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Chemistry of Enzymatic ATP Synthesis: An Insight through the Isotope Window

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1. INTRODUCTION

Among enzymes that are well-known for being perfectly arranged molecular devices of paramount importance are those generating the energy carrier adenosine triphosphate (ATP). A tremendous success has been reached in understanding the mechanical functioning of these enzymes. New technologies of single-molecule optics and mechanics are developed to visualize the mechanics of the most beautiful molecular motor, ATP synthase.[1−4] It has been shown that the direction of its rotor circulation—clockwise or counterclockwise—controls chemical function of ATP synthase and changes it reversibly from synthesis to hydrolysis.[5,6] Moreover, macroscopic reversibility in ATP synthesis does not mean microscopic reversibility, i.e., the pathway for ATP hydrolysis is not the same as for ATP synthesis.[2,7−9]

Despite the great progress in the knowledge of structure and understanding of molecular dynamics and functioning of ATP synthesizing enzymes,[10] far less is known about the chemical mechanism of ATP synthesis.[2,4] How does mechanical motion in molecular motors result in the chemical reaction of P−O bond formation? How does mechanical energy transform into the energy of the newly born chemical P−O bond in ATP? What is the limiting step—entering of reactants into the catalytic site, release of products, or the chemical reaction itself? All these questions are under discussion.

A universal property of the phosphorylating enzymes is their magnesium dependence. A generally accepted mechanism implies a nucleophilic addition of inorganic phosphate (provided by ATP synthase) or phosphate group of substrate (kinases) to ADP. In terms of this mechanism, the main function of the Mg^{2+} ion was traditionally thought to coordinate phosphate reactants, to keep them in a position precisely within the reaction pathway of nucleophilic attack, and perhaps to slightly modify their chemical reactivity by redistribution of charges in a Mg(ADP) complex. The Mg^{2+} ion was always considered as an assistant and never assumed to participate in the ATP synthesis as a reactant. This simplistic nucleophilic mechanism was accepted as a paradigm, so that the chemistry of ATP synthesis itself did not attract much attention because there was nothing to discuss.

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The nucleophilic mechanism does not imply participation of any paramagnetic species in the ATP synthesis, so that the discovery of magnetic isotope and magnetic field effects[11,12] in enzymatic ATP synthesis catalyzed by magnesium was unprecedented and seemed to be an unbelievable result. The goal of this paper is to present the chemistry and physics of these unexpected effects, to formulate a new, ion-radical mechanism of ATP synthesis, to consider its energy and kinetic arguments, and to illustrate its biomedical applications based on the first-time observed in vivo isotope effect in living organisms. Finally we will show that the discovery of the ion-radical mechanism has also provided a background for the generally accepted but so far hypothetical nucleophilic mechanism of ATP synthesis.

2. ISOTOPE EFFECTS IN ATP SYNTHESIS

For the first time, heavy isotopes were used for studying ATP-generating enzymes in 1953.[13] The $^{18}O/^{16}O$ isotope exchange between ^{18}O inorganic phosphate and ^{16}O water in mitochondria was shown to occur only during the ATP functioning; no isotope exchange was observed when the ATP synthesis was suppressed, particularly by omission of Mg^{2+} ions. No certain conclusions have been derived from this observation. When mitochondria were incubated with P_i labeled with ^{18}O, ^{33}P, and ^{32}P and unlabeled ATP, a fast exchange of P_i oxygen with water and rapid P_i—ATP exchange were detected.[14,15] Moreover, the terminal bridge oxygen in ATP formed by oxidative phosphorylation was shown to come from ADP, not P_i[16] (for a review of the early studies of isotopes in ATP synthesis, see ref 17). Measuring ^{18}O isotope effects, it was shown that the dissociation of P—O bond is the rate-limiting step in the Ras-catalyzed hydrolysis of GTP.[18,19]

The first example of the *magnetic* (mass-independent) *isotope effect* operating in a biochemical system was the reaction of methyl mercury chloride with creatine kinase.[20] The effect manifests itself both in the nuclear spin dependence of enzymatic activity and the fractionation of magnetic and nonmagnetic mercury isotopes. These observations demonstrate that the interaction of methyl mercury chloride, a hazardous environment pollutant, with creatine kinase is a radical spin-selective process; being *electron spin-selective*, it inevitably results in the *nuclear spin selectivity*. However, methyl mercury chloride does not intervene in enzymatic transfer of phosphate; it just chemically modifies the structure of the site and suppresses the catalytic activity of kinase. This magnetic (mass-independent) isotope effect with particular emphasis of its importance in environmental chemistry was later observed in many studies (for review, see ref 21).

3. ISOTOPE EFFECTS IN CREATINE KINASE REACTION

As has been shown by Ivanov, the reaction of photosynthetic CO_2 fixation, which is catalyzed by rubiloso-5-bisphosphate carboxylase/oxidase (RuBisCo), is strongly activated by the presence of $^{25}MgCl_2$.[22] This result stimulated Kouznetsov et al.[23] to develop a preparative electrophoretic technique to substitute Mg^{2+} ions with natural isotope composition by $^{25}Mg^{2+}$ ions. By using this technique, the samples of creatine kinase from the *Vipera xanthia* venom with high content of $^{25}Mg^{2+}$ ions (86% versus 10% of natural abundance) were obtained. In the experiments, these samples were shown to exhibit enormously high ATP-generating activity of enzyme: an 8-fold increase in the share of $^{25}Mg^{2+}$ ions in a total pool of magnesium ions was accompanied by a 2.4-fold increase in the ATP yield. This unbelievable result indicated that the presence of magnetic isotope nuclei ^{25}Mg in the catalytic site of the enzyme somehow promotes ATP synthesis. In a series of later studies, this new and unpredictable phenomenon was investigated in detail, and the results are summarized and discussed later.

Table 1. Magnesium, Calcium, and Zinc Isotopes[26]

isotope	spin	magnetic moment, mB	natural abundance, %
^{24}Mg	0	0	79
^{25}Mg	5/2	0.85	10
^{26}Mg	0	0	11
^{40}Ca	0	0	96.94
^{43}Ca	7/2	1.317	0.135
^{64}Zn	0	0	48.6
^{66}Zn	0	0	27.9
^{67}Zn	5/2	0.875	4.1
^{68}Zn	0	0	18.8

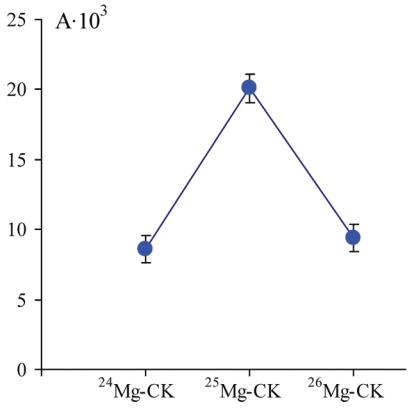

Figure 1. Rates of ATP synthesis by creatine kinases as a function of magnesium isotopes. The yield Y is given as radioactivity of [^{32}P]ATP measured as a number of scintillations/min/mg of total amounts of protein (pure CK); concentration of $MgCl_2$ in all experiments was 15 mM. The lines are drawn for guiding the eyes. Reprinted with permission from ref 25. Copyright 2005 Springer.

3.1. Magnesium Isotopes

Because isotope effects in ATP synthesis are unexpected, unusual, and even unbelievable phenomena, it is appropriate to describe in brief all key materials and technologies used in the biochemical experiments.[24,25] Isotope-containing $MgCl_2$, $CaCl_2$, and $ZnCl_2$ samples (Table 1) were obtained using the treatment of magnesium, calcium, and zinc oxides with analytically pure HCl. Monomeric creatine kinase was purified from *Vipera xanthia* venom, phosphoglycerate kinase was purified from pig skeletal muscle, and pyruvate kinase was isolated and purified from rabbit muscles.

The catalytic activities of enzymes were measured conventionally as the amounts of labeled ATP formed in the presence of [^{32}P]phosphocreatine, [^{32}P]phosphoglycerate, or [^{32}P]phosphopyruvate in 1 min at optimal incubation conditions and corrected to 1 mg of pure enzyme tested. For loading or substitution of magnesium, calcium, and zinc ions into the enzyme active sites, the original electrophoretic technique was employed. The procedure of ion substitution as such does not change the enzyme activity; this has been proven in special experiments, when magnesium ions

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$^{*}Mg^{2+}$ were substituted by the $^{*}Mg^{2+}$ ions of the same composition (*Mg means magnesium with natural abundance of the three isotopes; see Table 1). Such a replacement has no influence on the enzymatic activity. For monitoring total ion contents, atomic absorption spectrometry has been employed. For isotope composition control, isotope mass spectrometry was applied. For analytical purposes, some routine high-performance liquid chromatography (HPLC) procedures were used. The enzyme samples were incubated at 30 °C for 40 min; this time was required for the ATP yield to reach an equilibrium mode, i.e., a limiting magnitude in all cases (for all metal ions and all isotopes). Further, the enzymatic activity values were determined as mentioned above (for details, see ref 25).

Creatine kinases (CKs) loaded with different ions and isotopes will be further specified as ^{24}Mg-CK, ^{25}Mg-CK, ^{26}Mg-CK, and *Mg-CK, respectively. The first question the authors[25] were faced with was the nature of the observed isotope effect. To answer it, a special series of experiments were carried out in which CK was loaded with almost pure magnesium isotope ions $^{24}Mg^{2+}$ (isotope content 99.6%), $^{25}Mg^{2+}$ (95.75%), and $^{26}Mg^{2+}$ (96.26%) using identical techniques and conditions of the loading. The results are shown in Figure 1. They unambiguously demonstrate that the kinases in which magnesium are substituted by nonmagnetic isotopic nuclei ^{24}Mg and ^{26}Mg were shown to be identical in their activity whereas ^{25}Mg-CK is almost twice as efficient. This is convincing evidence that magnetic isotope effects do occur in ATP synthesis[27−31] while the classical, mass-dependent effect may be ignored.

Figure 2 demonstrates the yield of ATP synthesized by ^{25}Mg-CK and *Mg-CK as a function of $MgCl_2$ concentration. At concentrations around 10 mM, it exhibits a large isotope effect similar to that shown in Figure 1, although experimental techniques were different. The loss of activity at high concentrations of Mg^{2+} ions does not mean that the enzyme is paralyzed. Its activity may be recovered by removing the metal ions and loading the enzyme repeatedly with the new portions of the ions (see Supporting Information). Figure 3 shows a linear relationship between the rate of ATP synthesis and the fraction of ^{25}Mg in a total pool of magnesium ions in CK;[32] a similar relationshiop is also valid for the phosphoglycerate kinase (PGK),[33] as will be specified later.

