

Thiacalix[4]arene-tetraphosphonate Eliminates Inhibitory Effects of Heavy Metals on Smooth Muscle Myosin S1 ATPase Activity

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Abstract: Numerous female reproductive abnormalities are caused by uterine smooth muscle (myometrium) dysfunctions. Heavy metals have an adverse effect on the contractility of uterine smooth muscle. Thus, methods recovering normal contractile activity of myometrium are needed to be developed to overcome this negative impact. It has been found an inhibitory effect of Ni^{2+} , Pb^{2+} , and Cd^{2+} on enzymatic hydrolysis of ATP catalyzed by myosin subfragment-1 (S1) obtained from smooth muscle of swine uterus. It was demonstrated that tetrahydroxythiacalix[4]arene-tetraphosphonate (C-800) restored the normal myosin S1 ATPase activity in the presence of heavy metal cations. One of the most probable mechanisms of tetrahydroxythiacalix[4]arene-tetraphosphonate protective effect is based on its ability to chelate heavy metal cations from the incubation medium. Also, we speculated that protective activity of C-800 might be the result of weakening the interaction between heavy metal ions and amino acid residues near the active site of myosin ATPase.

Keywords: Myosin S1, Heavy Metals, Thiacalix[4]arene, ATPase Activity, Docking, Smooth Muscle, Uterus

1. Introduction

The primary functions of the uterus in the female organism are pregnancy and delivery. Accumulation of heavy metals in the environment is the cause of a large number of women reproductive system pathologies [1-8]. The situation is deteriorating with the growing pollution of the environment by heavy metals ions.

The harmful effects of heavy metals on female reproduction may lead to miscarriages [4, 5], preterm childbirth [1], infertility [6], and cancer [7, 8]. Pb^{2+} and Cd^{2+} are most common pollutants of the environment among heavy metal cations [9, 10]. These cations have the highest ability to accumulate in tissues of living organisms [11]. Also, they are toxic to many physiological systems in vertebrates, including reproductive ones. The uterus is an organ that can accumulate these metals [12]. Ni^{2+} , being biometal, in excess amounts is toxic to living organisms,

causing some pathologies. Chronic intoxication with Ni^{2+} increases the risk of developing tumors of the lungs, kidneys, skin, and stomach [13]. Also, it is known for its adverse impact on the reproductive system of humans and animals [14].

Women reproductive system pathologies associated with disorders of the uterine smooth muscle contraction. The uterine contractile function is associated with the activity of the protein complex – actomyosin, in which myosin exhibits enzyme activity, namely the ability to hydrolyze ATP. Myosin ATPase localized in the catalytic domain of myosin subfragment-1 (S1, head) transforms chemical energy deposited in macroergic bonds of ATP into mechanical movement. As a result, myosin moves along the actin filament causing the muscle contraction. Therefore, ATP hydrolysis, catalyzed by myosin, is considered as one of the essential processes in the molecular mechanism of the myometrium function [15].

It was demonstrated that Ni^{2+} , Pb^{2+} , and Cd^{2+} have inhibitory effects on the ATP-hydrolase activity of swine actomyosin complex of myometrium [16, 17] which can lead to a disturbance of uterine smooth muscle contractility.

The negative impact of Pb^{2+} , Cd^{2+} , and Ni^{2+} on the contractile properties of the myometrium requires the development of pharmacological substances that can eliminate these harmful effects. A promising class of such compounds is water-soluble cup-shaped calixarenes [18] and thiacalixarenes [19] possessing the metal complexing groups at the macrocyclic molecular platform [20, 21]. Derivatives of calixarenes due to their ability to form the supramolecular complexes with (bio)metal cations have also been used in biomedical research as modulators of ATP-dependent calcium pump [22], metalloenzyme inhibitors [23, 24],

sensors [25], extractants of heavy metals [26, 27] and radionuclides [28].

Previously, it was shown that thiacalix[4]arene-tetrasulphonate (C-798) eliminated adverse effects of Pb^{2+} , Cd^{2+} , and Ni^{2+} on ATP hydrolysis catalyzed by myosin S1 from swine myometrium [16].

Tetrahydroxythiacalix[4]arene-tetraphosphonate C-800 (code is shown) is the macrocyclic substances which have a lipophilic intramolecular highly-structured cavity formed by four aromatic cycles modified by phosphonic groups (Figure 1, A). Similar to other thiacalixarenes (for example, C-798) C-800 has hydroxyl groups and divalent sulfur atoms on the lower rim of macrocycle that can chelate transition and heavy metals to form metal complexes [21]. (Figure 1, B).

