Magnesium Isotope Effects in Enzymatic Phosphorylation

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Recent discovery of magnesium isotope effect in the rate of enzymatic synthesis of adenosine triphosphate (ATP) offers a new insight into the mechanochemistry of enzymes as the molecular machines. The activity of phosphorylating enzymes (ATP-synthase, phosphocreatine, and phosphoglycerate kinases) in which Mg^{2+} ion has a magnetic isotopic nucleus ^{25}Mg was found to be 2−3 times higher than that of enzymes in which Mg^{2+} ion has spinless, nonmagnetic isotopic nuclei ^{24}Mg or ^{26}Mg. This isotope effect demonstrates unambiguously that the ATP synthesis is a spin-dependent ion-radical process. The reaction schemes, suggested to explain the effect, imply a reversible electron transfer from the terminal phosphate anion of ADP to Mg^{2+} ion as a first step, generating ion-radical pair with singlet and triplet spin states. The yields of ATP along the singlet and triplet channels are controlled by hyperfine coupling of unpaired electron in $^{25}Mg^{+}$ ion with magnetic nucleus ^{25}Mg. There is no difference in the ATP yield for enzymes with ^{24}Mg and ^{26}Mg; it gives evidence that in this reaction magnetic isotope effect (MIE) operates rather than classical, mass-dependent one. Similar effects have been also found for the pyruvate kinase. Magnetic field dependence of enzymatic phosphorylation is in agreement with suggested ion-radical mechanism.

1. Introduction

Phosphorylating enzymes are known to be perfectly arranged molecular devices generating energy carrier P−O chemical bond in adenosine triphosphate (ATP) and triphosphates of other nucleotides. The great progress in the knowledge of structure and understanding of molecular dynamics and mechanical functioning of ATP synthesizing enzymes are now attained.[1−5] Generally accepted and quite evident mechanism of ATP synthesis implies a nucleophilic addition of inorganic phosphate (in ATP synthase) or phosphate group of phosphorylating substrates (in kinases) to adenosine diphosphate (ADP). This mechanism seems to satisfactorily explain how molecular motion in these enzymes results in chemical reaction of P−O bond formation, and how mechanical energy accumulated in enzyme and dispersed over numerous unequilibrium conformations of its macromolecule transforms into the energy of chemical bond by mechanical compression of reagents in the catalytic site.

Nevertheless, despite the great progress in our knowledge of structure and understanding of molecular dynamics and functioning of ATP synthesizing enzymes, a chemical mechanism of phosphorylation remains enigmatic.[1] A remarkable and intriguing property of the phosphorylating enzymes is that they are magnesium dependent. The main function of Mg^{2+} ion was traditionally thought to coordinate reagents, to keep them along the reaction pathway, and perhaps to slightly modify their chemical reactivity by complexation accompanied by redistribution of charges in a complex. The Mg^{2+} ion was always considered as an assistant, and it was never assumed that the ion participates directly in the phosphorylation reaction as a reagent. A new insight into the chemical mechanism of the phosphorylation stems from the recently discovered unusual and surprisingly large isotope effects of magnesium on the phosphorylating activity of enzymes.[6−9] The goal of the presented paper is to summarize these results and discuss new ideas that follow from the observation of magnesium isotope effects in enzymatic phosphorylation.

TABLE 1: Magnesium Isotopes

isotope	Spin	magnetic moment (μ_B)	natural abundance, %
^{24}Mg	0	0	79
^{25}Mg	5/2	0.85	10
^{26}Mg	0	0	11

2. Materials And Experimental Techniques

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 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; see Table 1).

Myocardial mitochondria, many enzymes of which are Mg^{2+}-dependent, were tested in vitro.[7] 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

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TABLE 2: Elemental Composition (in Mass %) of Magnesium Oxides

element	^{24}MgO	^{25}MgO (1)	^{25}MgO (2)	^{26}MgO	*MgO
Mg	99.6 (^{24}Mg)	95.8 (^{25}Mg)	92.0 (^{25}Mg)	96.3 (^{26}Mg)	100
Mn	6×10^{-3}	$<2 \times 10^{-3}$	1×10^{-3}	1.5×10^{-5}	1×10^{-3}
Fe	3×10^{-3}	$<3 \times 10^{-3}$	1×10^{-3}	4.2×10^{-3}	1.7×10^{-2}
Cu	$<2 \times 10^{-3}$	3×10^{-3}	1×10^{-3}	5.6×10^{-4}	9×10^{-4}
Ni	2×10^{-3}	3×10^{-3}	$<2 \times 10^{-3}$	1.4×10^{-3}	
Zn		3×10^{-3}	1×10^{-3}	$<4.2 \times 10^{-2}$	2×10^{-2}
Pb	2×10^{-3}	3×10^{-4}	1×10^{-4}	1.3×10^{-3}	8×10^{-4}
Cr	$<3 \times 10^{-3}$			$<4 \times 10^{-3}$	1×10^{-3}
Ti				$<1.4 \times 10^{-3}$	2×10^{-3}
K	2×10^{-3}				3.6×10^{-2}
Sr	$<2 \times 10^{-3}$		$<2 \times 10^{-2}$		
Ba	$<5 \times 10^{-3}$			$<1.4 \times 10^{-2}$	
Ca	6×10^{-3}	3×10^{-3}	1×10^{-3}	1.1×10^{-2}	5.7×10^{-2}

^{32}P-phosphocreatine. To estimate a contribution of oxidative phosphorylation into the total ATP yield, 1-methylnicotine amide (MNA) or KCN have been used, which are known to suppress ATP synthase activity.

In a separate series of experiments, an active 40 kDa creatine kinase purified from *V.xanthia* venom were employed. The activity of phosphoglycerate kinase, purified from pig skeletal muscle, was measured as the amount of ^{32}P-ATP using ^{32}P-phosphoglycerate.[9] Pyruvate kinase was isolated and purified from rabbit muscles according to ref 10, and its activity was characterized by the yield of ATP.[11] Electrophoretic replacement of magnesium ions in the enzyme active sites with $^{24}Mg^{2+}$, $^{25}Mg^{2+}$, and $^{26}Mg^{2+}$ ions was employed according to ref 12. The procedure of the ion substitution as itself does not change enzyme activity; it was proven by special testing experiments when magnesium ions $*Mg^{2+}$ were substituted by the $*Mg^{2+}$ ions of the same composition. Such a replacement produced no influence on the enzymatic activity.

For a total Mg^{2+} content monitoring, atomic absorption spectrophotometry was employed. For the control of magnesium isotopic composition, the isotope mass spectrometry has been applied. For the chemical analysis, the various techniques of high-performance liquid chromatography (HPLC) were used. Elemental and isotopic composition of magnesium oxides used in experiments are presented in Table 2.

