Calcium induced ATP synthesis: Isotope effect, magnetic parameters and mechanism

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\textbf{A B S T R A C T}
ATP synthesis by creatine kinase with calcium ions is accompanied by \(^{43}\text{Ca}/^{40}\text{Ca}\) isotope effect: the enzyme with \(^{43}\text{Ca}^2+\) was found to be 2.0 ± 0.3 times more active than enzymes, in which \(^{25}\text{Mg}^2+\) ions have nonmagnetic nuclei \(^{43}\text{Ca}\). The effect demonstrates that primary reaction in ATP synthesis is electron transfer between reaction partners, \(\text{Ca(H}_2\text{O}_4\text{)}_{\text{n}}^+\), \((n \leq 3)\) and \(\text{Ca}^{(\text{ADP})^3}^+\). It generates ion-radical pair, in which spin conversion results in the isotope effect. Magnetic parameters (g-factors and HFC constants of \(^{43}\text{Ca}\) and \(^{31}\text{P}\)) confirm that namely terminal oxygen atom of the ADP ligand in the complex \(\text{Ca}^{(3\text{ADP})^3}^+\) donates electron to the \(\text{Ca(H}_2\text{O}_4\text{)}_{\text{n}}^+\) ion.

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1. Introduction
The observation of magnesium isotope effect in the rate of ATP synthesis unambiguously demonstrates that the ATP synthesis is spin-dependent ion-radical process [1–4]. It switches on at high concentration of \(^{25}\text{Mg}^2+\) ions (by 20–50 times higher than intracellular one) and provides additional and considerable enzymatic source of ATP. The starting reaction of this mechanism is electron transfer from the complexes \(\text{Mg}^{(\text{ADP})^2}^+\) or \(\text{Mg}^{(\text{ADP})^3}^+\) to \(\text{Mg(H}_2\text{O}_4\text{)}_{\text{n}}^+\) ion. It generates ion-radical pair, in which populations of singlet and triplet states and the rate of singlet–triplet spin conversion are controlled by hyperfine coupling of unpaired electrons with magnetic \(^{25}\text{Mg}\) and \(^{31}\text{P}\) nuclei and by Zeeman interaction. Due to these two interactions, the yield of ATP is a function of nuclear magnetic moment and magnetic field. Both these effects were observed experimentally [2,5] and confirmed by spin theory [6,7]. Nucleophytic and ion–radical mechanisms coexist independently and contribute additively into ATP synthesis. The former dominates in vivo, the latter can be switched on artificially by targeted delivering \(\text{MgCl}_2\) (or, even better, \(\text{MgCl}_2\)) in heart muscle to stimulate ATP synthesis and prevent pathologies related to deficiency of ATP [8–10].

In this Letter we will show, both theoretically and experimentally, that enzymatic ATP synthesis is also catalyzed by calcium ions. The contribution of \(^{25}\text{Ca}^2+\) ions in enzymatic ATP synthesis in native cells seems to be negligible because, first, \(^{25}\text{Ca}^2+\) ions are bound in catalytic site more weakly than \(^{25}\text{Mg}^2+\) ions and, second, their concentration in mitochondria is by order of magnitude lower than that of magnesium ions. Natural calcium contains mostly even isotopes with spinless, nonmagnetic nuclei \(^{40}\text{Ca}\) (96.6%), \(^{42}\text{Ca}\) (0.65%) and \(^{44}\text{Ca}\) (2.1%); for brevity we will further specify natural calcium as \(^{40}\text{Ca}\). We substituted magnesium ions in catalytic sites of creatine kinase, first, by calcium ions with natural abundance of \(^{43}\text{Ca}\) (0.135%) and then by calcium ions, strongly (by 86.7%) enriched with isotope \(^{43}\text{Ca}\) (nuclear spin 7/2, magnetic moment 1.3 \(\mu\)\text{b}\)). These two samples of calcium creatine kinase (further we will denote them as \(^{40}\text{Ca}–\text{CK}\) and \(^{43}\text{Ca}–\text{CK}\), respectively) were shown to exhibit enormous isotope effect in enzymatic ATP synthesis similar to that found earlier for magnesium creatine kinases \(^{40}\text{Mg}–\text{CK}\) and \(^{43}\text{Mg}–\text{CK}\) [11].