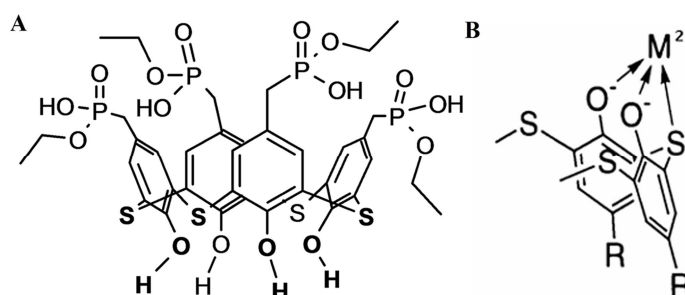


Figure 1. The chemical structure of the tetrahydroxythiacalix[4]arene-tetraphosphonate C-800 (A) and the scheme of the chelating complex of the thiacalixarene with metal cation on the lower rim (inverted position)(B).

The aim of this work to study the influence of heavy metals cations (Pb^{2+} , Cd^{2+} and Ni^{2+}) and their joint action with thiacalix[4]arene C-800 on myosin S1 ATPase activity from the uterus. Myosin S1 was used in our research as a model of a functional myosin unit since it fully retains the ATPase activity of the whole myosin and the ability to interact with the actin

2. Materials and Methods

2.1. Chemicals

The following reagents were used: serum albumin, EGTA, EDTA, ATP, ascorbic acid, tris, tricine, dithiothreitol, acrylamide, (Sigma, USA), glycine (Merck, Germany), N, N'-methylenebisacrylamide (Acros Organics, Belgium) N,N,N',N'-tetramethylethylenediamine (Reanal, Hungary), and reagents of domestic production (R grade). The solutions were prepared in water purified on Crystal Bio system (Adrona, Latvia). The water conductance was less than 0.1 μS . The concentration of the divalent metal cations in solution was determined by Mohr method Tetrahydroxythiacalix[4]arene-tetraphosphonate (C-800) was synthesized in the Department of Phosphorane Chemistry, Institute of Organic Chemistry, National Academy of Sciences of Ukraine. Infrared and NMR spectroscopy confirmed the structure of the synthesized calix[4]arene C-800.

2.2. Actomyosin Complex and Myosin S1 Isolation

The actomyosin complex was isolated from swine uterine smooth muscle by the method described in our previous works [17]. The myosin S1 was prepared by Suzuki method [29]. Polyacrylamide gel electrophoresis controlled the purity of isolated actomyosin and myosin S1 under denaturing conditions [30]. All experiments with animals were performed by the guidelines of the European Convention for the Protection of the Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and approved by local governmental authorities within the recommendations of First National Congress of Ukraine on Bioethics and Legislation of Ukraine 2001.

2.3. ATPase Activity Assay

ATPase activity of myosin S1 was determined in a 96-well plate at 37°C in an incubation medium (total volume 0.1 ml) of the following composition (mM): tris-HCl buffer (pH 7.2) – 20, KCl – 100, CaCl_2 – 0.01, MgCl_2 – 5, ATP – 3 (standard conditions). Protein (myosin S1) concentration was 20 $\mu\text{g/ml}$. Incubation time – 5 min. Samples containing all components of the incubation medium without myosin S1 were taken as control of non-enzyme ATP hydrolysis. The amount of inorganic phosphate cleaved from ATP during hydrolase reaction was determined by the Chen method [31], by measurement of optical absorbance of the solution at 820 nm using a microplate reader μQuant (Biotek® Instruments,

Inc., USA) and expressed as P_i nmol/min per mg of protein.

2.4. Superprecipitation of Actomyosin Complex

Superprecipitation of actomyosin was determined in a 96-well plate at 25°C in an incubation medium (total volume 0.3 ml) of the following composition (mM): tris-HCl buffer (pH 7.2) - 20, KCl - 100, $CaCl_2$ - 0.01, $MgCl_2$ - 5, protein - 500 mg (standard conditions). The reaction was started by adding ATP to a final concentration of 3 mM. The actomyosin superprecipitation was recorded using point measurements at 3 sec intervals for 3 minutes on a μ Quwant plate reader (Biotek @ Instruments, Inc., USA) at 550 nm. The value of actomyosin superprecipitation in the standard incubation environment was taken as 100% (zero points).

The effect of heavy metal cations or/and thiocalix[4]arene C-800 on the myosin S1 ATPase activity and actomyosin superprecipitation were investigated in standard incubation medium, to which aliquot of heavy metal cations or/and thiocalixarene of the corresponding concentration was added.