The impurities of metals, determined by atomic absorption spectroscopy and electron spectroscopy for chemical analysis (ESCA), do not exceed 10−30 ppm in ^{24}MgO and ^{26}MgO as well as in ^{25}MgO(1) and ^{25}MgO(2), which refer to the two different lots of ^{25}MgO. Only in *MgO, the content of calcium, potassium, zinc, and iron is higher and accounts for 100−500 ppm.

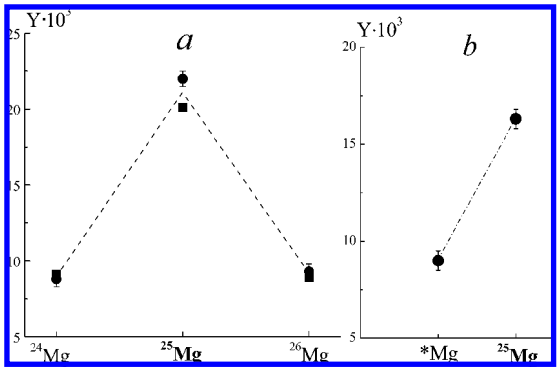
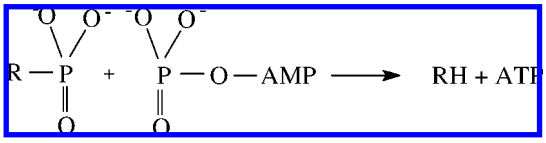


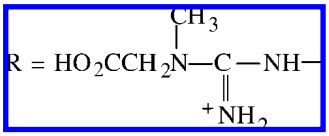
Figure 1. (a) The rate of ATP synthesis by mitochondrial PCK as a function of magnesium isotopes. The rate is measured by using ^{32}P-phosphocreatine; circles refer to mitochondrial PCK with coexisting oxidative and substrate phosphorylation; squares refer to mitochondrial PCK with oxidative phosphorylation suppressed by methylnicotine amide. In these experiments, $^{25}Mg^{2+}$ ions were prepared from ^{25}MgO (1). *Y* is a radioactivity of ^{32}P-ATP as a number of scintillations in minutes per milligrams of enzyme. (b) The rate of ATP synthesis by PCK isolated from *V.xanthia* venom; the rate is measured by using ^{32}P-phosphocreatine. Here, $^{25}Mg^{2+}$ ions were prepared from ^{25}MgO (2). The lines are drawn for the eyes.

3. Experimental Detection of Magnesium Isotope Effects

Phosphocreatine and Phosphoglycerate Kinases. These both kinases, phosphocreatine kinase (PCK) and phosphoglycerate kinase (PGK) are known to transfer phosphoryl group from phosphocreatine and phosphoglycerate to ADP, generating ATP



Here, R is a residue of the phosphorylating substrate



and R = $HO_2CCHOHCH_2O-$ for the phosphocreatine and phosphoglycerate, respectively, (AMP stands for adenosine monophosphate residue). The yield of ATP produced by mitochondrial PCK as a function of magnesium isotopy is shown in Figure 1. There is no difference in activity of PCK-^{24}Mg and PCK-^{26}Mg with spinless ions $^{24}Mg^{2+}$ and $^{26}Mg^{2+}$; however, substitution of Mg^{2+} ions by isotopic $^{25}Mg^{2+}$ ions with magnetic nuclei amazingly increases the rate of phosphorylation (Figure 1a).

Note that the rate of ATP synthesis by mitochondrial PCK does not depend on whether oxidative phosphorylation in mitochondria is suppressed or not (Figure 1). It means that the two enzymes, ATP synthase and PCK, function independently in mitochondria.

In a separate series of experiments with PCK isolated from *V.xanthia* venom, the isotopic dependence of the ATP yield was shown to be in a perfect agreement with that for the mitochondrial PCK. In these experiments, we have compared the activity of PCK-*Mg with that of PCK-^{25}Mg; in the latter case $^{25}Mg^{2+}$ ions were prepared from ^{25}MgO (2) (see Table 2). The results are shown in Figure 1 and demonstrate that the substitution of $*Mg^{2+}$ ions with $^{25}Mg^{2+}$ ions almost doubles the yield of ATP.

Special attention was paid to the influence of Mn^{2+} ions, which are known to easily substitute Mg^{2+} ions in catalytic sites. In the independent experiments, we replaced Mg^{2+} ions in PCK

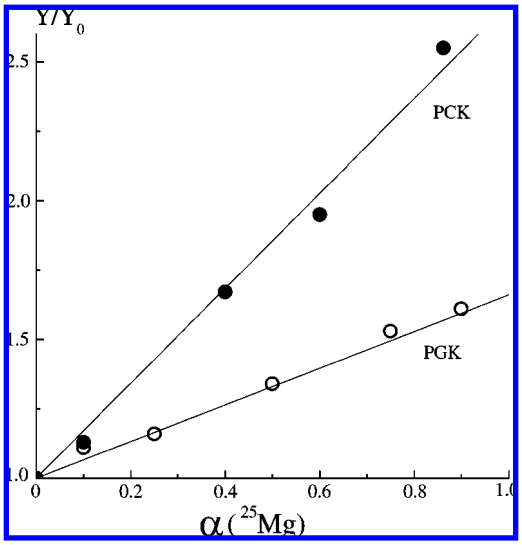


Figure 2. The rate Y of phosphorylation by PCK and PGK, referred to the rate Y_0 of phosphorylation by PCK-^{24}Mg and PGK-^{24}Mg, as a function of α(^{25}Mg) the share of ^{25}Mg in a total magnesium pool.

by Mn^{2+} and measured the rate of ADP phosphorylation by PCK-Mn at the conditions completely identical to those of phosphorylation by PCK-Mg. The ATP synthesis with PCK-Mn was shown to take place, however the yield of ATP was one-fifth as large as that with PCK-Mg. So we conclude that the influence of manganese impurities (see Table 2) on the ATP synthesis may be ignored. Moreover, despite the difference in elemental compositions of ^{25}MgO (1) and ^{25}MgO (2), the difference in PCK activities with ions $^{25}Mg^{2+}$ prepared from these two lots of ^{25}MgO does not exceed an accuracy of the ATP yield measurements. These tests prove that the only reason for the magnesium isotope effects in enzymatic phosphorylation is an influence of magnesium-25 nuclear spin.

We have also studied the yield of ATP generated by PCK as a function of the contents of $^{25}Mg^{2+}$ in a total magnesium pool; the yield was shown to linearly depend[9] on the fraction α of $^{25}Mg^{2+}$ ion (Figure 2).

Similarly to PCK, PGK exhibits magnesium isotope effects: enzymes with $^{25}Mg^{2+}$ ions are more active than those with $^{24}Mg^{2+}$ or $^{26}Mg^{2+}$ ions. A linear dependence between the yield of ATP and the fraction of $^{25}Mg^{2+}$ in the total pool of magnesium ions (Figure 2) is similar to that for PCK. Again PGK-^{24}Mg and PGK-^{26}Mg reveal no difference in the rate of ATP synthesis.

