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Mitochondrial F1FO-ATPase and permeability transition pore response to sulfide in the midgut gland of Mytilus galloprovincialis

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:
Algieri C., Nesci S., Trombetti F., Fabbri M., Ventrella V., Pagliarani A. (2021). Mitochondrial F1FO-ATPase and permeability transition pore response to sulfide in the midgut gland of Mytilus galloprovincialis. BIOCHIMIE, 180, 222-228 [10.1016/j.biochi.2020.11.012].

Availability:
This version is available at: https://hdl.handle.net/11585/783596 since: 2021-07-20

Published:
DOI: http://doi.org/10.1016/j.biochi.2020.11.012

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29 June 2024

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The final published version is available online at: https://doi.org/10.1016/j.biochi.2020.11.012

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Mitochondrial F_1F_O-ATPase and permeability transition pore response to sulfide in the midgut gland of *Mytilus galloprovincialis*

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Declaration of interest

None.

Abbreviations: CRC, calcium retention capacity; EDTA, ethylenediammine tetraacetic acid; DMSO, Dimethylsulphoxide; HEPES, (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid); mPTP, mitochondrial permeability transition pore; Tris, Tris(hydroxymethyl)-aminomethane.

Abstract

The molecular mechanisms which rule the formation and opening of the mitochondrial permeability transition pore (mPTP), the lethal mechanism which permeabilizes mitochondria to water and solutes and drives the cell to death, are still unclear and particularly little investigated in invertebrates. Since Ca^{2+} increase in mitochondria is accompanied by mPTP opening and the participation of the mitochondrial F_1F_O-ATPase in the mPTP is increasingly sustained, the substitution of the natural cofactor Mg^{2+} by Ca^{2+} in the F_1F_O-ATPase activation has been involved in the mPTP mechanism. In mussel midgut gland mitochondria the similar kinetic properties of the Mg^{2+}- or Ca^{2+}-dependent F_1F_O-ATPase activities, namely the same affinity for ATP and bi-site activation kinetics by the ATP substrate, in spite of the higher enzyme activity and coupling efficiency of the Mg^{2+}-dependent F_1F_O-ATPase, suggest that both enzyme activities are involved in the bioenergetic machinery. Other than being a mitochondrial poison and environmental contaminant, sulfide at low concentrations acts as gaseous mediator and can induce post-translational modifications of proteins. The sulfide donor NaHS, at micromolar concentrations, does not alter the two F_1F_O-ATPase activities, but desensitizes the mPTP to Ca^{2+} input. Unexpectedly, NaHS, under the conditions tested, points out a chemical refractoriness of both F_1F_O-ATPase activities and a failed relationship between the Ca^{2+}-dependent F_1F_O-ATPase and the mPTP in mussels. The findings suggest that mPTP role and regulation may be different in different taxa and that the F_1F_O-ATPase insensitivity to NaHS may allow mussels to cope with environmental sulfide.

Keywords

F_1F_O-ATPase; mitochondria; midgut gland, *Mytilus galloprovincialis*; permeability transition pore; sulfide.

1. Introduction

Sulfide is an important environmental agent for a variety of aquatic and terrestrial organisms. In recent years studies on sulfide effects have been mainly focused on mammals, due to the emerging sulfide role as physiological gaseous modulator and its involvement in cardiovascular [1] and liver [2] protection. Marine habitats have received most attention for the occurrence of hydrothermal vents and hypoxic habitats, where high sulfide concentrations impose biological adaptations to allow survival [3]. However, sulfide is a naturally produced compound which affects and drives biochemical events from bacteria to mammals, and, depending on the dose and the available targets, may be a respiratory and neurological poison, a signaling molecule [4], and also have therapeutic potential [1]. In aquatic biota sulfide toxicology has common features, especially at the biochemical level, which affect health, survival, productivity and distribution of aquatic species [5].

In aqueous solutions, H_2S reversibly dissociates into the hydrosulfide (HS^-) and bisulfide (S^{2-}) anions, whose proportion is pH and temperature dependent. At physiological pH (7.4) and temperature (37 °C), the most abundant forms are HS^- (nearly 70%) and H_2S (about 30%). The latter is moderately lipophilic and can cross biological membranes. At increasing pHs, as in seawater, the H_2S level decreases, while HS^- and S^{2-} concentrations increase and may somehow contribute to sulfide bioactivity, even if the negative charges most likely prevent membrane crossing. However, the quantification of the actual concentration of these three species is not easy and often controversial [6,7], so the term sulfide is currently used to embrace the three interconverting forms [8].

Mitochondria provide the main defense against environmental sulfide [5] and also constitute one of its preferred targets [9]. Sulfide can affect mitochondrial proteins by directly producing post translational modifications of aminoacid residues such as cysteine, and/or by indirectly affecting redox homeostasis [10]. Controversial effects were reported on the pro-oxidant and anti-oxidant effects of H_2S and on its modulation of mitochondrial bioenergetics [11].