2.5. Kinetics Study

For the study of the effect of different concentrations of Pb^{2+} and Cd^{2+} on the enzyme activity, inhibition coefficients $I_{0.5}$ and Hill coefficients n_H were calculated using linear Hill plots according to equation $\lg[(A_0-A)/A] = -n_H \lg I_{0.5} + n_H \lg [I]$, where A_0 and A – specific enzyme activities in the absence of Pb and Cd cations (“zero point”) and in the presence of metal cations at a concentration $[I]$ in the incubation medium.

2.6. Statistical Analysis

Statistical processing of the obtained data was performed using standard methods of variation statistics. Experimental data were analyzed by using the standard software “MS Office” and “Statistica 4.5”. The statistical comparisons were performed using two-way analysis of variances (ANOVA).

2.7. Computer Modeling

Computer modeling of the interaction between ligands (thiacalix[4]arene C-800 and heavy metals cations of Pb^{2+} , Cd^{2+} , and Ni^{2+} , model bindings) and receptor (myosin S1) was performed using AutoDock software, version 4.2 [32]. It was used standard parameters of the AMBER power field for metal ions and the three-dimensional enzyme structure with the 1b7t identifier in RSCB PDB [33]. Computer modeling of the thiocalix[4]arene C-800 structural peculiarities was carried out using Chimera [34].

Program AutoDockTools was employed for preliminary “processing” of interacting molecules. 100 runs of Lamarckian genetic algorithms (population size - 100, the maximal number of energy evaluations - 10^6) were conducted. We apply the programs Chimera [35] and Yassara to analyze and visualize the docking results [36]. Calculation of the minimal total binding energy was performed considering Van der Waals forces, electrostatic and hydrophobic interactions, and hydrogen bonds. The optimal ligand positions in the complex

“receptor-ligand” were determined according to the energy values obtained by docking software calculator for binding energy in the receptor-ligand complex. Thus, we selected a series of complexes with the lowest total energy, and then calculated the optimal geometry of the complexes and determined the most energetically preferred arrangement of the ligands in the space of myosin S1 binding domain.

3. Results and Discussion

It was shown the inhibitory effect of heavy metal cations Pb^{2+} , Cd^{2+} , and Ni^{2+} on the uterine smooth muscle myosin S1 ATPase activity at the concentration ranging from 30 to 300 μ M for Pb^{2+} , Cd^{2+} , and Ni^{2+} (Figure 2). It was observed that cations of Pb and Cd exhibited a more considerable inhibitory effect on the myosin S1 ATPase activity than Ni cations. Inhibitory impact for Pb^{2+} and Cd^{2+} at concentration 30 μ M was approximately 25% and 20% compared to control, respectively. In particular, the inhibitory effect of Pb^{2+} and Cd^{2+} was the most notable at the 300 μ M concentration in comparison with control ATPase activity on average 88% and 56%, respectively. The values of inhibition coefficient $I_{0.5}$ for these cations were 0.08 ± 0.01 mM for Pb^{2+} and 0.30 ± 0.03 mM for Cd^{2+} . The less significant inhibitory effect was demonstrated for Ni^{2+} . Namely, Ni cations at a concentration of 300 μ M inhibited the myosin S1 ATPase activity on 30%.

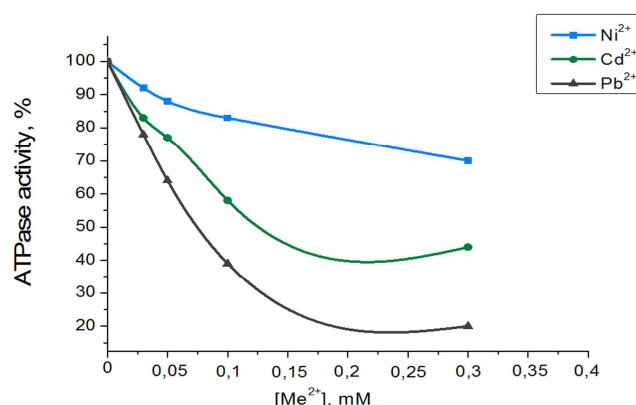


Figure 2. Myosin S1 ATPase activity from myometrium in the presence of different concentrations of Pb, Cd, and Ni cations. 100% is the value of ATPase activity without the addition of heavy metal cations. The typical sample size was $n = 3-4$.

100 μ M C-800 solution in 50 mM Tris-HCl buffer (pH 7.2) was added to the incubation medium containing heavy metal cations to remove the negative impact of Pb^{2+} , Cd^{2+} , and Ni^{2+} on the enzymatic activity of uterine smooth muscle myosin.

