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How mechanical energy of phosphorylating enzymes transforms into the energy of chemical bonds?

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Recent discovery of huge magnesium isotope effect in the rate of enzymatic synthesis of ATP offers a new insight on the mechanochemistry of enzymes as the molecular machines. The activity of magnesium-dependent enzymes (ATPase, phosphocreatine, phosphoglycerate kinases), in which Mg^{2+} ion has a magnetic isotope nucleus ^{25}Mg, was found to be two or three times higher than that of enzymes in which the Mg^{2+} ion has nonmagnetic, spinless isotopic nuclei ^{24}Mg or ^{26}Mg. This isotope effect demonstrates unambiguously that the ATP synthesis is a spin-dependent process in which the Mg^{2+} ion is a reagent. It transforms mechanical energy of unequilibrium conformations of enzyme macromolecule into the energy of the P–O bond in ATP.

Despite the great progress in our knowledge of structure and understanding of molecular dynamics and functioning of ATP synthesizing enzymes,[1–4] chemical mechanism of phosphorylation remains enigmatic,[1] there is no understanding how mechanical motion in these molecular machines results in chemical reaction of P–O bond formation, what is a mechanism of transformation of the mechanical energy accumulated in enzyme and dispersed over numerous unequilibrium conformations of its macromolecule into the energy of a newly born chemical P–O bond in ATP.

The remarkable and universal property of the phosphorylating enzymes is that they are magnesium dependent; the ability to generate ATP is lost if catalytic site of enzyme has no magnesium ions Mg^{2+}. Generally accepted mechanism of phosphorylation implies a nucleophilic addition of inorganic phosphate (in ATP synthase) or phosphate group of substrate (in kinases) to ADP. In terms of this mechanism, the Mg^{2+} ion is supposed to be an organizer of the catalytic site, it holds reagents on the reaction pathway and functions as an assistant.[5] The intrigue is that many other ions (calcium, for instance) can serve this function even better than the magnesium ion. Why evolution has selected namely magnesium for the synthesis of the energy carriers in living organisms? In order to elucidate this problem, we have used pure isotopic forms of magnesium ion: $^{24}Mg^{2+}$, $^{25}Mg^{2+}$ and $^{26}Mg^{2+}$. In this isotopic triad, only the ^{25}Mg nucleus is magnetic (nuclear spin of 5/2; magnetic moment of –0.855 Bohr magneton), two others, $^{24,26}Mg$, are spinless, nonmagnetic.

Experimental

So far as magnesium isotope effect in phosphorylation is an unexpected and unusual phenomenon it is worthy to shortly describe materials and technologies used in isotopic biochemical experiments. Isotope-containing $MgCl_2$ samples were obtained using the treatment of magnesium oxides ^{24}MgO, ^{25}MgO, ^{26}MgO and *MgO with analytically pure HCl (*Mg means magnesium with natural abundance of the three isotopes).

Myocardial mitochondria, many enzymes of which are Mg^{2+}-dependent, were tested *in vitro*. Mitochondrial pellets were suspended in a homogenization buffer and then divided into two portions: the first one was used for further estimation of the total ATP production in isolated mitochondria, in the second one the mitochondrial creatine kinase activity was measured as the amount of ^{32}P-ATP formed at the incubation with ^{32}P-phosphocreatine. To estimate a contribution of oxidative phosphorylation into the total ATP yield, 1-methylnicotineamide (MNA) or KCN have been used, which are known to suppress ATP synthase activity.

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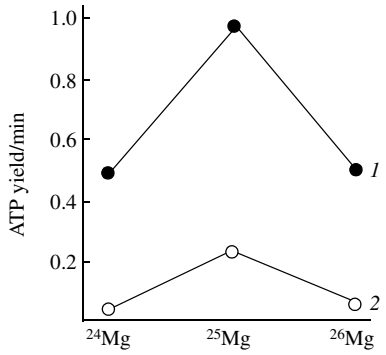


Figure 1 The rate of ATP synthesis as a function of magnesium isotopy (*1*) in native mitochondria and (*2*) in mitochondria with oxidative phosphorylation suppressed by addition of 1-methylnicotineamide. The yield of ATP is given in mmol g^{-1} total protein. Standard error in the ATP yield does not exceed 8% (six experiments for each point).

In a separate series of experiments, an active 40 kDa creatine kinase purified from *V.xanthia* venom was employed. The activity of phosphoglycerate kinase, purified from pig skeletal muscle, was measured as the amount of ^{32}P-ATP using ^{32}P-phosphoglycerate. Pyruvate kinase was isolated and purified from rabbit muscles; its activity was characterized by the yield of ATP. Electrophoretic replacement of magnesium ions in the enzyme active sites with $^{24}Mg^{2+}$, $^{25}Mg^{2+}$ and $^{26}Mg^{2+}$ ions was employed.