As far as we are aware no study has dealt with a direct effect of sulfide on the mitochondrial F_1F_O-ATPase, which plays a central role in the production of ATP under aerobic conditions and, as recently emerged, in the cell lifespan [12]. The mitochondrial F_1F_O-ATPase has been increasingly involved in the formation of the permeability transition pore (mPTP), which dramatically increases the inner mitochondrial membrane permeability and drives the cell to death. This lethal task, triggered by an increase in Ca^{2+} concentration in the mitochondrial matrix which activates the hydrolytic activity of the F_1F_O-ATPase [13,14], is established in mammals [15,16], but still uncertain in invertebrates where it could depend on the so called lipidome, namely the lipid composition of the inner mitochondrial membrane [17,18], which may affect the membrane flexibility required to form the pore.

Accordingly, the formation of the mPTP was only described in sea urchin gametes [19], hypothesized in the opistobranch mollusk *Aplysia* [20] and undetected in crustaceans [21]. Clues of pore forming properties of F_1F_O-ATPase dimers were found in model organisms, namely *Drosophila* and yeast [22]. To our knowledge, there are no reports in bivalve mollusks, whose mitochondrial F_1F_O-ATPase mechanism exhibits some astonishing similarities to mammals [23]. Sulfide accumulates below mussel farms [24] and has a great impact on benthic populations [5].

The present work aims at casting light in a quite unexplored field, by testing sulfide effects on the mitochondrial F_1F_O-ATPase activated either by the natural cofactor Mg^{2+} or by Ca^{2+} in the midgut gland of *Mytilus galloprovincialis*, a widely cultivated species in the Mediterranean Sea. Interestingly, bivalve mollusks constitute emerging animal models to study molecular mechanisms, such as those involved in cancer [25], muscle contraction [26], inflammation [27] and aging [28], other than having a recognized role in ecotoxicology [29]. The results may also contribute to improve the knowledge of sulfide physio-pathological roles and of the mitochondrial responsiveness to sulfide in invertebrates under aerobic conditions.

2. Materials and Methods

2.1. Chemicals

NaHS, oligomycin (a mixture of oligomycins A, B and C) and Fura-FF were purchased from Vinci-Biochem (Vinci, Italy). Na_2ATP was obtained from Sigma–Aldrich (Milan, Italy). Quartz double distilled water was used for all reagent solutions.

2.2. Animals

Adult specimen of commercial size (mean average weight 20 g, >25 mm shell length) of mussels *Mytilus galloprovincialis* Lamark were obtained from coastal culture plants in the Northern Adriatic Sea and transported alive in aerated seawater tanks to the laboratory. Approximately 60 mussels were used, divided into pools of 10-15 animals each. According to the Italian law, the use of commercially available bivalve shellfish for research purpose does not require any approval.

2.3. Preparation of mitochondrial fractions

From dissected mussels, the digestive glands (hepatopancreas) were quickly removed, pooled (10-15 animals for each pool), repeatedly rinsed in ice-cold medium A (0.25 M sucrose, 5 mM

Tris(hydroxymethyl)-aminomethane (Tris), 5 mM ethylenediammine tetraacetic acid (EDTA), pH 7.4) and gently dried on blotting paper. Whenever detected, the crystalline stylus was promptly removed. Then tissues were weighted and stored in small vials in small amounts of medium A in liquid nitrogen until use. Immediately after thawing, excess medium was decanted and the digestive glands were homogenized in medium B (0.25 mM sucrose, 0.5 g/L fatty acid-free bovine serum albumin (BSA), Tris 24 mM, pH 7.4), in the proportion 11 mL medium B for each g (wet mass) of tissue, by Braun homogenizer Type 853202 at 450 rpm for 1 min. The mitochondrial fraction was obtained by stepwise centrifugation (Sorvall RC2-B, rotor SS34). The homogenate was centrifuged at 1,100xg for 8 min; the obtained supernatant was filtered through four gauze layers and further centrifuged at 16,800xg for 10 min to yield the raw mitochondrial pellet. The latter was resuspended in medium B and further centrifuged at the same speed for 10 min to obtain the final mitochondrial pellet which was resuspended by gentle stirring using a Teflon Potter Elvejehm homogenizer in a small volume of medium B, thus obtaining a protein concentration of 10-12 mg/mL. All steps were carried out at 0-4 °C. Protein concentration was determined by Bio-Rad Protein Assay kit II with BSA as standard according to the colorimetric method of Bradford [30]. Mitochondrial preparations were then stored in liquid nitrogen until use. The stability of mitochondrial preparations in liquid nitrogen was previously evaluated as a function of storage time. Results indicated that the mitochondrial Mg-ATPase activity was unaffected even after a year [31].

