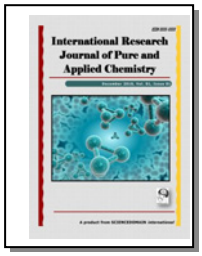

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Phosphate Transfer Enzymes as the Nuclear Spin Selective Nanoreactors

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Research Article

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ABSTRACT

Magnesium isotope effect manifests itself in the enzymatic ATP synthesis at relatively high concentrations of Mg^{2+} ions. At low concentrations, there is no isotope effect at all so the nucleophylic mechanism of the ATP synthesis dominates. Concentration of Mg^{2+} ions exceeds intracellular one by 50-100 times, a huge isotope effect appears which means that the new ion-radical mechanism of ATP synthesis is switched on. This provides an additional and considerable source of ATP. This mechanism implies the electron transfer from Mg^{2+}(ADP) complex to the $Mg(H_2O)n^{2+}$ complex generating an ion-radical pair as a starting reaction of the ATP synthesis. Populations of both singlet and triplet states and the rate of singlet-triplet conversion in the pair are controlled by hyperfine coupling of the unpaired electrons with magnetic ^{25}Mg and ^{31}P nuclei and by Zeeman interaction. Due to these two interactions, the yield of ATP is a function of nuclear magnetic moment and magnetic field. Electron transfer reaction does not depend on m but strongly depends on n. It is exoergic and energy allowed at ($0 \le n << \infty$) for the deprotonated pyrophosphate anions and at ($0 \le n < 4$) for the protonated ones; for other values of n, the reaction is energy deficient and forbidden. The boundary between exoergic and endoergic regimes corresponds to the trigger magnitude n^* ($n^* = 4$ for protonated anions and $6 < n^* << \infty$ for deprotonated ones). These results explain why the ATP synthesis occurs only in some special nanodevices, i.e.,

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within a very few molecular enzymatic machines, but not in water ($n = \infty$). Biomedical and biotechnological consequences of the ion-radical enzymatic ATP synthesis as well as its protein (catalytic site) nanotopology background are under discussion.

Keywords: ATP synthesis; kinases; ^{25}Mg – magnetic isotope effect; nuclear spin selective biocatalysis;

1. INTRODUCTION

ATP synthesizing enzymes are known to be perfectly arranged molecular nanodevices transferring and/or generating energy carrier, P̃O chemical bond in the ATP (Buchachenko et al., 2005a,b; Buchachenko and Kuznetsov, 2008a,b).

Another universal property of the phosphorylating molecular machines is squeezing water molecules out of the catalytic site when protein domains of enzyme approach each other to unite substrate and ADP and so to stimulate ATP synthesis. Particularly, this process was discussed in detail for the ATP synthase, a rotary nanomachine, capable to synthesize and hydrolyze ATP depending on the clockwise or anticlockwise rotation of the shaft (Buchachenko et al., 2005a,b; Buchachenko and Kuznetsov, 2008a,b,c). However, it is still unclear, does the release of water is accompanied by destruction of the Mg^{2+} ion hydrate shell and does this process result in the change of the ion reactivity?

The rate of enzymatic ATP synthesis was shown to strongly depend on the magnesium isotopes. The activities of ATP synthase and phosphocreatine kinase 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. There is no difference in the ATP yield for enzymes with ^{24}Mg and ^{26}Mg; it gives evidence that this reaction is the magnetic isotope effect operated one rather than classical, mass-dependent process.

It has been found recently that besides the classical mechanism of enzymatic ATP synthesis which implies a direct nucleophylic addition of the substrate phosphate group to ADP, there is an ion-radical mechanism of ATP synthesis, also promoted by magnesium ions. An ion-radical mechanism has been recently discovered by substitution of natural magnesium in catalytic sites of the phosphorylating enzymes by pure isotopes ^{24}Mg, ^{25}Mg, and ^{26}Mg (Buchachenko et al., 2005a,b; Buchachenko and Kuznetsov, 2008a,b). It includes an electron transfer from the Mg^{2+}(ADP) complex to hydrated $Mg(H_2O)_2^{2+}$ ion as a primary reaction (Buchachenko and Kuznetsov, 2008c). Electron transfer generates ion-radical pair with singlet and triplet spin states differing in chemical reactivity. Populations of the spin states and the rate of singlet-triplet spin conversion are controlled by hyperfine coupling of unpaired electrons with magnetic nuclei ^{31}P and ^{25}Mg and by Zeeman interaction. Due to these two interactions, the ATP yield is a function of nuclear magnetic moment field; both these effects were experimentally detected (Buchachenko and Kuznetsov, 2008b,c; Buchachenko et al., 2008).

Now we are faced with a problem, whether these two mechanisms are independent and contribute additively into the ATP synthesis or they are competitive? What conditions are required for these mechanisms to function? To find answers, we have studied a dependence

of the ATP yield on the magnesium concentration and magnesium isotopes for two typical phosphorylating enzymes, phosphoglycerate kinase (PGK) and pyruvate kinase (PK).

Another task we're dealing with is to formulate a chemical mechanism of the ATP synthesis and to choose some specific reactions in catalytic site which would demonstrate all these fundamental properties of the phosphorylating molecular machines. This mechanism should include radical or ion-radical steps with participation of Mg^{2+} ion as a reagent; its chemical reactivity is supposed to be dependent on the number of water molecules in its hydrate shell. The reaction mechanism proceeded inside the catalytic site is not accessible to direct experimental testing; this is a reason why our work is focused on a theoretical inspection of the reaction mechanism.

2. MATERIALS AND METHODS

Isotope containing $MgCl_2$ samples were obtained using a treatment of magnesium oxide ^{24}MgO, ^{25}MgO, ^{26}MgO with analytically pure HCl. The impurities of metals, determined by atomic absorbtion spectroscopy and ESCA, do not exceed 40 ppm in ^{24}MgO, ^{26}MgO as well as in ^{25}MgO.