3.2. Calcium and Zinc Isotopes

We will further specify CK loaded with calcium and zinc isotopes as ^{43}Ca-CK and ^{40}Ca-CK, ^{64}Zn-CK, and ^{67}Zn-CK. Concentration dependences of the ATP yield for ^{40}Ca-CK and ^{43}Ca-CK are shown in Figure 4; they are similar to those for Mg-CK (Figure 2). Again, at a low concentration of $CaCl_2$ there is no difference in the ATP yield; however, at $[CaCl_2] \geq 40$ mM, ^{43}Ca-CK produces ATP more efficiently than ^{40}Ca-CK. In maximum, at $[CaCl_2] \approx 120$ mM, the isotope effect, i.e., the ratio of the yields, is 1.8 ± 0.2. Taking into account that the content of ^{43}Ca in ^{43}Ca-CK is 86.7% only, it is easy to estimate a net, referred to as 100% of ^{43}Ca, isotope effect as 2.0 ± 0.2.[34]

ATP synthesis by CK is accompanied by generation of creatine and creatinine; they originate from the substrate, phosphocreatine. The yield of creatine is shown in Figure 5; evidently, both the concentration dependence and isotope effect on the creatine yield satisfactorily (not perfectly precise because the creatinine is not taken into account) reproduce those for the ATP yields (Figure 4).[34]

Figure 6 demonstrates concentration dependence of the ATP yield for ^{64}Zn-CK and ^{67}Zn-CK. It exhibits features very similar

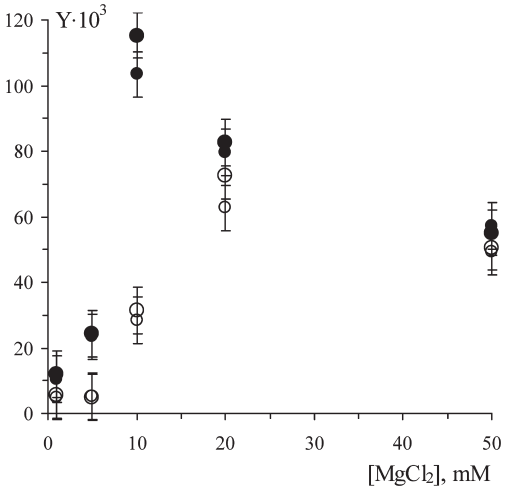

Figure 2. Yield of ATP synthesized by ^{25}Mg-CK (filled circles) and *Mg-CK (open circles) as a function of $MgCl_2$ concentration. The fraction of ^{25}Mg isotope in ^{25}Mg-CK is 78%. Y is the amount of ATP produced for 40 min and expressed in mM/g of CK.

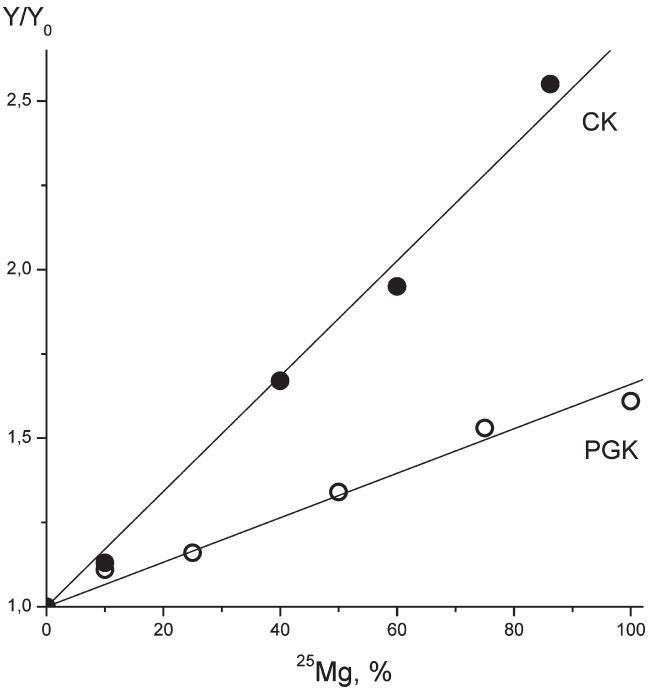

Figure 3. Catalytic activity Y of ^{25}Mg-CK and ^{25}Mg-PGK referred to Y_0l the activity of ^{24}Mg-CK and ^{24}Mg-PGK, respectively, as a function of the fraction of ^{25}Mg in a total magnesium pool. Reprinted with permission from ref 33. Copyright 2005 PNAS.

to those for magnesium and calcium CK. First, Zn^{2+} ions actually catalyze ATP synthesis, like Ca^{2+} ions, with the efficiency comparable with that of Mg^{2+} ions; second, ATP yield increases as concentration of $ZnCl_2$ increases, and then reaches maximum and gradually decreases.[35]

4. ARE ISOTOPE EFFECTS TRUSTWORTHY?

Being unexpected and unprecedented, isotope effects meet with distrust (note that the use of Mg, Ca, and Zn isotopes in

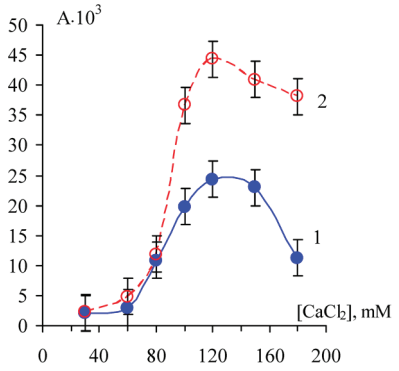

Figure 4. Yields of ATP produced by ^{40}Ca-CK (1) and ^{43}Ca-CK (2). A is the radioactivity of ^{32}P-ATP (in scintillations/min/mg CK). Reprinted with permission from ref 34. Copyright 2011 Elsevier.

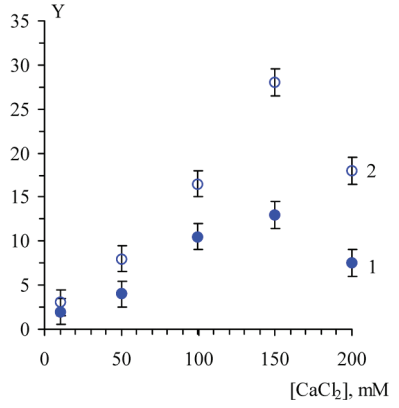

Figure 5. Yield Y of creatine (in mM/min/g of enzyme) produced by ^{40}Ca-CK (1, filled circles) and ^{43}Ca-CK (2, open circles) as a function of $CaCl_2$ concentration. Reprinted with permission from ref 34. Copyright 2011 Elsevier.

enzymatic ATP synthesis also has no precedent). To get convinced of the reality of the detected isotope effects, it is not enough to claim that all experimental conditions and procedures were identical. Independent proofs are required. The arguments listed below will serve this aim.

(1) Using different sources of magnesium isotopes, no differences in enzymatic activity values measured in the presence of ^{24}Mg and ^{26}Mg were ever found—in contrast to the experiments involving ^{25}Mg.

(2) Impurities of metals, other than magnesium, determined by atomic absorption spectroscopy and electron spectroscopy for chemical analysis (ESCA) in ^{24}Mg-CK, ^{25}Mg-CK, ^{26}Mg-CK, and *Mg-CK, did not exceed 10−30 ppmm and their influence on the ATP yield independently tested was found to be negligible.[36] Among the impurities, the most suspicious was manganese. It was in fact shown to catalyze ATP synthesis (see Supporting Information, Table S1) with the efficiency much lower than that of magnesium, so that its contribution into the total production of ATP is absolutely ignorable when it is presented in tracer amounts. However, even detailed and careful analysis of traces of impurities does not guarantee the absence of unexpected, accidental impurities.

(3) To exclude accidental impurities and unpredictable factors, an independent series of experiments was carried out.

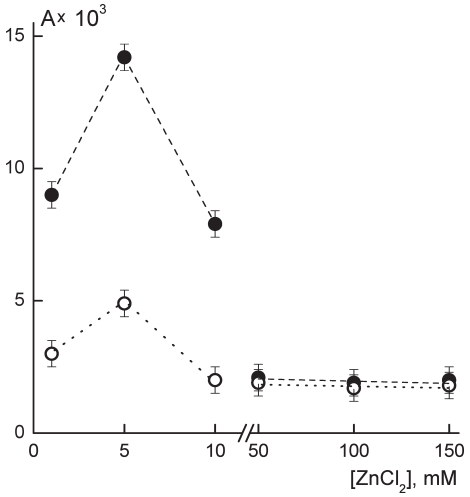

Figure 6. Yield of ATP produced by Zn-CK as a function of $^{64}ZnCl_2$ (open circles) and $^{67}ZnCl_2$ (filled circles) concentration. A is the radioactivity of [^{32}P]ATP (in scintillations/min/mg CK).

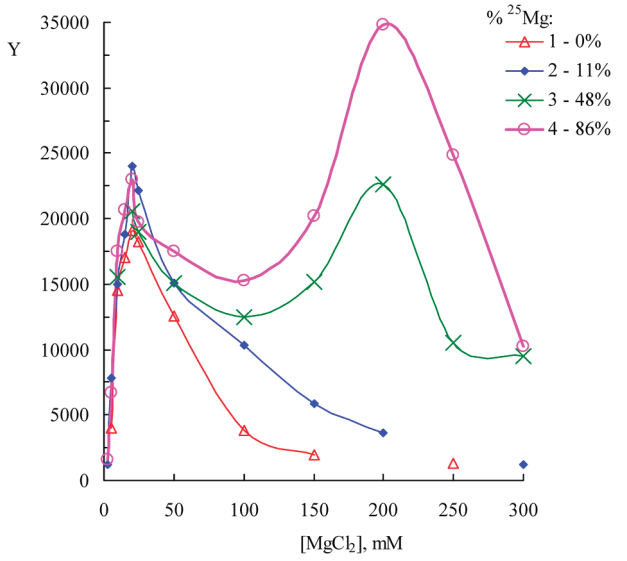

Figure 7. Rate of ATP synthesis by Mg-PK as a function of $MgCl_2$ concentration. Y is the radioactivity of ^{32}P-ATP (in scintillations/min/mg PK). The contents of $^{25}Mg^{2+}$ are shown in the right upper corner. Reprinted with permission from ref 36. Copyright 2008 American Chemical Society.

The yields of ATP in identical conditions were estimated as 9 100 ± 200, 20 100 ± 200, 8 900 ± 200, and 12 600 ± 200 scintillations/min/mg for ^{24}Mg-CK, ^{25}Mg-CK, ^{26}Mg-CK, and *Mg-CK, respectively. (The yield of ATP was measured as radioactivity of [^{32}P]ATP generated from the [^{32}P]creatine phosphate precursor.) The total (summarized) activity of the first three kinases, taken in fractions equivalent to those in natural abundance (9 100 × 0.79 + 20 100 × 0.10 + 8 900 × 0.11 = 10 200 ± 400 scintillations/min/mg) would be expected to coincide with that of *Mg-CK. In fact, it is very close to the measured activity of *Mg-CK (12 600 ± 400 scintillations/min/mg).[25]

(4) Then the experiment was inverted. In another series, a mixture of $^{24}MgCl_2$, $^{25}MgCl_2$, and $^{26}MgCl_2$ was taken in

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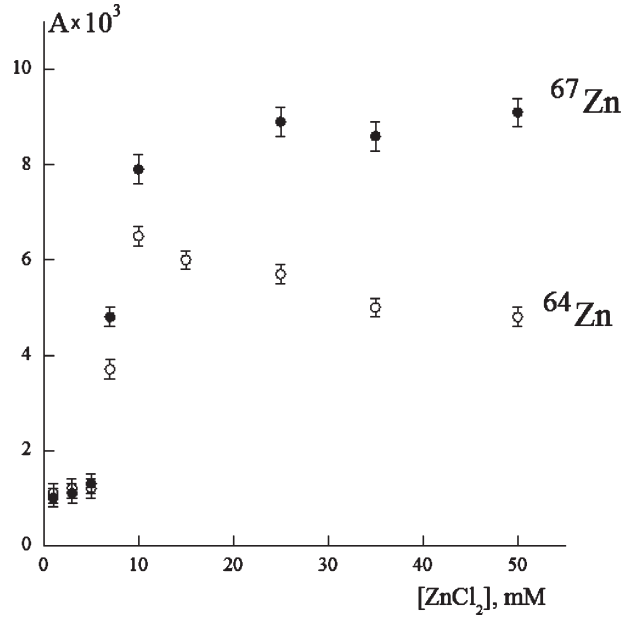
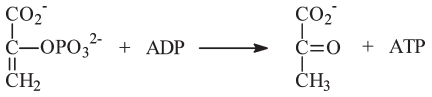


Figure 8. Yield of ATP produced by PK as a function of $^{64}ZnCl_2$ and $^{67}ZnCl_2$ concentrations. A is the radioactivity of ^{32}P-ATP (in scintillations/min/mg CK). ^{67}Zn-PK contains 78.4% of ^{67}Zn; ^{64}Zn-PK has a natural abundance of ^{67}Zn.

proportion of 0.79:0.10:0.11 identical to that in natural $^*MgCl_2$. The yield of ATP produced by CK loaded with this mixture was measured to be 11 400 ± 400 scintillations/min/mg, whereas the yield of ATP produced by *Mg-CK was 12 100 ± 400 scintillations/min/mg. These experiments leave no doubts in the validity of the detected magnesium isotope effect and exclude any possible artifacts.