The same quantity of Tris-HCl buffer was included in control samples. The Pb and Cd cations were added at a concentration of 100 μ M, whereas the Ni cations were included in concentrations 300 μ M.

As shown in Figure 3, compound C-800 almost does not affect myosin S1 ATPase activity from myometrium. It was shown that 100 μ M C-800 removed inhibitory effects of 100

$\mu\text{M Pb}^{2+}$ and Cd^{2+} on the process of hydrolysis of ATP catalyzed by myosin S1. (Figure 3). Thiocalix[4]arene C-800

had a similar restoration effect on ATPase activity of myosin S1 in the presence of $300 \mu\text{M Ni}^{2+}$ (Figure 4).

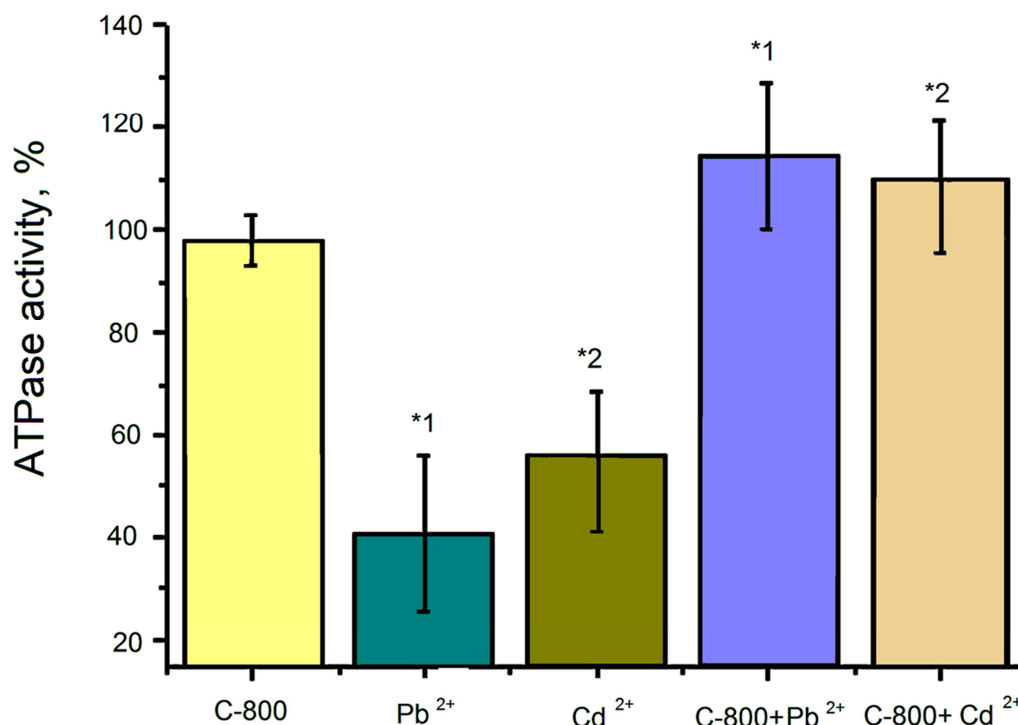


Figure 3. Effect of $100 \mu\text{M}$ C-800 on myosin S1 ATPase activity in the presence of $100 \mu\text{M Pb}^{2+}$ and Cd^{2+} ($M \pm SD$, $n = 5-6$). 100% is the value ATPase activity without the addition of heavy metal cations. The difference between the "Pb" and "Pb + C-800", as well as between the values of "Cd" and "Cd + C-800" is statistically significant ($p < 0.05$) and shown as *1 and *2.

