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When too much ATP is bad for protein synthesis

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Abstract

Adenosine triphosphate (ATP) is the energy currency of living cells. Even though ATP powers virtually all energy-dependent activity, most cellular ATP is utilized in protein synthesis via tRNA aminoacylation and GTP regeneration. Magnesium (Mg^{2+}), the most common divalent cation in living cells, plays crucial roles in protein synthesis by maintaining the structure of ribosomes, participating in the biochemistry of translation initiation and functioning as a counter-ion for ATP. A non-physiological increase in ATP levels hinders growth in cells experiencing Mg^{2+} limitation because ATP is the most abundant nucleotide triphosphate in the cell and Mg^{2+} is also required for the stabilization of the cytoplasmic membrane and as a cofactor for essential enzymes. We propose that organisms cope with Mg^{2+} limitation by decreasing ATP levels and ribosome production, thereby reallocating Mg^{2+} to indispensable cellular processes.

Graphical Abstract

Keywords

magnesium; PhoP/PhoQ; protein synthesis; ribosome; stress response

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Introduction

Growth and replication requires energy in all organisms. Although organisms often differ in the ways they obtain energy, all energy is ultimately converted into adenosine triphosphate (ATP), which serves as the universal energy currency of the cell. ATP powers the synthesis of proteins, nucleic acids and all other building blocks that make up organisms. In addition, ATP fuels transport of molecules across the membrane, cell movement and cell division [1].

Given the central role that ATP plays in a cell, it seems paradoxical that high levels of ATP can be detrimental. However, in the central nervous system, binding of extracellular ATP to purinergic receptors triggers an inflammatory response that leads to homeostatic regulation in small doses but to neuron dysfunction and death in large doses [2,3]. And in the bacterial pathogen *Salmonella enterica* serovar Typhimurium, high levels of intracellular ATP can result in decreased virulence and growth arrest in media containing low magnesium (Mg^{2+}) [4-6]. Because ATP has a high affinity for Mg^{2+} [7] (Table 1), a non-physiological increase in ATP levels can deplete free cytosolic Mg^{2+} pools, and thus, disrupt essential Mg^{2+} -dependent cellular processes including protein synthesis [8,9].

Here we explore the connection between ATP, Mg^{2+} and protein synthesis, which uses most of the Mg^{2+} and energy in the cell. We suggest that cells respond to Mg^{2+} limitation resulting from high ATP levels by reducing the production of ATP and ribosomes, which constitute the two largest depots of chelated Mg^{2+} (Table 1). By sacrificing a portion of its energy and protein synthesis potential, a cell may regain growth by repurposing Mg^{2+} from ATP complexes and dysfunctional ribosomes to essential biological processes (Fig. 1).

Protein synthesis, ATP and Mg^{2+}

Proteins carry out the vast majority of the biochemical activities in living cells. Despite the increased appreciation of RNA's enzymatic and regulatory abilities [10], proteins are largely responsible for the structural, transport and catalytic properties of all cells [1]. Consuming over 70% of the ATP pools utilized by biosynthetic processes, translation is the most expensive anabolic activity [11]. In bacteria, the rate of protein synthesis is determined by the number of ribosomes [12]. Cells coordinate ribosome production with ATP availability [13] because translation is an energy-consuming process [14,15]. In other words, there is a connection between ATP, ribosome biogenesis and protein synthesis.

In 1954, Zamecnik and colleagues demonstrated that the majority of protein synthesis occurs at the ribosome [16]. It was later shown that Mg^{2+} removal from the growth medium of cultures from the bacterium *Escherichia coli* resulted in the loss of ribosomes, concomitantly with a halt in translation and growth arrest [17]. Mg^{2+} depletion did not reduce cell viability, and the re-addition of Mg^{2+} to the growth medium led to the regeneration of ribosomes, re-initiation of protein synthesis and growth [17]. *In vitro* studies established that ribosomes are composed of subunits that dissociate upon Mg^{2+} removal [18-20].