All MgO species used were of about 92.8 – 98.4% isotopic purity level (Gamma-Lab Corp., Alicante, Spain).

Table 1. Electron affinity ΔE (in eV) of the $Mg(H_2O)_n^{2+}$ complexes as a function of *n*.

N	0	1	2	3	4	5	6	∞
ΔE	15.4	13.2	11.1	9.5	7.8	7.3	6.5	0.32

PGK – The purified pig skeletal muscle PGK samples (EC 2.7.1.08) were kindly donated by Dr. L. Gergely, Institute of Biophysics, Hungarian Academy of Sciences (Szeged, Hungary). The enzyme samples used were electrophoretically homogeneous as tested by a non denaturating 7.5 – 12.0% PAAG gradient separation method according to Davis (Davis, 1979). α[^{32}P]phosphoglycerate (21 – 28 Ci/mmol, Amersham, UK) was used in all measurements of the PGK specific activity which has been expressed in [^{32}P]ATP c.p.m./mg protein (Arno et al., 1994).

For protein estimations, a conventional Bradford procedure was applied (Arno et al., 1994). To isolate the ATP fraction from post- and pre-incubation mixtures, our HPLC technique (ODS-S5CN, 10 – 60% pyridine linear gradient) has been employed (Kuznetsov et al., 2004). All incubation mixtures used were balanced for the catalysis optimal conditions according to (Gergely and Bardizh, 1999).

For magnesium isotope ratio monitoring, an isotope mass spectrometry has been applied using the VGS Prism 2000 elemental analyzer/gas-isotope-ratio mass spectrometer (Carlo Erba) (Dupont et al., 2002). To determine a total amount of magnesium, atomic absorption spectrometry was performed in QL 400 AA-spectrophotometer (Carl Zeiss Jena) (Orchaev et al., 2003).

For all data presented, mean values of six measurements were estimated by the Dunnett's non-parametric method suitable in $n \leq 8$ cases (Dunnett and Rosenbrought, 2001).

PK – A pure crystalline PK sample (EC 2.7.1.40, ATP: pyruvate 2-0-phosphotransferase B) isolated from the rabbit skeletal muscle has been employed; the enzyme specific catalytic activity was equal to 500-600 IU/mg protein. Being electrophoretically homogenous (7.5 – 12% PAAG linear gradient), the enzyme does not contain any traces of either creatine kinase or myokinase. The PK incubation was carried out for 40 min (+37°C) in the following reaction mixture: 100IU enzyme per 1mL; 25mM sodium phosphate buffer (pH 7,60); 1.5 mM ATP; 20 mM ADP; 15 mM [^{32}P]-phosphoenol pyruvate (4,8-6,4 Ci/mmol, Amersham Radiochemical Centre, UK); 2.5 mM EDTA, 15mM $MgCl_2$. This composition was found to be an optimal one for the ATP yield level reached. In a separate series of testes, the ATP-producing activities were measured in these reaction mixtures within a wider $MgCl_2$ concentration range (2.5 – 300 mM). In these tests, all isotopically different $MgCl_2$ forms were studied separately using the following samples: $^{25}MgCl_2$ (98.4% isotopic purity), $^{24}MgCl_2$ and $^{26}MgCl_2$ (94.2% and 92.8% respectively).

The ATP synthesis activity was evaluated as an amount of γ-[^{32}P]-ATP formed in 1 min under the above specified conditions and corrected to 1,0 mg of pure PK used. For all protein measurements, a routine Bradford colorimetric method has been employed (Bradford, 1976). To quantify the labeled ATP formation level, the reaction was terminated by addition of 10 volumes of ice-cold acetone (0°C) and then all samples were mixed extensively and incubated overnight at +4°C. Then the acetone-insoluble fraction was carefully collected by centrifugation at 20,000 r.p.m. (10 min, +4°C). The resulting supernatants were lyophilized and the reverse phase HPLC procedure (ODS-S5CN, 1.8x22.5 cm column, 10% water-methanol based 10-60% (v/v) pyridine linear elution gradient, 2,000 p.s.i., 22-25°C) (Culter, 2002) has been employed to estimate both ATP amounts and the ATP/ADP ratio values.

For radioactivity quantitative estimation, the UNICOUNT-M600 dioxane scintillation liquid (Amersham Radiochemical Centre, UK) and the Wallac SL600-E liquid scintillation counter (Wallac O.Y., Finland) were employed. For the ATP chromatographic detection/measurements, the Altex 2000 (Altex Corp., Ireland) columns and the QL4000 Analytic System (Shimadzu Co., Japan) were used.

Full optimization of the geometries and energy characteristics was performed with the Gaussian-03 program package using the density functional theory (DFT) with the B3LYP three-parameter exchange-correlation functional and conventional 6-31G* split-valence basis sets.

