, whereas the mitochondrial matrix exhibited a transient elevation in ATP followed by a marked decline. The authors proposed that the initial rise in mitochondrial ATP level results from the activity of hexokinases associated with the outer mitochondrial membrane. Although the hexokinase-catalyzed phosphorylation of glucose to glucose-6-phosphate is generally regarded as irreversible under physiological conditions, the authors speculate that rapid mitochondrial ATP uptake in the absence of cytosolic glucose could make the reverse reaction feasible. This hypothesis, however, requires further confirmation. Another study combined the fluorescent dye TMRE with a mitochondria-targeted ATeam probe to assess the correlation between *pmf* and ATP levels in the neuronal mitochondria [74].

ATeam sensors have also been applied successfully to monitor mitochondrial ATP dynamics in intact tissues and even whole organs. Analysis using a mitochondria-targeted probe in the mouse hearts demonstrated that empagliflozin, an inhibitor of the sodium–glucose cotransporter 2 (SGLT2), elevates mitochondrial ATP levels [75].

In our laboratory, an ATeam-derived sensor was successfully employed to quantify ATP concentrations in yeast cells and in isolated mitochondria. We selected the yAT1.03 variant, optimized for expression in *Saccharomyces cerevisiae* and exhibiting reduced pH sensitivity relative to the original ATeam sensors [76]. We isolated mitochondria from the yeast expressing this probe fused to the mitochondrial localization signal. Permeabilization with the channel-forming antibiotic alamethicin – which renders the inner membrane permeable to low-molecular-weight solutes (up to 1.5 kDa) but not to proteins [77] – enabled calibration of the matrix yAT1.03 signal by adding ATP at defined concentrations to the incubation medium. These experiments demonstrated that ATP concentration in the matrix of isolated yeast mitochondria increases to millimolar levels upon the addition of respiratory substrates [78]. This method also appears promising for quantitative *in vitro* studies of intramitochondrial ATP concentration dynamics under various conditions.

Besides the FRET-based ATeam family, additional protein sensors comprising a single fluorescent protein with ATP-dependent spectral shifts have been developed. These ‘single-color’ sensors include QUEEN [79] and MaLion [80]. QUEEN has been applied with moderate success to quantify ATP levels in yeast mitochondria [81], where – consistent with observations in HeLa cells – mitochondrial ATP was found to be slightly lower than cytosolic ATP. Another study employing QUEEN demonstrated that, in yeast, addition of the protonophore FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), which collapses the *pmf* across the inner mitochondrial membrane, leads to an increase in ATP within the matrix and a decrease in the cytosol [82]. To account for this unexpected behavior, the authors proposed that FCCP stimulates ATP import from the cytosol into mitochondria via the ATP-Mg^{2+}/HPO_4^{2-} carrier, although the presented data provide insufficient evidence to support this conclusion. In a separate investigation, use of the mitochondria-targeted MaLion probe revealed that when hepatocytes transition from glucose deprivation to active glucose utilization, the mitochondrial ATP concentration declines, implying a potential role of the mitochondrial adenine-nucleotide dynamics in regulating cellular metabolism [83].

Another family of fluorescent probes includes the Perceval sensor and its analogs. These proteins have very high affinity for both ATP and ADP, so inside the cells they are always bound to one of these nucleotides. The spectral properties of Perceval differ depending on which nucleotide is bound, and the overall fluorescence signal reliably reflects the ATP/ADP ratio [84]. However, the signal of this probe is sensitive to pH changes between 6 and 8, which makes quantitative interpretation more difficult. A later variant, PercevalHR, was developed with improved spectral characteristics [85], but we found no reports of its successful use for measuring ATP/ADP ratios in mitochondria. Recently, a protein fluorescent probe capable of detecting the GTP/GDP ratio in mitochondria was developed [86].

Although the studies described above demonstrate varying degrees of success in applying the protein-based fluorescent sensors to mitochondrial ATP measurements, several substantial methodological limitations remain. All such probes are influenced to some extent by pH and by ionic composition, display partial cross-reactivity with other nucleotides, and exhibit temperature-dependent fluorescence behavior. Moreover, probes of the ATeam family are subject to asynchronous maturation of their two fluorescent proteins, potentially leading to the accumulation of an unknown – yet possibly considerable – population of non-functional probe species within the cell. These aberrant molecules lack either the FRET donor or acceptor and could, therefore, compromise the composite signal. Consequently, rigorous calibration of the probe output directly in the experimental sample (e.g., through permeabilization followed by the addition of defined ATP concentrations)

and cross-validation of ATP measurements using independent approaches (distinct fluorescent probes, luciferin–luciferase assays, HPLC) are critically important.

