THE JOURNAL OF PHYSICAL CHEMISTRY A

 VOLUME 108, NUMBER 5, FEBRUARY 5, 2004

LETTERS

Spin Biochemistry: Magnetic Isotope Effect in the Reaction of Creatine Kinase with CH_3HgCl

Anatoly L. Buchachenko,* Dmitri A. Kouznetsov, and Alexandre V. Shishkov

Institute of Chemical Physics, Russian Academy of Sciences, Moscow 119991, Russia

Received: April 7, 2003; In Final Form: August 20, 2003

The inhibition of enzymatic activity of the ATP-synthesizing creatine kinase by CH_3HgCl is shown to strongly depend on the mercury isotope substitution. The efficiency of inhibition does not depend on the nuclear mass of the mercury isotopes; however, it is different for magnetic (^{199}Hg, ^{201}Hg) and nonmagnetic (^{200}Hg, ^{202}Hg) nuclei. When mercury isotopes in CH_3HgCl are presented in natural abundance, the reaction of creatine kinase with CH_3HgCl fractionates magnetic and nonmagnetic mercury isotopes. These observations demonstrate that the reaction between creatine kinase and CH_3HgCl is nuclear spin selective; that is, isotope fractionation is induced by the magnetic isotope effect. According to the suggested reaction scheme, it operates in the ion−radical pairs generated in enzyme active site from CH_3HgCl and cysteine residue as the reaction partners.

Introduction

Creatine kinase (CK) is a phosphorylation enzyme that catalyzes reversible conversion of phosphocreatine and ADP−Mg^{2+} to creatine and ATP−Mg^{2+} playing a crucial role in energy homeostasis control.[1,2] The catalyzing site includes a water-coordinated Mg^{2+}−ADP complex surrounded by aspartate, arginine, valine, triptophan, glycine, glutamate, histidine, and cysteine amino acid residues; chemical modification of any of them affects the CK enzymatic activity.[3]

One of the most efficient CK inhibitors, methylmercury chloride (MMC), is now considered an environmental and occupational hazard, promoting the hardest neurological disorder known as a Minamata disease.[4,5] A key element in biochemical pathogenesis of the MMC toxic effect is a poison-induced suppression of phosphorylation processes in vivo,[6] although a slight activation of the latter has also been observed in the low MMC dosage toxicity research.[7]

To elucidate the mechanism of the MMC toxic effect, we have studied a variation of the CK enzymatic activity by different isotopic forms of MMC: $H_3C^{199}HgCl$, $H_3C^{200}HgCl$, $H_3C^{201}HgCl$, and $H_3C^{202}HgCl$. The two mercury isotopes, ^{200}Hg and ^{202}Hg, have neither nuclear spin nor magnetic moment; two others, ^{199}Hg and ^{201}Hg, have nuclear spins $^1/_2$ and $^3/_2$ and magnetic moments $+0.5029$ and -0.5602 μ_B, respectively. Further, we will refer to these two groups of mercury isotopes as *nonmagnetic* and *magnetic* isotopes.

Our idea is to test the reaction between CK and MMC with respect to the magnetic isotope effect (MIE), which is known to exist in radical reactions and results in fractionation of magnetic and nonmagnetic isotopes.[8] Here we report the first results on the behavior of CK in the presence of isotopically different MMC molecules.

Experimental Section

Methyl mercury chloride, MMC, synthesized from isotopically pure (99.99%) metallic ^{199}Hg, ^{200}Hg, ^{201}Hg, and ^{202}Hg (Cambridge Isotope Labs) was purchased from the Ferein Pharmaceuticals Research Centre. A salt-free preparation of the lyophylized electrophoretically homogeneous 40 kDa CK monomer has been employed. The *V. xanthia* enzyme was isolated and purified followed by chromatography on hydrophobic (Phenyl Sepharose 6B-CL) anion exchange (Mono Q)

* Corresponding author.