3. RESULTS

3.1 GENERAL SCHEME OF THE NUCLEAR SPIN DEPENDENT PHOSPHORYLATION

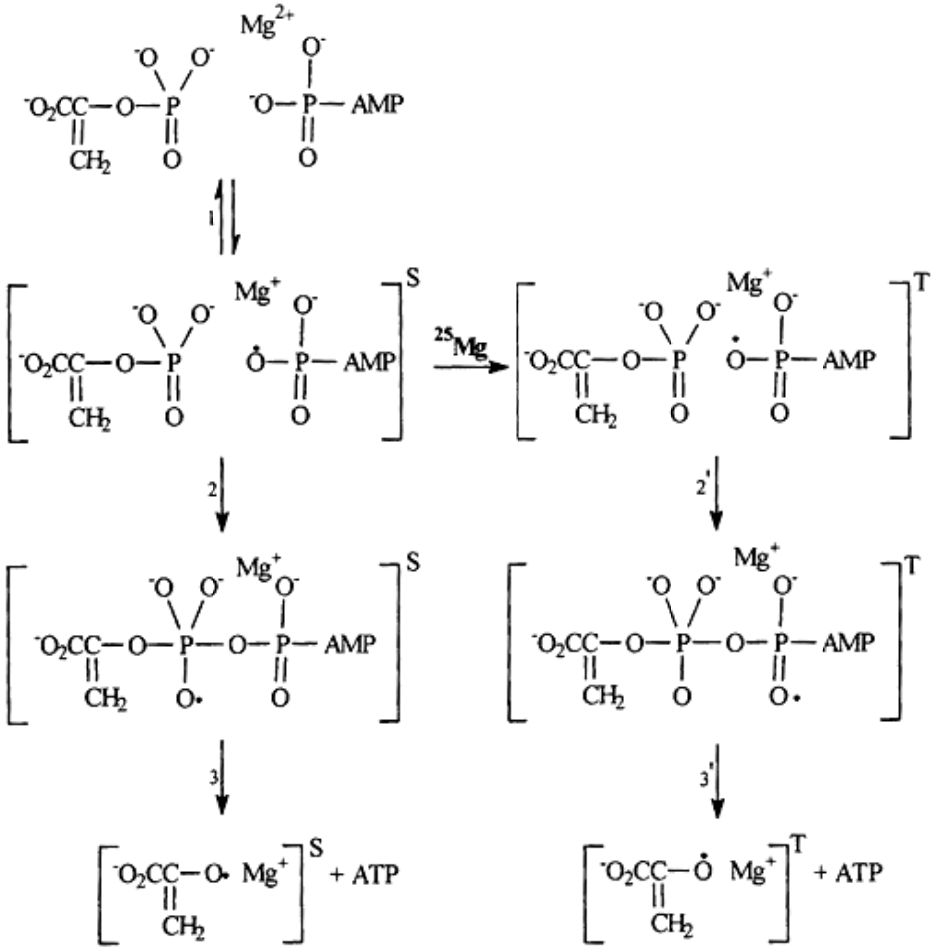
As seen from the Introduction, a classical paradigm of the ATP synthesis is based on the nucleophylic addition of the phosphate group to ADP. Magnesium nuclear spin dependence of the ATP synthesis is a new, paradigm-shifting phenomenon. It means that besides the generally accepted nucleophylic mechanism there is another mechanism, which should be in conformity to three postulates derived from the experiment:

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

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

(iii) the relative contribution of these spin channels into the ATP yield 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 the phosphorylation.

Such a mechanism was formulated earlier in the very general form (Buchachenko, 2009); for the particular case of ATP synthase it is shown in Scheme 1, where AMP is an adenosine monophosphate residue of ADP.



Scheme 1. ATP synthesis directed by pyruvate kinase

As a first step the reaction scheme implies a transfer of electron from terminal phosphate group of ADP to Mg^{2+} ion, generating a primary ion-radical pair, composed of monovalent radical cation Mg^{+} and oxyradical of ADP (Scheme 1, reaction 1), 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 inorganic phosphate by ADP oxyradical (reaction 2). Generated in this addition reaction is another oxyradical that decomposes via β-scission of P–O chemical bond (reaction 3) and generates ATP and final ion-radical pair $(\dot{HO}\quad \dot{M}g^{+})$, which regenerates Mg^{2+} in the reaction like this:

$$(\dot{HO}\quad \dot{M}g^{+}) \xrightarrow{H^{+}} H_2O + Mg^{2+}$$

The rate of phosphorylation along a singlet channel (reactions 1-3 in Scheme 1) is suppressed by spin. This allowed back electron transfer in the primary ion-radical pair, which regenerates starting reagents and decreases ATP yield. However, in the presence of $^{25}Mg^{2+}$ ion hyperfine coupling of unpaired electron with the ^{25}Mg nucleus in Mg^{+} 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 increases by 2-3 times the total production of ATP. The final ion-radical pair in triplet channel undergoes fast triplet-singlet conversion due to electron spin relaxation in OH radical (relaxation time is about 10^{-11} s) and again regenerates Mg^{2+} ion in the reaction.

This reaction scheme explains magnesium isotope and magnetic field effects in ATP synthesis. Moreover, from the experimentally measured yield of ATP as a function of $^{25}Mg^{2+}$ content and the kinetic scheme, equivalent to the reaction Scheme 1, the rate constants in the catalytic site were estimated[i]: $1.2\cdot10^{8}$ s^{-1} for the reaction 1 in Scheme 1, $1.2\cdot10^{9}$ s^{-1} for the back electron transfer in the primary ion-radical pair, and $6\cdot10^{8}$ s^{-1} for the rate of the singlet-triplet spin conversion in the site with $^{25}Mg^{2+}$ ion. However, the most intriguing problem is that ATP synthesis as a redox reaction shown in Scheme 1, occurs only in enzymes and does not take place in water solutions. In this paper we will focus on the key reaction 1 in Scheme 1. Our task is to answer the question, why this reaction proceeds only in enzymes and what conditions are required for this reaction to be exoergic and energy allowed.

The calculated values of $\Delta E(n)$ for n = 0-6 are given in Table 1. At n = 0 calculated ΔE = 15.41 eV quite satisfactorily agrees with experimental magnitude 15.14 eV.

For the reaction

$$Mg(H_2O)_{\infty}^{2+} + e \rightarrow Mg(H_2O)_{\infty}^{+} + \Delta E(\infty)$$

(in water), the energy $\Delta E(\infty)$ was derived from the thermodynamics of the reaction. Energy diagram for the hydrated magnesium ions Mg^{2+} and Mg^{+} taken from is shown in Figure 1. It results to $\Delta E(\infty)$ = 0.32 eV and this value was used in calculations of energies for the reactions in water. The energies do not depend on whether adenosine residue is replaced by hydrogen atom or by methyl group. It means that these energies may be certainly attributed to the reactions of the $Mg^{2+}(ADP^{3-})$ in native enzymes. The energies only slightly depend on m (m = 0-4), the number of water molecules in the first coordination sphere of the ions $Mg(H_2O)^{2+}_m$(pyrophosphate) and $Mg(H_2O)^{+}_m$(pyrophosphate); therefore, they are almost independent on the hydration of $Mg^{2+}(ADP^{3-})$ complex in catalytic site. The energies of all

reactions are strongly dependent on the *n*, the number of water molecules in hydrate shell of the $Mg(H_2O)_n^{2+}$ and $Mg(H_2O)_n^{+}$.