Ribosomes are the largest and the most abundant nucleoprotein complexes in the cell [21]. During ribosome biogenesis, strands of ribosomal RNAs (rRNAs) carrying a strong negative

charge from phosphate groups need to be compacted into a stable structure, further stabilized by ribosomal proteins. To reduce electrostatic repulsion, rRNAs chelate an unprecedented amount of cations, mostly Mg^{2+} [22]. A single ribosome chelates > 170 Mg^{2+} ions [23] and a bacterial cell can contain up to 70,000 ribosome particles [24] (Table 1). Assuming a cell volume of $1 \mu m^3$ [25], the entire pool of 70,000 ribosomes chelates at least 12 mM Mg^{2+} , the equivalent of 25% of the total Mg^{2+} in the cell (Table 1 and Chart 1). Indeed, experiments carried out in *Bacillus subtilis* indicate that there is a direct correlation between Mg^{2+} content and number of ribosomes [26]. Given that total Mg^{2+} levels vary from 30 mM in mammalian tissues [8] to 75 -100 mM in bacteria [26,27] (of which 50 -65 mM are intracellular, and 25 -35 mM, or one third, are bound to the components of the cell wall [28]), the translation apparatus traps a significant fraction of the intracellular Mg^{2+} . In addition to maintaining the structural integrity of the ribosomal subunits, Mg^{2+} is required for all steps in translation, especially initiation [22], as well as for several biochemical reactions generating substrates for protein synthesis [29,30]. Mg^{2+} ions directly involved in catalysis are often tightly bound by the corresponding enzymes [31-33]. However, many enzymes require Mg^{2+} ions at low affinity sites [27]. Loosely bound Mg^{2+} can only stay in such sites if the concentration of free cytoplasmic Mg^{2+} remains in the 1- 5 mM range [8,27]. Even though Mg^{2+} is the most abundant divalent cation in living cells, only 5% is present as free Mg^{2+} [34-36]. Approximately 50% of the total Mg^{2+} is bound to nucleotide triphosphates (NTPs), primarily ATP [8] Table 1 and Chart 1). A significant fraction of intracellular Mg^{2+} is associated with DNA and non-ribosomal RNAs and proteins [8] (Chart 1).

ATP plays a central role in cellular physiology. As the chemical energy of cells, ATP powers hundreds of energy-dependent enzymatic reactions, including those giving rise to other NTPs [37-39]. Remarkably, the majority of the ATP in the cell fuels protein synthesis: ATP is used directly in the charging of tRNAs [30] and indirectly as a pyrophosphate donor in the synthesis of guanosine triphosphate (GTP) [37-39], which is used by elongation factors during the translocation of ribosomes along messenger RNAs (mRNAs; [40]).

The physiological connection between ATP and translation is clear from the way enteric bacteria regulate ribosome levels. Ribosomal promoters are uniquely sensitive to the cytosolic levels of the initiating NTP due to the short half-lives of their open complexes [13]. By using ATP as the initiating nucleotide of almost all rRNA transcripts, bacteria establish a physiological link between their energy status and rRNA synthesis, the rate-limiting step in ribosome biogenesis [14,15]. Synthesis of ribosomal proteins is coordinated with the availability of free rRNA. When the amount of ribosomal proteins exceeds that of rRNA, ribosomal proteins repress their own expression by binding to the mRNA that specifies them and preventing translation and/or attenuating transcription [41].

Bacteria also coordinate rRNA transcription with nutrient availability through another small molecule: guanosine penta/tetraphosphate ((p)ppGpp). Synthesis of (p)ppGpp is stimulated when cells experience a number of nutritional stresses [42-44]. For example, when an uncharged tRNA binds to the ribosomal A site, signaling a shortage of amino acids, (p)ppGpp is synthesized by the RelA protein [45]. In enteric bacteria, (p)ppGpp binds to RNA polymerase and destabilizes the transcription initiation complex [46-48]. Because the

open promoter complexes of genes encoding rRNA and ribosomal proteins are intrinsically unstable, they are particularly sensitive to inhibition by (p)ppGpp [47,49]. This mechanism prevents the accumulation of ribosomes that would not be able to carry out protein synthesis if amino acids were not available, even when the energy requirements of the cell were met.

In the gram-positive bacterium *Bacillus subtilis*, GTP is the initiating nucleotide in the majority of the rRNA operons. Therefore, ATP appears to regulate rRNA transcription initiation in *B. subtilis* indirectly, that is, by virtue of its effect on GTP biosynthesis [37-39]. In addition, (p)ppGpp production depletes cytosolic GTP pools, which would also decrease rRNA transcription [50].