Mitochondria. Figure 3a shows the total yield of ATP in isolated mitochondria with selectively substituted magnesium ions (see Section 2) as a function of magnesium isotopy. There is no difference in ATP yield in mitochondria with $^{24}Mg^{2+}$ or $^{26}Mg^{2+}$ but the presence of $^{25}Mg^{2+}$ ions again almost doubles the ATP yield. The activity of mitochondria-*Mg increases with respect to that of mitochondria-^{24,26}Mg proportionally to the portion of ^{25}Mg in *Mg (10%).

The addition of MNA to mitochondria is known to completely suppress oxidative phosphorylation produced by ATP synthase. As a result, the total yield of ATP strongly decreases by MNA; however, magnesium isotope effect is conserved (Figure 3a). It convincingly demonstrates that both oxidative phosphorylation, produced by ATP synthase, and substrate phosphorylation, driven by PCK, PGK, and may be other kinases presented in native mitochondria, are magnesium nuclear spin dependent.

In another series of experiments $^{25}Mg^{2+}$ ions prepared from the ^{25}MgO (2) were used; the total yields of ATP, produced by

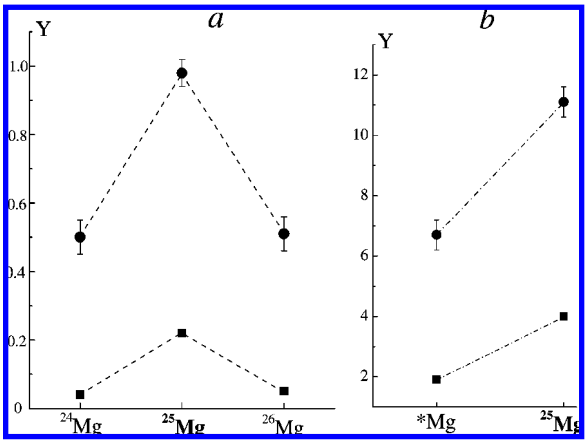
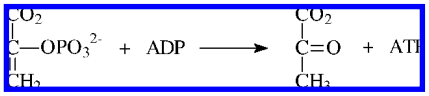


Figure 3. (a) The rate of ATP synthesis by mitochondria as a function of magnesium isotopy. The rate is measured at 25 °C in mmol/min/g mitochondria in native mitochondria (circles) and in mitochondria with oxidative phosphorylation suppressed by methylnicotine amide (squares). Mg^{2+} ions were prepared from ^{25}MgO (1); (b) The rate of ATP synthesis by mitochondria with natural $*Mg^{2+}$ and $^{25}Mg^{2+}$ ions from ^{25}MgO (2). The circles refer to the native mitochondria, and squares refer to mitochondria with oxidative phosphorylation suppressed by KCN; temperature of incubation 37 °C. The lines are drawn for the eyes.

SCHEME 1



mitochondria-*Mg and by mitochondria-^{25}Mg, are shown in Figure 3b. To suppress oxidative phosphorylation and to switch off ATP synthase, an addition of KCN was used in this case; the yield of ATP strongly decreases, like in the presence of MNA. Nevertheless, isotope effect remains and proves that both oxidative and substrate phosphorylation in mitochondria are nuclear spin dependent.

Pyruvate Kinase. Pyruvate kinase (PK) phosphorylates ADP by phosphoenolpyruvate producing ATP and pyruvate according to Scheme 1.

As in the case of PCK and PGK, the transfer of phosphate group is mediated by magnesium ion. The rate of ATP synthesis by PK as a function of magnesium concentration is shown in Figure 4.

It exhibits two remarkable features: first, its dependence on the concentration reveals two maxima; second, it demonstrates unusual dependence on the magnesium isotope composition.[13]

The existence of the two maxima indicates that PK functions in the two forms, PK-1 and PK-2. The former exists at rather low, physiologically acceptable concentrations of Mg^{2+} ions (10−50 mM); the latter manifests itself at high concentrations of Mg^{2+} (100−300 mM), its efficiency in the ATP production is even larger than that of the former.

To the best of our knowledge, the nature of PK-2 is unknown; it can differ from PK-1 by details in structure of catalytic site or by the number of Mg^{2+} ions in the site. It is also absolutely different from PK-1 in its response to isotopic composition. In contrast to PK-1, which is isotopically independent, PK-2 demonstrates enormously strong nuclear spin dependence of the phosphorylation. Step-by-step replacement of spinless$^{24,26}Mg^{2+}$ ions in the catalytic sites by magnetic $^{25}Mg^{2+}$ ions strongly increases ATP yield (Figure 4).

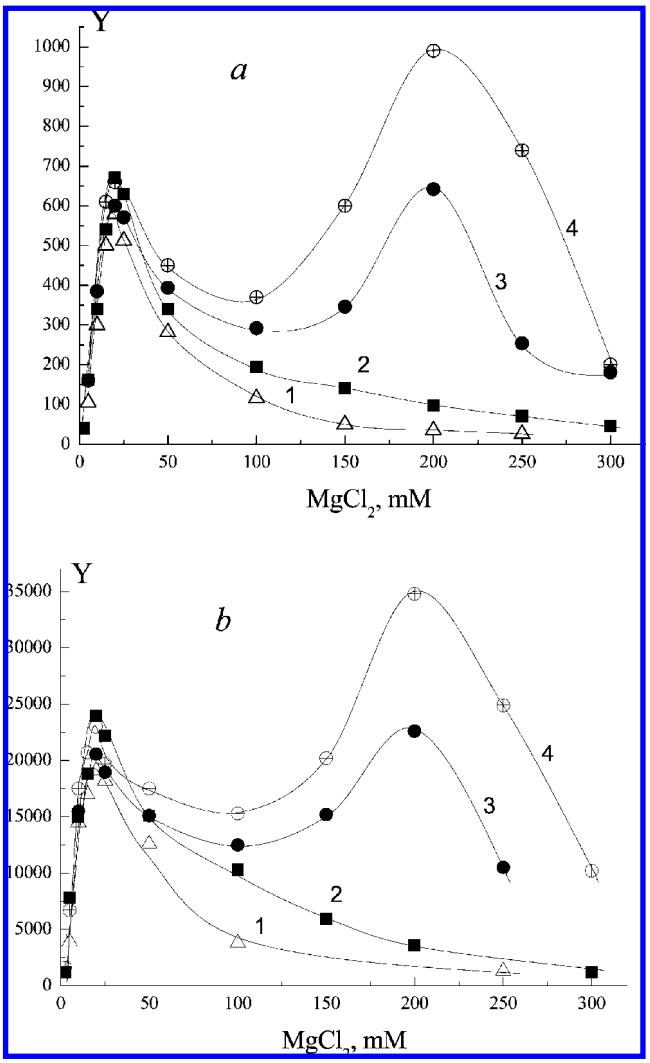


Figure 4. The rate of ATP synthesis by PK as a function of $MgCl_2$ concentration. (a) *Y* is the yield of ATP in millimoles in minutes per milligrams of enzyme. (b) *Y* is the yield of ^{32}P-ATP as a number of scintillations in minutes per milligrams of enzyme. The content of $^{25}Mg^{2+}$ (in %): 0 (1), 0.11 (2), 0.50 (3), and 0.86 (4). The $^{25}Mg^{2+}$ samples were prepared from ^{25}MgO (2).