2. Experimental procedures and computational technique
Both \(^{40}\text{CaCl}_2\) and \(^{43}\text{CaCl}_2\) were prepared by treatment of \(^{40}\text{CaO}\) (0.135\% of \(^{43}\text{Ca}\)) and \(^{43}\text{CaO}\) (86.7\% of \(^{43}\text{Ca}\)) with analytically pure HCl. The impurities of metals (Na, K, Zn, Mg, Fe, Ni, Ba) determined by atomic absorption spectroscopy did not exceed 30–50 ppm in both \(^{40}\text{CaCl}_2\) and \(^{43}\text{CaCl}_2\) samples. Creatine kinase CK (E.C.2.7.3.2), 40.0 kDa active monomer, purified from \(V. xanthia raddei\) venom [2,5] and \(^{32}\text{P}\) phosphocreatine with 6400–7200 Ci/mmol initial specific activity (from Amersham, UK) have been employed. For CK activity measurements, enzyme samples were incubated at 37 °C in Mg-free media containing Tris–HCl (pH 6.35)/\(\text{CaCl}_2\) (either \(^{43}\text{Ca}\) or \(^{40}\text{Ca}\)) 10–160 mM/12.5 \(\mu\)M ATP/80 \(\mu\)g CK/15 mM potassium phosphate/160 \(\mu\)M phosphocreatine/160 \(\mu\)M ADP for 40 min. This
time is enough for the yield of ATP or creatine to reach a limiting value. In control tests, in the absence of CaCl2, the yield of ATP was negligible.

Two series of experiments were performed. First, enzymatic activities of 40Ca–CK and 43Ca–CK were measured with unlabeled phosphocreatine used. The former includes HPLC-analysis of the acetone-soluble pool of post-incubated mixture [3], while the latter is based on the determination of ATP yield by measuring radioactivity of [32P] ATP [2].

Theoretical calculations of the energy characteristics with full optimization of geometry was performed with the Gaussian-03 program package [12] using density functional theory (DFT) with B3LYP three-parameter exchange-correlation functional and conventional 6-31G* and 6-31+G* split-valence basis sets [13]. Magnetic parameters, \( g_{iso} \) and \( g_{xy} \), for the optimized structures were calculated with the ORCA program [14] using the PBE0 functional and the built-in Ahlrichs triple-zeta valence basis set (TZV(P)+ in the ORCA manual designations). For HFC calculations, we have also used more flexible in the core region uncontracted Partridge basis set [16,17] (Partridge-1) since the specialized EPR and IGLO basis sets are not implemented for P and Mg atoms.

![Figure 1](chart1.png)

**Figure 1.** Schematic presentation of the calcium pyrophosphate complexes. Calcium atoms are gold, phosphorus is green, oxygen is red, carbon is yellow, and hydrogen is blue. The figures are inter-atomic distances in Å. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Structure of calcium complexes

The idea to search isotope effect in the calcium catalyzed ATP synthesis was stemmed from DFT calculations of the energy of electron transfer reactions:

\[
\begin{align*}
\text{Ca}(H_2O)_m^{2+} + \text{Ca}(H_2O)_n^{2+} (\text{HOPO}_2\text{OPO}_3\text{CH}_3)_{2-} & - \rightarrow \text{Ca}(H_2O)_m^{2+} (\text{HOPO}_2\text{OPO}_3\text{CH}_3)_{2-} (\text{HOPO}_2\text{OPO}_3\text{CH}_3)^- (1)\text{Ca}(H_2O)_n^{2+} \\
+ \text{Ca}(H_2O)_m^{2+} (\text{HOPO}_2\text{OPO}_3\text{CH}_3)_{2-} & - \rightarrow \text{Ca}(H_2O)_m^{2+} + \text{Ca}(H_2O)_m^{2+} (\text{HOPO}_2\text{OPO}_3\text{CH}_3)^2 (2)
\end{align*}
\]

We have calculated structure and energy of complexes 1 (Chart 1) in which \( Ca^{2+}(ADP)^2 \) is modeled by hydrated pyrophosphate complex \( Ca(H_2O)_m^{2+} (\text{HOPO}_2\text{OPO}_3\text{CH}_3)^2 \) with methyl group instead of adenosine monophosphate residue. Calcium ion is supposed to be added to oxygen atoms of pyrophosphate anion as shown in Chart 1 and donates two coordination bonds. The other four coordination bonds may be used for addition of the \( m \) water molecules, \( m \) being in the range from 0 to 4.

The value \( m = 4 \) corresponds to the completely filled six-coordinated shell of \( Ca^{2+} \) ion. Besides of complexes 1 we have also calculated structure and energy of the ion–radicals 1a (Chart 1) generated from 1 by electron detachment. Figure 1 shows some typical structures for selected values of \( m \). The withdrawal of electron from complexes 1 results in redistribution of the electron density and slightly changes inter-atomic distances in complexes 1a with respect to those in 1.