Organisms harbor a variety of proteins that promote ribosome shut down or reprogramming as well as the rescue of ribosomes stalled due to environmental stresses or properties of the mRNA being translated [51]. Moreover, they have evolved a number of mechanisms to coordinate translation status and ribosome levels with the availability of both energy and amino acid precursors for protein synthesis [13]. However, it remains unclear how cells adjust ribosome biosynthesis with the availability of Mg^{2+} ions, which are required for both ribosome stabilization and function.

A regulatory system that responds to extracytoplasmic Mg^{2+}

Some 40 years after the physiological connection between Mg^{2+} and protein synthesis was established, the first signal transduction system that responds to Mg^{2+} as primary signal was identified in *S. enterica* serovar Typhimurium [52]. This system consists of the sensor of extracytoplasmic Mg^{2+} PhoQ and its cognate transcriptional regulator PhoP [53,54]. When *Salmonella* experiences a low Mg^{2+} environment, PhoQ promotes the active (i.e., phosphorylated) state of PhoP (PhoP-P), which binds to its target promoters with higher affinity than unphosphorylated PhoP [52,55,56]. Binding of PhoP-P to target promoters enables recruitment of RNA polymerase and transcription initiation of PhoP-activated genes [57]. By contrast, when *Salmonella* experiences high Mg^{2+} , PhoQ promotes the non-phosphorylated state of PhoP, which does not bind to DNA *in vivo* [55].

The PhoP/PhoQ system plays an essential role in Mg^{2+} homeostasis because, first, *phoP* and *phoQ* null mutants fail to grow in low Mg^{2+} [52,58]. Second, PhoP-P promotes transcription of two of the three Mg^{2+} transporters encoded in the *Salmonella* genome [52,58], and negatively regulates the activity of the third Mg^{2+} transporter [59]. Third, as discussed above, the sensor PhoQ is activated when surrounding Mg^{2+} decreases beyond a certain level [52,60]. And fourth, the PhoP/PhoQ system governs expression of genes responsible for a variety of chemical modifications of the phosphate residues in the lipopolysaccharide (LPS) portion of the outer membrane [28,61-64]. These modifications would make the Mg^{2+} ions normally neutralizing the phosphate residues available for other Mg^{2+} -dependent functions [65].

The PhoP/PhoQ system is found in a wide range of gram-negative bacterial species [66-73]. Its role in governing the adaptation to low Mg^{2+} conditions is conserved within the family Enterobacteriaceae [57]. That is, in all species where PhoQ responds to low Mg^{2+} by activating PhoP-dependent transcription, the PhoP protein controls expression of one or

more Mg^{2+} transporter gene(s). However, the identity of the Mg^{2+} transporter regulated by PhoP often differs across species [58,66,73,74].

The *phoP* and *phoQ* genes are ancestral to enteric bacteria [53,74]. By contrast, the majority of the genes regulated by PhoP are horizontally acquired and thus differ across related enteric species [57]. Some of these genes encode proteins mediating virulence [75,76] or novel forms of surface motility [77] whereas others specify Mg^{2+} transporters or proteins participating in other aspects of Mg^{2+} homeostasis [5,78].

RNA sensors for cytoplasmic Mg^{2+}

How does an organism achieve differential expression of PhoP-activated genes between those that play a role in Mg^{2+} homeostasis and those that don't? A way *Salmonella* does so is by having the leader regions of individual PhoP-dependent mRNAs respond to cytosolic signals that determine whether transcription terminates within the leader or continues into the associated coding region. For example, the leader region of the transcript corresponding to the PhoP-activated Mg^{2+} transporter gene *mgtA* operates as a Mg^{2+} -sensing device that enables bacteria to express a gene directly involved in cytoplasmic Mg^{2+} homeostasis (i.e., the Mg^{2+} transporter MgtA) in response to cytoplasmic Mg^{2+} [79]. Low extracytoplasmic Mg^{2+} sensed by the PhoQ protein promotes PhoP-dependent transcription initiation at the *mgtA* promoter. However, RNA polymerase does not proceed into the *mgtA* coding region unless cytoplasmic Mg^{2+} levels drop below a certain threshold. When this happens, the *mgtA* leader mRNA adopts a conformation that advances transcription elongation into the *mgtA* coding region. By contrast, when cytosolic Mg^{2+} remains high, the conformation of the *mgtA* leader mRNA promotes transcription termination within the leader by the Rho protein [80,81].