4. Enzymatic Phosphorylation as a Spin-Selective Reaction

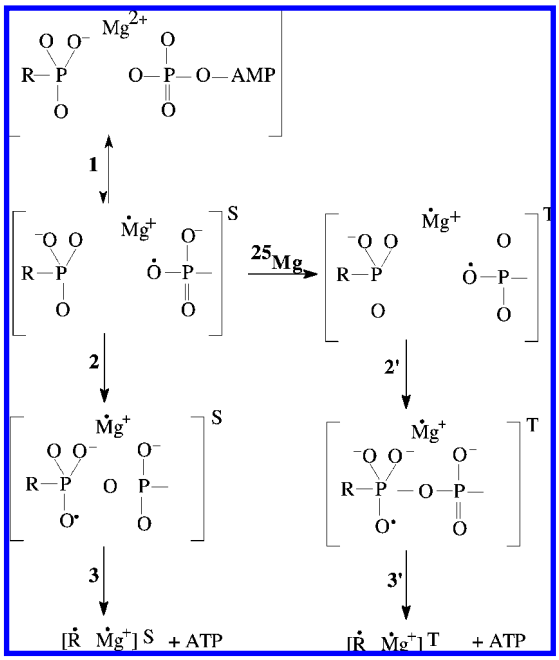
We are perfectly aware that the nuclear spin dependence of the phosphorylation is an unusual and unpredictable phenomenon but it is a fact of irrefutable reliability. It means that besides the generally accepted nucleophilic mechanism of enzymatic ATP synthesis there is another mechanism based on the following arguments:

(i) phosphorylation is an electron-transfer reaction that generates an ion-radical pair comprised of Mg^+ ion and phosphate radical-anion of ADP as the partners;

(ii) because of spin conservation in reactions, chemical reactivity of triplet and singlet spin states of the ion-radical pair is different and results in the difference in the yield of ATP along these reaction channels;

(iii) the relation between spin channels in the ion-radical pair is controlled by electron−nuclear (hyperfine) magnetic coupling of unpaired electrons with magnetic nucleus ^{25}Mg in the Mg^+ ion and with ^{31}P in phosphate radical; it induces singlet−triplet spin conversion and results in the nuclear spin selectivity of phosphorylation.

SCHEME 2: Reaction Scheme of the Phosphorylation by PCK and PGK



Any proposed mechanism should be in conformity to these postulates. It is necessary to emphasize an important point: magnetic isotope effect of magnesium is unambiguous evidence that the limiting step of the ATP synthesis is a chemical reaction as itself rather than release of ATP from the catalytic site. This conclusion is strongly supported by comparison of ^{25}Mg isotope effects in the two processes, phosphorylation and complexation. In the former, it reaches about 200% and even more (see Figures 1−3); in the latter, it is due to the classical, mass-dependent isotope effect and should not exceed ∼1%. Indeed, isotope effect on the binding of magnesium ions to crown ethers is 0.71% for the $^{24}Mg^{2+}/^{25}Mg^{2+}$ pair and 0.68% for the pair $^{25}Mg^{2+}/^{26}Mg^{2+}$.[14] If the limiting step of the ATP synthesis were release of ATP from the catalytic site, magnesium isotope effect would be of the same order of magnitude as in the case of crown ethers and would not exceed 1−2%.

Now we are faced with a problem to formulate a mechanism of phosphorylation.

PCK and PGK. For the PCK and PGK, we suggest a mechanism shown in Scheme 2. In the catalytic site, Mg^{2+} ion withdraws an electron from the terminal phosphate anion group of ADP, generating a primary ion-radical pair, composed of a monovalent radical cation $\dot{M}g^+$ and oxyradical of ADP (reaction 1). This pair is in a singlet spin state due to the total spin conservation in this process. The next step is the phosphorylation itself, which occurs as an attack of P═O chemical bond of phosphocreatine (or phosphoglycerate) by ADP oxyradical (reaction 2). Generated in this addition reaction another oxyradical decomposes via *β*-scission of R−P chemical bond (reaction 3) and generates ATP as a product and a final ion-radical pair of radical $\dot{R}$ and $\dot{M}g^+$ ion.

For the phosphocreatine radical the reaction in final pair $(\dot{R}\ \dot{M}g^+)^S$ is shown in Scheme 3; it regenerates Mg^{2+} ion and synthesizes creatine by spin-allowed reverse electron transfer.

Similarly, for the glycerate radical the reaction in final pair $(\dot{R}\ \dot{M}g^+)^S$ regenerates Mg^{2+} ion by transfer of unpaired electron from $\dot{M}g^+$ to $\dot{R}$:

$$(HO_2CCHOH-CH_2\dot{O}\ Mg^{+}) \xrightarrow{H^+} HO_2CCHOHCH_2OH + Mg^{2+} \quad (I)$$

The rate of ADP phosphorylation along a singlet channel (reactions 1, 2, 3) is restricted by spin-allowed reverse electron transfer in the primary ion-radical pair. This reaction regenerates starting reagents and decreases the yield of ATP. The remarkable feature of PCK-^{25}Mg and PGK-^{25}Mg is that they provide a new, additional channel of phosphorylation; ion-radical pairs, generated in the catalytic sites of PCK-^{25}Mg and PGK-^{25}Mg, undergo singlet−triplet spin conversion induced by hyperfine coupling between the magnetic moment of unpaired electron and the nuclear magnetic moment of ^{25}Mg. Spin conversion transforms the singlet pair into the triplet one in which the reverse electron transfer is spin forbidden; this channel is open for the phosphorylation only and provides an additional yield of ATP.

The reaction path along the triplet channel (reactions 2′ and 3′) is identical to that along the singlet channel. The only difference is that in the final pair $(\dot{R}\ \dot{M}g^{+})^{T}$ the reverse electron transfer is spin forbidden, so that the inverted, from triplet to singlet, spin conversion is required to allow generation of creatine RH and regeneration of Mg^{2+} by electron transfer. In the case of PGK, the final pair $(\dot{R}\ \dot{M}g^{+})^{T}$ in triplet channel transforms into the singlet pair because of the fast electron spin relaxation in oxyradical $HO_2CCHOH-CH_2\dot{O}$, then this pair produces glycerate and regenerates Mg^{2+} by spin-allowed electron transfer from $\dot{M}g^{+}$ to $\dot{R}$.