Taking into account that in catalytic site both ADP molecules, protonated and deprotonated, may be presented, we have calculated structure and energy of the complexes \( Ca(H_2O)_m^{2+} (\text{HOPO}_2\text{OPO}_3\text{CH}_3)^2 \)– (they model \( Ca^{2+}(ADP)^2 \)) and of those with detached electrons. At last, the structure and energy of ions \( Ca(H_2O)_m^{2+} \) and \( Ca(H_2O)_m^{2+} \) were also calculated as a function of \( n \), the number of water molecules in the coordination sphere of calcium ion; \( n \) is supposed to be in the range 0–6.

3.2. Energy of electron transfer reactions

A total energy of the reactions 1 and 2, which model reactions 3 and 4, is determined as a difference between summary energy of reactants and products. The reaction is exothermic if the difference is positive; on the contrary, the reaction is endoergic and energy forbidden if the difference is negative. Such an approach has an important advantage in that it cancels any errors and compensates possible inaccuracy in energies of individual reagents and products. Both reactions 1 and 2 generate ion–radical pairs, the source of the magnetic isotope effect.

The energies \( E \) of reactions 1 and 2 are presented in Figure 2 as the functions of \( m \) and \( n \) and result in the following conclusions:

1. The energies are almost independent on \( m \) (\( m = 0–4 \)), the number of water molecules in the first coordination sphere...
3.3. Calcium isotope effect

The yields of ATP, synthesized by $^{40}$Ca–CK and $^{43}$Ca–CK, respectively, are shown in Figure 3 as a function of CaCl$_2$ concentration (they were reproduced in the three independent series of experiments). The yields increase as concentration of CaCl$_2$ increases, then reach maximum and decrease at [CaCl$_2$] $>$ 120 mM. At low concentration of CaCl$_2$ there is almost no difference in the ATP yields, however, at [CaCl$_2$] $>$ 40 mM $^{43}$Ca–CK produces ATP more efficiently than $^{40}$Ca–CK. In maximum, at [CaCl$_2$] = 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 100% of $^{43}$Ca, isotope effect; it equals to 2.0 ± 0.3.

ATP synthesis by CK is accompanied by generation of creatine; it originates from the substrate, phosphocreatine. The yield of creatine is shown in Figure 4. Evidently, both the dependence of creatine yield on the CaCl$_2$ concentration and isotope effect, reproduce those for the ATP yields (Figure 3).

3.4. Chemical mechanism of the ATP synthesis

First of all, we should emphasize that Ca$^{2+}$ ion catalyzes ATP synthesis with almost the same efficiency as it does Mg$^{2+}$ ion [1,11]. Like in the case of Mg$^{2+}$, at low concentration of Ca$^{2+}$ ions there is no isotope effect, i.e. classical, nucleophylic mechanism of ATP synthesis dominates. However, at high content of Ca$^{2+}$ another, ion–radical nuclear spin dependent and very effective mechanism is switched on; it is even much more effective than nucleophylic one. It is based on the following postulates which are dictated by experiment and DFT calculations:

(i) Phosphorylation starts as an electron transfer reaction which generates an ion–radical pair comprised of hydrated Ca$^+_1$ radical–cation and phosphate radical–anion of ADP as the partners;

(ii) Due to the spin conservation, chemical reactivity of triplet and singlet spin states of the ion–radical pair is different and results in the difference of ATP yield 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 $^{43}$Ca in the hydrated Ca$^+$ ion and with $^{31}$P in the radical pyrophosphate fragment of ADP; this coupling induces singlet–triplet spin conversion and results in the nuclear spin dependence of ATP yield.

Such a mechanism was formulated earlier for the Mg–CK [11,18]; for the Ca–CK it is shown in Scheme 1, where AMP is an adenosine monophosphate residue of ADP.

As a first step the reaction scheme implies transfer of electron from terminal phosphate group of ADP to the Ca$^{2+}$ ion; it generates primary ion–radical pair, composed of radical–cation Ca$^+$ and...
oxy-radical of ADP (reaction 1 in Scheme 1), in the 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 nucleophilic phosphate by ADP oxy-radical (reaction 2). Generated in this addition reaction another oxy-radical decomposes via β-scission of P=O chemical bond of creatine phosphate by ADP oxy-radical (reaction 3) and results in ATP and final ion-radical pair, which generates creatine and regenerates Ca²⁺ by reverse electron transfer. Note, that both Ca²⁺ and Ca⁺ ions are hydrated (Scheme 1).