No one reaction occurs in water, when hydrate shell of the $Mg(H_2O)_n^{2+}$ is fully completed ($n = \infty$). This result is in a perfect agreement with a fact that the ATP synthesis from ADP and substrate does not take place in water.

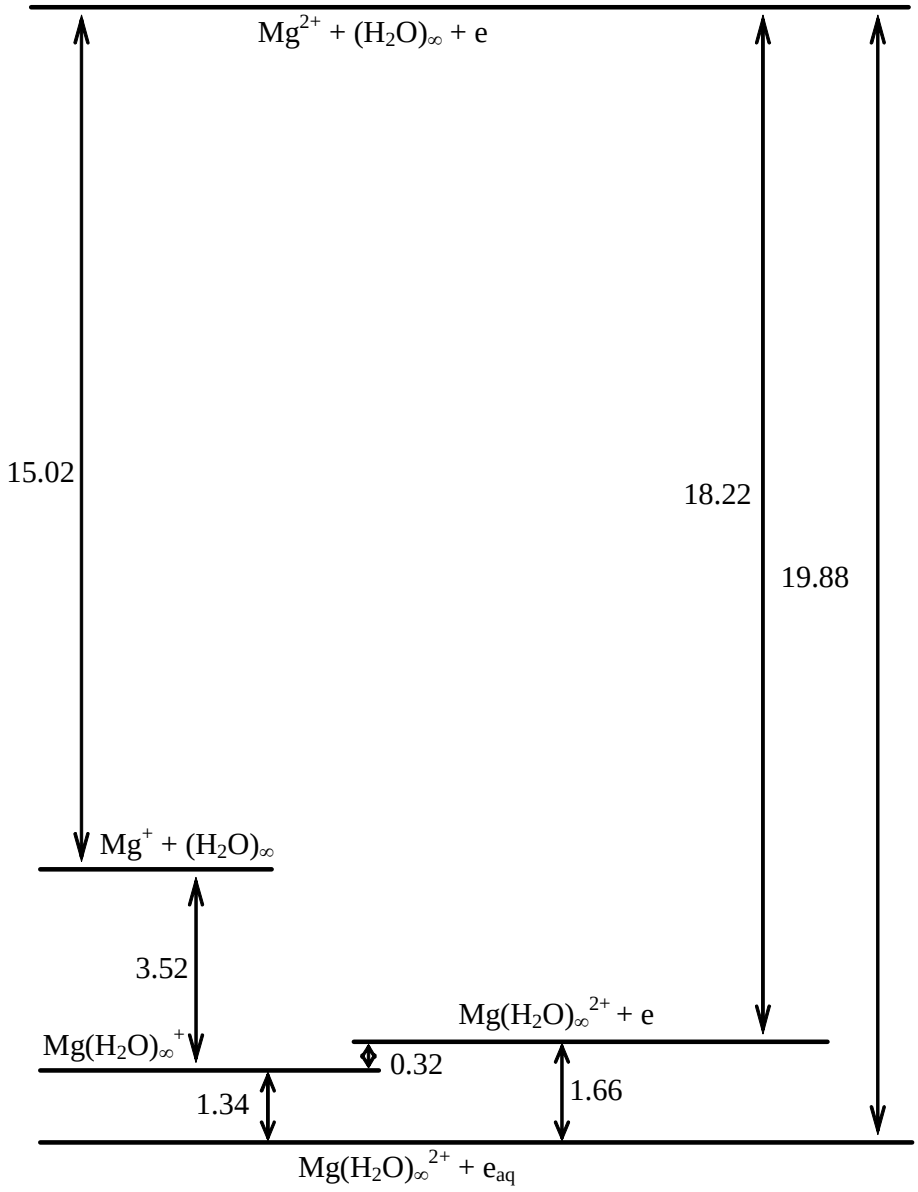


Figure 1. Energy diagram for the hydrated ions Mg^{2+} and Mg^{+}.

(Energies are given in *eV*, zero level refers to Mg^{2+} in water, e_{aq} and e denote hydrated and dry electron, respectively).

In order ATP synthesis to proceed in catalytic site it is necessary to partly remove water from the site and hydrate shell of the $Mg(H_2O)_n^{2+}$. Only at this condition the key reaction – electron transfer from $Mg^{2+}(ADP^{3-})$ to $Mg(H_2O)_n^{2+}$, the reaction 1 in Scheme 1, becomes energy allowed.

The switching over the reaction from exoergic regime to endoergic one 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 overcame as the compression of catalytic site squeezes water molecules out of the site.

For the reactions which include protonated pyrophosphate anions $HP_2O_7H^{2-}$ and $CH_3P_2O_7H^2$, with the $Mg(H_2O)_n^{2+}$ the trigger value of n^* is estimated quantitatively, $n^* = 4$; at $n > 4$ the reactions are endoergic, at $n < 4$ they are allowed by energy.

3.2 REACTIONS OF THE SUBSTRATE MAGNESIUM COMPLEXES

Three types of magnesium complexes may be presented and react in enzymatic site: hydrated complexes $Mg(H_2O)_n^{2+}$, $Mg^{2+}(ADP^{3-})$ and Mg^{2+}(substrate) complexes; in the latter case a substrate may be inorganic phosphate (as in ATP synthase) or phosphates of creatine, glycerate, pyruvate (in phosphorylating kinases).