Scheme 2 elucidates the main features of the phosphorylation driven by PCK and PGK: magnesium isotope effect, nuclear spin dependence, generation of ATP, creatine and glycerate, and regeneration of Mg^{2+}. It indicates that the role of magnesium ion is not as modest as it was traditionally assumed. Ion-radical mechanism of enzymatic phosphorylation has nothing to do with the generally accepted one; however, nuclear spin dependence of ATP synthesis forces us to accept this mechanism, which is unbelievable at first sight..

Scheme 2 needs two comments. First, spin conversion in the primary ion-radical pair is a reversible process. It is induced by hyperfine coupling of unpaired electrons with ^{25}Mg and ^{31}P nucleus in $\dot{M}g^{+}$ and phosphate radical, respectively. Its magnitude is 214 G in $\dot{M}g^{+}$[15] and ∼30 G in phosphate radical.[16] Hyperfine coupling stimulates reversible triplet-singlet conversion with the rate 6×10^8 and 8×10^7 s^{-1}, respectively, in pairs containing ^{25}Mg and in pairs with spinless, nonmagnetic ^{24,26}Mg. Nuclear spin dependence of the ATP yield exhibits itself if the rate of spin conversion is comparable with or exceeds the rate of back electron transfer in the primary ion-radical pair. This qualitative and kinetically quite evident conclusion is also supported by calculations of the spin dynamics in the ion-radical pair.[17]

The second important comment concerns the reversible electron transfer between Mg^{2+} and the phosphate anion of ADP. It is well known that this reaction does not occur in water where 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.[18] As a result, the hydrate shell of the Mg^{2+} ion is partly destroyed resulting in the increase of both positive charge and electron affinity of Mg^{2+} ion. Of course, it is an open question how many water molecules should be released from $Mg(H_2O)_n^{2+}$ cluster to overcome an energy forbiddance for the electron transfer (reaction 1, Scheme 2). Water molecules in the first, inner shell ($n \leq 6$) of the $Mg(H_2O)_n^{2+}$ cluster are known to be tightly bound (for review, see ref 19), while the molecules in outer shells ($n > 6$) are weekly bound. We have calculated energies of the $Mg(H_2O)_n^{2+}$ and $Mg(H_2O)_n^{+}$ clusters in terms of density functional theory DFT theory at B3LYP/6-31G* level; their differences are presented in Table 3. The conversion of $Mg(H_2O)_n^{2+}$ into $Mg(H_2O)_n^{+}$ is strongly exothermic even for $n = 6$; however, in the bulk water this conversion is endothermic,[20] as shown in the last column of Table 3.

SCHEME 3: The Reaction in the Final Radical Pair

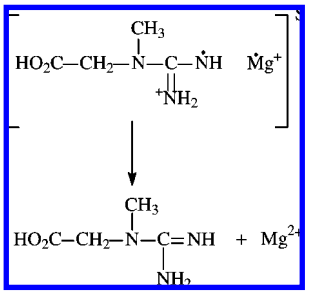


TABLE 3: Electron Affinity ΔE of $Mg(H_2O)_n^{2+}$ Complexes as a Function of *n*

n	0	1	2	3	4	5	6	∞
ΔE, eV	+15.41	+13.24	+11.14	+9.50	+7.80	+7.26	+6.46	−1.3

We have also calculated energies of the two reactions

$$Mg(H_2O)_n^{2+} + HPO_4^{2-} \rightarrow Mg(H_2O)_n^{+} + HPO_4^{-}$$

and

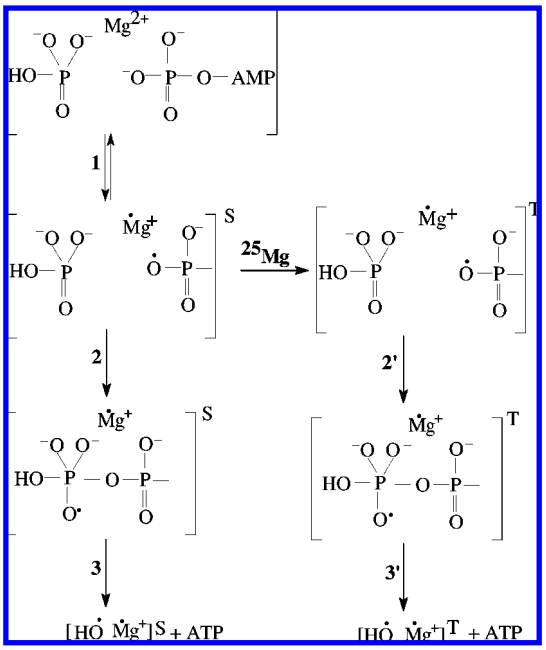
$$[Mg(H_2O)_n^{2+}\ (HPO_4^{2-}] \rightarrow [Mg(H_2O)_n^{+}\ (HPO_4^{-})]$$

The former implies intermolecular electron transfer between $Mg(H_2O)_n^{2+}$ cluster and HPO_4^{2-}, which models terminal phosphate anion in ADP. The latter considers electron transfer as an intramolecular process in a complex in which Mg^{2+} ion is directly complexed with a pair of oxygen atoms of phosphate anion (this structure is shown to be the most stable). The former reaction is strongly exothermic; its energy falls in the range from +18.6 to +12.7 eV (if n changes from 0 to 3). The latter reaction is endothermic; its energy is −1.5, −2.3, −3.0, and −3.7 eV for $n = 0, 1, 2, 3$, respectively.

Of course, these results cannot be considered as a proof of the reaction 1 in Scheme 2, because many important details of the structure and interactions in phosphate−magnesium complex in a catalytic site remain to be unknown. Nevertheless, the general idea of the electron transfer between phosphate anion and partly dehydrated magnesium ion seems to be correct. It is also in accordance with a general property of phosphorylating enzymes to release water molecules from the catalytic sites, when they function as the molecular machines. Our efforts are now directed to investigate magnesium−phosphate complexes by means of quantum chemistry to elucidate their structures, energy, charges, and spin states as a function of hydrated shell of magnesium ion. 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 realized in enzymes. It seems to be enough to remove outer shell water of the complex to allow for reaction 1 to proceed.

ATP Synthase. This enzyme embodies two of the major cellular functions: it can synthesize and hydrolyze ATP, and it operates as a mechanochemical molecular motor. The enzyme

SCHEME 4: Reaction Scheme of the Phosphorylation by ATP Synthase



is composed of the two parts, F_0 and F_1, connected by a common rotor shaft, and employs mechanical rotation to convert the energy of the electrochemical transmembrane potential into the chemical energy of ATP. Basic physical principles of its functioning are now quite well understood and described in many excellent papers (for instance, see refs 1−4).