CONCLUSION

When discussing adenine nucleotide levels in eukaryotic cells, it can be stated that under most conditions, cytosolic ATP concentration lies in the millimolar range, while the concentrations of ADP and AMP are approximately 10-fold and 100-fold lower, respectively. In the mitochondrial matrix, the proportion of ATP is lower and the proportions of ADP and AMP are higher compared with the cytosol; this indicates that the so-called 'state 4', in which oxidative phosphorylation is inhibited in the resting cells due to the lack of ADP in the matrix, is very unlikely to occur *in vivo* under physiological conditions.

To clarify causal relationships between the activities of bioenergetic enzymes, changes in *pmf*, and adenine nucleotide concentrations in the mitochondrial matrix and cytosol, reliable methods are needed for quantitative measurements of ATP concentrations *in vivo*. Fluorescent protein sensors are excellent tools for monitoring dynamic changes in ATP concentration in the cytosol and mitochondria, but they cannot provide precise values for absolute nucleotide concentrations because their calibration *in vivo* is difficult. Therefore, an optimal strategy is to combine dynamic measurements obtained with several different fluorescent protein probes – calibrated using standard ATP solutions after cell permeabilization – with traditional nucleotide quantification methods (HPLC, luciferin–luciferase assays) and newer analytical approaches such as single-cell metabolomics, mass-spectrometry imaging, and high-resolution respirometry within the same experimental framework.

Abbreviations

AEC	adenylate energy charge
AMPK	AMP-activated protein kinase
FRET	Förster resonance energy transfer
NDPK	nucleoside diphosphate kinase
pmf	proton motive force
TCA	tricarboxylic acid cycle

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Ethics approval and consent to participate
This work does not contain any studies involving human and animal subjects.

Conflict of interest
The authors of this work declare that they have no conflicts of interest.

REFERENCES

1. Zheng, W., Chai, P., Zhu, J., and Zhang, K. (2024) High-resolution in situ structures of mammalian respiratory supercomplexes, *Nature*, **631**, 232-239, https://doi.org/10.1038/s41586-024-07488-9.
2. Burgess, D. R. (2004) NIST SRD 46. Critically Selected Stability Constants of Metal Complexes: Version 8.0 for Windows, National Institute of Standards and Technology [Internet], https://doi.org/10.18434/M32154.
3. Maeshima, K., Matsuda, T., Shindo, Y., Imamura, H., Tamura, S., Imai, R., Kawakami, S., Nagashima, R., Soga, T., Noji, H., Oka, K., and Nagai, T. (2018) A transient rise in free Mg^{2+} ions released from ATP-Mg hydrolysis contributes to mitotic chromosome condensation, *Curr. Biol.*, **28**, 444-451.e6, https://doi.org/10.1016/j.cub.2017.12.035.
4. Babot, M., Birch, A., Labarbuta, P., and Galkin, A. (2014) Characterisation of the active/de-active transition of mitochondrial complex I, *Biochim. Biophys. Acta*, **1837**, 1083-1092, https://doi.org/10.1016/j.bbabio.2014.02.018.