10.1021/jp030450o CCC: $27.50
Published on Web 01/13/2004

TABLE 1: Isotope Composition of MMC, Incubated with Creatine Kinase, before and after Reaction

fractions	protein, μg/mL	MMC, μM	Hg isotope composition, %[a]			
			^{199}Hg	^{200}Hg	^{201}Hg	^{202}Hg
starting MMC molecules (preincubation control)	85.0	60.0	17.0 ± 0.1	23.1 ± 0.1	13.2 ± 0.1	26.6 ± 0.1
unbound MMC (acetone soluble pool)	traces	48.3	16.7 ± 0.1	28.0 ± 0.4	14.8 ± 0.6	20.7 ± 0.3
CK-bound MMC	84.6	12.0	34.0 ± 0.3	7.4 ± 0.7	28.2 ± 0.7	10.3 ± 0.6

[a] Mean data of nine tests estimated by SEM.

and affinity gel (ADP-Sepharose 4B-CL) sorbents, as we have described earlier.[9] All sorbents listed were purchased from Pharmacia Fine Chemicals AB.

The CK activity was assayed in a total of 100 μL of reaction mixture containing 15 mM sodium phosphate (pH 6.35) supplemented with 20 μM enzyme, 160 μM creatine phosphate, 160 μM ADP (Upstate Biotechnology), 20 μM $MgCl_2$, and 12.5 μM ATP (Bio-Rad). The resulting enzyme nucleotide-binding sites concentration was then about 1.0 μM.[9] After 30 min incubation at 30 °C, the reaction was terminated by addition of ice-cold acetone (10:1, v/v). Then the acetone soluble pool samples were subjected to ADP and ATP quantitative measurements carried out by the reversed phase HPLC automatized analysis using the Altex 2000 DL column, S5CN-ODS stationary phase, linear 10−65% pyridine elution gradient formed on the methanol/water (9:1, v/v) mobile phase, 22 °C.[10] The CK specific activity was expressed in amounts of nanomoles ATP synthesized per one minute by one mg of CK protein. Protein concentration was assessed by the Bradford assay (Bio Rad PM kit) using the bovine serum albumin as a standard. The incubation conditions stated above were controlled to be sufficient for the direct, ATP-generating, reaction processing.[9,11]

To quantify the MMC inhibitory effect, the IC_{50} index was introduced; it is a concentration of MMC that suppress enzymatic activity by 50%.[12] MMC was added to the reaction mixture 10 min after the start of incubation; then incubation was continued for 20 min. In control tests, samples were incubated in the absence of MMC. All chemical used were of analytical grade (Bio-Rad).

In a separate series of experiments, MMC with a natural abundance of mercury isotopes was added in a concentration sufficient to completely suppress the enzyme activity. Then, after incubation, an unbound inhibitor was washed out by the cold acetone (0 °C) with the following acetone washing of protein pellets on the Millipore fiberglass filters. Both pre- and post-incubated samples were then chemically and isotopically analyzed.

To determine a total mercury amount, an atomic adsorption spectrophotometry was used (the QL 400 Spectrophotometer, Karl Zeiss, Jena). An isotope mass spectrometry has been employed for mercury isotope ratio estimations, combusted samples were analyzed in the modified VGS Prism 2000 elemental analyzer gas-isotope-ratio mass spectrometer (Carlo Erba). All samples analyzed were lyophylized first and then redissolved in methanol. Aliquots were injected into a mass spectrometer combusting unit (1100 °C, 20% O_2, 10 min), and then the thermoionic emission-produced ionized gas (the MK 400 single-band ion emission source) was subjected to electromagnetic separation in the Dollax SR.15 vacuum tube at 15.0 kV. The isotope ratio analysis was carried out in the on-line coupled Farrand XB6 diffraction detector (Farrand Opticals). All data on the mercury isotopic composition were treated by a processing of the resulting mass spectra in the Olivetti AS 640 computation system.