Prior to storage, the respiratory activities were polarographically evaluated [32] on freshly prepared mitochondrial membranes as previously described [33], to check their functionality. These tests, combined with the failed detection of the Na,K-ATPase activity, a known marker of plasma membranes [34], witnessed the quality and the virtual absence of contamination of mitochondrial preparations [35].

2.4. Mitochondrial F_1F_O-ATPase activity assays

Immediately after thawing, mitochondrial preparations were used to evaluate the F_1F_O-ATPase activity. The ATP hydrolysis capability was assayed in a reaction medium (1 mL) containing 0.15 mg mitochondrial protein and 75 mM ethanolammine–HCl buffer pH 8.9, 5.0 mM Na_2ATP and 2.0 mM $MgCl_2$ for the Mg^{2+}-activated ~~F~~F_1F_O-ATPase assay, and in the same buffer at pH 8.9 plus 5.0 mM Na_2ATP and 2.0 mM $CaCl_2$ to evaluate the Ca^{2+}-activated F_1F_O-ATPase activity. After 5 min preincubation at 30 °C, the reaction, carried out at the same temperature, was started by adding the substrate Na_2ATP and stopped after 5 min by adding 1 mL of ice-cold 15% (w/w) trichloroacetic acid aqueous solution. Once the reaction was blocked, vials were centrifuged for 15 min at 3,500 rpm

(Eppendorf Centrifuge 5202). The concentration of inorganic phosphate (Pi) hydrolyzed by known amounts of mitochondrial protein in the supernatant, which indirectly detects the F_1F_O-ATPase activity, was spectrophotometrically evaluated [36]. To this aim, 1.0 µL from a stock solution of 4.0 mg/mL oligomycin in dimethylsulfoxide (DMSO) was directly added to the reaction mixture before starting the reaction. The total F_1F_O-ATPase activity was calculated by the Pi evaluation in control tubes run in parallel and containing 1.0 µL DMSO per mL reaction system. Control tubes were alternated to the condition to be tested in each set of experiments. The dose of 4.0 mg/mL oligomycin, specific inhibitor of F_1F_O-ATPase, which selectively blocks the F_O subunit, which is currently used in F_1F_O-ATPase assays [37], ensured maximal F_1F_O-ATPase inhibition. The F_1F_O-ATPase activity, also defined as mitochondrial oligomycin-sensitive ATPase activity, was routinely measured by subtracting, from the Pi hydrolyzed by total mitochondrial ATPase activity, the Pi hydrolyzed in the presence of oligomycin (mitochondrial oligomycin-insensitive ATPase), and expressed as µmol Pi·mg protein^{-1}·min^{-1} in all experiments. Even if the mitochondrial ATPase activity in mussels referred to F_1F_O-ATPase could be only confirmed by SDS-gel and Western Blot analyses, the ways to confirm enzyme purity and integrity as done in *E. coli* F_1F_O-ATP synthase by other lab [38], at present we could not do it, due to the lack of adequate protocol and reagents.

The effects of the NaHS were tested by adding 4 µl aliquots of NaHS in DMSO (control in NaHS-free medium with 4 µl DMSO) to the reaction mixture immediately prior to the addition of the mitochondrial suspensions. To this aim, NaHS concentrations, obtained by dilution from the 25 mM NaHS DMSO stock solution, were added to the reaction mixture to obtain final NaHS concentrations in the range 0.1–100 µM NaHS in the reaction system.

2.5. Kinetic analyses

In all plots the specific enzyme activity, evaluated as µmoles P_i·mg protein^{-1}·min^{-1} was taken as the expression of the initial reaction rate *v*. To evaluate the enzyme activation kinetics by the ATP substrate, Hill plots were built [39]. To this aim Mg^{2+} and Ca^{2+}-ATPase assays were carried out at various stated ATP millimolar concentrations in the reaction medium, by keeping constant all other assay conditions. The linear transformation of Hill equation was used:

$$log\frac{v_0}{V_{max}-v_0} = -n_{Hi}\, log[ATP] + log\, K'$$

where V_{max} and v_0 represent the enzyme reaction rates, respectively in the presence of substrate concentration which gives the maximal rate and in the presence of a stated substrate concentration

[ATP], K' is a constant value. By plotting log $v_0/(V_{max}-v_0)$ versus log[ATP] a straight line is obtained, whose slope is Hill coefficient ($-n_{Hi}$). In case $|n_{Hi}| \neq 1$ multiple binding sites for the substrate can be involved [39] even if the n_{Hi} value cannot stoichiometrically correspond to the binding sites.