5. PYRUVATE KINASE: MAGNESIUM AND ZINC ISOTOPES

Pyruvate kinase (PK) transfers a phosphate group from phosphoenolpyruvate to ADP producing ATP and pyruvate according to the reaction



Like in the case of CK, the transfer of the phosphate group is mediated by a magnesium ion. The rate of ATP synthesis by PK as a function of magnesium concentration is shown in Figure 7.[36]

It exhibits two remarkable features. First, its dependence on the concentration reveals two maxima; second, it demonstrates an unusual dependence on the magnesium isotope composition. At low concentrations of Mg^{2+} ions (10−50 mM), there is no dependence on the isotopes. At high concentrations (100−300 mM), Mg-PK demonstrates an enormously strong nuclear spin dependence of the ATP yield. Step-by-step replacement of spinless $^{24,26}Mg^{2+}$ ions in the catalytic sites by nuclear magnetic $^{25}Mg^{2+}$ ions gradually increases the ATP yield (Figure 7). Among other enzymes, PK demonstrates a very specific behavior. It is not excluded that, at high concentration of $MgCl_2$, its structure is somehow disturbed; nevertheless, it continues to function. Moreover, the higher the content of ^{25}Mg, the higher is the extent of its survival. It means that PK is a strongly sustained enzyme

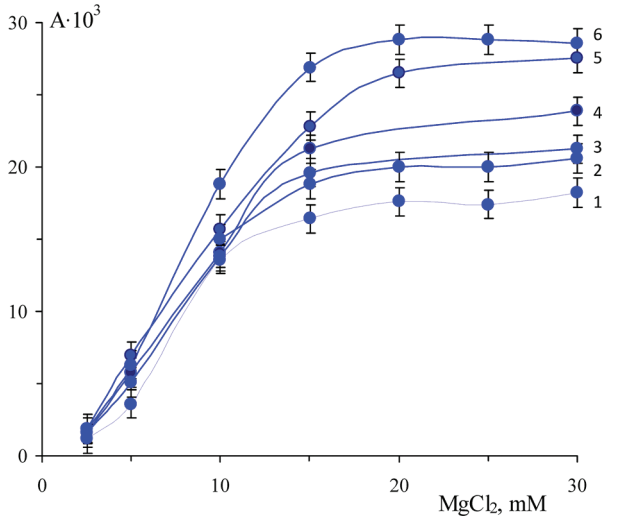


Figure 9. Activity of Mg-PGK as a function of $MgCl_2$ concentration. A stands for radioactivity of [^{32}P]ATP (in scintillations/min/mg PGK). The contents of ^{25}Mg in series 1−6 are equal to 0, 9, 25, 50, 75, and 98%, respectively. The lines are drawn to guide the eyes. Reprinted with permission from ref 33. Copyright 2005 American Chemical Society.

and its stability and functioning may be increased by magnetic isotope ^{25}Mg. The functioning of PK at high concentrations of $MgCl_2$ certainly has no importance in terms of biochemistry and physiology; however, it deserves attention as a purely chemical phenomenon in which magnetic isotope effect functions.

Figure 8 demonstrates the anomalous behavior of Zn-PK. Again a nuclear spin dependence of the ATP synthesis exists only at high concentrations of Zn^{2+} ions (at 50 mM of $ZnCl_2$ isotope effect is 2.2 ± 0.3).[35]

6. PHOSPHOGLYCERATE KINASE: MAGNESIUM ISOTOPES

Figure 9 shows the dependence of the ATP yield produced by phosphoglycerate kinase (PGK) on the $MgCl_2$ concentration. The activity of PGK depends on the isotope composition: the limiting yield of ATP synthesized by ^{25}Mg-PGK at [$MgCl_2$] ≥ 20 mM is by 1.7 ± 0.2 times higher than that synthesized by ^{24}Mg-PGK. There is also a linear correlation between the yield of ATP and the share of ^{25}Mg in the total magnesium pool (Figure 3).[33]

7. MITOCHONDRIA: MAGNESIUM AND ZINC ISOTOPES

Myocardial mitochondria were tested in vitro. To estimate the contribution of oxidative phosphorylation to the total ATP yield, methyl nicotine amide (MNA) or KCN were used. Acting differently, both inhibitors are nonetheless known to suppress the activity of ATP synthase. The rates of ATP production by isolated mitochondria (Figure 10) incubated in media containing separately ions $^{24}Mg^{2+}$ (99.6% of ^{24}Mg), $^{25}Mg^{2+}$ (95.8% of ^{25}Mg), and $^{26}Mg^{2+}$ (96.3% of ^{26}Mg) strongly depend on the nuclear spin and magnetic moment: mitochondria with magnetic nuclei ^{25}Mg produce twice as much ATP as mitochondria with spinless, nonmagnetic nuclei ^{24,26}Mg.[25,37] When oxidative ATP synthesis is selectively blocked by treatment with MNA, the yield of ATP strongly decreases. The remaining part refers to the ATP produced by mitochondrial creatine kinase (m-CK), which also demonstrates an isotope effect: $^{25}Mg^{2+}$ ions are about twice more

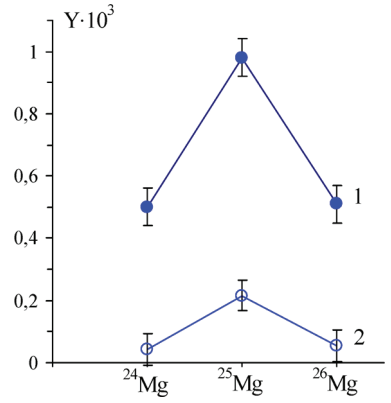


Figure 10. Rates of ATP synthesis by mitochondria as a function of magnesium isotope. 1, intact mitochondria; 2, mitochondria subjected to selective blockade of oxidative phosphorylation by MNA. The yield of ATP is given in μmol/min/g of total amounts of protein; concentration of $MgCl_2$ in all experiments was 15 mM, and temperature was 37 °C. The lines are drawn to guide the eyes. Reprinted with permission from ref 25. Copyright 2005 Springer.

active than $^{24}Mg^{2+}$ or $^{26}Mg^{2+}$ ions. There is no difference between $^{24}Mg^{2+}$ and $^{26}Mg^{2+}$ ions in both oxidative and substrate ATP synthesis. Moreover, the activity of m-CK was shown to be almost identical to that of creatine kinase isolated from *Vipera xanthia* venom.[25] These observations demonstrate that enzymatic ATP synthesis in mitochondria is a spin-selective process exhibiting magnetic isotope effect.

Additional experiments were carried out to compare the ATP formation capabilities of mitochondria with natural magnesium (*$MgCl_2$) and with magnesium enriched with ^{25}Mg ($^{25}MgCl_2$) (Table 2). They demonstrate high efficiency of mitochondrial ^{25}Mg-ATP synthase, proportional to the content of $^{25}MgCl_2$.

Rat heart muscle mitochondrial m-CK was tested with [^{32}P]phosphocreatine and $ZnCl_2$ of different isotope compositions. After 60 min incubation of mitochondria, the total ATP yield and oxygen consumption were measured. The ATP yield as a function of $ZnCl_2$ concentration is shown in Figure 11. It exhibits the following features: First, Zn^{2+} ions were indeed found to catalyze mitochondrial ATP synthesis with the efficiency comparable with that of Mg^{2+} ions. Second, the ATP yield increases as the concentration of $ZnCl_2$ increases, reaches maximum at ~5 mM, and then decreases. Third, even at low concentration of $ZnCl_2$ (~1 mM), there is a large isotope effect in the ATP synthesis. Fourth, there is no isotope effect in the oxygen consumption of mitochondria. The latter means that m-CK in mitochondria functions independently of the ATP synthase.[33]

It is necessary to note that the concentration of Mg^{2+} and Zn^{2+} ions in the catalytic sites of mitochondria does not correspond to that in the reaction media because the distribution of ions between the reaction media and mitochondria is not known. However, the distribution of isotopes in catalytic sites perfectly corresponds to that in the reaction media because neither binding of ions nor their diffusion is supposed to depend on the isotopes. At least they cannot account for the experimentally observed isotope effects.

8. NUCLEAR SPIN-DEPENDENT CHEMISTRY OF ATP SYNTHESIS

To summarize what has been presented so far, the rate of enzymatic ATP synthesis strongly depends on the nuclear

Table 2. Rate of ATP Synthesis Catalyzed by $MgCl_2$ in Mitochondria[a]

$MgCl_2$	Y × 10^3
$MgCl_2$ (20 mM, 10% ^{25}Mg)[a]	6.7
$^{25}MgCl_2$ (20 mM, 92% ^{25}Mg)	9.8
$^{25}MgCl_2$ (20 mM, 95.8% ^{25}Mg)	11.1

[a] Y is the yield of ATP in μm/min/g of protein; the accuracy is 10%.

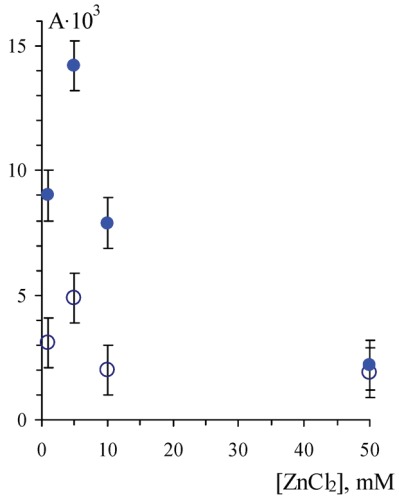


Figure 11. Rates of ATP synthesis by m-CK in mitochondria as a function of zinc isotopes: $^{64}ZnCl_2$ (open circles) and $^{67}ZnCl_2$ (filled circles). A is the radioactivity of ATP (in scintillations/min/mg of protein).

magnetic moments of the metal ions: ATP synthase and kinases, in which metal ions have magnetic nuclei, are found to be by 2−3 times more efficient than enzymes with spinless, nonmagnetic isotopic nuclei. This phenomenon clearly and unambiguously manifests that the ATP synthesis is a process in which the mass-independent, magnetic isotope effect operates rather than the classical, mass-dependent one.[31]

The nuclear spin-dependence of ATP synthesis is a new, paradigm-shifting phenomenon. It reliably indicates that the mechanism of the ATP synthesis includes paramagnetic intermediates and that, besides the generally accepted nucleophilic mechanism, there is also another mechanism that should be in accordance with the following physical postulates:

(1) ATP synthesis is a radical or, more exactly, an ion-radical process.

(2) The starting, key reaction of the process is an electron transfer from ADP to the M^{2+} ion, which generates an ion-radical pair composed of an M^+ ion and phosphate radical-anion of ADP as the partners; here M is Mg, Ca, Zn.

(3) Because of spin conservation, the chemical reactivity of triplet and singlet spin states of the ion-radical pair is different. This leads to a different yield of ATP along the singlet and triplet phosphorylation channels.