5. Zubareva, V. M., Lapashina, A. S., Shugaeva, T. E., Litvin, A. V., and Feniouk, B. A. (2020) Rotary ion-translocating ATPases/ATP synthases: diversity, similarities, and differences, *Biochemistry (Moscow)*, **85**, 1613-1630, https://doi.org/10.1134/S0006297920120135.
6. Klingenberg, M. (2008) The ADP and ATP transport in mitochondria and its carrier, *Biochim. Biophys. Acta*, **1778**, 1978-2021, https://doi.org/10.1016/j.bbamem.2008.04.011.
7. Penin, F., Deléage, G., Godinot, C., and Gautheron, D. C. (1986) Efficient reconstitution of mitochondrial energy-transfer reactions from depleted membranes and F1-ATPase as a function of the amount of bound oligomycin sensitivity-conferring protein (OSCP), *Biochim. Biophys. Acta*, **852**, 55-67, https://doi.org/10.1016/0005-2728(86)90056-3.
8. Matsuno-Yagi, A., and Hatefi, Y. (1988) Estimation of the turnover number of bovine heart F0F1 complexes for ATP synthesis, *Biochemistry*, **27**, 335-340, https://doi.org/10.1021/bi00401a050.
9. Morgenstern, M., Peikert, C. D., Lübbert, P., Suppanz, I., Klemm, C., Alka, O., Steiert, C., Naumenko, N., Schendzielorz, A., Melchionda, L., Mühlhäuser, W. W. D., Knapp, B., Busch, J. D., Stiller, S. B., Dannenmaier, S., Lindau, C., Licheva, M., Eickhorst, C., Galbusera, R., Zerbes, R. M., Ryan, M. T., Kraft, C., Kozjak-Pavlovic, V., Drepper, F., Dennerlein, S., Oeljeklaus, S., Pfanner, N., Wiedemann, N., and Warscheid, B. (2021) Quantitative high-confidence human mitochondrial proteome and its dynamics in cellular context, *Cell Metab.*, **33**, 2464-2483.e18, https://doi.org/10.1016/j.cmet.2021.11.001.
10. Lapashina, A. S., and Feniouk, B. A. (2018) ADP-Inhibition of H+-FOF1-ATP Synthase, *Biochemistry (Moscow)*, **83**, 1141-1160, https://doi.org/10.1134/S0006297918100012.
11. Campanella, M., Casswell, E., Chong, S., Farah, Z., Wieckowski, M. R., Abramov, A. Y., Tinker, A., and Duchen, M. R. (2008) Regulation of mitochondrial structure and function by the F1Fo-ATPase inhibitor protein, IF1, *Cell Metab.*, **8**, 13-25, https://doi.org/10.1016/j.cmet.2008.06.001.
12. Galkina, K. V., Zubareva, V. M., Kashko, N. D., Lapashina, A. S., Markova, O. V., Feniouk, B. A., and Knorre, D. A. (2022) Heterogeneity of starved yeast cells in IF1 levels suggests the role of this protein *in vivo*, *Front. Microbiol.*, **13**, 816622, https://doi.org/10.3389/fmicb.2022.816622.
13. Lapashina, A. S., Kashko, N. D., Zubareva, V. M., Galkina, K. V., Markova, O. V., Knorre, D. A., and Feniouk, B. A. (2022) Attenuated ADP-inhibition of FOF1 ATPase mitigates manifestations of mitochondrial dysfunction in yeast, *Biochim. Biophys. Acta*, **1863**, 148544, https://doi.org/10.1016/j.bbabio.2022.148544.
14. Pfaff, E., and Klingenberg, M. (1968) Adenine nucleotide translocation of mitochondria. 1. Specificity and control, *Eur. J. Biochem.*, **6**, 66-79, https://doi.org/10.1111/j.1432-1033.1968.tb00420.x.