Besides reactions of hydrated complexes $Mg(H_2O)_n^{2+}$ with the $Mg^{2+}(ADP^{3-})$, we have also calculated energies of reactions modeling interaction between $Mg(H_2O)_n^{2+}$ and Mg^{2+}(substrate) complexes. It is not known exactly, whether substrate is presented in enzymatic site as a free molecule or as a magnesium complex, however the latter possibility is very probable, taking into account a high strength of magnesium ion bonding with phosphate anions. For this reason we have calculated energies of the electron transfer reactions (A) and (B):

$$Mg(H_2O)_n^{2+} + Mg(H_2O)_m^{2+}(HPO_4^{2-}) \longrightarrow Mg(H_2O)_n^{+} + Mg(H_2O)_m^{2+}(H\dot{P}O_4^{-}) \quad \text{(A)}$$

$$Mg(H_2O)_n^{2+} + Mg(H_2O)_m^{2+}(CH_3PO_4^{2-}) \longrightarrow Mg(H_2O)_n^{+} + Mg(H_2O)_m^{2+}(CH_3\dot{P}O_4^{-}) \quad \text{(B)}$$

The former refers to the ATP synthase, the latter models ATP synthesis by pyruvate kinase.

The energies of both reactions (A) and (B) are almost identical and at all values of m and n are much more endoergic (by ~4 eV, see curve 1 in Fig. 2, 3) than the similar reactions of $Mg^{2+}(ADP^{3-})$. If the reactions with the substrates even happen they do not result in ATP synthesis and would only decrease an efficiency of the phosphorylating enzyme. In particular, for ATP synthase with $^{24,26}Mg^{2+}$ in catalytic site the efficiency is about 10%, but it is twice higher for enzyme with $^{25}Mg^{2+}$ ion in the site, as derived from the kinetic analysis of magnesium isotope effect in ATP synthesis by ATP synthase.

These data show that the reactions of hydrated complexes $Mg(H_2O)_n^{2+}$ with Mg^{2+}(substrate) complexes may be neglected and the dominating reaction is an electron transfer from the $Mg^{2+}(ADP^{3-})$ to $Mg(H_2O)_n^{2+}$ complex as a starting reaction of the ion-radical ATP synthesis.

3.3 PGK

Phosphoglycerate kinase is a typical two-domain enzyme catalyzing transfer of the phosphate group from phosphoglycerate to ADP to produce ATP. Figure 2 shows the yield of ATP as a function of the magnesium chloride concentration. The data presented in Figure 2 are nothing but a result of the reaction mixture incubation in a number of tests with several pre-programmed $MgCl_2$ concentrations, 60 min incubation per one run. After the 40 min incubation the yield of ATP reaches its limit and remains almost constant, only slightly decreasing. It is just these limiting, top yields are presented in Fig. 2.

The activity of PGK, as seen in Figure 1, does not depend on the magnesium isotope composition at low concentration of $MgCl_2$. However, at high concentration of $MgCl_2$ the top yields of ATP increase as the share of $^{25}Mg^{2+}$ ions in the total pool of magnesium increases , that is isotope effect in the ATP synthesis progressively increases with the concentration of $MgCl_2$ and reaches maximum at $[MgCl_2] \approx 30mM$.

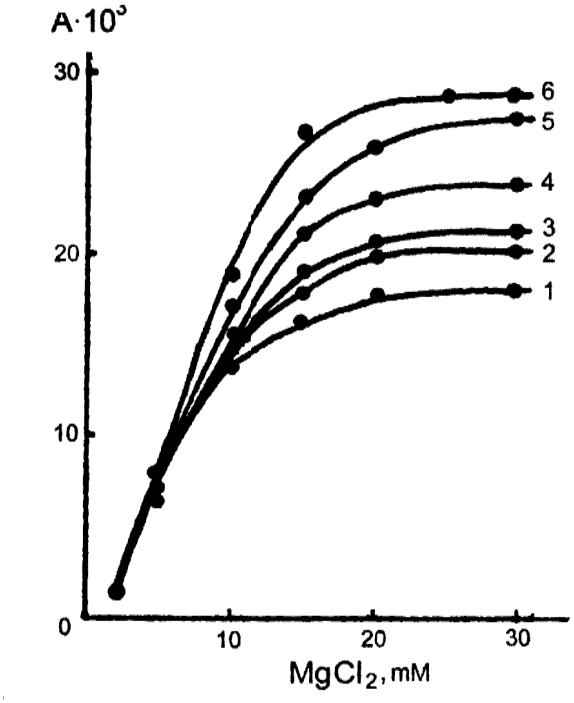


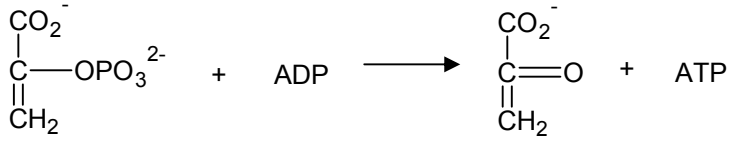
Figure 2. PGK activity A as a function of $MgCl_2$ concentration.

(In series 1-6, the share of ^{25}Mg in a total pool of magnesium increases as 0, 9, 25, 50, 75 and 98%, respectively. *A* is estimated as the amount of ATP formed in 1 min by mg of PGK and measured as radioactivity of ^{32}P-ATP).

A dependence of the PGK activity on the magnesium isotope composition (at high concentration of $MgCl_2$) indicates that the functioning of PGK in excess of $MgCl_2$ is controlled by nuclear spin of ^{25}Mg and that the magnetic isotope effect operates in enzymatic ion-radical reaction of ATP synthesis. At $[MgCl_2]$≈30mM, the magnitude of isotope effect is 2.6, that is enzymes with $^{25}Mg^{2+}$ ions in catalytic sites produce ATP by 2.6-fold more effectively than enzymes with $^{24}Mg^{2+}$ ions.

3.4 PK

Pyruvate kinase phosphorylates ADP by phosphoenolpyruvate producing ATP and pyruvate:



Like in the case of PGK, the transfer of phosphate group is mediated by magnesium ion. The yield of ATP as a function of $MgCl_2$ concentration reveals two maxima; second, it demonstrates unusual magnesium isotope effect (Fig. 3).