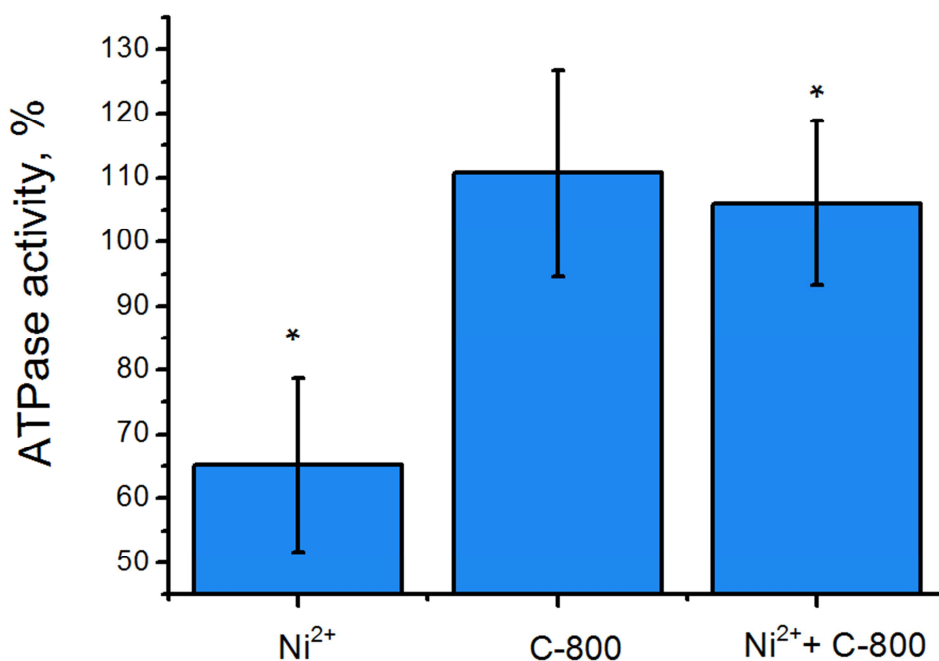


Figure 4. Effect of $100 \mu\text{M}$ C-800 on myosin S1 ATPase activity in the presence of $300 \mu\text{M Ni}^{2+}$ ($M \pm SD$, $n = 7-8$). 100% is the value of ATPase activity without the addition of heavy metal cations. The difference between the Ni^{2+} and $\text{Ni}^{2+} + \text{C-800}$ is statistically significant ($p < 0.05$) and marked with *.

It was observed the restorative effect of thiocalix[4]arene C-800 and $300 \mu\text{M Ni}^{2+}$ on the actomyosin superprecipitation reaction of the myometrium (Figure 5). This reaction imitates contractile ability of actomyosin complex with a certain approximation.

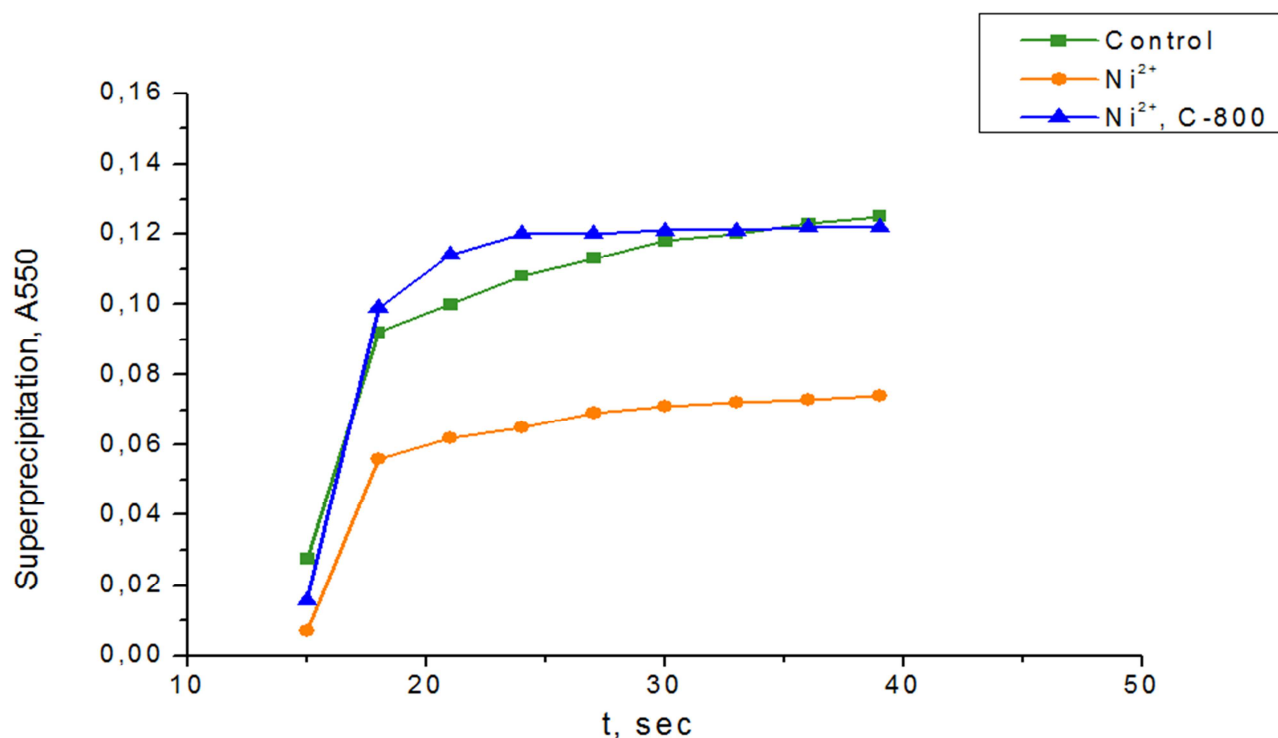


Figure 5. The combined effect of 300 μM Ni^{2+} and 100 μM C-800 on superprecipitation reaction of actomyosin from myometrium. The representative graph, $n = 4$.