Magnesium isotope effect

Isolated mitochondria with selectively substituted magnesium isotopes were prepared and the rate of phosphorylation as a function of magnesium isotopy was measured (Figure 1).[6–8] The results convincingly demonstrate that both oxidative phosphorylation, produced by ATP synthase, and substrate phosphorylation, driven by kinases presented in native mitochondria, are nuclear spin dependent. These two types of phosphorylation were separated by addition to mitochondria of methylnicotineamide which is known to completely suppress oxidative phosphorylation.[9] Figure 1 shows that both oxidative and substrate phosphorylation are nuclear spin dependent and demonstrate enormously large magnetic isotope effect, *i.e.*, the dependence of the reaction rates on the nuclear spin and magnetic moments of reagents.[10,11]

We have measured the rate of ATP synthesis by mitochondrial creatine kinase using ^{32}P-phosphocreatine (Figure 2). This enzyme exhibits the similar isotope effect: there is no difference between enzymes with $^{24}Mg^{2+}$ and $^{26}Mg^{2+}$ in catalytic sites; however, replacement of natural Mg^{2+} by isotopic $^{25}Mg^{2+}$ ions was shown to amazingly increase the rate of phosphorylation. Moreover, this effect is reproduced by creatine kinase isolated from *V.xanthia* venom (Figure 2): both the rates of ATP synthesis and isotope effects are identical for these two kinases.

A similar isotope effect was found in the rate of ATP synthesis by phosphoglycerate kinase;[8] the rate was shown for both kinases to linearly depend on the $^{25}Mg^{2+}$ content of a total magnesium pool (Figure 3).

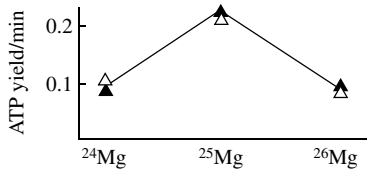


Figure 2 The rates of ATP synthesis by mitochondrial creatine kinase (black triangles) and by creatine kinase from venom of *V.xanthia* (empty triangles). The yield of ATP is given in mmol g^{-1} total protein.

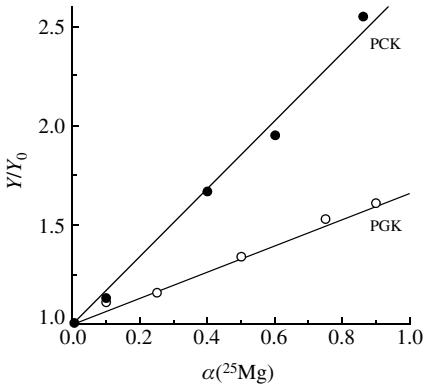
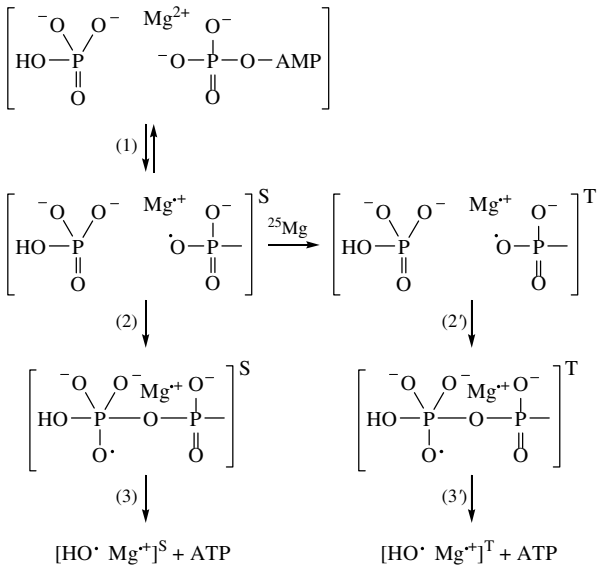


Figure 3 The rate of phosphorylation *Y*, referred to the rate Y_0 of ATP production by creatine kinase (PCK) and phosphoglycerate kinase (PGK) with $^{24}Mg^{2+}$ only, as a function of the contents of $^{25}Mg^{2+}$ in a total magnesium pool.

The discovery of this attention-catching and surprising isotope effect convincingly and unequivocally demonstrates that enzymatic phosphorylation is an ion-radical *electron spin selective process* in which Mg^{2+} participates as a reagent rather than a modest assistant.