15. Winkler, H. H., Bygrave, F. L., and Lehninger, A. L. (1968) Characterization of the atractyloside-sensitive adenine nucleotide transport system in rat liver mitochondria, *J. Biol. Chem.*, **243**, 20-28, https://doi.org/10.1016/s0021-9258(18)99320-8.
16. Matsuno-Yagi, A., and Hatefi, Y. (1990) Studies on the mechanism of oxidative phosphorylation. Positive cooperativity in ATP synthesis, *J. Biol. Chem.*, **265**, 82-88, https://doi.org/10.1016/S0021-9258(19)40198-1.
17. Panayiotou, C., Solaroli, N., and Karlsson, A. (2014) The many isoforms of human adenylate kinases, *Int. J. Biochem. Cell Biol.*, **49**, 75-83, https://doi.org/10.1016/j.biocel.2014.01.014.
18. Fujisawa, K. (2023) Regulation of adenine nucleotide metabolism by adenylate kinase isozymes: physiological roles and diseases, *Int. J. Mol. Sci.*, **24**, 5561, https://doi.org/10.3390/ijms24065561.
19. Miyoshi, K., Akazawa, Y., Horiguchi, T., and Noma, T. (2009) Localization of adenylate kinase 4 in mouse tissues, *Acta Histochem. Cytochem.*, **42**, 55-64, https://doi.org/10.1267/ahc.08012.
20. Liu, R., Ström, A.-L., Zhai, J., Gal, J., Bao, S., Gong, W., and Zhu, H. (2009) Enzymatically inactive adenylate kinase 4 interacts with mitochondrial ADP/ATP translocase, *Int. J. Biochem. Cell Biol.*, **41**, 1371-1380, https://doi.org/10.1016/j.biocel.2008.12.002.
21. Panayiotou, C., Solaroli, N., Johansson, M., and Karlsson, A. (2010) Evidence of an intact N-terminal translocation sequence of human mitochondrial adenylate kinase 4, *Int. J. Biochem. Cell Biol.*, **42**, 62-69, https://doi.org/10.1016/j.biocel.2009.09.007.
22. Wilson, D. E., Povey, S., and Harris, H. (1976) Adenylate kinases in man: evidence for a third locus, *Ann. Hum. Genet.*, **39**, 305-313, https://doi.org/10.1111/j.1469-1809.1976.tb00134.x.
23. Nam, K., Thodika, A. R. A., Tischlik, S., Phoeurk, C., Nagy, T. M., Schierholz, L., Ådén, J., Rogne, P., Drescher, M., Sauer-Eriksson, A. E., and Wolf-Watz, M. (2024) Magnesium induced structural reorganization in the active site of adenylate kinase, *Sci. Adv.*, **10**, eado5504, https://doi.org/10.1126/sciadv.ado5504.
24. Blair, J. M. (1970) Magnesium, potassium, and the adenylate kinase equilibrium. Magnesium as a feedback signal from the adenine nucleotide pool, *Eur. J. Biochem.*, **13**, 384-390, https://doi.org/10.1111/j.1432-1033.1970.tb00940.x.
25. Gellerich, F. N. (1992) The role of adenylate kinase in dynamic compartmentation of adenine nucleotides in the mitochondrial intermembrane space, *FEBS Lett.*, **297**, 55-58, https://doi.org/10.1016/0014-5793(92)80326-c.
26. Tomasselli, A. G., Schirmer, R. H., and Noda, L. H. (1979) Mitochondrial GTP-AMP phosphotransferase. 1. Purification and properties, *Eur. J. Biochem.*,