The most probable mechanism of protective action of C-800 is based on its ability to chelate cations of Pb, Cd, and Ni with hydroxyl groups and divalent sulfur at the lower rim from incubation medium [19]. Also, we speculated that C-800 was able to pull heavy metals cations from some binding sites of the myosin S1.

Ionic radius of Mg^{2+} , Ni^{2+} , Cd^{2+} and Pb^{2+} in solution represent 0.070; 0.067; 0.102 and 0.126 nm, respectively [37]. Since Cd and Pb are much larger cations compared to Mg then interaction between Cd^{2+} , Pb^{2+} and Mg^{2+} -binding sites of myosin is challenging. Nevertheless, experimentally it was shown their inhibitory effect on the myosin S1 ATPase activity. Therefore, we can assume that these cations can bind to other functionally important sites of S1, which facilitates binding and hydrolysis of ATP.

The computer simulation was used to visualize these possible binding sites in the structure of myosin S1. It was defined that the Pb, Cd and Ni cations may interact with myosin S1 amino acid residues in several sites related to the catalytic activity of the enzyme. Moreover, the binding sites of these cations on the surface of myosin S1 differ from those for Mg^{2+} .

It is known that P-loop (Gly176-Lys187), switch 1 (Gly230-Phe243) and switch 2 ((Ile461-Ser471), 50-kDa

upper and lower subdomains, relay (Leu475-Glu506) participate in the formation of ATPase active site [33, 34]. Therefore, much attention was paid to most likely interaction regions for each of the cations (Cd^{2+} , Pb^{2+} , and Ni^{2+}) close to ATP-binding sites of myosin S1.

The ion's radius of Mg^{2+} and Ni^{2+} in solutions is similar. Therefore, the interaction of Ni^{2+} with Mg^{2+} binding sites of myosin is possible. Ni cations may occupy these sites at the relatively high concentrations.

Ni cations can cooperate with myosin S1 in a binding site related to the switch 2 involved in the binding of ATP and its hydrolysis. Ni^{2+} interaction with Glu 465 (bond length 0.22 nm), Asp468 (bond length 0.35 nm) and Leu653 (bond length 0.37 nm) in this area close to the cleft between the upper and lower 50 kDa subdomains forming the ATP-binding pocket [38].

It was selected the two most probable binding sites for Cd and Pb cations in the myosin S1 molecule. Cd^{2+} interacts with Arg236 (bond length 0.3 nm) in 1 binding site (Figure 6, A), located between the ATP-binding site and the lower 50-kDa subdomain (Ser652-Leu666) close to the P-loop. Also, the Cd cation coordinates with the oxygen atoms of Glu177 (bond length of 0.27 and 0.36 nm) and with the oxygen atom of Ser178 (bond length 0.35 nm).

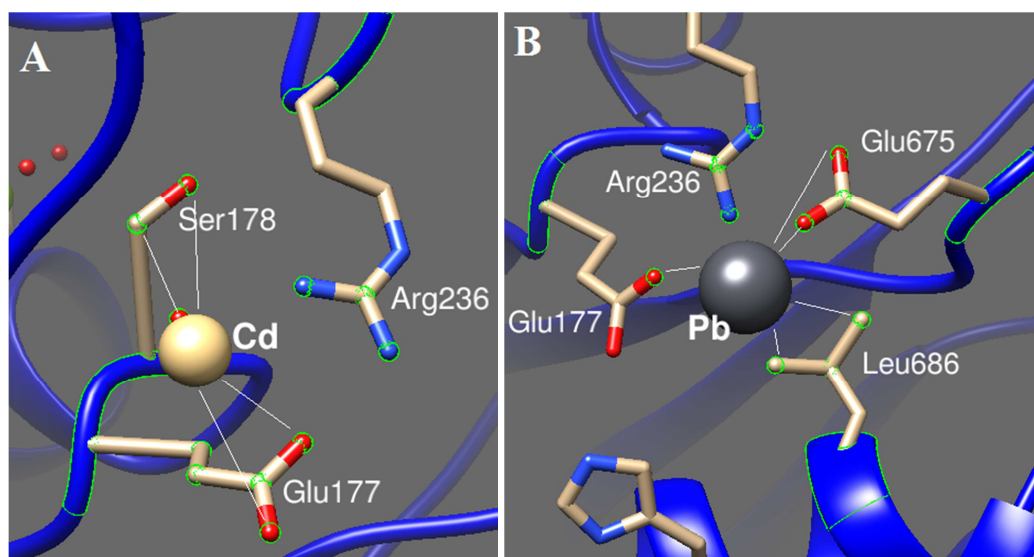


Figure 6. The Cd (A) and Pb (B) cation interaction with the 1 ligand-binding site of the myosin S1. The lines show ionic interactions.