Mg^{2+} -responding leader RNAs have been identified in transcripts corresponding to other Mg^{2+} transporter genes. For example, the M-box Mg^{2+} riboswitch that precedes the *mgtE* coding region in the gram-positive bacterium *B. subtilis* adopts a compact structure in the presence of Mg^{2+} , thereby reducing transcription elongation into the coding region [82]. The M-box riboswitch has been identified in the leader regions of transcripts corresponding to other Mg^{2+} transporters in a variety of bacterial species [82,83], including the PhoP-activated *mgtE* homolog of the gram-negative species *Serratia marcescens* [73].

The leader region of the PhoP-dependent *mgtCBR* operon from *Salmonella* also responds to cytosolic Mg^{2+} [84], which makes physiological sense given that the *mgtB* gene specifies a Mg^{2+} transporter [85] and the *mgtC* gene product inhibits *Salmonella*'s own F_1F_0 ATP synthase [5], which is responsible for making the majority of the ATP in the cell. (The *mgtR* gene specifies a short peptide that promotes the degradation of the MgtC protein; [86]). It is presently unclear whether the *mgtCBR* leader mRNA senses cytoplasmic Mg^{2+} directly, like the *mgtA* leader mRNA [79], or indirectly. That is, cytoplasmic Mg^{2+} may modulate the translation of one or both of the two short open reading frames (ORFs) present within the *mgtCBR* leader region, which, in turn, would determine which of the alternative RNA conformation forms to favor transcription elongation into the associated coding region or termination within the leader [4,87]. Located near the 5' end of the leader, the *mgtM* ORF is part of a region mediating the response to cytosolic ATP (i.e., high ATP promotes

transcription elongation into the associated coding region) [87]. The other ORF, designated *mgtP*, harbors three consecutive proline codons that enable the response to conditions that lower the levels of proline-charged tRNA^{Pro}, such as hyperosmotic stress [87,88].

In sum, the use of mRNA leaders to sense Mg²⁺ enables bacteria to vary expression of Mg²⁺ transporters in response to cytoplasmic Mg²⁺ even under conditions of constant extracytoplasmic Mg²⁺.

Cytosolic Mg²⁺ tunes the activity of a regulatory system that responds to extracytoplasmic Mg²⁺

By responding to cytoplasmic Mg²⁺, the *mgtA* leader mRNA makes a critical contribution to Mg²⁺ homeostasis beyond that exerted directly on the expression of the Mg²⁺ transporter MgtA. This is because MgtA-mediated Mg²⁺ transport from the periplasm into the cytoplasm reduces the periplasmic Mg²⁺ concentration, thereby removing an inhibitory signal for the sensor PhoQ, which, in turn, increases the levels of PhoP-P [89]. In this way, when cytosolic Mg²⁺ levels drop below a certain threshold, the *mgtA* leader mRNA promotes a significant increase in PhoP-P levels [89], and the resulting transcription of a subset of PhoP-activated genes harboring promoters with low affinity binding sites for PhoP-P [90].

Some PhoP-activated promoters are fully activated by the amount of PhoP-P generated shortly after *Salmonella* experiences the PhoQ-inducing condition low Mg²⁺ [56]. By contrast, other PhoP-activated promoters require higher levels of PhoP-P, which are only achieved when the Mg²⁺ transporter MgtA is made [89]. The MgtA-dependent PhoP-activated genes include *pagM*, which enables surface migration and replication in low Mg²⁺ conditions [77]. This regulatory strategy enables the specific response to cytoplasmic Mg²⁺ because: first, mildly acidic pH does not promote high PhoP-P levels [89] despite also activating PhoQ [91]; second, the *mgtA* leader mRNA does not respond to protons; and third, the MgtA protein is not known to transport protons. This regulatory design enables bacteria to incorporate information about the cytoplasmic Mg²⁺ status into the activation level of the major regulator of Mg²⁺ homeostasis.