The main chemical reactions, ATP synthesis and hydrolysis, take place in β-subunits of the enzyme where catalytic sites are located. Synchronization of the motor rotation and conformational displacements in β-subunits in the vicinity of the catalytic site with the cycles of the binding of reagents (ATP or ADP + P_i), their chemical conversion, and the following release of products is now also well understood.[1] The binding of the reagents (ADP + P_i) proceeds in a way that was called the "binding zipper".[3] This mechanism considers the binding of ADP and inorganic phosphate P_i as a sequential, progressive, bond-by-bond, formation of hydrogen bonds between the catalytic site and captured reagents. Transition-state analogs show that the tight binding state involves 15−20 hydrogen bonds. As each hydrogen bond forms, it releases two hydration water molecules, so that ultimately when the catalytic site occupied by reagents is closed, it squeezes water molecules out of the site and probably dehydrates the $Mg(H_2O)_n^{2+}$ cluster. Because the isotope effects observed in mitochondrial phosphorylation are similar to those for PCK and PGK, the mechanism of the addition of inorganic phosphate to ADP catalyzed by ATP synthase is assumed to be analogous to that for PCK and PGK. It is displayed in Scheme 4, which is similar to Scheme 2 for PCK and PGK. The only difference is that in this reaction, the hydroxyl radical instead of creatine and glycerate radicals is formed by β-scission (reaction 3). The final ion-radical pair ($H\dot{O}$ $\dot{M}g^+$) again regenerates Mg^{2+} in the reaction

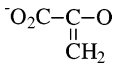
$$(H\dot{O}\ \dot{M}g^+) \xrightarrow{H^+} H_2O + Mg^{2+} \qquad \text{(II)}$$

As in the case of PCK and PGK, the rate of phosphorylation along a singlet channel (reactions 1−3 in Scheme 4) is suppressed by spin-allowed reverse electron transfer. However, in the presence of $^{25}Mg^{2+}$ ion, the hyperfine coupling of unpaired electron with the ^{25}Mg nucleus in $\dot{M}g^+$ stimulates singlet−triplet conversion of the primary ion-radical pair and transforms it into the triplet pair in which back electron transfer is spin forbidden. This new, triplet channel of phosphorylation provides an additional yield of ATP, which almost doubles a total production of ATP by ATP synthase in mitochondria. A final ion-radical pair in triplet channel undergoes a very fast triplet-singlet conversion due to electron spin relaxation in OH radical (the relaxation time is about 10^{-11} s) and regenerates Mg^{2+} ion in the reaction (II).

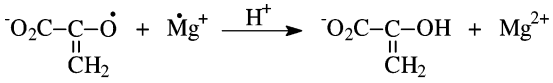
Note that Scheme 4 predicts and explains also the transfer of isotopic label ^{18}O from inorganic phosphate to water. This phenomenon, which was observed in 1953[21] and since this time, remains enigmatic. The point is that no ^{18}O isotope exchange between H_2O and ^{18}O-labeled phosphate was observed in the presence of nonfunctioning ATP synthase; isotope exchange occurred only during the phosphorylation process, that is, ATP synthesis is necessarily accompanied by ^{18}O isotope transfer from phosphate to water. Now in terms of Scheme 4, this phenomenon is no longer enigmatic; moreover, it strongly supports the ion-radical mechanism of phosphorylation.

Pyruvate Kinase. At low concentration of magnesium ions, the functioning of pyruvate kinase depends neither on the mass, nor on the spin and magnetic moment of magnesium isotope (see Figure 4). However, the excess of Mg^{2+} ions seems to modify somehow the structure of enzyme and results to the nuclear spin selectivity of the functioning. One can suppose that at low concentration of Mg^{2+} ions enzyme acts as nucleophilic molecular machine; however, its modified form PK-2 operates via ion-radical, nuclear spin-dependent, mechanism. The contribution of the latter into the phosphorylation increases as the share of $^{25}Mg^{2+}$ grows (Figure 4).

The ion-radical mechanism of phosphorylation by PK-2 is expected to be similar to that by PCK and PGK and may be described by Scheme 2, where R is now the pyruvate residue of the pyruvate phosphate

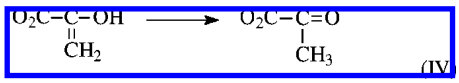


The scheme again implies reversible electron transfer between phosphate anion of ADP and Mg^{2+} ion (reaction 1), which generates ion-radical pair of ADP phosphate oxyradical and radical-cation $\dot{M}g^+$. Further two reactions, 2 and 3, produce ATP and the final ion-radical pair. The latter regenerates Mg^{2+} ion according to the reaction



(III)

Enol pyruvate is well known to transform into the pyruvate molecule



(IV)

Reaction 1, being thermally induced, generates ion-radical pair in singlet spin state. In the presence of $^{25}Mg^{2+}$ ion, hyperfine coupling of unpaired electron with magnetic nucleus ^{25}Mg in $\dot{M}g^+$ radical-cation stimulates spin conversion of singlet state into the triplet spin state from which regeneration of initial reagents is spin forbidden.

Triplet channel of ATP production is chemically identical to the singlet one. Enol pyruvate and Mg^{2+} are formed in the final triplet pair by reaction (III) because fast electron relaxation in oxyradical is known to remove spin prohibition and allows reaction (III) to occur.

4. Quantitative Measure of Magnesium MIE

The reaction in Schemes 2 and 4 of enzymatic phosphorylation may be easily transformed into the kinetic Scheme 5.

It describes a formation of the ion-radical pair (IRP) within catalytic site (CS) of enzyme with a rate constant k_0 owing to the electron transfer from ADP phosphate group to Mg^{2+}. Further this IRP may regenerate initial reagents by reverse electron transfer with the rate constant k_{-1} or it may generate ATP along the two additive channels with rate constants k_n and k_m. These channels are chemically identical: the first one refers to the ions $^{24,26}Mg^{2+}$, while the second one relates to $^{25}Mg^{2+}$-containing enzymes. A total rate of ATP synthesis is determined as a sum of the rates along both channels

$$Y = d[ATP]/dt = [\alpha k_m + (1 - \alpha)k_n][IRP] \quad (1)$$

where α is a share of catalytic sites occupied by $^{25}Mg^{2+}$, which is suppose to be equal to $^{25}Mg^{2+}$ share in a whole catalytic system, because isotope effect in the magnesium ion-binding constant is negligibly small and can be ignored. The [ATP] and [IRP] values are related to ATP concentration and IRP stationary concentration, respectively. The latter is determined by the kinetic equation

$$d[IRP]/dt = k_0[CS] - [k_{-1} + \alpha k_m + (1 - \alpha)k_n][IRP] = 0 \quad (2)$$

By solving these two equations one can derive

$$Y = \frac{[\alpha(k_m - k_n) + k_n]k_0}{k_{-1} + \alpha[k_m - k_n] + k_n}[CS] \quad (3)$$

When ^{25}Mg is absent in a reaction mixture ($\alpha = 0$), $Y = Y_0$ and eq 3 results in eq 4:

$$Y_0 = \frac{k_n k_0}{k_{-1} + k_n}[CS] \quad (4)$$

From eqs 3 and 4, the following ratio easy to find:

$$\frac{Y}{Y_0} = \frac{[\alpha(k_m - k_n) + k_n][k_{-1} + k_n]}{[k_{-1} + \alpha(k_m - k_n) + k_n]k_n} \quad (5)$$

This equation predicts a rather complicated dependence of Y/Y_0 on α; however, experimental dependences (Figure 2) are found to be linear with a high accuracy. A linear dependence follows from eq 5 only at the condition

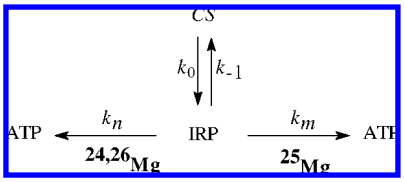
$$k_{-1} \gg \alpha(k_m - k_n) + k_n \quad (6)$$

which implies that the reverse electron transfer in ion-radical pair is the fastest process and that the rate of ATP synthesis is controlled by reactions 2 and 3 in Schemes 2 and 4.