93, 257-262, https://doi.org/10.1111/j.1432-1033.1979.tb12818.x.

27. Seifert, E. L., Ligeti, E., Mayr, J. A., Sondheimer, N., and Hajnóczky, G. (2015) The mitochondrial phosphate carrier: role in oxidative metabolism, calcium handling and mitochondrial disease, *Biochem. Biophys. Res. Commun.*, **464**, 369-375, https://doi.org/10.1016/j.bbrc.2015.06.031.
28. Küster, U., Bohnensack, R., and Kunz, W. (1976) Control of oxidative phosphorylation by the extra-mitochondrial ATP/ADP ratio, *Biochim. Biophys. Acta*, **440**, 391-402, https://doi.org/10.1016/0005-2728(76)90073-6.
29. Davis, E. J., and Davis-van Thienen, W. I. (1978) Control of mitochondrial metabolism by the ATP/ADP ratio, *Biochem. Biophys. Res. Commun.*, **83**, 1260-1266, https://doi.org/10.1016/0006-291x(78)91357-8.
30. Lemasters, J. J., and Sowers, A. E. (1979) Phosphate dependence and atractyloside inhibition of mitochondrial oxidative phosphorylation. The ADP-ATP carrier is rate-limiting, *J. Biol. Chem.*, **254**, 1248-1251, https://doi.org/10.1016/s0021-9258(17)34194-7.
31. Slater, E. C., Rosing, J., and Mol, A. (1973) The phosphorylation potential generated by respiring mitochondria, *Biochim. Biophys. Acta*, **292**, 534-553, https://doi.org/10.1016/0005-2728(73)90003-0.
32. Groen, A. K., Wanders, R. J., Westerhoff, H. V., van der Meer, R., and Tager, J. M. (1982) Quantification of the contribution of various steps to the control of mitochondrial respiration, *J. Biol. Chem.*, **257**, 2754-2757, https://doi.org/10.1016/s0021-9258(19)81026-8.
33. Fiermonte, G., De Leonardis, F., Todisco, S., Palmieri, L., Lasorsa, F. M., and Palmieri, F. (2004) Identification of the mitochondrial ATP-Mg/Pi transporter, *J. Biol. Chem.*, **279**, 30722-30730, https://doi.org/10.1074/jbc.M400445200.
34. Hardie, D. G., and Hawley, S. A. (2001) AMP-activated protein kinase: the energy charge hypothesis revisited, *Bioessays*, **23**, 1112-1119, https://doi.org/10.1002/bies.10009.
35. Ramaiah, A., Hathaway, J. A., and Atkinson, D. E. (1964) Adenylate as a metabolic regulator. Effect on yeast phosphofructokinase kinetics, *J. Biol. Chem.*, **239**, 3619-3622, https://doi.org/10.1016/S0021-9258(18)91181-6.
36. Atkinson, D. E. (1968) Energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers, *Biochemistry*, **7**, 4030-4034, https://doi.org/10.1021/bi00851a033.
37. Hardie, D. G., Carling, D., and Sim, A. T. R. (1989) The AMP-activated protein kinase: a multisubstrate regulator of lipid metabolism, *Trends Biochem. Sci.*, **14**, 20-23, https://doi.org/10.1016/0968-0004(89)90084-4.
38. Hardie, D. G., Ross, F. A., and Hawley, S. A. (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis, *Nat. Rev. Mol. Cell Biol.*, **13**, 251-262, https://doi.org/10.1038/nrm3311.
39. Park, J. O., Rubin, S. A., Xu, Y.-F., Amador-Noguez, D., Fan, J., Shlomi, T., and Rabinowitz, J. D. (2016) Metabolite concentrations, fluxes and free energies imply efficient enzyme usage, *Nat. Chem. Biol.*, **12**, 482-489, https://doi.org/10.1038/nchembio.2077.
40. Gauthier, S., Coulpier, F., Jourdren, L., Merle, M., Beck, S., Konrad, M., Daignan-Fornier, B., and Pinson, B. (2008) Co-regulation of yeast purine and phosphate pathways in response to adenylic nucleotide variations, *Mol. Microbiol.*, **68**, 1583-1594, https://doi.org/10.1111/j.1365-2958.2008.06261.x.
41. Traut, T. W. (1994) Physiological concentrations of purines and pyrimidines, *Mol. Cell Biochem.*, **140**, 1-22, https://doi.org/10.1007/BF00928361.
42. Johnson, J. D., Mehus, J. G., Tews, K., Milavetz, B. I., and Lambeth, D. O. (1998) Genetic evidence for the expression of ATP- and GTP-specific succinyl-CoA synthetases in multicellular eucaryotes, *J. Biol. Chem.*, **273**, 27580-27586, https://doi.org/10.1074/jbc.273.42.27580.
43. Stark, R., Pasquel, F., Turcu, A., Pongratz, R. L., Roden, M., Cline, G. W., Shulman, G. I., and Kibbey, R. G. (2009) Phosphoenolpyruvate cycling via mitochondrial phosphoenolpyruvate carboxykinase links anaplerosis and mitochondrial GTP with insulin secretion, *J. Biol. Chem.*, **284**, 26578-26590, https://doi.org/10.1074/jbc.M109.011775.
44. Milon, L., Meyer, P., Chiadmi, M., Munier, A., Johansson, M., Karlsson, A., Lascu, I., Capeau, J., Janin, J., and Lacombe, M. L. (2000) The human nm23-H4 gene product is a mitochondrial nucleoside diphosphate kinase, *J. Biol. Chem.*, **275**, 14264-14272, https://doi.org/10.1074/jbc.275.19.14264.
45. Tokarska-Schlattner, M., Boissan, M., Munier, A., Borot, C., Mailleau, C., Speer, O., Schlattner, U., and Lacombe, M.-L. (2008) The nucleoside diphosphate kinase D (NM23-H4) binds the inner mitochondrial membrane with high affinity to cardiolipin and couples nucleotide transfer with respiration, *J. Biol. Chem.*, **283**, 26198-26207, https://doi.org/10.1074/jbc.M803132200.
46. Boissan, M., Dabernat, S., Peuchant, E., Schlattner, U., Lascu, I., and Lacombe, M.-L. (2009) The mammalian Nm23/NDPK family: from metastasis control to cilia movement, *Mol. Cell Biochem.*, **329**, 51-62, https://doi.org/10.1007/s11010-009-0120-7.
47. Muhonen, W. W., and Lambeth, D. O. (1995) The compartmentation of nucleoside diphosphate kinase in mitochondria, *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **110**, 211-223, https://doi.org/10.1016/0305-0491(94)00123-c.
48. Lacombe, M.-L., Tokarska-Schlattner, M., Boissan, M., and Schlattner, U. (2018) The mitochondrial nucleoside diphosphate kinase (NDPK-D/NME4), a moonlighting protein for cell homeostasis, *Lab Invest*, **98**, 582-588, https://doi.org/10.1038/s41374-017-0004-5.