(4) The relative contribution of these spin channels to the ATP yield is controlled by electron−nuclear (hyperfine) magnetic coupling of unpaired electrons with the magnetic nucleus ^{25}Mg (^{43}Ca, ^{67}Zn) in the Mg^+ (Ca^+, Zn^+)

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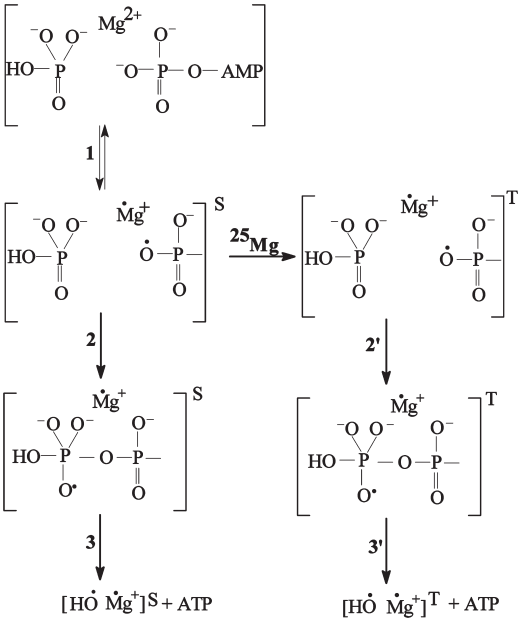
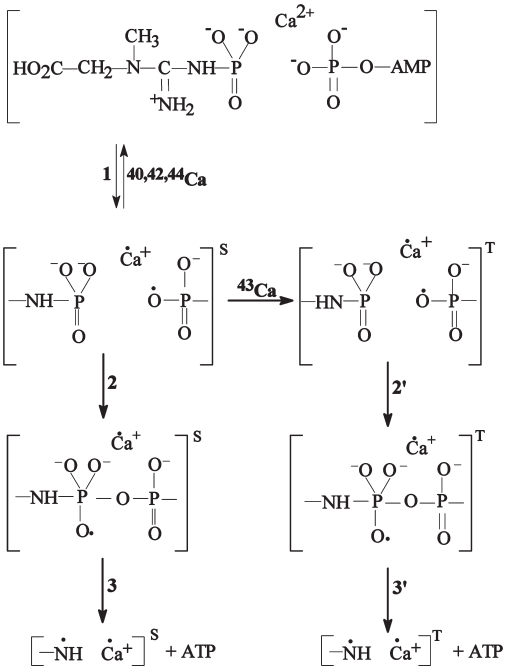
Scheme 1. Mechanism of ATP Synthesis by ATP Synthase



Scheme 2. Mechanism of ATP Synthesis by Ca-CK



ion and with ^{31}P in the ADP phosphate radical. It induces singlet−triplet spin conversion and results in a nuclear spin-dependence of the ATP synthesis.

A reaction scheme was suggested to account for this unusual but firmly established phenomenon.[36] Schemes 1 and 2 illustrate such a mechanism for particular cases of ATP synthase (oxidative phosphorylation) and CK (substrate phosphorylation). The schemes are exemplified by magnesium and calcium ions but are valid also for zinc as well as for other enzymes. As a first step, Scheme 1 implies transfer of an electron from the terminal phosphate group of ADP to Mg^{2+} ion, which generates the primary ion-radical pair, composed of the radical-cation Mg^+ and oxyradical of ADP (reaction 1). Because of the total spin conservation in the process, this pair is in a singlet spin state. The next step is the phosphorylation itself in which the ADP oxyradical attacks the P═O chemical bond of inorganic phosphate (reaction 2). The oxyradical that has been additionally generated in this reaction decomposes via β-scission of the P−O chemical bond (reaction 3), generating ATP and a final ion-radical pair (HO Mg^+); the latter regenerates Mg^{2+} in the reaction of back electron transfer:

$$(\mathrm{HO\ Mg^+}) \xrightarrow{\mathrm{H^+}} \mathrm{H_2O} + \mathrm{Mg^{2+}} \qquad \text{(I)}$$

The rate of phosphorylation along the singlet channel (reactions 1-3 in Schemes 1and 2) is suppressed by spin-allowed reverse electron transfer in the primary ion-radical pair, which regenerates the starting reactants and decreases the ATP yield. However, in the presence of $^{25}Mg^{2+}$, $^{43}Ca^{2+}$, and $^{67}Zn^{2+}$ ions, hyperfine coupling of the unpaired electron with the magnetic nuclei ^{25}Mg, ^{43}Ca, and ^{67}Zn in the Mg^+, Ca^+, and Zn^+ ions stimulates singlet−triplet spin conversion of the primary ion-radical pair and transforms it into a triplet pair, in which back electron transfer is spin-forbidden. This new triplet channel of phosphorylation (reactions 2′ and 3′) provides an additional yield of ATP that increases the total production of ATP by 2−3 times. The final ion-radical pair in the triplet channel undergoes fast triplet−singlet conversion due to electron spin relaxation (in OH the spin relaxation time is $\sim 10^{-11}$ s) and again regenerates Mg^{2+}, Ca^{2+}, and Zn^{2+} ions in the reactions identical to (I).

Schemes 1 and 2 elucidate nuclear spin control of the ATP yield as the main feature of ATP synthesis. However, they are not adequate to real enzymatic chemistry as long as they do not reflect the role of metal ions as catalysts. The following will be exemplified by the case of magnesium ions. Numerous studies convincingly demonstrate that the functioning of protein kinase requires at least two Mg^{2+} ions in the catalytic site.[8,38−40] PGK was shown to function only if two Mg^{2+} ions are presented in the catalytic site; one of them is tightly bound in the MgADP complex, the other is "free", not bound with phosphate groups, but solvated by water molecules and protein groups.[41] Even RNA synthesis by RNA polymerase requires assistance of the two Mg^{2+} ions.[42]

Moreover, detailed kinetic studies of ATP synthesis by ATP synthase convincingly prove that the presence of at least two magnesium ions is indeed required for ATP synthesis to occur: one is tightly bound to ADP, the other is present as a free but hydrated complex $Mg(H_2O)_n^{2+}$.[43] Thus, in the modified Scheme 1, the starting electron transfer reaction 1 should be written as follows:

$$\mathrm{Mg(H_2O)_n^{2+} + Mg(H_2O)_m^{2+}(ADP^{3-}) \rightarrow Mg(H_2O)_n^{+} + Mg(H_2O)_m^{2+}(ADP^{2-})} \qquad (1)$$

$$\mathrm{Mg(H_2O)_n^{2+} + Mg(H_2O)_m^{2+}(ADP^{2-}) \rightarrow Mg(H_2O)_n^{+} + Mg(H_2O)_m^{2+}(ADP^{-})} \qquad (2)$$

They imply that the MgADP complexes are also hydrated by m water molecules (their structures and energies will be discussed later). Reaction 1 refers to fully deprotonated ADP, and reaction 2 refers to partly protonated ADP because at physiological pH in cells and mitochondria both forms of ADP are supposed to be present almost equally. These two reactions generate an

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ion-radical pair composed of $Mg(H_2O)_n^+$ ion and the phosphate radical anion of ADP as partners.

Another important consequence in the case of CK is the formation of creatine and creatinine from the substrate residue. The yield of ATP was shown to coincide with the total yield of creatine and creatinine.[32] It means that every chemical act of ADP phosphorylation quantitatively transforms the creatine residue into either creatine or creatinine and the yield of creatine is expected to exhibit nuclear spin dependence identical to that for the ATP yield. This prediction is in a perfect agreement with experiment (see Figures 4 and 5). Note that only the ion-radical mechanism is able to properly explain isotope as well as magnetic field effects.

9. IN VIVO ISOTOPE EFFECTS AND MEDICINE

The great advantage of the ion-radical mechanism is that it can be switched on artificially by insertion of $MgCl_2$ (or, even better, of $^{25}MgCl_2$) in excess to stimulate ATP synthesis and hence to prevent some pathological disorders related to the deficiency of ATP in vivo such as ischemia caused local tissue hypoxia cases, heart muscle metabolic disbalance of all sorts, drug and toxins cardiotropic side effects, electrical trauma consequences, inhalation deplete hypoxia, etc. For these specific purposes, a magnesium ion carrier (nanocationite) based on some new porphyrin adduct of fullerene-C_{60} (PMC16) (see Supporting Information, Figure 1S) has been designed.[44] This novel product was found to be suitable for the targeted delivery of Mg^{2+} nanoamounts toward the heart muscle. This nanocarrier gets accumulated predominantly in the heart muscle because myocardial cells have a high-affinity protein receptor to porphyrin residue of PMC16 situated in the external membrane of myocardiocyte mitochondria. Another attractive property of PMC16 as a potential drug is its day-long retention in the heart tissue, particularly inside the heart mitochondria membranes.[45,46] Being membranotropic cationites, these "smart" nanocontainers release Mg^{2+} ions only in response to the metabolic acidosis, i.e., to the slight acidic pH shift known as a direct and inevitable consequence of a tissue hypoxia of any sort, but take them back once the cell metabolism gets recovered.

The ability to stimulate ATP synthesis in the heart muscle of living rats was demonstrated by in vivo experiments. Injection of doxorubicin was to promote a significant damage to the local myocardial ATP synthesis, suppressing it by ~70%. Then the PCM16 loaded with $^{24}MgCl_2$ or $^{25}MgCl_2$ was injected the same way, and after that a recovery of the ATP production up to the initial, predoxorubicin, level was observed. The extent of recovery as a function of magnesium concentration is shown in Figure 12. Evidently, there is a large isotope effect: delivery of [^{25}Mg]PMC stimulates ATP synthesis by 2−3 times more efficiently than [^{24}Mg]PMC. This is the first report ever on the isotope effect manifesting itself in a living organism and hence showing the potential of this specific effect to be applied as an efficient remedy for the treatment of heart disease—particularly for minimizing some known drug cardiotoxic side effects.[47−52]

The [$^{25}Mg^{2+}$]PMC16 nanocationites were carefully tested in vivo to estimate all major pharmacokinetics and pharmacodynamics patterns in a variety of mammals including mice, rats, rabbits, dogs, and goats. One may conclude that the stimulation of ATP synthesis by a magnetic isotope of magnesium is a new phenomenon of fundamental biomedical importance; it represents a breakthrough in the design of new remedies for treatment

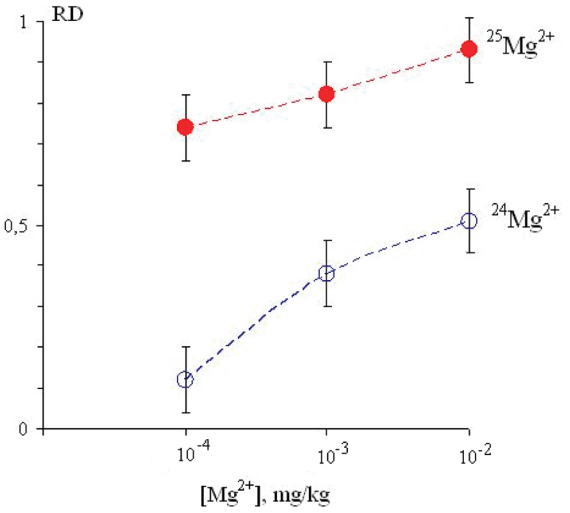

Figure 12. Recovery degree RD of ATP production in living rats as a function of delivered amounts of Mg^{2+} ions.