Under condition 6, eq 5 transforms into eq 7

$$\frac{Y}{Y_0} = \alpha\left[\frac{k_m}{k_n} - 1\right] + 1 \quad (7)$$

SCHEME 5: Kinetic Scheme of Phosphorylation



By comparing this ratio with the experimentally detected dependences (Figure 2) the magnitudes of the k_m/k_n ratio are found to be 1.6 for PGK and 2.6 for PCK, respectively. This result indicates that the rate of ATP synthesis by enzymes with $^{25}Mg^{2+}$ ion in a catalytic site is 2−3 times as high as in the same sites with $^{24}Mg^{2+}$ or $^{26}Mg^{2+}$ ions.

5. ATP Hydrolysis: No Isotope Effect

Both the ATPase and the above-mentioned kinases function as the reversible molecular machines, and they synthesize ATP and hydrolyze it. The intriguing question is whether the latter reaction is also nuclear spin controlled similarly to the former reaction. To answer this question, the authors[22] have measured the rate of ATP hydrolysis by creatine kinases in which magnesium ions were substituted by pure isotopic ions $^{24}Mg^{2+}$, $^{25}Mg^{2+}$, $^{26}Mg^{2+}$. Figure 5 demonstrates decay of ATP (curve 1, black points) and accumulation of ADP (curve 2, empty points) as a function of incubation time. The sum of ATP and ADP does not depend on time but remains constant; that is, ATP quantitatively transforms to ADP. It is remarkable that the ATP decay (and therefore ADP accumulation) are identical for all kinases as they do not depend on the magnesium isotopes. It is a direct indication that the ATP → ADP enzymatic reaction is not spin selective. No paramagnetic species are formed in this reaction, and it is in a perfect agreement with classical insight into the hydrolysis as a protolytic reaction. In this reaction, there is no magnetic isotope effect but classical, mass-dependent isotope effect being negligibly small is not detected.

Thus, we meet a situation when direct reaction, ATP synthesis, depends on the magnesium isotopes, while the reverse reaction, ATP hydrolysis, does not depend on the isotopes. It means that the equilibrium constants as well as free energy for the enzyme functioning are nuclear spin dependent.

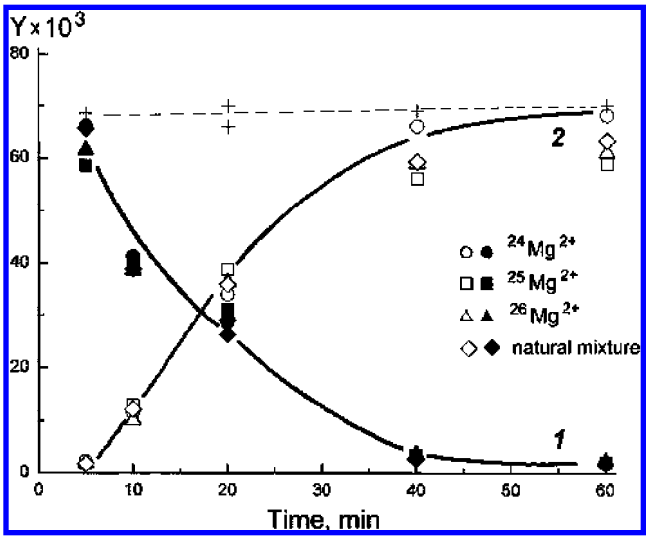


Figure 5. ATP decay (curve 1) and ADP formation (curve 2), induced by PCK, as a function of incubation time. Crosses and dotted line refer to the sum ATP + ADP; the yields Y of ATP and ADP are given in millimoles per grams of enzyme.

To derive an isotope contribution into the thermodynamic of phosphorylation, one can consider, as an example, a reversible phosphorylation by creatine kinase

$$PC + ADP \underset{k_h}{\overset{k_S}{\rightleftharpoons}} ATP + C$$

where PC and C are phosphocreatine and creatine, k_S and k_h are the rate constants of ATP synthesis and hydrolysis, respectively. Then the equilibrium constant is determined by simple relation

$$K = \frac{k_S}{k_h} = \frac{[C][ATP]}{[PC][ADP]} \quad (8)$$

where k_S depends on the magnesium isotopes but k_h does not. k_S for the magnetic isotope ^{25}Mg is denoted as $^{25}k_S$, and for nonmagnetic isotopes the corresponding constants are $^{24}k_S$ and $^{26}k_S$. As $^{24}k_S = {}^{26}k_S$, the ratio of the equilibrium constants for isotopic forms ^{25}Mg and ^{24}Mg is

$$\frac{^{25}K}{^{24}K} = \frac{^{25}k_S}{^{24}k_S} > 1 \quad (9)$$

The substitution of nonmagnetic isotope $^{24}Mg^{2+}$ by magnetic $^{25}Mg^{2+}$ in creatine kinase increases both equilibrium constant and ATP stationary concentration in the same ratio as it increases the rate constant $^{25}k_S$.

In terms of free energy from eq 9 follows the equation

$$\frac{^{25}K}{^{24}K} = \frac{^{25}k_S}{^{24}k_S} = e^{\Delta F/RT} \quad (10)$$

where $\Delta F = {}^{25}F - {}^{24}F$, which is the difference of free energies of processes driven by creatine kinases with ^{25}Mg and ^{24}Mg. For PCK, the ratio $^{25}k_S/^{24}k_S = 2.6$ (see Figure 2); it corresponds to $\Delta F = 2.4$ kJ/mol at 300 K. For PGK, this ratio is 1.6, and it corresponds to $\Delta F = 1.3$ kJ/mol at 300 K. These magnitudes determine isotopically dependent part of total free energy. They characterize an increase of free energy induced by the very simple procedure: the replacement of spinless isotopic nucleus ^{24}Mg with magnetic nucleus ^{25}Mg and specify a new nuclear spin dependent channel of phosphorylation, introduced by $^{25}Mg^{2+}$ ion instead of $^{24}Mg^{2+}$.