49. Gordon, D. M., Lyver, E. R., Lesuisse, E., Dancis, A., and Pain, D. (2006) GTP in the mitochondrial matrix plays a crucial role in organellar iron homoeostasis, *Biochem. J.*, **400**, 163-168, https://doi.org/10.1042/BJ20060904.
50. Mansurova, S. E., Drobyshev, V. I., and Kulaev, I. S. (1972) Nucleotides of beef heart mitochondria and submitochondrial particles, *J. Bioenerg.*, **3**, 499-507, https://doi.org/10.1007/BF01539058.
51. Brierley, G., and O'Brien, R. L. (1965) Compartmentation of heart mitochondria, *J. Biol. Chem.*, **240**, 4532-4539, https://doi.org/10.1016/s0021-9258(18)97095-x.
52. Meisner, H., and Klingenberg, M. (1968) Efflux of adenine nucleotides from rat liver mitochondria, *J. Biol. Chem.*, **243**, 3631-3639, https://doi.org/10.1016/S0021-9258(19)34186-9.
53. Haynes, R. C., Jr., Picking, R. A., and Zaks, W. J. (1986) Control of mitochondrial content of adenine nucleotides by submicromolar calcium concentrations and its relationship to hormonal effects, *J. Biol. Chem.*, **261**, 16121-16125, https://doi.org/10.1016/s0021-9258(18)66686-4.
54. Gout, E., Rébeillé, F., Douce, R., and Bligny, R. (2014) Interplay of Mg2+, ADP, and ATP in the cytosol and mitochondria: unravelling the role of Mg2+ in cell respiration, *Proc. Natl. Acad. Sci. USA*, **111**, E4560-E4567, https://doi.org/10.1073/pnas.1406251111.
55. Zuurendonk, P. F., and Tager, J. M. (1974) Rapid separation of particulate components and soluble cytoplasm of isolated rat-liver cells, *Biochim. Biophys. Acta*, **333**, 393-399, https://doi.org/10.1016/0005-2728(74)90022-x.
56. Akerboom, T. P. M., Bookelman, H., Zuurendonk, P. F., Meer, R., and Tager, J. M. (1978) Intramitochondrial and extramitochondrial concentrations of adenine nucleotides and inorganic phosphate in isolated hepatocytes from fasted rats, *Eur. J. Biochem.*, **84**, 413-420, https://doi.org/10.1111/j.1432-1033.1978.tb12182.x.
57. Tischler, M. E., Hecht, P., and Williamson, J. R. (1977) Determination of mitochondrial/cytosolic metabolite gradients in isolated rat liver cells by cell disruption, *Arch. Biochem. Biophys.*, **181**, 278-293, https://doi.org/10.1016/0003-9861(77)90506-9.
58. Elbers, R., Heldt, H. W., Schmucker, P., Soboll, S., and Wiese, H. (1974) Measurement of the ATP/ADP ratio in mitochondria and in the extramitochondrial compartment by fractionation of freeze-stopped liver tissue in non-aqueous media, *Hoppe Seylers Z. Physiol. Chem.*, **355**, 378-393, https://doi.org/10.1515/bchm2.1974.355.1.378.
59. Sutton, R., and Pollak, J. K. (1978) The increasing adenine nucleotide concentration and the maturation of rat liver mitochondria during neonatal development, *Differentiation*, **12**, 15-21, https://doi.org/10.1111/j.1432-0436.1979.tb00985.x.