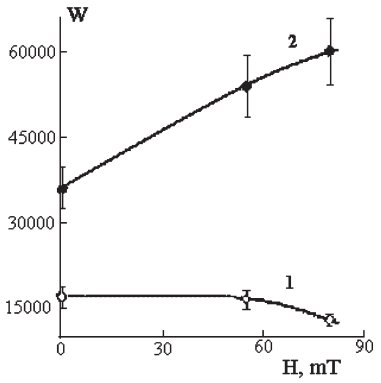

Figure 13. Rate W of ATP synthesis by ^{24}Mg-CK (1) and by ^{25}Mg-CK (2) as a function of the magnetic field. The rate was measured as radioactivity of [^{32}P]ATP. The lines are drawn to guide the eye. Reprinted with permission from ref 12. Copyright 2008 American Chemical Society.

of cell/tissue hypoxia and some heart pathologies related to ATP deficiency. PMC16 is shown to be a safe, easy-to-eliminate, and efficient medicinal agent with a prolonged pharmacological activity and with no sign of harmful metabolites formed.

10. MAGNETIC FIELD EFFECTS

According to the ion-radical mechanism, the enzyme catalytic site is a nuclear spin-dependent nanoreactor with the two competing reaction channels, singlet and triplet ones. Their relative contributions to the ATP synthesis are controlled by hyperfine coupling of unpaired electrons with magnetic nuclei ^{25}Mg, ^{43}Ca, ^{67}Zn, and ^{31}P. However, spin conversion of the ion-radical pair is controlled not only by internal magnetic fields (hyperfine coupling) of the magnetic nuclei but also by an external magnetic field. To verify this statement, the yield of ATP synthesized by Mg-CK as a function of an external magnetic field was studied.[12] The yield of ATP produced by enzyme with $^{24}Mg^{2+}$ ions was shown to decrease by ~10% in magnetic field 80 mT (Figure 13), whereas for enzyme with $^{25}Mg^{2+}$ ions it

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Chart 1. Magnesium Complexes of the Pyrophosphates

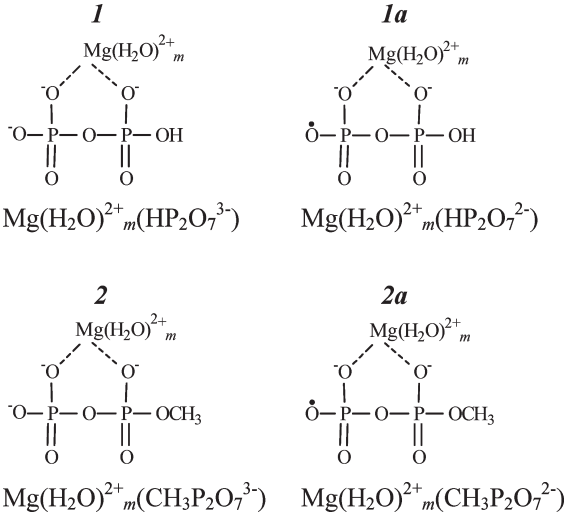
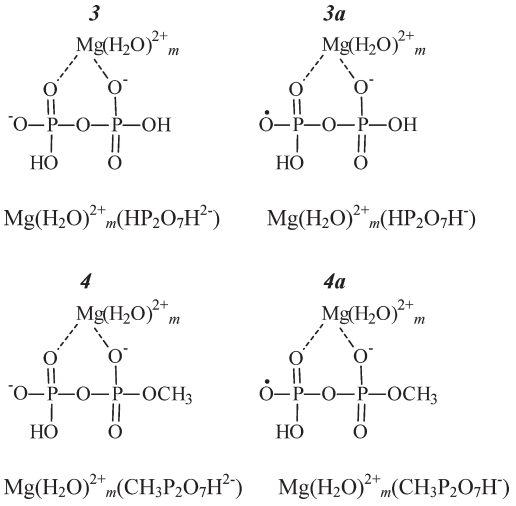


Chart 2. Magnesium Complexes of Protonated Pyrophosphates



increases by 50 and 70% in the fields 55 and 80 mT, respectively. In the Earth field, the rate of ATP synthesis by enzyme with $^{25}Mg^{2+}$ ions is 2.5 times higher than that by enzymes with nonmagnetic nuclei ^{24}Mg or ^{26}Mg. Both magnetic field and magnetic isotope effects are in perfect agreement with the physical postulate[12] and demonstrate that the ATP synthesis is an ion-radical process affected by Zeeman interaction and hyperfine coupling in the intermediate ion-radical pair. The experimental results are in perfect agreement with theoretical predictions.[53,54]

11. ENERGETICS OF ATP SYNTHESIS

Schemes 1 and 2 explain isotope and magnetic field effects in ATP synthesis. However, the intriguing problem is that the key reaction 1 of ATP synthesis shown in Schemes 1 and 2 occurs only in enzymes and does not take place in water. To answer this question, it is necessary to know the structure of the metal complexes and the energies of their reactions. They were calculated using density functional theory (DFT).[55−57]

11.1. Complexes of Metal Ions and their Reactions

In the catalytic site, ADP is present as complex $M^{2+}(ADP)$; we will specify the metal as M where M implies Mg, Ca, or Zn. Further, its structure is modeled by the hydrated pyrophosphate complexes $M(H_2O)_m^{2+}(HP_2O_7^{3-})$ and $M(H_2O)_m^{2+}$-$(CH_3P_2O_7^{3-})$ with a hydrogen atom and a methyl group, respectively, instead of an adenosine residue. Chart 1 illustrates their structure by examples of magnesium complexes **1** and **2** in which a Mg^{2+} ion is supposed to be coordinated to the oxygen atoms of the pyrophosphate anion and accepts two coordinate bonds. The other coordinate bonds may be used for addition of *m* water molecules, with *m* being in the range from 0 to 4. The value *m* = 4 corresponds to the fully completed six-coordinated shell of the Mg^{2+} ion. Besides complexes **1** and **2**, ion-radicals **1a** and **2a** (Chart 1) generated from **1** and **2** by electron detachment were also calculated. In complexes **1** and **2**, **1a** and **2a** the pyrophosphate anions are deprotonated. However, in cells and mitochondria ADP is presented in both forms—deprotonated and partly protonated—almost equally, on a par so that the structures of partly protonated complexes **3** and **4**, presented in Chart 2, also have been calculated, as well as their ion-radicals **3a** and **4a** generated from **3** and **4** by electron detachment.[58]

Typical structures for selected values of *m* are shown in Figure 2S (Supporting Information). The withdrawal of electron from complexes **1**−**4** results in redistribution of the electron density and slightly changes interatomic distances in complexes **1a**−**4a** with respect to those in **1**−**4**. The structures of calcium and zinc complexes were shown to be identical to those of magnesium[34,59] (see Figures 3S and 4S). The energies of the hydrated complexes $M(H_2O)_n^{2+}$ and $M(H_2O)_n^{+}$ as a function of *n*, the number of water molecules in coordination sphere of ions, also have been calculated[58,59] at the B3LYP/6-31G* level of DFT theory[55,56] (see Figure 5S in Supporting Information).

Knowing the energies of individual complexes, one can calculate energies of the electron transfer from hydrated pyrophosphate complexes **1**−**4** shown in Charts 1 and 2 to the complexes $Mg(H_2O)_n^{2+}$ as a function of *n* and *m*:

$$M(H_2O)_n^{2+} + M(H_2O)_m^{2+}(OPO_2OPO_3CH_3)^{3-} \rightarrow M(H_2O)_n^{+} + M(H_2O)_m^{2+}(OPO_2OPO_3CH_3)^{2-} \quad (1a)$$

$$M(H_2O)_n^{2+} + M(H_2O)_m^{2+}(OPO_2OPO_3CH_3)^{3-} \rightarrow M(H_2O)_n^{+} + M(H_2O)_m^{2+}(OPO_2OPO_3CH_3)^{2-} \quad (2a)$$

$$M(H_2O)_n^{2+} + M(H_2O)_m^{2+}(HOPO_2OPO_3CH_3)^{2-} \rightarrow M(H_2O)_n^{+} + M(H_2O)_m^{2+}(HOPO_2OPO_3CH_3)^{-} \quad (3)$$

$$M(H_2O)_n^{2+} + M(H_2O)_m^{2+}(HOPO_2OPO_3CH_3)^{2-} \rightarrow M(H_2O)_n^{+} + M(H_2O)_m^{2+}(HOPO_2OPO_3CH_3)^{-} \quad (4)$$

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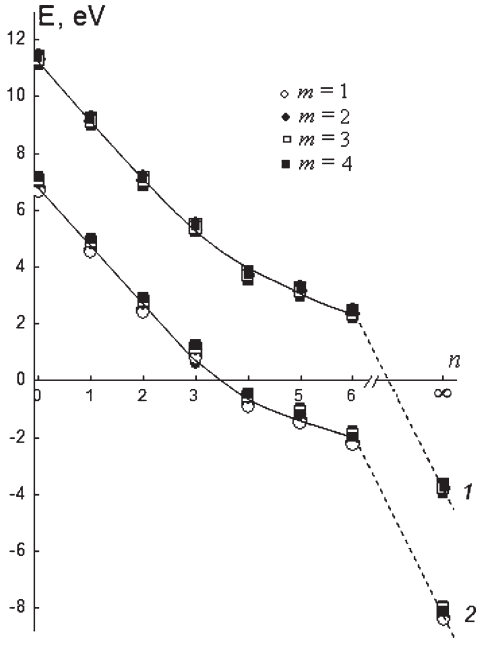

Figure 14. Energies of reactions 1 (curve 1) and 2 (curve 2) as a function of the number of water molecules n in hydrated complex $Mg(H_2O)^{2+}_n$. Reprinted with permission from ref 58. Copyright 2010 American Chemical Society.

These reactions model reaction 1 in Schemes 1 and 2 as starting reaction of electron transfer in the ATP synthesis:

$$M(H_2O)_n^{2+} + M^{2+}(ADP)^{3-} \rightarrow M(H_2O)_n^{+} + M^{2+}(ADP)^{2-} \quad (5)$$

$$M(H_2O)_n^{2+} + M^{2+}(ADP)^{2-} \rightarrow M(H_2O)_n^{+} + M^{2+}(ADP)^{-} \quad (6)$$

The total energy for reactions 1−4 is defined as the difference between the energy of products and reactants. The reaction is exoergic if the energy of reactants exceeds that of products. On the contrary, the reaction is endoergic and energy-forbidden if the energy of products is higher than that of reactants. Such an approach has an important advantage because it cancels any errors and compensates possible inaccuracy in energy calculations of individual reactants and products.

The energies of reactions 1−4 for magnesium are shown in Figure 14 as a function of n. For n from 0 to 6, they were calculated by DFT theory; the energies of reactions in water, i.e., under conditions of the total hydration of magnesium ion (we will assume for this case $n = \infty$), were derived from the thermodynamics of the $Mg(H_2O)_n^{2+}$ and $Mg(H_2O)_n^{+}$ ions.[36] Figures 15 and 16 demonstrate energy of electron transfer as a function of n for calcium and zinc, respectively.[59]

11.2. Energy-Forbidden and Energy-Allowed Electron Transfer

The total energies of the electron transfer reactions presented in Figures 14−16 as functions of m and n result in the following conclusions.

(1) The energies do not depend on whether an adenosine residue is replaced by hydrogen atom (in complexes **1** and **3**) or by a methyl group (in complexes **2** and **4**). It means that these energies may be certainly attributed to the reactions of $Mg^{2+}(ADP)$ in native enzymes.