A standard root mean scattering has been used for a routine SEM calculations using the Dunnett's PC software for a nonparametric ($n \leq 8$) multivariate statistical treatment as described by Zar.[13]

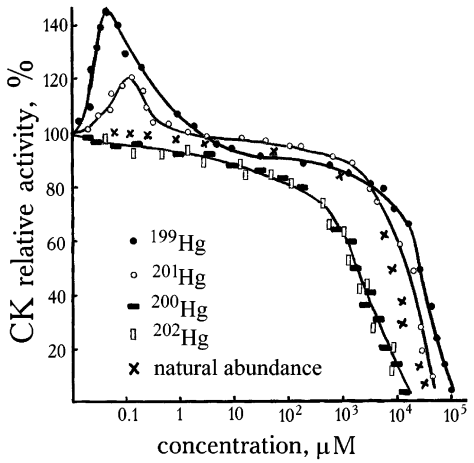


Figure 1. Creatine kinase activity as a function of methylmercury chloride (MMC) concentration for different isotopic forms of MMC.

Results

The relative CK activity as a function of MMC concentration is plotted in Figure 1. First, a significant stimulation of CK activity is observed at low concentrations of $CH_3{}^{199}HgCl$ and $CH_3{}^{201}HgCl$, whereas there is no such effect for $CH_3{}^{200}HgCl$ and $CH_3{}^{202}HgCl$. It is also not observed for MMC with a natural pool of mercury isotopes. In this case the total content of magnetic isotopes does not exceed 30%; i.e., the contribution of MMC molecules with magnetic Hg nuclei is not large enough to be manifested in the stimulation of CK activity. Second, the suppression of CK activity induced by high concentrations of MMC is very sensitive to the mercury isotopes (Figure 1). Thus, the loss of the half of enzymatic activity occurs at $IC_{50} = 42$ mM for MMC with ^{199}Hg and ^{201}Hg, whereas for MMC with ^{200}Hg and ^{202}Hg IC_{50} is much lower, about 2.8 mM. For MMC with a natural abundance of Hg isotopes IC_{50} falls between these two limits in approximate proportion of magnetic and nonmagnetic isotopes in natural abundance.

The effect of mercury isotopes on the CK activity (Figure 1) clearly indicates the difference in reactivity of MMC molecules with magnetic and nonmagnetic mercury nuclei. Phenomenologically the inhibition effect looks like an irreversible nuclear spin-dependent CK decay induced by reaction of enzyme with MMC.

To elucidate what kind of CK−MMC interaction takes place, we have compared mercury isotope composition of three fractions of MMC molecules: (i) the starting, preincubation material as a control; (ii) acetone soluble fraction extracted by cold acetone from postincubated system (see Experimental Section); (iii) the fraction of MMC bound to CK and insoluble in acetone.

The chemical and isotope analysis of the fractions is presented in Table 1.

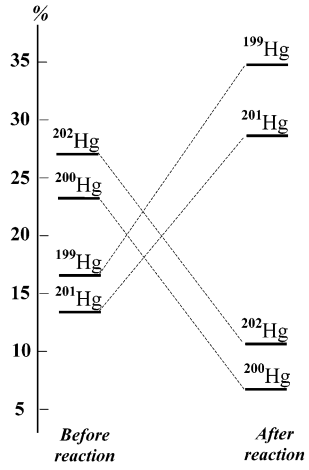


Figure 2. Mercury isotope contents in MMC (before reaction) and in creatine kinase (after reaction).

The majority of incubated MMC molecules (about 80%) are acetone-soluble and easily extractable. The isotope composition of this fraction differs slightly from that of the control (the first line in Table 1). The remaining 20% of MMC molecules bound to CK are strongly, by 15−17% to control, enriched with magnetic isotopes and impoverished with nonmagnetic nuclei (by 16−18% to control). Within the limits of experimental accuracy, the total balance of isotope contents between acetone-soluble and enzyme-bound fractions of MMC is satisfactorily conserved. The data presented in Table 1 are visualized in Figure 2, which convincingly demonstrates isotope fractionation in the reaction of CK with MMC.