Pb cooperates with the switch 1 and the P-loop, close to the spiral of the relay (Leu475-Glu506) in 1 binding sites of myosin subfragment-1 (Figure 6, B). Pb cation likewise interacts with Arg236 (bond length 0.25 nm) and Leu686 (bond length of 0.26 and 0.296 nm) in the 2 binding site (Figure 7, A), located in an area close to the upper 50-kDa subdomain (Leu218-Asp463, Glu605-Phe621) and to the switch 1 (Gly233-Phe246). Similarly, the Cd cation is

coordinated with the oxygen atom of Glu327 (bond length 0.24 nm), with the oxygen atom Glu326 (bond length 0.32 nm) and the oxygen atom of Asp323 (bond length 0.35 nm) of the interaction site related to the binding and hydrolysis of ATP. Also, Pb cation is coordinated to oxygen atom Glu177 (bond length 0.32 nm) and oxygen atoms of Glu675 (bond length of 0.27 and 0.35 nm).

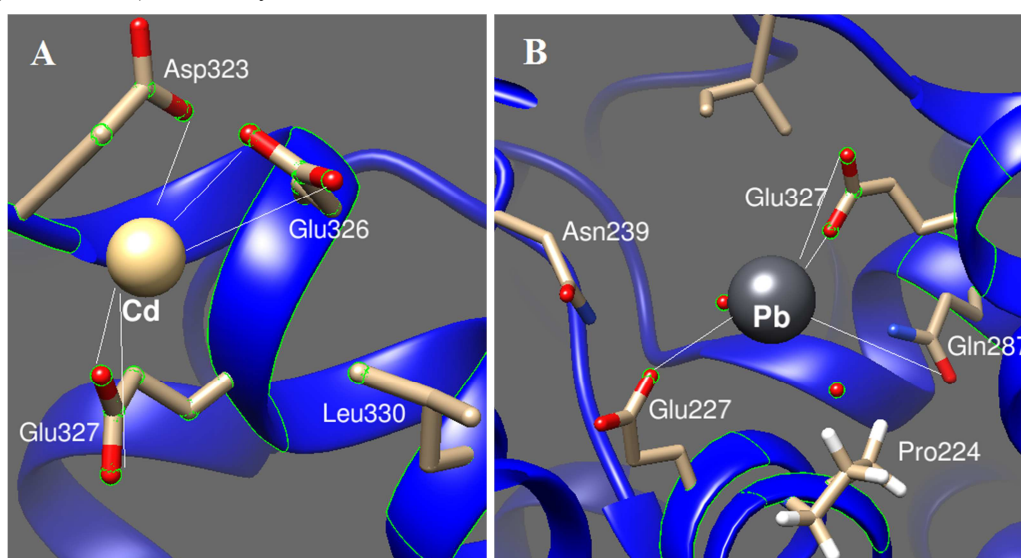


Figure 7. The Cd (A) and Pb (B) cation interaction with the 2 ligand-binding site of the myosin S1. The lines indicate ionic interactions.

Pb cation coordinates with the oxygen atoms of Glu327 (bond length of 0.25 and 0.34 nm) (Figure 7, B) in another binding site, with the oxygen atom of Glu227 (bond length 0.35). Also, Pb cation interacts with Pro224 (bond length 0.4 nm) and Gln287 (bond length 0.45 nm) of the interaction site connected with the switch 1 which involved in binding and ATP hydrolysis.

It was studied the interaction of calix[4]arene C-800 with the ligand-binding site of heavy metal cations on myosin subfragment-1. C-800 molecule is in the cone conformation,

which is stabilized with internal hydrogen bonds between the phenolic groups. First, we got the energy-minimized structure of C-800. The presence of ionized groups at the rim (including bottom) of thiacalix[4]arene significantly increases the contribution of electrostatic interactions to the total energy of guest-host interaction [36]. Further, we used computer docking to investigate the possible capability of calix[4]arene C-800 to change the geometry of the positioning of Cd and Pb cations in the 1 binding site of myosin S1 (Figure 8). Thus, Cd²⁺ (Figure 8, A) are

coordinated tetrahedrally with His689 (bond length 0.26 nm), and with Glu177 (bond length 0.37 nm) of myosin subfragment-1, and with atoms of oxygen and sulfur at the

lower rim of thiacalixarene (S4 – 0.15 nm; O4 – 0.27 nm; O1 – 0.33 nm).