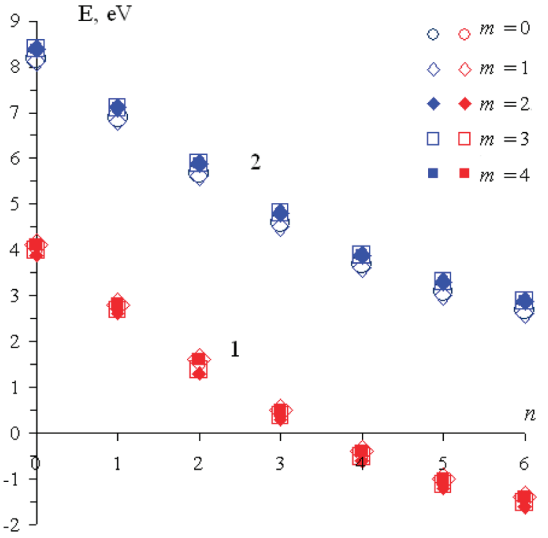

Figure 15. Energies of reactions 3 and 4 as a function of the number of water molecules n in the $Ca(H_2O)^{2+}_n$ ions for different m, the number of water molecules in $Ca(H_2O)_m^{2+}(HOPO_2OPO_3CH_3)^{2-}$ (curve 1) and $Ca(H_2O)_m^{2+}(OPO_2OPO_3CH_3)^{3-}$ (curve 2). Reprinted with permission from ref 59. Copyright 2010 Russian Academy of Science.

(2) The energies depend only slightly on m (m = 0−4), the number of water molecules in the first coordination sphere of ions $M(H_2O)^{2+}_m$(pyrophosphate) and $M(H_2O)^{+}_m$(pyrophosphate); therefore, they are almost independent of the hydration of the $Mg^{2+}(ADP)$ complex in the catalytic site.

(3) The energies of all reactions are strongly dependent on n, the number of water molecules in the hydration shell of $M(H_2O)_n^{2+}$ and $M(H_2O)_n^{+}$ (see Figures 14−16).

(4) No reaction occurs in water, when the hydrate shells of the reactants are fully completed ($n = \infty$). This result is in perfect agreement with the fact that ATP synthesis from ADP and substrate does not take place in water.

(5) For ATP synthesis to proceed in the catalytic site, it is necessary to partly remove water from the site and hydration shell of $M(H_2O)_n^{2+}$. Only under this condition the key reaction—electron transfer from $M^{2+}(ADP)$ to $M(H_2O)_n^{2+}$, reaction 1 in Schemes 1 and 2—becomes energy-allowed.

(6) The reaction of complexes **1** and **2** with $Mg(H_2O)_n^{2+}$ is exoergic at n = 0−6 and even at $n > 6$ (Figure 14). The switching from exoergic to endoergic reaction takes place at n^*, which lies somewhere between $n = 6$ and $n = \infty$. This magnitude $6 < n^* < \infty$ functions as a trigger; it determines the boundary between energy-allowed and energy-forbidden regimes, and the boundary is overcome as the compression of the catalytic site squeezes water molecules out of the site and partly removes it from the $Mg(H_2O)_n^{2+}$ ion. The same is valid for calcium and zinc ions (Figures 15 and 16).

(7) For the reactions of complexes **3** and **4**, which include protonated pyrophosphate anions $HP_2O_7H^{2-}$ and $CH_3P_2O_7H^{2-}$, with $Mg(H_2O)_n^{2+}$ the trigger value of n^* is estimated quantitatively as $n^* = 4$ (Figure 11); at $n > 4$

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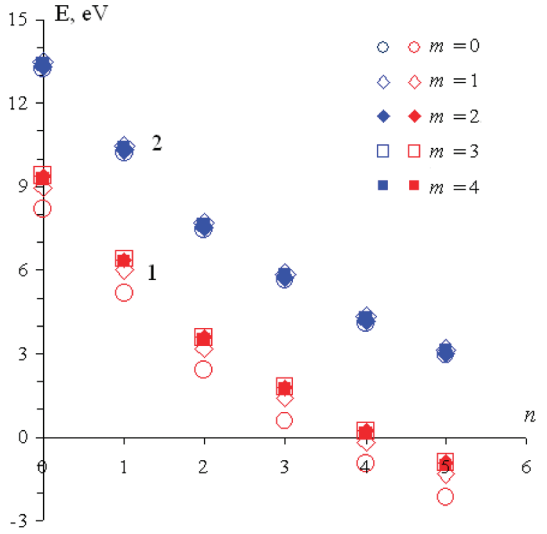


Figure 16. Energies of reactions 3 and 4 as a function of the number of water molecules n in the $Zn(H_2O)^{2+}{}_n$ ion for different m, the number of water molecules in $Zn(H_2O)_m{}^{2+}(HOPO_2OPO_3CH_3)^{2-}$ (curve 1) and $Zn(H_2O)_m{}^{2+}(OPO_2OPO_3CH_3)^{3-}$ (curve 2). Reprinted with permission from ref 59. Copyright 2010 Russian Academy of Science.

the reactions are endoergic, at $n < 4$ they are energy-allowed. For the calcium ions the trigger value is $n^* = 3$; for zinc ions it is $n^* = 4$ (Figures 15 and 16).

12. HOW DO ATP-SYNTHESIZING ENZYMES FUNCTION?

Despite the difference in enzyme mechanics (ATP synthase is a rotary molecular motor, kinases are molecular pumps), a general principle of the functioning of the ATP-generating enzymes can be formulated as follows. Theoretical inspection of the reactions between pyrophosphate and hydrated complexes reveals their general properties. These reactions perfectly model those in catalytic sites of enzymes:

$$M(H_2O)_n{}^{2+} + M(H_2O)_m{}^{2+}(ADP^{3-}) \rightarrow M(H_2O)_n{}^{+} + M(H_2O)_m{}^{2+}(ADP^{2-})$$

$$M(H_2O)_n{}^{2+} + M(H_2O)_m{}^{2+}(ADP^{2-}) \rightarrow M(H_2O)_n{}^{+} + M(H_2O)_m{}^{2+}(ADP^{-})$$

Here M = Mg, Ca, or Zn. These reactions generate ion-radical pairs in which subsequent reactions 2′ and 3′ in Schemes 1 and 2, similar to reactions 2 and 3, accomplish ATP synthesis.

The modified scheme implies the presence of at least two magnesium ions in the catalytic site: one is tightly bound to ADP, the other is present as a hydrated complex $M(H_2O)_n{}^{2+}$. Detailed kinetic studies of the ATP synthesis by ATP synthase convincingly prove that the presence of two magnesium ions is indeed required for the ATP synthesis to occur: one of them is bound ADP, the other belongs to hydrated ion.[37−43] This experimental result is in perfect agreement with our theoretically derived conclusions.

The ion-radical mechanism presented by Schemes 1 and 2 seems to be unbelievable, because electron transfer (reaction 1) does not occur in water where metal ions are highly hydrated. However, the remarkable property of the enzymes is that, in the reactive state, when the enzyme domains are drawn together to unite substrate and ADP, they squeeze water molecules out of the catalytic site[60] and partly dehydrate the $M(H_2O)_n{}^{2+}$ ion.[61]

Now the question is what complexes of ADP are responsible for the ATP synthesis, fully deprotonated $Mg^{2+}(ADP)^{3-}$ or partly protonated $Mg^{2+}(ADP)^{2-}$. As follows from the energy consideration, it depends on the state of the partners, the $Mg(H_2O)_n{}^{2+}$ complexes. The binding energies of the outer, sixth water molecules in the complexes $Mg(H_2O)_6{}^{2+}$, $Ca(H_2O)_6{}^{2+}$, and $Zn(H_2O)_6{}^{2+}$ were calculated to be 24.5, 24.7, and 21.8 kcal/mol, respectively.[62] The energies of releasing the fifth water molecules are slightly larger, −28.0, 27.7, and 24.0 kcal/mol for the same complexes, respectively. To activate $M(H_2O)_n{}^{2+}$ complexes, i.e., to increase their electron affinity and to reach the threshold values of n^*, it is necessary to release at least two water molecules, the sixth and fifth. The total energy cost of this "undressing" process is ∼53−46 kcal/mol for M = Mg, Ca, Zn.[62] These values are rather high, so that the escape of coordinated water from the hydrated shell of ions seems to be the most energy-expensive process in the ion-radical mechanism. It means that the protonated complexes $Mg^{2+}(ADP)^{2-}$ are hardly involved in the ATP synthesis. The main sources of ATP seem to be complexes $Mg^{2+}(ADP)^{3-}$, i.e., complexes of the deprotonated ADP, for which $n^* \gg 6$. For these complexes to react there is no need to remove tightly bound water from the first coordination shell of $Mg(H_2O)_6{}^{2+}$. The release of the outer, weakly bound water of the third or even fourth coordination sphere is enough to activate the complex $Mg(H_2O)_n{}^{2+}$ as an electron acceptor and stimulate ATP synthesis.

The removal of water from the coordination sphere of a metal ion activates it by increasing its electron affinity, so that at some threshold value n^* electron transfer becomes exoergic and energy allowed. The water molecule with number n^* in complex $M(H_2O)_n{}^{2+}$ functions as a trigger; it switches the reaction between endoergic and exoergic regimes. At $n > n^*$ electron transfer is endoergic; at $n < n^*$ it is energy-allowed and exoergic (see Figures 14−16). Note that energy of electron transfer is independent of the m in the $M(H_2O)^{2+}{}_m$(pyrophosphate) complex; it is almost identical for $m = 0$−4.

A huge isotope effect in the rate of ATP synthesis is a direct and irrefutable proof of the proposed mechanism. It is evidence that the M^{2+} ion is a central reactant, which functions reversibly and transforms mechanical energy of the enzyme into the energy of the P−O bond in ATP.[58,61] It is a point where mechanics meets chemistry, the key point of the functioning of enzymes as mechano-chemical molecular motors, where energy of conformational mechanics of the enzyme macromolecule transforms into the energy of a chemical bond.

13. MAGNETIC PARAMETERS AND SPIN DYNAMICS OF THE ION-RADICAL PAIR

Electron transfer generates an ion-radical pair composed of paramagnetic complexes $M^{2+}(ADP)^{2-}$ and $M(H_2O)_n{}^{+}$. The contributions of singlet and triplet spin states of the pair to ATP production are different because the reverse electron transfer regenerates the starting reactants; it is spin-allowed in the singlet state but spin-forbidden in the triplet state. The populations of these states and the rates of singlet−triplet spin conversion are controlled by magnetic parameters, g-factors, and hyperfine coupling (HFC) of the unpaired electrons with

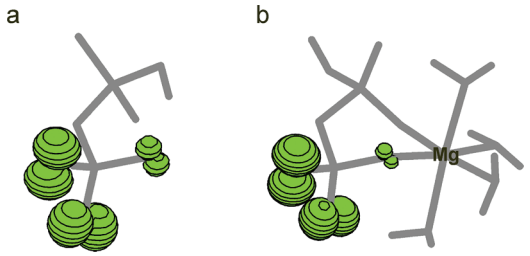


Figure 17. Spin densities (green color) in pyrophosphate radical $(HOPO_2OPO_3)^{2-}$ (a) and $Mg(H_2O)_4^{2+}(HOPO_2OPO_3)^{2-}$ complex (b). Reprinted with permission from ref 63. Copyright 2009 Elsevier.

magnetic nuclei ^{31}P and ^{25} Mg, ^{43}Ca, ^{67}Zn in the paramagnetic complexes, the partners of the ion-radical pair.

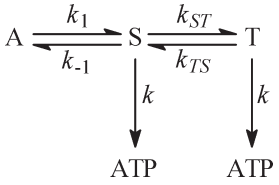
The magnetic parameters (g-factors and HFC constants $a(^{25}Mg)$, $a(^{31}P)$) of the paramagnetic pyrophosphate complexes $Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{2-}$, which model those in the catalytic site of enzymes, are identical to the parameters of the pyrophosphate radical.[63] It means that the pyrophosphate ligand in the complex is present as a pyrophosphate radical, in which spin density is almost completely localized on the terminal oxygen atom and only slightly propagates to the $Mg(H_2O)_m^{2+}$ fragment. This conclusion is illustrated in Figure 17 by direct comparison of spin densities calculated for isolated pyrophosphate radical and $Mg(H_2O)_4^{2+}(HOPO_2OPO_3)^{2-}$ complex.