The physiological response to a decrease in cytosolic Mg²⁺

Many cellular processes display a strict dependency on Mg²⁺ including enzymatic reactions and translation initiation. Then, how can a cell continue to carry out these processes when it runs out of free cytosolic Mg²⁺? On the one hand, it can use Mg²⁺ transporters to import extracytoplasmic Mg²⁺ into the cytosol [92]. That the expression of the *mgtA*, *mgtCBR* and *mgtE* Mg²⁺ transporter genes responds to cytosolic Mg²⁺ suggests that the corresponding gene products help bacteria cope with low cytosolic Mg²⁺. In this context, the PhoP-dependent modification of the LPS is proposed to make the Mg²⁺ ions that normally neutralize the LPS available for transport into the cytoplasm by the PhoP-dependent Mg²⁺ transporter(s) [65]. It has been estimated that about one third of the total Mg²⁺ content of *E. coli* is bound to the LPS, suggesting that the LPS serves as a Mg²⁺ reservoir [28].

On the other hand, a cell can redistribute the Mg^{2+} that it already has in the cytosol (Fig. 1). ATP and other NTPs exist as Mg^{2+} salts in living cells [93,94]. We now propose that by lowering the levels of ATP and, consequently, of other ATP-derived NTPs, organisms free cytosolic Mg^{2+} for use in other cellular processes (Fig. 1). In support of this notion, the MgtC protein inhibits the F_1F_o ATP synthase thereby reducing ATP levels [5]. Moreover, spontaneous mutations that restored growth to a mutant *B. subtilis* requiring unusually high Mg^{2+} concentrations for growth due to mutations in multiple Mg^{2+} transporters mapped to the genes specifying subunits of the F_1F_o ATP synthase [95].

Because ribosomes require large amounts of Mg^{2+} ions for their structural stabilization and function [16,18-20,22,23], a reduction of ATP would also turn off ribosome biosynthesis [13], thereby preventing wasteful allocation of free Mg^{2+} for ribosome stabilization (Fig. 1). Furthermore, a decrease in *de novo* ribosome biosynthesis followed by the degradation of existing ribosomes [96] would liberate additional Mg^{2+} ions, allowing cells to redistribute Mg^{2+} to indispensable cellular processes such as translation, membrane transport and biogenesis, and Mg^{2+} -dependent enzymes including kinases, phosphatases, adenylate cyclases, enolases, isocitrate lyases and glutamate synthases (see table 2 in [8]).

The hypothesis presented above implies that an inability to lower ATP levels would have dire repercussions for cells experiencing low cytosolic Mg^{2+} . Given that ATP has a high binding affinity for Mg^{2+} ions (Table 1), high cytosolic ATP would chelate Mg^{2+} ions, making them unavailable for other cellular processes, particularly translation. The decrease in protein synthesis would reduce the rate of ATP consumption [11,15], thus creating a cycle of ATP accumulation and further promoting chelation of cytosolic Mg^{2+} .

Organisms appear to use a variety of strategies to overcome the burden of too much ATP. For example, the PhoP-dependent MgtC protein directly inhibits the activity of the F_1F_o ATPase in *Salmonella* [5]. The MgtC protein is present in evolutionarily distant organisms that share two properties: the ability to grow in low Mg^{2+} and the capacity to survive within a mildly acidic phagosome inside eukaryotic cells [78,97-100]. The latter condition can increase the proton motive force and result in non-physiologically high ATP levels. And in *E. coli*, the PhoP/PhoQ system represses cellular respiration indirectly [101]. A switch from the highest ATP-producing metabolic pathway to an alternative, low ATP-yielding form of metabolism would prevent the accumulation of toxic levels of ATP. Thus, cells modify their metabolism and modulate the activity of the main ATP-generating machinery when experiencing low Mg^{2+} .

In sum, organisms choose to surrender a part of its ATP and ribosome pools to recover free Mg^{2+} for essential cellular processes.

Concluding remarks

The physiological connection between ATP, ribosome biogenesis and protein synthesis indicates that cells coordinate their translation status, ATP levels and cytosolic Mg^{2+} availability. The identification of multi-layered, genetically encoded adaptive responses to Mg^{2+} limitation provides an explanation to the 50-year old discovery that *E. coli* cells undergo ribosome depletion when experiencing Mg^{2+} deprivation [17]. We postulate that

while its implementation may differ across species, the solution to cytosolic Mg^{2+} limitation will be highly conserved: enhanced importation of extracellular Mg^{2+} and redistribution of the intracellular Mg^{2+} pool.