60. Schwenke, W. D., Soboll, S., Seitz, H. J., and Sies, H. (1981) Mitochondrial and cytosolic ATP/ADP ratios in rat liver *in vivo*, *Biochem J*, **200**, 405-408, https://doi.org/10.1042/bj2000405.
61. Soboll, S., Akerboom, T. P., Schwenke, W. D., Haase, R., and Sies, H. (1980) Mitochondrial and cytosolic ATP/ADP ratios in isolated hepatocytes. A comparison of the digitonin method and the non-aqueous fractionation procedure, *Biochem. J.*, **192**, 951-954, https://doi.org/10.1042/bj1920951.
62. Chen, W. W., Freinkman, E., Wang, T., Birsoy, K., and Sabatini, D. M. (2016) Absolute quantification of matrix metabolites reveals the dynamics of mitochondrial metabolism, *Cell*, **166**, 1324-1337.e11, https://doi.org/10.1016/j.cell.2016.07.040.
63. Soboll, S., and Bünger, R. (1981) Compartmentation of adenine nucleotides in the isolated working guinea pig heart stimulated by noradrenaline, *Hoppe Seylers Z. Physiol. Chem.*, **362**, 125-132, https://doi.org/10.1515/bchm2.1981.362.1.125.
64. Soboll, S., Scholz, R., and Heldt, H. W. (1978) Subcellular metabolite concentrations. Dependence of mitochondrial and cytosolic ATP systems on the metabolic state of perfused rat liver, *Eur J Biochem*, **87**, 377-390, https://doi.org/10.1111/j.1432-1033.1978.tb12387.x.
65. Pfaller, W., Guder, W. G., Gstraunthaler, G., Kotanko, P., Jehart, I., and Pürschel, S. (1984) Compartmentation of ATP within renal proximal tubular cells, *Biochim. Biophys. Acta*, **805**, 152-157, https://doi.org/10.1016/0167-4889(84)90162-9.
66. Yoshida, T., Alfaqaan, S., Sasaoka, N., and Imamura, H. (2017) Application of FRET-based biosensor "ATeam" for visualization of ATP levels in the mitochondrial matrix of living mammalian cells, *Methods Mol. Biol.*, **1567**, 231-243, https://doi.org/10.1007/978-1-4939-6824-4_14.
67. Kioka, H., Kato, H., Fujikawa, M., Tsukamoto, O., Suzuki, T., Imamura, H., Nakano, A., Higo, S., Yamazaki, S., Matsuzaki, T., Takafuji, K., Asanuma, H., Asakura, M., Minamino, T., Shintani, Y., Yoshida, M., Noji, H., Kitakaze, M., Komuro, I., Asano, Y., and Takashima, S. (2014) Evaluation of intramitochondrial ATP levels identifies G0/G1 switch gene 2 as a positive regulator of oxidative phosphorylation, *Proc. Natl. Acad. Sci. USA*, **111**, 273-278, https://doi.org/10.1073/pnas.1318547111.
68. Morciano, G., Imamura, H., Patergnani, S., Pedriali, G., Giorgi, C., and Pinton, P. (2020) Measurement of ATP concentrations in mitochondria of living cells using luminescence and fluorescence approaches, *Methods Cell Biol.*, **155**, 199-219, https://doi.org/10.1016/bs.mcb.2019.10.007.
69. Imamura, H., Nhat, K. P. H., Togawa, H., Saito, K., Iino, R., Kato-Yamada, Y., Nagai, T., and Noji, H. (2009) Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators, *Proc. Natl. Acad. Sci. USA*, **106**, 15651-15656, https://doi.org/10.1073/pnas.0904764106.