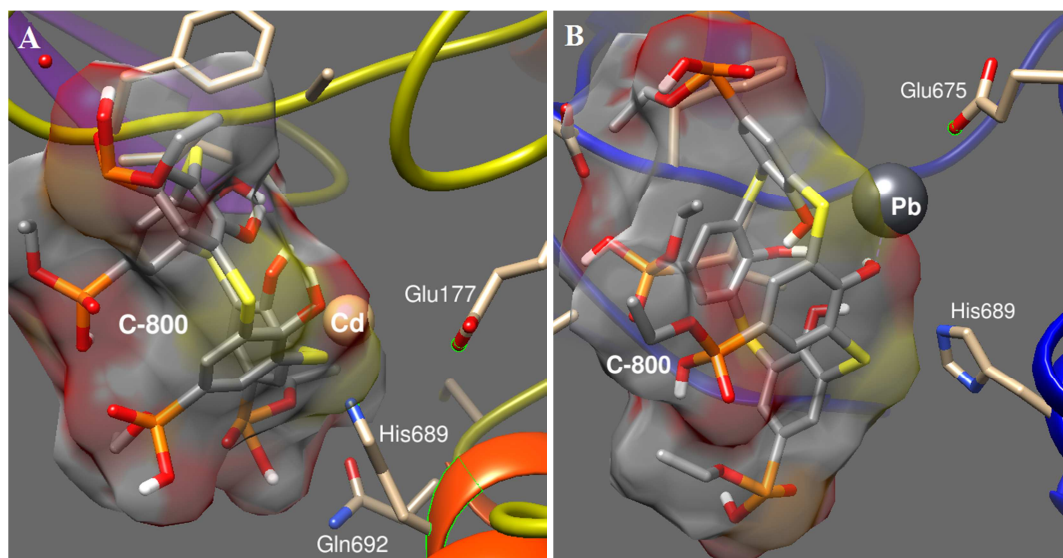


Figure 8. Effect of the C-800 on the interaction of cations Cd (A) and Pb (B) with the amino acid residues in 1 binding of the myosin S1.

The Pb cation is associated with myosin subfragment-1 interaction with calixarene C-800 (Figure 8, B). As a result, the cation is removed from the previous binding sites in region 1. Pb²⁺ interacts with several atoms of oxygen and sulfur at the lower rim of the C-800 (O1 - 0.22 nm; S2 – 0.26 nm O3 - 0.3 nm).

Thus, interaction between Cd²⁺, Pb²⁺, and C-800 within myosin S1 could mitigate the inhibitory effect of these cations. Functional groups of the C-800 cooperating with the Pb and Cd cations may lead to significant weakening of bonds between cations and amino acid residues increasing the distances between them in the myosin S1.

Hence, the inhibitory effect of Ni²⁺, Pb²⁺, and Cd²⁺ on the myosin S1 ATPase from pig myometrium was identified. The myosin subfragment-1 ATPase activity is restored to the normal level in the presence of the C-800 and heavy metal cations. The possible binding sites of Ni²⁺, Pb²⁺ and Cd²⁺ with the myosin S1 were identified using computer modeling method. Also, it was determined the type of relations between the cations and the amino acid residues, their length, and geometrical characteristics.

Protective effect of C-800 towards myosin S1 ATPase activity in the presence of heavy metal cations may be the result of the weakening of the interaction between these cations and myosin amino acid residues near the ATPase active center.

4. Conclusions

Heavy metals have an adverse effect on the contractility of uterine smooth muscle. It has been found an inhibitory effect of Ni²⁺, Pb²⁺, and Cd²⁺ on enzymatic hydrolysis of ATP catalyzed by myosin S1 obtained from smooth muscle of swine uterus. It was demonstrated that

tetrahydroxythiacalix[4]arene-tetraphosphonate (C-800) restored the normal myosin S1 ATPase activity in the presence of heavy metal cations. Therefore, obtained results can be used for further research aimed at using C-800 as a pharmacological compound.

One of the most probable mechanisms of C-800 protective effect is based on its ability to chelate heavy metal cations from the incubation medium. Also, it was assumed that protective activity of C-800 might be the result of weakening the interaction between heavy metal ions and amino acid residues near the active site of myosin ATPase.

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