Discussion

Both the toxic effect and isotope fractionation unambiguously exhibit the influence of mercury nuclei in MMC on the CK functioning. It is absolutely unbelievable that the difference in nuclear mass would play any role in these effects. First, the mass differences are too small to cause any pronounced classical mass-dependent isotope effect. Second, experimentally measured effects (Figures 1 and 2) are not selective to the mass of the Hg nuclei but highly sensitive to the nuclear magnetism.

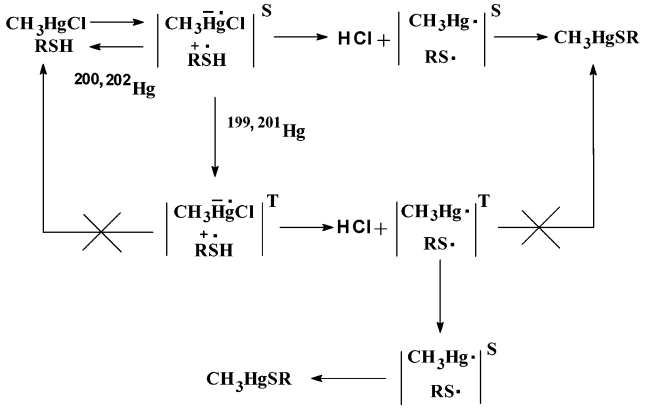
Fractionation of magnetic and nonmagnetic isotopes reliably demonstrates that the interaction of methylmercury chloride with creatine kinase is a radical and spin-selective process; being electron spin-selective reaction, it inevitably results in the nuclear spin selectivity.[8]

The Hg−C bond is known to be nonpolar and resistant to nucleophilic or electrophilic attack; however, it is reactive in oxidation. In particular, MMC is very reactive to SH groups, even the extraction and quantitative analysis of MMC is based on this reaction with specially designed cotton enriched with SH groups.[14]

One of the key structural elements of the active site in CK enzymes is the cysteine residue, which keeps creatine anchored and positions it precisely along the enzyme reaction coordinate.[2,3] There is much evidence and direct experimental proof that the Cys residue in the CK active site is the most vulnerable with respect to MMC. Therefore one can suppose that the interaction between MMC and the Cys-belonging SH group (denote it as RSH) in the active site is responsible for the nuclear spin selectivity in the CK functioning.

The sequence of chemical events in the interaction of CK with MMC is shown in Scheme 1. The first step in the thermally induced process is generation of ion−radical pair by electron

SCHEME 1: Routes of Mercury Isotope Fractionation in the Reaction of Cystein (RSH) with CH_3HgCl



transfer in singlet spin state. Coulomb attraction between the cation−radical $R^{+\bullet}SH$ and anion−radical $CH_3{}^{-\bullet}HgCl$ prevents dissociation of the pair. It can be transformed into a secondary radical pair ($CH_3Hg^{\bullet}\ {}^{\bullet}SR$) by release of HCl; this pair may recombine with the following grafting of CH_3Hg to the Cys residue. However, this channel of transformation of the primary ion−radical pair is hardly competitive as compared to other, more energetically preferable routes.

One of them is the back electron transfer regenerating the starting ion−radical pair; the second is a barrierless singlet−triplet spin conversion, which is induced by magnetic interaction of unpaired electron in $CH_3{}^{-\bullet}HgCl$ anion−radical with magnetic nuclei ^{199}Hg and ^{201}Hg. Due to this electron−nuclear (hyperfine, or Fermi) interaction, the primary singlet ion−radical pairs with magnetic nuclei experience fast transformation into the triplet spin state pairs. Singlet−triplet conversion of the pairs with nonmagnetic isotopes (^{200}Hg,^{202}Hg) is delayed and these pairs by back electron transfer regenerate starting reagents, which are now carrying an excess of ^{200}Hg and ^{202}Hg nuclei in CH_3HgCl.