70. Gnaiger, E., Aasander Frostner, E., Abdul Karim, N., Abdel-Rahman, E. A., Abumrad, N. A., Acuna-Castroviejo, D., Adiele, R. C., et al. (2020) Mitochondrial physiology, *Bioenerg. Commun.*, https://doi.org/10.26124/bec:2020-0001.v1.
71. Korshunov, S. S., Skulachev, V. P., and Starkov, A. A. (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria, *FEBS Lett.*, **416**, 15-18, https://doi.org/10.1016/s0014-5793(97)01159-9.
72. Skulachev, V. P. (1996) Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants, *Q. Rev. Biophys.*, **29**, 169-202, https://doi.org/10.1017/s0033583500005795.
73. Depaoli, M. R., Karsten, F., Madreiter-Sokolowski, C. T., Klec, C., Gottschalk, B., Bischof, H., Eroglu, E., Waldeck-Weiermair, M., Simmen, T., Graier, W. F., and Malli, R. (2018) Real-time imaging of mitochondrial ATP dynamics reveals the metabolic setting of single cells, *Cell Rep.*, **25**, 501-512.e3, https://doi.org/10.1016/j.celrep.2018.09.027.
74. Suzuki, R., Hotta, K., and Oka, K. (2018) Transitional correlation between inner-membrane potential and ATP levels of neuronal mitochondria, *Sci. Rep.*, **8**, 2993, https://doi.org/10.1038/s41598-018-21109-2.
75. Choi, J., Matoba, N., Setoyama, D., Watanabe, D., Ohnishi, Y., Yasui, R., Kitai, Y., Oomachi, A., Kotobuki, Y., Nishiya, Y., Pieper, M. P., Imamura, H., Yanagita, M., and Yamamoto, M. (2023) The SGLT2 inhibitor empagliflozin improves cardiac energy status via mitochondrial ATP production in diabetic mice, *Commun. Biol.*, **6**, 278, https://doi.org/10.1038/s42003-023-04663-y.
76. Botman, D., van Heerden, J. H., and Teusink, B. (2020) An improved ATP FRET sensor for yeast shows heterogeneity during nutrient transitions, *ACS Sens.*, **5**, 814-822, https://doi.org/10.1021/acssensors.9b02475.
77. Gostimskaya, I. S., Grivennikova, V. G., Zharova, T. V., Bakeeva, L. E., and Vinogradov, A. D. (2003) In situ assay of the intramitochondrial enzymes: use of alamethicin for permeabilization of mitochondria, *Anal. Biochem.*, **313**, 46-52, https://doi.org/10.1016/s0003-2697(02)00534-1.
78. Lapashina, A. S., Galkina, K. V., Markova, O. V., Knorre, D. A., and Feniouk, B. A. (2023) Observations of ATP levels in mitochondria and whole yeast cells using fluorescent probes, in *Receptors and Intracellular Signaling Collected Articles* (Berezhnov A. V., and Zinchenko, V. P., eds) pp. 352-357 [in Russian].
79. Yaginuma, H., Kawai, S., Tabata, K. V., Tomiyama, K., Kakizuka, A., Komatsuzaki, T., Noji, H., and Imamura, H. (2014) Diversity in ATP concentrations in a single bacterial cell population revealed by quantitative single-cell imaging, *Sci. Rep.*, **4**, 6522, https://doi.org/10.1038/srep06522.
80. Arai, S., Kriszt, R., Harada, K., Looi, L.-S., Matsuda, S., Wongso, D., Suo, S., Ishiura, S., Tseng, Y.-H., Raghunath, M., Ito, T., Tsuboi, T., and Kitaguchi, T. (2018) RGB-color intensiometric indicators to visualize spatiotemporal dynamics of ATP in single cells, *Angew. Chem. Int. Ed. Engl.*, **57**, 10873-10878, https://doi.org/10.1002/anie.201804304.
81. Takaine, M., Ueno, M., Kitamura, K., Imamura, H., and Yoshida, S. (2019) Reliable imaging of ATP in living budding and fission yeast, *J. Cell Sci.*, **132**, jcs230649, https://doi.org/10.1242/jcs.230649.
82. Sawai, A., Taniguchi, T., Noguchi, K., Seike, T., Okahashi, N., Takaine, M., and Matsuda, F. (2025) ATP supply from cytosol to mitochondria is an additional role of aerobic glycolysis to prevent programmed cell death by maintenance of mitochondrial membrane potential, *Metabolites*, **15**, 461, https://doi.org/10.3390/metabo15070461.
83. Tsuno, S., Harada, K., Horikoshi, M., Mita, M., Kitaguchi, T., Hirai, M. Y., Matsumoto, M., and Tsuboi, T. (2024) Mitochondrial ATP concentration decreases immediately after glucose administration to glucose-deprived hepatocytes, *FEBS Open Bio*, **14**, 79-95, https://doi.org/10.1002/2211-5463.13744.
84. Berg, J., Hung, Y. P., and Yellen, G. (2009) A genetically encoded fluorescent reporter of ATP:ADP ratio, *Nat. Methods*, **6**, 161-166, https://doi.org/10.1038/nmeth.1288.
85. Tantama, M., Martínez-François, J. R., Mongeon, R., and Yellen, G. (2013) Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio, *Nat. Commun.*, **4**, 2550, https://doi.org/10.1038/ncomms3550.
86. Zhang, M., Yang, B., Zhang, J., Song, Y., Wang, W., Li, N., Wang, Y., Li, W., and Wang, J. (2022) Monitoring the dynamic regulation of the mitochondrial GTP-to-GDP ratio with a genetically encoded fluorescent biosensor, *Angew. Chem. Int. Ed. Engl.*, **61**, e202201266, https://doi.org/10.1002/anie.202201266.