At $[MgCl_2]$ < 30mM, there is no dependence of the ATP yield on the magnesium isotopes in accordance with nycleophylic mechanism. However, at $[MgCl_2]$ > 30mM ATP yield demonstrates a huge isotope effect with a maximum at $[MgCl_2]$ = 200mM which strongly, by three orders of magnitude, exceeds physiological level of $MgCl_2$ in cells (Isel et al., 1999). Moreover, PK with $^{24}Mg^{2+}$ ions in catalytic sites contributes almost nothing in ATP yield (curve 1, Fig. 3), but increasing the share of $^{25}Mg_{2+}$ ions progressively and strongly stimulates ATP synthesis (curves 2-4) along the new, ion-radical channel.

A boundary between nucleophylic and ion-radical regimes deserves of particular interest. At the intermediate of Mg^{2+}, in the range 30-100mM, the ATP yield strongly decreases; for PK with $^{24}Mg^{2+}$ in catalytic sites it falls even to nothing. One may suggest at low concentration of $MgCl_2$ the complexation Mg^{2+} with ADP increases the rate of ATP synthesis in the nucleophylic channel, but the next portion of Mg^{2+} ions forms complexes with substrate, pyruvate phosphate. The simultaneous complexation of reagents, ADP and pyruvate phosphate, inhibits their nucleophylic reaction and decreases ATP yield.

This conclusion is in a perfect agreement with that derived independently from the kinetic analysis of ATP synthesis by ATP synthase (Galkin and Syroejkin, 1999). The rate of ATP synthesis was shown to increase as concentration of $MgCl_2$ increases, and then it starts to decrease as concentration of Mg^{2+} ions reaches a magnitude sufficient for Mg^{2+} ions to couple with inorganic phosphate as reagent. This coupling deactivates substrate and prevents nucleophylic route of ATP synthesis.

Nucleophilic mechanism is well known and convincingly documented by theoretical DFT calculations (Diaz and Field, 2004). Ion-radical mechanism has been discussed early (Buchachenko and Kuznetsov, 2008b,c); here we will illustrate it by PK-stimulated ATP synthesis presented in Scheme 1.

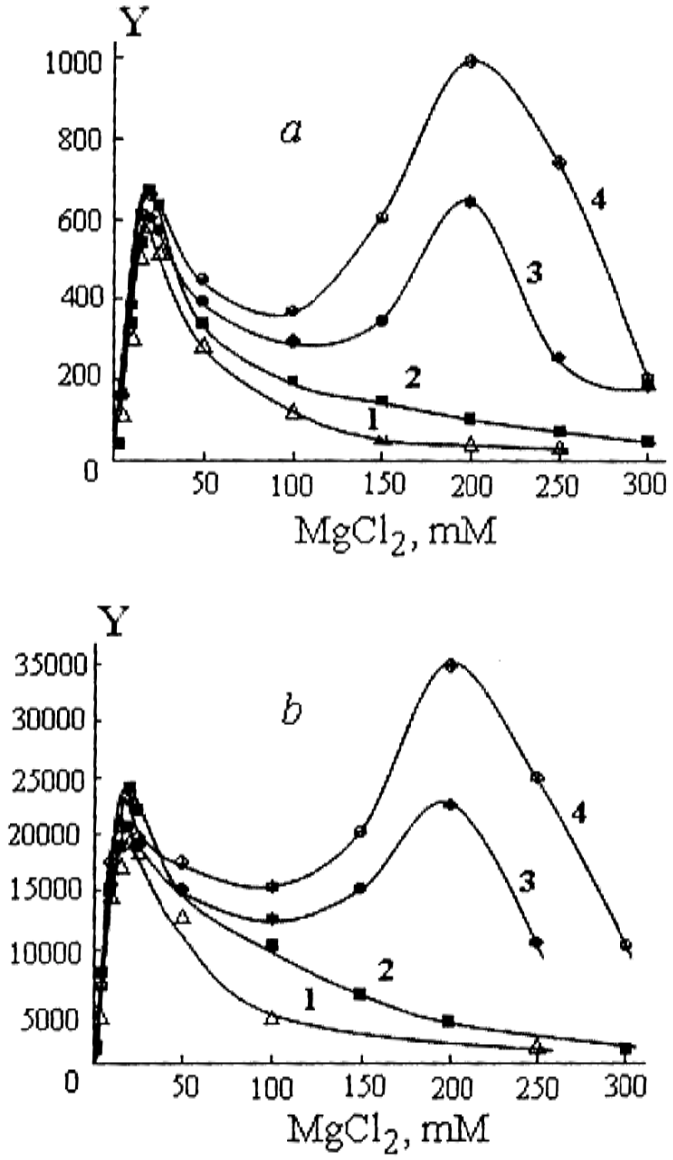
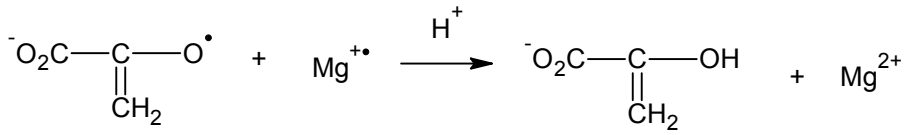


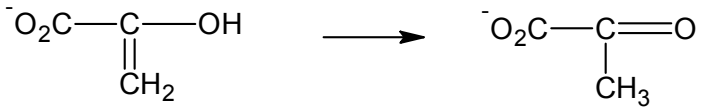
Figure 3. The yield Y of ATP synthesized by PK as a function of $MgCl_2$ concentration.