A triplet ion−radical pair with an excess of ^{199}Hg and ^{201}Hg nuclei cannot regenerate CH_3HgCl because back electron transfer is spin forbidden. The only chemically available channel is a release of a HCl-generating triplet pair of neutral radicals ($CH_3Hg^{\bullet}\ {}^{\bullet}SR$). Due to large electron−nuclear interaction (hyperfine coupling constant on the ^{199}Hg nuclei in the mercury-centered radicals is about 400 G, which corresponds to the rate of spin conversion of about 10^9 s^{-1} [15]) this pair experiences fast and reversible spin conversion, which stimulates the recombination of the pair. The other way is a dissociation of the pair with the following reactions of escaped radicals with the residues of CK. In both cases the radical $CH_3Hg^{\bullet}$ appears to be chemically bound to CK.

The data presented in Figure 1 look inconsistent with the data of Table 1: thus, the MMCs with the magnetic Hg isotopes are less efficient inhibitors, being more reactive, however, toward the enzyme molecule. This clearly recalls a so-called paradoxical activation of mitochondrial enzymes promoted by low doses of MMC that we observed earlier.[6,7] It is hardly possible to offer any no-doubt explanation for such a phenomenon. The only statement we may come up with now is that a strong and irreversible interaction between CK and magnetic MMC species modifies the enzyme in a way that makes it *more active* as compared to effects of ^{200}Hg- and ^{202}Hg-containing inhibitors. The activating impact, more obvious in cases of the low concentrations of the magnetic nuclei-based MMC molecules application, seems to be a new example of the hormetic model in molecular toxicology, i.e., the model that includes an

unexpected and marked *activation* of biosystems in vitro and in vivo (enzymes, isolated mitochondria, etc.) in response to low doses of toxins known as inhibitors of numerous biochemical reactions when administrated at the 0.1−1.0 DL_{50} dose range.[16] One of the most intriguing problems in current molecular toxicological research, *the hormesis*, requires further extensive study.

In terms of the reaction scheme, a spin-selective nanoreactor responsible for the fractionation of magnetic and nonmagnetic mercury isotopes is the primary ion−radical pair. The competition between two coexisting channels, the regeneration of the MMC molecule via back electron transfer and triplet−singlet spin conversion, depends on the nuclear spin and nuclear magnetic moment. Ultimately, the primary ion−radical pair produces a sorting of isotopes: nonmagnetic nuclei return to the starting MMC molecules, and magnetic nuclei stimulate spin conversion of the primary pair and remain chemically bound to creatine kinase.

A difference between the measured values of isotopic content of ^{202}Hg in the starting MMC molecules (26.6%, Table 1) and the natural abundance of this isotope (29.8%) is, most likely, a common consequence of technological peculiarities of the metallic Hg industrial purification/accumulation procedure, a Lutzoff−Rattenau multistep evaporation condensate collection technique, which has already been reported to affect the final product isotopic order.[17]

Conclusion

We have presented the first example of the magnetic isotope effect operating in a biochemical system. The effect manifests itself in nuclear spin dependent enzyme activity and isotope fractionation. An up-to-date magnetic isotope effect was demonstrated for carbon, sulfur, oxygen, silicon, germanium, and uranium;[8] now it is found for mercury isotopes. Its magnitude is rather large, and it is determined by favorable combination of the parameters characterizing molecular, spin, and chemical dynamics of the ion−radical pair which fractionates isotopes: fast spin conversion induced by large hyperfine coupling, reversibility of the electron transfer, and commensurability of the rates of spin conversion and chemical transformation of the primary pair into the starting reagents and secondary radical pair. It is a happy chance of combination of dynamic parameters that were theoretically predicted[8] to result in highly efficient isotope sorting.