The rate of ATP synthesis along the singlet channel (reactions 1–3 in Scheme 1) is suppressed by spin allowed reverse electron transfer in primary ion-radical pair, which regenerates starting Ca²⁺ by reverse electron transfer. Note, that both Ca²⁺ and Ca⁺ ions are hydrated (Scheme 1).

It should be noted that the reaction 1 in Scheme 1 is indeed electron transfer from Ca²⁺(ADP)²⁻ or Ca⁺(ADP)⁻ complexes to the Ca(H₂O)₆²⁺ ion and occurs only at n ≤ p. Two important consequences follow from this result. First, ion–radical mechanism of ATP synthesis is switched on at the excess of Ca²⁺ ions only, when free, not involved in complex with ADP and substrate, Ca(H₂O)₆²⁺ ions appear in the catalytic site. Second, it explains why ATP synthesis occurs only in special devices, molecular enzymatic machines [19]. Their functioning includes compression of reactants and partial squeezing water molecules out of the catalytic site; the latter partly dehydrates Ca²⁺ ion and activates attachment of electron to Ca(H₂O)₆²⁺ ion, when n ≤ p. This is a reason why ATP synthesis does not occur in water where Ca²⁺ ions as well as Mg²⁺ ions are highly hydrated (n >> 4). Compression of reactants in catalytic site stimulates both nucleophilic and ion–radical reactions, however, mechanisms of the stimulation are different. Despite the fact, that calcium and magnesium ions are presented in mitochondria almost equally the former is hardly important in native ATP synthesis because the affinity of magnesium ions to phosphate groups of ADP and substrate is larger than that of calcium ions, so that in the competition for the phosphate groups magnesium ions wins.

3.5. Magnetic parameters
Isotropic g-factors of the Ca(H₂O)₆²⁺ ions are very close to the g-factor of the free electron (2.0023) and only slightly and irregularly deviate from its value as a function of n (Table 1). HFC constants α(43Ca) are shown in Table 1 as a function of n. They are negative due to the negative magnetic moment of ⁴³Ca nucleus [20].

The decreasing of |α(43Ca)| as a function of n illustrates the leakage of the n-electron spin density from the central calcium ion to the ligand water molecules. These results are very similar to those for Mg(H₂O)₆²⁺ ions [21,22]; the only difference is that the HFC constants for ⁴⁳Mg are slightly (by ≈30%) lower than those for Ca(H₂O)₆²⁺ ions.

g-Factors of the pyrophosphate complexes Ca(H₂O)₆²⁺(HOPO₂OPO₃CH₃) are almost independent on the number of water molecules m in the coordination sphere of calcium ion. Their magnitudes are in the limits 2.013–2.015 and identical to the g-factors [20] of the free electron (2.0023) and only slightly and irregularly deviate from its value as a function of n (Table 1). HFC constants α(43Ca) of the Ca(H₂O)₆²⁺(31P) in the Ca(H₂O)₆²⁺(31P) complexes unambiguously indicate that the pyrophosphate ligand in the complex is presented as a pyrophosphate radical, in which spin density is almost completely localized on the terminal oxygen atom and only slightly propagates to the Ca(H₂O)₆²⁺(31P) fragment like in the similar paramagnetic complexes Mg(H₂O)₆²⁺(HOPO₂OPO₃)²⁻ [22]. It unambiguously demonstrates that namely terminal oxygen atom of the pyrophosphate ligand in the complex Ca(H₂O)₆²⁺(HOPO₂OPO₃CH₃)²⁻ donates electron to the Ca(H₂O)₆²⁺ ion. In general, magnetic parameters are in agreement with reaction Scheme 1.

4. Conclusion
Thus, general property of the Ca–CK and Mg–CK is that their functioning depends neither on calcium, nor magnesium isotopes at low concentration of Ca²⁺ or Mg²⁺ ions, in accordance with generally accepted nucleophilic mechanism of enzymatic ATP synthesis in vivo. However, at high concentration of ions it unambiguously and strongly depends on the nuclear magnetic moment of calcium and magnesium, indicating that the novel, ion–radical mechanism is switched on, which provides additional and significant enzymatic source of ATP. Recently this ion-radical mechanism was shown to function in the ATP synthesis.
catalyzed by Zn$^{2+}$ ions [23]. This universal mechanism is sup-
posed to be promising in biomedical applications as was men-
tioned in the Section 1.

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