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Abbreviations

Mg^{2+}	Magnesium
ATP	adenosine triphosphate
GTP	guanosine triphosphate
NTP	nucleoside triphosphate
(p)ppGpp	guanosine penta/tetraphosphate
rRNA	ribosomal RNA
tRNA	transport RNA
mRNA	messenger RNA
LPS	lipopolysaccharide
PhoPP	phosphorylated PhoP protein

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Highlights

- Cell's energy and protein synthesis potentials depend on the availability of Mg^{2+}
- Bacteria sense and respond to various degrees of Mg^{2+} limitation
- Non-physiological increase in ATP disrupts essential Mg^{2+} -dependent processes
- Cell restores free Mg^{2+} levels by shutting down synthesis of ATP and ribosomes

Fig. 1.

The interconnection between translation, ATP levels, ribosome biogenesis and Mg^{2+} under different physiological states.

The leftmost panel shows the distribution of Mg^{2+} between the free state (blue) and bound (grey) to ATP or ribosomes when Mg^{2+} is not limited. Ribosomes and ATP are plentiful, protein synthesis is active, and cells grow fast. The middle panel shows distribution of Mg^{2+} when a cell experiences Mg^{2+} starvation. Mg^{2+} is trapped within high-affinity complexes with ATP and inactive (grey) ribosomes. Translational arrest leads to accumulation of ATP. Despite the large amount of ribosomes and high levels of energy in the cell, protein synthesis is suspended due to lack of free Mg^{2+} , and cells stop growing. The rightmost panel shows distribution of Mg^{2+} when cell adapt to Mg^{2+} limitation. As the levels of ATP and ribosomes decrease, the free Mg^{2+} necessary for translation is recovered. Protein synthesis and cell growth are resumed. The size of the various geometrical figures reflects the concentrations and/or numbers of total and free Mg^{2+} , ATP and ribosomes.

Chart 1. Distribution of intracellular Mg^{2+} in the cell

The relative amounts of Mg^{2+} chelated by various cellular components was calculated based on the following assumptions: the total intracellular Mg^{2+} - 50 mM (two thirds of 75 mM, the amount of the total Mg^{2+} calculated for bacteria [26]; assuming that the remaining one third is associated with components of the cell wall, such as LPS [28] or teichoic acids [110]); Mg^{2+} chelated by nucleotide triphosphates – 24 mM (~9 mM bound by ATP, ~8 mM bound by UTP, ~4.5 mM bound by GTP and ~2.5 mM bound by CTP, assuming rapidly growing bacterial cell, see Table 1); Mg^{2+} chelated by ribosomes – 12 mM (assuming rapidly growing bacterial cell, see Table 1); free Mg^{2+} - 2mM [35, 27].

Table 1Intracellular levels, affinities to Mg^{2+} and calculated amount of chelated Mg^{2+} for selected compounds

	Intracellular concentration	Affinity to Mg^{2+} (Km)	Chelated Mg^{2+}
Ribosomes	7 to 70 μ M, or 7,000 to 70,000 particles per cell [102]	N/A. ~ 2 mM are required for maximum activity <i>in vitro</i> [103]	1.2-12 mM [102]
ATP	9.6 mM (Glucose - fed <i>E. coli</i> , [104])	38- 80 μ M [105] [106]	> 9 mM
ADP	0.55 mM (Glucose - fed <i>E. coli</i> , [104])	300 μ M [106]	< 0.2 mM
AMP	0.28 mM (Glucose - fed <i>E. coli</i> , [104])	10 mM [106]	N/A
UTP	8.3 mM (Glucose - fed <i>E. coli</i> , [104])	69 μ M [107]	> 8 mM
GTP	4.9 mM (Glucose - fed <i>E. coli</i> , [104])	Similar to the one of ATP [106]	> 4.5 mM
CTP	2.7 mM (Glucose - fed <i>E. coli</i> , [104])	Similar to the one of ATP [106]	> 2.5 mM
PPi (inorganic pyrophosphate)	~0.2 mM [108] to 0.5 mM [109]	~ 24 μ M [107]	0.2 to 0.5 mM