The dominating contribution to the spin conversion of the ion-radical pair results from the $Mg(H_2O)_n^{+}$ ions. The HFC constants for these ions are rather large; they are in the limits of 180−40 G for n in the range from 0 to 4[63] (see Figure 6S in Supporting Information) and responsible for the fast spin conversion. The hyperfine coupling constant for ^{31}P in the oxyradical is ∼45 G so that the dominating contribution of the triplet channel into the ATP production is provided by catalytic sites with ^{25}Mg.

Similar conclusions were derived for the calcium pyrophosphate complexes $Ca(H_2O)^{2+}{}_m(HOPO_2OPO_3CH_3)^{-}$.[32] Their g-factors are almost independent the number of water molecules m in the coordination sphere of the calcium ion; their magnitudes are in the limits 2.013−2.015 and identical to the g-factor of the pyrophosphate radical $HOPO_2OPO_3CH_3^{-}$. The HFC constants $a(^{43}Ca)$ were calculated to be small: 1.1 and 1.0 G for m = 0 and 1, respectively, and negligibly small for m = 2−4 (see Figure 7S in Supporting Information). No doubts that they contribute almost nothing into the spin conversion of the ion-radical pair as well as the isotope effect. The g-factors as well as the HFC constants $a(^{43}Ca)$ in the $Ca(H_2O)^{2+}{}_m(HOPO_2OPO_3CH_3)^{-}$ complexes (Table 2S in Supporting Information) unambiguously indicate that the pyrophosphate ligand in the complex is present as a pyrophosphate radical, in which spin density is almost completely localized on the terminal oxygen atom and only slightly propagates to the $Ca(H_2O)_m^{2+}$ fragment, like in the similar paramagnetic complexes $Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{2-}$. It unambiguously demonstrates that the terminal oxygen atom of the pyrophosphate ligand in the complex $Ca(H_2O)^{2+}{}_m(HOPO_2OPO_3CH_3)^{2-}$ donates the electron from its lone pair to the $Ca(H_2O)_n^{2+}$ ion.

In contrast to the pyrophosphate complexes, the HFC constants $a(^{43}Ca)$ in $Ca(H_2O)_n^{+}$ ions are in the limits of 270−50 G for n in the range from 0 to 5,[32] i.e., they ensure fast singlet−triplet spin conversion of the ion-radical pairs with magnetic nuclei ^{43}Ca. No doubt, the same conclusions are valid for the zinc-induced ATP synthesis.

Scheme 3. Kinetic Scheme of Spin-Dependent ATP Synthesis



14. KINETICS AND QUANTITATIVE MEASURE OF ISOTOPE EFFECTS

In order to quantitatively determine the isotope effect, one would need to analyze a detailed kinetic scheme of reactions in catalytic site equivalent to chemical Schemes 1 and 2, as seen in Scheme 3:

It implies generation of the ion-radical pair S in the singlet state with a rate constant k_1; this pair may be annihilated via back electron transfer (rate constant k_{-1}) or produce ATP with a rate constant k. In the presence of a $^{25}Mg^{2+}$ ion, it may experience reversible spin conversion to the triplet state T and back with the rate constants k_{ST} and k_{TS}, respectively. Triplet state T is supposed to generate ATP with the same rate constant k as the singlet pair because reactions 2, 2′ and 3, 3′ in Schemes 1 and 2 are not spin-dependent.

It is easy to show that $k_{ST} = k_{TS}$ despite the fact that the triplet state is composed of the three spin substates, T_+, T_0, and T_-. Taking into account these substates, the rate constants k_{ST} and k_{TS} may be presented as

$$k_{ST} = k^0_{ST_+} + k^0_{ST_0} + k^0_{ST_-}$$
$$k_{TS} = k^0_{T_+S} + k^0_{T_0S} + k^0_{T_-S}$$

Here the rate constants with superscripts zero characterize the rate constants of S−T and T−S spin conversion along the individual spin channels. Because $k^0_{T_+S} = k^0_{ST_+}$, $k^0_{T_0S} = k^0_{ST_0}$, $k^0_{T_-S} = k^0_{ST_-}$, it immediately follows that $k_{ST} = k_{TS}$. The physical reason is that the spin conversion occurs along the three channels with equal rates in both directions.

The kinetic scheme is described by a system of kinetic equations:

$$\frac{d[A]}{dt} = k_1[A] - k_{-1}[S] \quad (1aa)$$

$$\frac{d[S]}{dt} = k_1[A] + k_{ST}[T] - (k_{-1} + k + k_{ST})[S] = 0 \quad (2aa)$$

$$\frac{d[T]}{dt} = k_{ST}[S] - (k + k_{ST})[T] = 0 \quad (3a)$$

$$\frac{d[ATP]}{dt} = k([S] + [T]) \quad (4a)$$

where [A], [S], [T], and [ATP] are concentrations of the catalytic sites, singlet and triplet pairs, and ATP, respectively. For the short-lived S and T pairs, the well-known method of

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quasi-stationary concentrations may be used, which implies that stationary concentrations of the pairs do not change, so that their derivatives with respect to time can be set at zero. This is a usual, well-verified, and reliable approximation that gives accurate results for both chemical and physical kinetics.

The solution of the system of eqs 1 − 4 results in the following rate of ATP synthesis:

$$\left(\frac{d[ATP]}{dt}\right)_m = \frac{kk_1(2k_{ST} + k)}{(k_{ST} + k_{-1} + k)(k_{ST} + k) - {k_{ST}}^2}[A] \quad (5a)$$

with the rate constant

$$k_m = \frac{kk_1(2k_{ST} + k)}{(k_{-1} + k + k_{ST})(k_{ST} + k) - {k_{ST}}^2} \quad (6a)$$

It is specified as the rate constant in the site with magnetic nucleus ^{25}Mg (subscript m). For the catalytic sites with nonmagnetic nuclei ^{24}Mg or ^{26}Mg, $k_{ST} = 0$ and the rate constant k_n (subscript n) of ATP synthesis in these sites follows from eq 6:

$$k_n = \frac{kk_1}{k_{-1} + k} \quad (7)$$

Their ratio represents the isotope effect

$$IE = \frac{k_m}{k_n} = \frac{(k_{-1} + k)(2k_{ST} + k)}{(k_{-1} + k + k_{ST})(k_{ST} + k) - {k_{ST}}^2} \quad (8)$$

Its magnitude is controlled by competition of the three elementary reactions in catalytic site: annihilation (k_{-1}), ATP synthesis (k), and spin conversion (k_{ST}).

For a quantitative estimation of the rate constants, the dependence of IE on the dimensionless ratios k_{-1}/k and k_{ST}/k was analyzed.[64] For the value IE = 1.7, which was observed experimentally in mitochondria, the relation $k/k_{ST}/k_{-1}$ = 1:5:10 was shown to reproduce this magnitude of IE. As shown in a previous section, ATP synthesis by the ion-radical mechanism starts with the complex $Mg(H_2O)_6^+$ (if its partner is $Mg^{2+}(ADP)^{3-}$) or $Mg(H_2O)_4^+$ (if its partner is protonated $Mg^{2+}(ADP)^{2-}$). As was shown before, a reaction with participation of the intermediate complex $Mg(H_2O)_6^+$ is the most probable. For this radical cation the hyperfine coupling constant $a(^{25}Mg)$ was calculated to be 40 MHz. Taking into account the hyperfine coupling constant for ^{31}P in the oxyradical, the total hyperfine coupling constant in the catalytic site with ^{25}Mg is expected to be ∼60 G (∼170 MHz) so that in the catalytic site the approximate rate constants k, k_{ST}, and k_{-1} are 3×10^7, 1.7×10^8, and 3.4×10^8 s^{-1}, respectively.

The fastest step in the kinetic scheme is the back electron transfer, i.e., $k_{-1} \gg k, k_{ST}$. Then from eq 8 follows

$$IE = \frac{2k_{ST} + k}{k_{ST} + k} \quad (9)$$

If singlet−triplet conversion is faster than ATP synthesis ($k_{ST} > k$), then the limiting value of IE corresponds to doubling of the ATP yield in sites with $^{25}Mg^{2+}$ ions with respect to those with $^{24,26}Mg^{2+}$ ions.

Now the total rate of ATP synthesis and ATP yield is determined as a sum of ATP produced independently by enzymatic sites with $^{25}Mg^{2+}$ and $^{24,26}Mg^{2+}$ ions:

$$Y = d[ATP]/dt = [ak_m + (1 - a)k_n][A] \quad (10)$$

Here α is the fraction of catalytic sites occupied by $^{25}Mg^{2+}$. If only $^{24,26}Mg^{2+}$ ions are presented in catalytic sites ($\alpha = 0$), then $Y_0 = k_n$ [A] and

$$\frac{\Upsilon}{\Upsilon_0} = \alpha\left[\frac{k_m}{k_n} - 1\right] + 1 \quad (11)$$

Comparing this ratio with the experimentally estimated dependence $Y/Y_0 = f(\alpha)$ (Figure 3), the ratio k_m/k_n for PGK is found to be equal to 1.5, i.e., the rate of ATP synthesis is 1.5 times higher in ^{25}Mg-PGK enzyme with respect to the same enzyme with a nonmagnetic isotope ^{24}Mg. For CK it is even higher and equal to 2.6.

15. RATE-LIMITING STEP OF ATP SYNTHESIS

The isotope effect seems to be the most convincing evidence that the limiting step in ATP synthesis is the reaction itself because neither entering of reactants into the catalytic site nor release of ATP are expected to be dependent on the isotope substitution. However this at a first glance quite evident conclusion is not correct. The catalytic site as a dynamic system may be subdivided into two subsystems, which can be conventionally specified as a fast and a slow one. The former stimulates the chemical transformations shown in Schemes 1 and 2 and requires short-range shifts and displacements of reactants to unite them and accomplish chemical synthesis. The latter includes long-range displacements of protein domains, which maintain entering of reactants and release of ATP. In contrast to the short-range subsystem that functions on the time scale of nanoseconds, the long-range subsystem operates on the time scale of micro- or even possibly of milliseconds. The former may be denoted the nanosystem, the latter the microsystem.

If the reaction, controlled by the nanosystem, is irreversible (later we will show that this is the case), the isotope effect arising in it may be detected even if the limiting step of the total ATP synthesis is the release of ATP from the catalytic site. The latter as well as the entering of reactants into the catalytic site are controlled by the microsecond molecular dynamics whereas ATP synthesis itself and, accompanying it, the isotope effect are controlled by the nanodynamics. The widely used parameters k_{cat} and K_M of enzymatic kinetics may be related to the microdynamics of the catalytic site. The Michaelis constant K_M seems to be almost independent of the isotope substitution; however, the isotope effect resulting from the reaction in the nanosystem may exhibit itself in the total rate constant k_{cat}.

16. EFFICIENCY OF ATP SYNTHASE AS A MOLECULAR MACHINE

Under the efficiency we will imply the probability that the ADP molecule, entering into the catalytic site, transforms into the ATP molecule. In terms of the rate constants, it is the ratio $k/(k + k_{ST} + k_{-1})$; see section 14. On the basis of the experimentally measured ^{25}Mg isotope effect on the rate of ATP synthesis by ATP synthase, the rate constants k, k_{ST}, and k_{-1} of the reactions in the catalytic site were estimated (see section 14). The limiting chemical step of the synthesis is shown to consist of an addition of the phosphoryl anion-radical of ADP to the P═O bond of phosphate with a rate constant of 3×10^7 s^{-1}. From the relationship between the rate constants, the efficiency of ATP synthase with $^{24}Mg^{2+}$ ions in which only hyperfine coupling with ^{31}P operates is estimated to be ∼8%.