(In series 1-4, share of ^{25}Mg equals to 0, 11, 50, 98 %, respectively. The yield is measured by quantitative chemical analysis (a) and by radioactivity of ^{32}P-ATP (b))

The key reaction is an electron transfer from the phosphate anion of ADP to the Mg^{2+} ion and generation of the Primary ion-radical pair composed of ADP oxyradical and radical-cation Mg^{+}. Then oxyradical undergoes an addition to the P=O bond generating another oxyradical; the latter decomposes by β-scission of P-O bond and results to ATP and oxyradical of pyruvate with Mg^{2+} ion and enol pyruvate according to reaction. Thus, the first run goes this way:



Then, enol pyruvate transforms into the pyruvate molecule:



Being thermally induced, the first of these reactions generates ion-radical pair in singlet spin state in which reverse electron transfer spin allowed. The latter reaction generates starting reagents and decreases an efficiency of phosphorylation. However, in the presence of ^{25}Mg hyperfine coupling of unpaired electron with magnetic moment of ^{25}Mg nucleus in Mg^{+} stimulates a fast spin conversion of singlet ion-radical pair into a triplet one in which reverse electron transfer is spin forbidden. Thus, $^{25}Mg^{2+}$ ion in the catalytic site provides a new, additional channel of phosphorylation; its contribution into the ATP synthesis increases (Fig. 2). Triplet channel is chemically completely identical to the singlet one. Regeneration of Mg^{2+} and enol pyruvate in the final ion-radical pair is not spin-forbidden because fast electron relaxation of oxyradical is known to remove spin forbiddance of this reaction.

Of course, Scheme 1 describing the nuclear spin selective ion-radical mechanism of phosphorylation is simplified. As shown by detailed DFT calculations (Buchachenko and Kuznetsov, 2008c), the reaction 1 in Scheme 1 is indeed the electron transfer from Mg(ADP) complex to the hydrated $Mg(H_2O)_n^{2+}$ complex and occurs only at $n \leq 4$. Two important consequences stem from this result. First, ion-radical mechanism of phosphorylation is switched on in the excess of Mg^{2+} ions only that is in the presence of free, uncomplexed with ADP and substrate, magnesium ions. Second, it explains why ATP synthesis occurs only in special nanodevices, molecular enzymatic machines. Their functioning includes compression of reagents and squeezing water molecules out of the catalytic site; the latter partly dehydrades Mg^{2+} ion and activates attachment of electron to $Mg(H_2O)_n^{2+}$ ion, when $n \leq 4$. This is a reason why ATP synthesis does not occur in water where Mg^{2+} ions are highly hydrated ($n >> 4$). It is evident that the compression of reagents in catalytic site stimulates both reactions, nucleophylic and ion-radical, however mechanisms of the stimulation are different. It is also worthy to note out that unlike nucleophylic, uncontrollable mechanism, ion-radical mechanism may be controlled by isotopic substitution of magnesium and by magnetic fields both permanent and oscillating (Buchachenko and Kuznetsov, 2008b,d; Buchachenko et al., 2008).

4. DISCUSSION

General property of the two phosphorylating enzymes is that their functioning does not depend on the magnesium isotopes at low concentration of Mg^{2+} ions. However, it unambiguously and strongly depends on the nuclear magnetic moment of magnesium at high Mg^{2+} ion concentration. One may conclude that under conditions of magnesium deficiency, a classical nucleophylic mechanism of ATP synthesis functions while in excess of

Mg^{2+} ions, the additional, ion-radical mechanism is switched on which provides additional and significant source of ATP.

The key, starting reaction of this mechanism is an electron transfer from Mg^{2+}(ADP) complex to $Mg(H_2O)_n^{2+}$ ion. To estimate its energy it was modeled by the following reaction:

$$Mg(H_2O)_n^{2+} + Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{3-} \rightarrow Mg(H2O)_n^{+} + Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{2-}$$

In this reaction, a pyrophosphate ligand $(HOPO_2OPO_3)^{3-}$ models ADP; the number of water molecules *m* in the coordination sphere of $Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{3-}$ complex takes on magnitudes from 1 to 4 (Buchachenko and Kuznetsov, 2008c). In this particular case, the pre-calculated reaction energy does not depend on *m* but strongly depends on *n* [5]. That means, at large *n* this reaction becomes endoergetic, i. e. energetically forbidden.

This is a reason why this reaction occurs only in enzymes but does not proceed in water. Energy transfer generates ion-radical pair composed of paramagnetic complexes $(MgH_2O)_n^{+}$ and $Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{2-}$. Magnetic parameters (g-factors and ^{31}P hyperfine coupling constants) of the $Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{2-}$ complex and $(HOPO_2OPO_3)^{2-}$ radical were shown to be almost identical (Buchachenko, 2010) [20]. This no doubt unambiguously demonstrates that the pyrophosphate ligand in the $Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{3-}$ complex donates electron to the $Mg(H_2O)_n^{2+}$ ion. This involves a detachment of electron from the lone pair of negatively charged terminal oxygen atom of pyrophosphate ligand in $Mg(H_2O)_m^{2+}(HOPO_2OPO_3)^{3-}$ to $Mg(H_2O)_n^{2+}$. The remaining unpaired electron is almost completely localized on the oxygen atom; the next step of the ATP synthesis is an addition of the complexed pyrophosphate radical to the double P=O bond of substrate phosphate group which is further accompanied by ATP synthesis as shown in Scheme 1.

Intracellular concentration of Mg^{2+} ions is in the limits 0.21-0.24 mM (Isel et al., 1999), so that the dominating source of ATP in the living organisms is the nucleophylic reaction. Ion-radical mechanism functions under condition when Mg^{2+} concentration is in a 50-100 fold excess over intracellular one. We consider this conclusion with great satisfaction because the discovery of nuclear spin dependence of phosphorylation did not dismiss a generally accepted, classical nucleophylic mechanism of intracellular ATP synthesis.

Noteworthy, the ion-radical mechanism of enzymatic ATP synthesis may also function in the living organisms due to statistical fluctuations in distribution of magnesium ions in cells and mitochondria. When the two Mg^{2+} ions are presented in catalytic site they both are complexed with phosphate groups of ADP and substrate. However, the casual presence of the third, free (uncomplexed with phosphates) Mg^{2+} switches on the ion-radical mechanism of the ATP synthesis. Therefore, the contribution of the ion-radical mechanism of ATP synthesis cannot be completely excluded even in the living organisms, i.e. at the intracellular concentration of Mg^{2+}.