This unambiguously indicates also that the spin−orbital coupling in mercury-organic radicals is almost completely quenched, so that the T_1, spin relaxation time in mercury-centered radicals, is much longer than 10^{-9} s, the spin conversion time in the radical pair.

The important point to note is that methylmercury chloride does not intervene in enzymatic transfer of phosphate in the creatine kinase active site per se; however, it chemically modifies the structure of the site violating the catalytic function of kinase. In this relation, it is worth recalling remarkable magnetic field effects revealed by Grissom[18] in the enzymatic reactions. Unlike the magnetic isotope effect presented in this paper, Grissom's effects were induced by radicals generated by enzymatic reactions themselves.

Acknowledgment. This work was supported by Cesare Alba Fundazione Biomedica (Varese-Rome, Italy; grant SJ4800614-02/03), by the Russian Fund for Fundamental Research, and by the Russian Ministry of Industry, Science, and Technology.

References and Notes

(1) Kenyon, G. L. *Nature* **1996**, *381*, 281.

(2) Fritz-Wolf, K.; Schneider, T.; Walliman, T.; Kabsch, W. *Nature* **1996**, *381*, 341.

(3) Lahiri, S. D.; Wang, P. F.; Babbit, P. C.; McLeish, M. J.; Kenyon, G. L.; Allen, K. N. *Biochemistry* **2001**, *41*, 13861.

(4) Bakir, F.; Damluji, S. F.; Amin-Zaki, L.; Murtadha, M.; Khalidi, A.; Al-Rawi, N. Y.; Tikrity, S.; Dhahir, H.; Clarkson, T. W.; Smith, J. C.; Doherty, R. A. *Science* **1973**, *181*, 230.

(5) Cheung, M.; Verity, M. A. *J. Neurochem.* **1985**, *44*, 1799.

(6) Kouznetsov, D. A.; Richter, V. *Int. J. Neurosci.* **1987**, *34*, 1.

(7) Kouznetsov, D. A. *Neurochem. Res.* **1986**, *12*, 751.

(8) Buchachenko, A. L. *J. Phys. Chem. A* **2001**, *105*, 9995.

(9) Kouznetsov, D. A.; Orlova, M. A.; Berdieva, A. G.; Khasigov, P. Z. *Biomed. Chem.* **2003**, *49*, 266.

(10) Kouznetsov, D. A.; Govorkov, A. V.; Zavijalov, N. V.; Sibileva, T. M.; Richter, V.; Drawczek, J. A. *J. Biochem. Biophys. Methods* **1986**, *13*, 53.

(11) Lavrik, O. I.; Nevinski, G. A.; Akopyan, Z. I. In *Mechanisms of Enzyme Action*; Skulachev, V. P., Ed.; Harwood Academic Publishers: Heidelberg, 1989.

(12) Kelman, G. J.; Zavijalov, N. V.; Govorkov, A. V.; Kouznetsov, D. A. *Cell Biol. Toxicol.* **1986**, *2*, 337.

(13) Zar, J. H. *Biostatistical Analysis*; Princeton Scientific Publishing Co.: Princeton, NJ, 1974.

(14) *Metal Ions in Biological Systems*; Siegel, F., Ed.; Marcel Dekker: New York, 1997; Vol. 34.

(15) Landolt-Börnstein. In *Magnetic Properties of Free Radicals*; Fischer, H., Ed.; Numerical Data and Functional Relationships in Science and Technology. New Series. Vol. 17; Springer-Verlag: Berlin, 1990.

(16) Calabrese, E. J.; Baldwin, L. A. *Nature* **2003**, *421*, 691.

(17) Shevkounov, O. V.; Mazour, S. S. *Heavy Metal Stable Isotopes in Environmental Health and Occupational Pathology Studies*; Naukova Dumka: Kiev-Lvov, 1998.

(18) Grissom, C. B. *Chem. Rev.* **1995**, *95*, 3.