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It means that only one molecule of ATP is formed from the 12 ADP molecules sequentially entering into the catalytic site. For the synthase with $^{25}Mg^{2+}$ ions in the catalytic site, the efficiency is almost doubled.

17. DO ENZYMATIC MOTORS FUNCTION REVERSIBLY?

It was shown that macroscopic reversibility in ATP synthesis does not mean microscopic reversibility, i.e., the pathway for ATP hydrolysis is not that for ATP synthesis.[8,9] A completely independent proof for this statement is seen through the isotope window. The authors[36] measured the rate of ATP hydrolysis by Mg-CK where magnesium ions were substituted by pure isotopic ions $^{24}Mg^{2+}$, $^{25}Mg^{2+}$, and $^{26}Mg^{2+}$. Figure 18 demonstrates the 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 remains constant, that is, ATP quantitatively transforms into ADP. It is remarkable that the ATP decay (and, therefore, ADP accumulation) is identical for all kinases and does not depend on the isotopes. It is a direct indication that the ATP → ADP enzymatic reaction is not spin-selective; no paramagnetic species are formed in this reaction. In this reaction there is no magnetic isotope effect, but the classical, mass-dependent isotope effect is negligibly small, so that it is not detected in enzymatic activity. It means that the reaction trajectories for the direct and reverse reactions are not equivalent.

18. COMPARATIVE ANALYSIS OF THE TWO MECHANISMS OF ATP SYNTHESIS

Energy

Nucleophilic addition of a phosphate group to ADP is a rather energy-consuming route. Even at long distances, the reaction partners as negatively charged ions repel each other—despite the fact that the charged amino acid residues, constituting the catalytic site, slightly compensate the Coulomb repulsion of the reactants. However, at short distances and in the transition state, the repulsion of the closed electron shells is rather strong and high energy is required to overcome the repulsion for the nucleophilic addition to occur. Indeed, the activation energy of phosphorylation by protein kinase was calculated to be 15 kcal/mol.[39] The energy barrier for the nucleophilic reaction of ATP hydrolysis is even higher, ~39 kcal/mol, and relates to the nucleophilic attack of HO^- on the phosphate group of ATP.[65]

Electron transfer as a starting reaction of the ion-radical mechanism is exoergic. There is no calculation of the energy profile for this reaction; nevertheless, one may assume that the energy barrier for electron transfer is rather low like in typical exoergic electron transfer reactions. This statement is in accordance with experimental results: at concentrations of Mg^{2+}, Ca^{2+}, Zn^{2+} ions where the ion-radical mechanism works, the yield of ATP is strongly increased and considerably exceeds the contribution coming from nucleophilic mechanism.

Ion Concentration

The efficiency of nucleophilic mechanism depends on the concentration of ions:[43] even a single ion in the catalytic site seems to be enough to do that. However, for the efficient functioning two magnesium ions are required: one is bound with ADP, and the other is considered as "free", not bound with phosphate groups but solvated by water molecules and protein residues.[8] The invasion of the additional ions in the catalytic site results in complexes with the substrate; the latter suppresses the

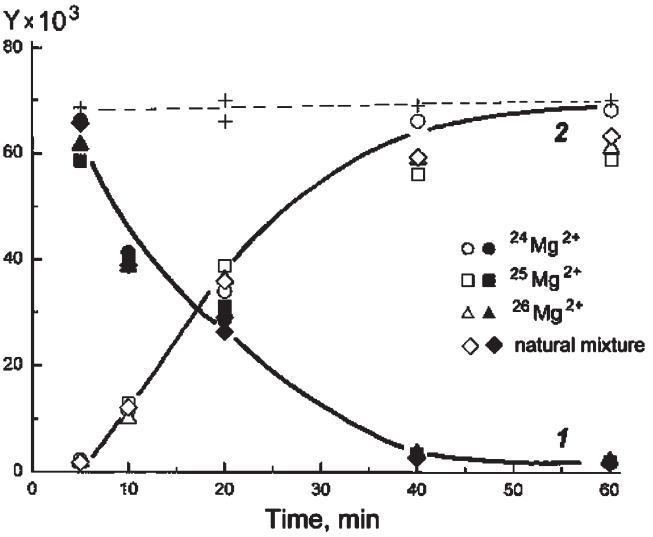

Figure 18. ATP decay (curve 1) and ADP formation (curve 2) induced by Mg-CK (concentration of $MgCl_2$ = 20 mM) as a function of incubation time. Crosses and dotted line refer to the sum (ATP + ADP); the amounts Y of ATP and ADP are given in mM per g of enzyme. Reprinted with permission from ref 36. Copyright 2008 American Chemical Society.

nucleophilic channel of ATP synthesis. This is convincingly proved by direct kinetic studies of ATP synthesis by ATP synthase.[43] Namely, in this transition region a switching over between two mechanisms occurs: the nucleophilic ATP synthesis is replaced by the ion-radical one. It provides an additional source of ATP, which is much more efficient than the nucleophilic one and is accompanied by the appearance of a magnetic isotope effect. Pyruvate kinase is an evident illustration of this statement (Figure 7): here nucleophilic and ion-radical mechanisms are clearly expressed and separated on the magnesium ion concentration scale. Thus, the discovery of isotope effects simultaneously discloses a new, ion-radical mechanism and proves classical, nucleophilic mechanism.

The intracellular concentration of metal ions is rather low,[60] so that the dominating source of ATP in living organisms is thought to be a nucleophilic reaction. The ion-radical mechanism functions under conditions when the concentration of metal ions is rather high. However, due to local concentrations induced by fluctuations of the distribution or inhomogeneous adsorption of metal ions in cells and mitochondria, the contribution of the ion-radical mechanism of ATP synthesis in living organisms is not negligible; it was tested by in vivo experiments (Figure 12). It demonstrates that both mechanisms, nucleophilic and ion-radical, coexist and function independently, producing comparable amounts of ATP even at low concentrations of the metal ions.

19. IS ATP SYNTHESIS A NUCLEOPHILIC OR AN ION-RADICAL PROCESS?

The generally accepted nucleophilic mechanism of ATP synthesis had no alternatives until magnetic isotope effects of Mg, Ca, and Zn were observed in enzymatic ATP synthesis driven by ATP synthase and kinases. To explain these effects as well as magnetic field effects, an ion-radical mechanism was formulated in which the starting reaction was suggested to be an electron transfer from the Mg(ADP) complex to

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$Mg(H_2O)_n^{2+}$ ion. It generates an ion-radical pair as an electron and nuclear spin-selective nanoreactor. By analysis of magnetic parameters (g-factors and hyperfine coupling constants $a(^{31}P)$ and $a(^{25}Mg)$) of the ion-radicals, it was shown that a terminal, negatively charged oxygen atom of ADP in the complex Mg-(ADP) donates an electron to the $Mg(H_2O)_n^{2+}$ ion. The same was proved to be valid for the calcium- and zinc-directed ATP synthesis.

This mechanism is unprecedented but firmly established; it is the only one that explains the whole body of experimental results.

(1) In the ion-radical pair, four reaction channels appear: the singlet one and three triplet ones. Their relative contribution to the ATP yield is controlled by the rate of singlet−triplet spin conversion induced by hyperfine electron−nuclear interaction. In pairs with nonmagnetic, spinless nuclei, spin conversion is slow and the contribution of triplet channels into the ATP production is not too significant. However, the magnetic nuclei ^{25}Mg, ^{43}Ca, and ^{67}Zn strongly stimulate spin conversion, resulting in an increase of ATP yield by 2−4 times.

(2) The magnetic field effects on ATP synthesis are another predictable and experimentally detected irrefutable argument in favor of the ion-radical mechanism.

(3) Theoretical calculations and thermodynamic data on the energy of electron transfer reactions between $M(H_2O)_n^{2+}$ ions and M(ADP) complexes reliably demonstrate that they are energy allowed only for the partly dehydrated ions $M(H_2O)_n^{2+}$. As shown in section 12, there is no need to destroy the first coordination sphere and release the sixth and fifth tightly bound water molecules. This important effect is in perfect accordance with the well-known observation that ATP synthesis does not occur in water (it is energy-forbidden by ~4 eV) from the same reactants and under the same conditions. ATP synthesis takes place only in specially designed molecular motors, enzymes that are known to squeeze water out of the catalytic sites when protein domains are approaching to unite phosphorylating substrate and ADP. The removal of water from the outer coordination sphere of $M(H_2O)_n^{2+}$ ion increases its positive charge and electron affinity, thereby activating the electron transfer reaction.

(4) In terms of the ion-radical mechanism, it is explicable why the efficiency of Ca^{2+} and Zn^{2+} ions in ATP production is almost the same as that of Mg^{2+} ions. Moreover, recent calculations of the electron transfer reactions convincibly predict that other ions, such as Ba^{2+}, Cd^{2+}, and Sn^{2+}, should be also efficient catalysts of ATP synthesis and show a magnetic isotope effect[59] similar to that of Mg^{2+}, Ca^{2+}, and Zn^{2+} ions.

The novel ion-radical mechanism of ATP synthesis is universal, but it is not the only one that is observed through the isotope window. The other outstanding result is the foundation of the classical, generally accepted, but so far hypothetical nucleophilic mechanism. A general property of ATP synthesis is that, at low concentration of magnesium, calcium, and zinc ions, there is no isotope effect in all enzymes. It unambiguously indicates that ATP synthesis does not involve paramagnetic intermediates and occurs as a nucleophilic reaction. The great advantage of the ion-radical mechanism is that it might be used for medicinal purposes to prevent and/or compensate some cell/tissue ATP losses caused be variable pathogenic factors and, therefore, to either treat or prevent a number of essential cardiology-related syndromes.

ASSOCIATED CONTENT

Supporting Information

Tables and figures of the optimized structures, energies, and magnetic parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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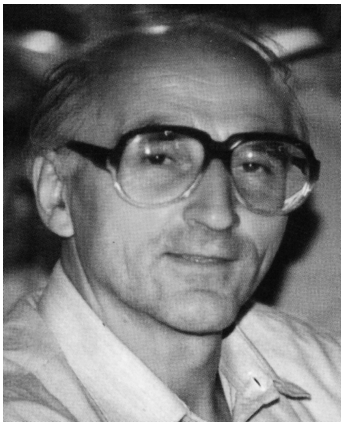

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While in proof, ATP production by two sorts of creatine kinase in presence of magnesium isotopes was reexamined (D. Grotty, G. Silkstone, S. Poddar, R. Ranson, A. Prina-Mello, M. Wilson, J.M.D. Coey. *PNAS*, DOI:10.1073/pnas.1117840108). Neither isotope effect nor magnetic field effect were found in contrast to our results. However, in both samples of $MgCl_2$ (*$MgCl_2$ with natural isotope composition and $^{25}MgCl_2$ with 97.7% of ^{25}Mg) used by authors of paper referenced above, the large amounts of contaminating Fe were presented (14.6 and 9.7μg/mL respectively). They correspond to the contents of Fe in Mg 3 and 2% respectively. Such a huge concentration of paramagnetic Fe^{3+} (or Fe^{2+}) ions does not kill ion-radical mechanism of the ATP synthesis but destroys nuclear spin selectivity and deletes magnetic isotope/magnetic field effects because in this case singlet-triplet spin conversion is controlled by spin relaxation rather than hyperfine coupling or Zeeman interaction. In our experiments concentration of Fe ions was 200−1000-fold less than in experiments described in paper cited above. The results presented in the *PNAS* paper are perfect but conclusions are erroneous (see A.L. Buchachenko, D.A. Kuznetsov. *Proc. Natl. Acad. Sci.*, submitted).