A mechanism of phosphorylation which is based on the magnesium isotope effect is shown in the Scheme 1 for the particular case of ATP synthase. It implies electron transfer from the terminal, nearest to Mg^{2+}, phosphate anion group of ADP, generating primary ion-radical pair, composed of the monovalent radical cation $Mg^{\cdot+}$ and oxyradical of ADP. This reaction is shown in Scheme 1 as a starting key reaction (1).



Scheme 1 Reaction scheme of the phosphorylation by ATP synthase.

The primary ion-radical pair, generated by electron transfer, is in a singlet spin state due to a total spin conservation in this process. The next step is the phosphorylation itself which occurs as an attack of P=O chemical bond of phosphate by ADP oxyradical [reaction (2)]. Generated in this addition reaction another oxyradical decomposes *via* β-scission of P–OH chemical bond [reaction (3)] and generates ATP as a product and final ion-radical pair ($HO^{\cdot}$ $Mg^{\cdot+}$) which regenerates Mg^{2+} in the reaction

$$(HO^{\cdot}\ Mg^{\cdot+}) \xrightarrow{H^+} H_2O + Mg^{2+}.$$

The rate of ADP phosphorylation along a singlet channel [reactions (1)–(3)] is restricted by spin allowed back electron transfer in the primary ion-radical pair. This reaction regenerates

starting reagents and, hence, decreases the yield of ATP. However, in the presence of ^{25}Mg a new, additional channel of phosphorylation appears. The primary ion-radical pair generated in active site with $^{25}Mg^{2+}$, experiences[5] singlet-triplet spin conversion, which is induced by hyperfine coupling between magnetic moment of unpaired electron and nuclear magnetic moment of $Mg^{\cdot+}$. It transforms primary singlet pair into a chemically identical triplet pair, in which back electron transfer is spin forbidden; this channel is open for the phosphorylation only and provides an additional yield of ATP.

This mechanism, alternative to the generally accepted nucleophilic mechanism, takes into account an important and irrefutable fact, magnesium isotope dependence of phosphorylation. Note that nothing but ion-radical mechanism is able to explain properly this effect.

The ion-radical mechanism presented in Scheme 1 seems unbelievable because electron transfer reaction (1), as well known, does not occur in water, where Mg^{2+} ion is highly hydrated. However, the remarkable property of the phosphorylating enzymes is that in a prereactive state of the phosphorylation, when the enzyme domains are drawn together to unite substrate and ADP, they squeeze water molecules out of enzyme intraglobular space surrounding substrates.[4,5] As a result, a hydrate shell of the Mg^{2+} ion partly destroys, positive charge and electron affinity of the ion increase and electron transfer [reaction (1)] becomes allowed by energy.[12] Our preliminary results indicate that there is no need to dehydrate completely a first shell, in which water molecules are tightly bound; this process is too energy expensive and hardly occurs in enzymes. It seems to be enough to remove outer shell water of complex to allow for the reaction (1) to proceed, its exoergicity is about 2–3 eV.

The important comment concerns the reversible electron transfer between Mg^{2+} and phosphate anion of ADP. This reaction, as well known, does not occur in water where the Mg^{2+} ion is highly hydrated. However, a peculiar and remarkable property of PCK (and, very probably, of PGK) is that in a prereactive state of the phosphorylation, when PCK protein domains are approaching to unite phosphocreatine and ADP, they squeeze the water molecules out of the catalytic site. As a result, the hydrate shell of the Mg^{2+} ion is partly destroyed resulting to increasing of both positive charge and electron affinity of Mg^{2+} ion. Of course, it is an open question of how many water molecules should be released from $Mg(H_2O)_n^{2+}$ cluster in order to overcome an energy forbiddance for the electron transfer [reaction (1), Scheme 1]. Water molecules in the first inner shell of the $Mg(H_2O)_n^{2+}$ cluster are known to be tightly bound, while the molecules in outer shells ($n > 6$) are weekly bound.

How do phosphorylating molecular machines function?

Now one can formulate a general principle of the phosphorylation by ATP producing enzymes. Despite the differences in mechanics (ATP synthase is a rotary molecular motor, kinases are molecular pumps), all enzymes operate as the mechanochemical machines converting energy of mechanical motion in the energy of chemical bond P–O in ATP. A magic mechanism of the energy conversion now seems to be clear. It includes the squeezing water molecules out of the catalytic site, when the motion of enzyme domains compress the reagents, which is accompanied by dehydration of the hydrate shell of the Mg^{2+} ion. Then, such a partly dehydrated Mg^{2+} ion rips off an electron from the terminal phosphate group of ADP resulting to a primary ion-radical pair of monovalent magnesium cation and phosphate oxyradical. Further reactions in the pair, an addition of oxyradical to P=O bond of substrate and dissociation of the resulting oxyradicals, complete a production of ATP and regeneration of Mg^{2+}.