As discussed above ion-radical mechanism implies electron transfer from Mg(ADP) complex to the hydrated $Mg(H_2O)_n^{2+}$ complex ($n \leq 4$), it functions in the presence of free, uncomplexed with ADP, hydrated magnesium complexes. Its great advantage is that it can be switched on artificially by injection of $^{25}MgCl_2$ in excess to stimulate ATP synthesis and prevent much pathology related to deficiency of ATP, such as hypoxia, heart diseases and other cardio toxic effects.

For these purposes, a specific nanocontainer (magnesium ion carrier) was designed for targeted delivery of $^{25}Mg^{2+}$ in nanoamounts to the heart muscle. Being membranotropic cationites, these "smart" nanocontainers release $^{25}Mg^{2+}$ ions only in response to the metabolic, induced by hypoxia, acidic shift in cells, but uptake back after the normal functioning of cells recovers. The energy stimulating effect of $^{25}Mg^{2+}$ delivered by nanocontainers was demonstrated in several Mammals (Amirshahi et al., 2008; Rezayat et al., 2009; Buchachenko, 2010; Kuznetsov et al., 2010).

At last, one can formulate a general idea how magnesium ions functions in enzymatic ATP synthesis. When Mg^{2+} ions are presented in low concentration they couple with ADP and stimulate nucleophylic addition of Mg(ADP) complex to substrate accompanied by ATP synthesis. The invasion of the second Mg^{2+} ion in catalytic site results in complexation of substrate; the latter decreases (more or less) it's chemical reactivity and suppresses nucleophylic channel of the ATP synthesis. However, the presence of Mg^{2+} in excess switches on another, very effective ion-radical reaction of ATP synthesis which may be controlled by magnesium isotope substitution and magnetic field. Shortly, both mechanisms, nucleophylic and ion-radical, coexist and function independently; the former dominates at low concentration of Mg^{2+} ions, the latter prevails at high content of magnesium ions.

Theoretical monitoring of reactions between magnesium complexes reveals such reactions which perfectly model those of $Mg^{2+}(ADP^{3-})$ complexes in catalytic site of phosphorylating enzymes and which unambiguously satisfy the full body of enzyme properties summarized in the introduction.

First, they are reactions of electron transfer from partly hydrated Mg^{2+}(pyrophosphate) complex to the $Mg(H_2O)_n^{2+}$ complex, so that in modified Scheme 1 the key reaction 1 could be replaced by reaction

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

It generates ion-radical pair in which subsequent reactions, similar to reactions 2 and 3 in Scheme 1, accomplish ATP synthesis.

Now one can formulate a general idea how magnesium ion functions in enzymatic ATP synthesis. When Mg^{2+} ions are presented in low concentration they couple with ADP and stimulate nucleophylic addition of the $Mg^{2+}(ADP^{3-})$ complex to substrate accompanied by ATP synthesis. The invasion of the second Mg^{2+} ion in catalytic site results in complexation of substrate; the latter decreases (more or less) its chemical reactivity and suppresses nucleophylic channel of the ATP synthesis (it was convincingly demonstrated for pyruvate kinase). However, the presence of the Mg^{2+} in excess switches on another, very effective ion-radical reaction of ATP synthesis which may be controlled by magnesium isotope substitution and magnetic fields, both permanent and oscillating.

In short, both mechanisms, nucleophylic and ion-radical, coexist and function independently; the former dominates at low concentration of the Mg^{2+} ions, the latter prevails at high content of magnesium ions.

5. CONCLUSION

We may conclude that the compression of reagents in catalytic site and squeezing water molecules out of the site is accompanied by partial dehydration of the $Mg(H_2O)_n^{2+}$ ion. The

reaction of electron transfer from the Mg^{2+}(pyrophosphate) complex to the fully hydrated Mg^{2+} ion does not occur in water, it is energy forbidden by 4 and 8 eV for the deprotonated and protonated pyrophosphate complexes respectively.Nonetheless, a removal of water from the coordination sphere of the $Mg(H_2O)_n^{2+}$ ion activates ion, so that at some threshold value n^* electron transfer reaction becomes exoergic and energy allowed. For the reaction of deprotonated pyrophosphate complex n^* ranges in limits $6 < n^* << \infty$; for the reaction of protonated complex $n^* = 4$.

The water molecule with number n^* in the complex $Mg(H_2O)_n^{2+}$ functions as a trigger, it switches over reaction between endoergic and exoergic regimes. At $n > n^*$ electron transfer is endoergic, at $n < n^*$ it is energy allowed and rather exoergic. Note that energy of electron transfer is independent on the hydration of the $Mg(H_2O)^{2+}_m$ (pyrophosphate) complex; it is almost identical for m = 0-4.

Magnetic parameters (g-factors, ^{25}Mg and ^{31}P hyperfine coupling constants) of the $Mg(H_2O)_m^{2+}(HOPO_2OPO_3^{2-})$ complex and $(HOPO_2OPO_3^{2-})$ radical were shown to be identical; it unambiguously demonstrates that namely pyrophosphate ligand (and, therefore, ADP ligand in the catalytic site of enzyme) in the $Mg(H_2O)_m^{2+}(HOPO_2OPO_3^{3-})$ complex donates electron to the $Mg(H_2O)_n^{2+}$ ion. It means that the electron transfer reaction occurs as a detachment of electron from the lone pair of negatively charged terminal oxygen atom of pyrophosphate ligand in the $Mg(H_2O)_m^{2+}(HOPO_2OPO_3^{3-})$ to the $Mg(H_2O)_n^{2+}$. The remaining unpaired electron is almost completely localized on the oxygen atom; the next step of the ATP synthesis is an addition of the complexed pyrophosphate radical to the double P=O bond of the substrate phosphate group which is further accompanied by ATP synthesis as shown in Scheme 1.

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