It is important to note that the different response of ATP synthesis and ATP hydrolysis to the isotopic magnesium ion substitution demonstrates that despite of macroscopic reversibility of these two reactions, they are microscopically not reversible; the pathways of direct and reverse reactions are different. This conclusion is in a perfect agreement with that derived from the completely different arguments.[23]

6. 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 internal magnetic field (hyperfine coupling) of ^{25}Mg nucleus but also by external magnetic field. This statement was verified by measuring the rates of ATP synthesis by PCK-^{24,26}Mg and PCK-^{25}Mg in the magnetic fields (Figure 6).

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 more as the magnetic field increases. On the contrary, the magnetic field strongly (by 50% at 550 G) stimulates activity of PCK-^{25}Mg. As a consequence, isotope effect (IE), that is, the ratio of the rates of ATP synthesis by PCK with magnetic ^{25}Mg nucleus and nonmagnetic nuclei ^{24}Mg and ^{26}Mg, increases as the magnetic field increases (Figure 7).

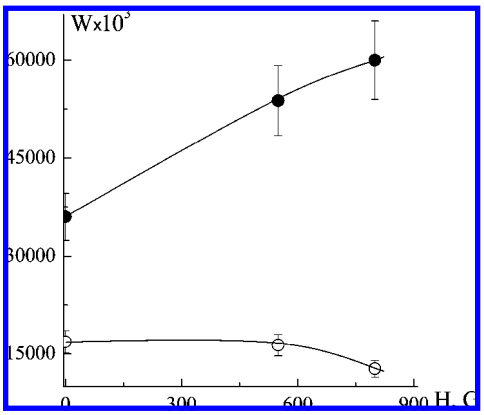


Figure 6. The rate of ATP synthesis by PCK-^{24,26}Mg (open circles) and by PCK-^{25}Mg (filled circles) as a function of magnetic field.

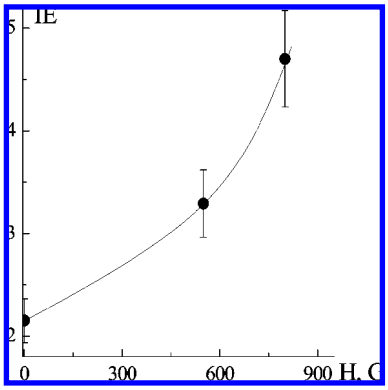


Figure 7. Magnesium isotope effect as a function of magnetic field.

These observations have a clear physical meaning in terms of spin chemistry;[28] they are also in general accordance with theoretical predictions.[17] Nevertheless, more detailed studies of the magnetic field dependence in a wide range of magnetic fields, from the low fields $H \approx a(^{31}P)$ to the high fields $H \gg a(^{25}Mg)$, are required.

7. Conclusion

A huge magnesium isotope effect in the rate of phosphorylation is a direct evidence that the $\dot{M}g^+$ ion is a key mediator in phosphorylation, a point where conformational mechanics of enzyme biomolecule crosses chemistry.[24] Despite the difference in mechanics (ATP synthase is a rotary molecular motor, PCK, PGK, and PK are linear motors), all these enzymes operate as the mechanochemical machines converting energy of mechanical motion into the energy of chemical bond P−O in ATP. A mechanism of the energy conversion includes release of water molecules from the catalytic site, when the motion of enzyme domains compresses reagents, which is accompanied by partial dehydration of the Mg^{2+} ion. A dehydrated Mg^{2+} ion withdraws an electron from the terminal phosphate group of ADP, resulting in a primary ion-radical pair of $\dot{M}g^+$ and phosphate oxyradical. Furthermore, two simple reactions in the pair, an addition of oxyradical to P═O bond of substrate and dissociation of the resulting oxyradical, complete synthesis of ATP as well as regeneration of Mg^{2+} and substrate fragments. Shortly, Mg^{2+} ion acts as a trigger of the phosphorylating reaction.

According to this mechanism, enzymatic site is a nuclear spin dependent nanoreactor with the two competing reaction channels, singlet and triplet. In the presence of $^{25}Mg^{2+}$ ion, hyperfine coupling of unpaired electron of the $\dot{M}g^{+}$ ion-radical with magnetic nucleus ^{25}Mg stimulates singlet−triplet spin conversion of the primary ion-radical pair and switches on a new, irreversible triplet channel, resulting in additional yield of ATP. The natural magnesium contains only 10% of ^{25}Mg; however, even in enzymatic sites with nonmagnetic magnesium nuclei ^{24}Mg and ^{26}Mg the triplet channel is presented, because in every site there is magnetic nucleus ^{31}P of the phosphate oxyradical, the partner of $\dot{M}g^{+}$ ion-radical. Hyperfine coupling constant for ^{31}P in oxyradical is about 30 G. It is much less than that for ^{25}Mg (214 G), 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 spinless magnesium isotopes.

Magnesium isotope effect is an unambiguous indicator of the reaction mechanism; its observation is direct evidence of the ion-radical mechanism; the absence of the effect means that the classical, nucleophilic mechanism dominates. As our experience shows, some enzymes (phosphocreatine and phosphoglycerate kinases, ATP synthase) demonstrate magnesium isotope effect, and they function as the nuclear spin-controlled molecular devices in which the magnetic isotope effect operates.[25−28] However, ATP hydrolysis by creatine kinase does not depend on the magnesium nuclear spin. Furthermore, pyruvate kinase exhibits both mechanisms of phosphorylation: at low concentration of magnesium, it functions according to the nucleophilic mechanism and reveals no isotope effect; however, at high concentration of magnesium ions it demonstrates isotope effect and functions as ion-radical nanoreactor.

The coexistence of both phosphorylation mechanisms may be understood in terms of the concept of "soft" and "rigid" molecular machines. Our idea is that the former compress reagents in a catalytic site only slightly inducing nucleophilic transfer of phosphate group without an essential violence of the magnesium water cluster, while the latter produce much more strong compression of reagents that is accompanied by squeezing water molecules out of the catalytic site and partial dehydration of magnesium water cluster, inducing electron transfer. The magnitude of the isotope effect seems to be a measure of the softness of enzymes as molecular machines. Thus, for the PGK the ratio of the rates of ATP synthesis by PGK-^{25}Mg and PGK-^{24}Mg is 1.6, for the ATP synthase it is 1.7, while for PCK this ratio is 2.6; the PCK seems to be the most rigid and powerful phosphorylating molecular machine. The competition of nucleophilic and ion-radical mechanisms is evidently controlled by protein structure and by molecular dynamics of enzyme macromolecule and its domains.

At last, it is worthy to point out that the enzymes that function by the ion-radical mechanism are expected to be susceptible to the magnetic fields, both permanent and oscillating; this consideration may be important as a physical basis of magnetobiology. Magnetic field dependence of the phosphorylation by PCK is proven experimentally (Section 6).

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