A huge magnesium isotope effect in the rate of phosphorylation is a direct and irrefutable proof of the proposed mechanism. It evidences that the Mg^{2+} ion is a central reagent that transforms mechanical energy of the unequilibrium conformations of enzyme macromolecule into the energy of the P–O bond in ATP.[13] It is a point where conformational mechanics meets chemistry, the key point of the functioning of enzymes as the mechanochemical molecular machines, where energy of conformational mechanics of enzyme macromolecule transforms into the energy of chemical bond.

According to the new mechanism, an enzymatic site is a nuclear spin dependent nanoreactor with the two competing reaction channels, singlet and triplet. In the presence of the $^{25}Mg^{2+}$ ion, hyperfine coupling of unpaired electron of the $Mg^{\cdot+}$ radical ion with the magnetic nucleus ^{25}Mg stimulates singlet-triplet spin conversion of the primary ion-radical pair and switches over reaction to the irreversible triplet channel, resulting in an additional yield of ATP. However, the natural magnesium contains only 10% ^{25}Mg, the rest of it consists of the nonmagnetic isotopic nuclei ^{24}Mg and ^{26}Mg. However, even in enzymatic sites with nonmagnetic magnesium ions the triplet channel is presented because in all sites there are magnetic nuclei ^{31}P of the phosphate oxyradicals, the partners of $Mg^{\cdot+}$ radical ions. Hyperfine coupling constant for ^{31}P in oxyradical is about 30 G. In comparison with that for ^{25}Mg, 212 G,[14] it is much less, so that the rate of singlet-triplet conversion, induced by ^{31}P in oxyradical, is about 9×10^7 s^{-1}, while in enzymatic sites with ^{25}Mg it is about 7×10^8 s^{-1}. It means that the contribution of the triplet channel into the ATP production is much more important in enzymes with ^{25}Mg than in enzymes with nonmagnetic magnesium isotopes.

Magnetic field dependence of the phosphorylation

Singlet–triplet spin conversion in the ion-radical pair, which switches on a triplet channel of phosphorylation, may be controlled not only by the internal magnetic field (hyperfine coupling) of ^{25}Mg nucleus but also by an external magnetic field. This statement was verified by measuring the rates of ATP synthesis by PCK-$^{24,26}Mg$ and PCK-^{25}Mg in magnetic fields (Figure 4).

Magnetic field slightly suppresses the activity of PCK with nonmagnetic nuclei ^{24}Mg and ^{26}Mg decreasing the ATP production by 3% at 550 G with a tendency to decrease with magnetic field. On the contrary, magnetic field strongly (by 50% at 550 G) stimulates activity of PCK-^{25}Mg. As a consequence, isotope effect IE, *i.e.*, the ratio of the rates of ATP synthesis by PCK with the magnetic ^{25}Mg nucleus and the nonmagnetic nuclei ^{24}Mg and ^{26}Mg, increases with magnetic field (Figure 5).

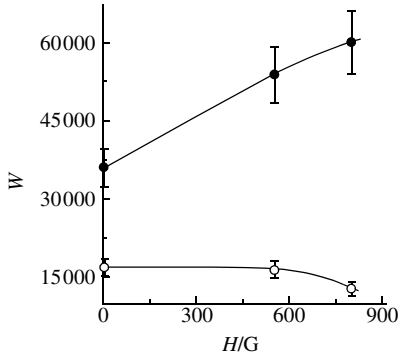


Figure 4 The rates of ATP synthesis by PCK–$^{24,26}Mg$ (open circles) and by PCK-^{25}Mg (solid circles) as a function of magnetic field.

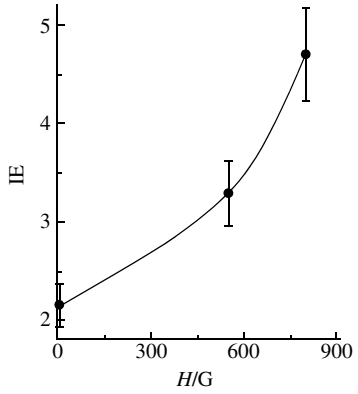


Figure 5 Magnesium isotope effect as a function of magnetic field.

These observations have a clear physical meaning in terms of spin chemistry; they are also in general accordance with theoretical predictions.[15] Nevertheless, more detailed studies of the magnetic field dependence in a wide range of magnetic fields, from the low fields $H \approx a(^{31}\mathrm{P})$ to the high fields $H >> a(^{25}\mathrm{Mg})$, are required.

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