To calculate the kinetic parameters (V_{max} and K_m) in the presence of different concentrations of ATP substrate, enzyme activity data were fitted to the Lineweaver-Burk equation in which the reciprocal of the reaction rate ($1/v$) was plotted as a function of the reciprocal concentration of ATP, raised to a power (n_{Hi}) which corresponds to Hill coefficient. This corrective procedure allows the building of a linear Lineweaver-Burk plot, and consequently the calculation of V_{max} and K_m values from the intercept with y and x axis, respectively, even when n_{Hi} is $\neq 1$.

Correlation coefficients were never lower than 0.96 thus confirming the linearity of all plots.

2.6. mPTP assay

Immediately after the preparation of mussel midgut gland mitochondrial fractions, fresh mitochondrial suspensions (1mg/mL) were energized in the assay buffer (130 mM KCl, 1 mM KH_2PO_4, 20 mM HEPES, pH 7.2 with TRIS), incubated at 30 °C with 1 μg/mL rotenone and 5 mM succinate. To evaluate NaHS effect, selected NaHS doses were added to the mitochondrial suspensions before mPTP evaluation. mPTP opening was induced by the addition of low concentrations of Ca^{2+} (10 μM) as $CaCl_2$ solution at fixed time intervals (1 min). The calcium retention capacity (CRC), whose lowering indicates mPTP opening, was spectrofluorophotometrically evaluated in the presence of 0.8 μM Fura-FF. The probe has different spectral properties in the absence and in the presence of Ca^{2+}, namely it displays excitation/emission spectra of 365/514 nm in the absence of Ca^{2+} (Fura-FF low Ca^{2+}) and shifts to 339/507 nm in the presence of high Ca^{2+} concentrations (Fura-FF high Ca^{2+}). mPTP opening, was evaluated by the increase in the fluorescence intensity ratio (Fura-FF high Ca^{2+})/(Fura-FF low Ca^{2+}), which indicates a decrease in CRC [40]. All measurements were processed by LabSolutions RF software.

2.7. Calculations and statistics

Statistical analyses were performed by SIGMASTAT software. The analysis of variance followed by Students–Newman–Keuls' test when F values indicated significance ($P \leq 0.05$) was applied. Percentage data were arc*sin*-transformed before statistical analyses to ensure normality.

3. Results and discussion

3.1. Ca^{2+}- and Mg^{2+}-dependent F_1F_O-ATPase kinetic parameters

As first approach, experiments aimed at pointing out the kinetic properties of the F_1F_O-ATPase in mussel digestive gland mitochondria either activated by the natural cofactor Mg^{2+} or by Ca^{2+}, being the latter involved in the lethal task of triggering the mPTP formation [14,40,41]. The F_1F_O-ATPase energy transduction mechanism converts the electrochemical gradient of a transmembrane proton motive force (Δp) by torque generation into ATP chemical energy during ATP synthesis and *vice versa* by ATP hydrolysis [42]. Both enzyme tasks of ATP synthesis/hydrolysis are sustained by the natural cofactor Mg^{2+} or by other metal divalent cations [14,43,44]. However, Ca^{2+} only sustains ATP synthesis and in this case, the rotor rotation was reported to be not coupled to Δp generation in beef heart submitochondrial particles [43], in *Rhodospirillum rubrum* [45], in chloroplast thylakoids [46] and in pea stem mitochondria [47]. Conversely, ATP complexed with Ca^{2+} can drive the pH gradient formation with nearly the same effectiveness as MgATP in *Escherichia coli* [48]. Moreover, in swine heart submitochondrial particles the Ca^{2+}-dependent F_1F_O-ATPase is inhibited by succinate-O_2 oxidoreductase activity, which generating the Δp contrast the H^+-pumping F_1F_O-ATPase. On the other hand, dinitrophenol makes the submitochondrial particles membrane permeable to H^+ and increases the hydrolytic activity of the Ca^{2+}-dependent F_1F_O-ATPase coupled to H^+ pumping [13]. The Ca^{2+}-dependent F_1F_O-ATPase of swine heart [13] and pea stem mitochondria [47] are fully sensitive to oligomycin, a specific inhibitor of F_O domain [49]. In mussel midgut gland mitochondria ATP hydrolysis can be sustained by Ca^{2+}, other than by Mg^{2+}, even if the oligomycin-sensitive ATPase activity only represents the 40% of total ATPase activity, while in presence of Mg^{2+} the oligomycin sensitive ATPase is as much as 85% of the total ATPase activity (Fig. 1). Moreover, the coupling index calculated as mitochondrial oligomycin-sensitive ATPase activity on the total mitochondrial ATPase activity ratio of the Mg^{2+}-dependent F_1F_O-ATPase shows a better value than the Ca^{2+}-dependent F_1F_O-ATPase (0.85±0.13 *vs* 0.37±0.08). Since we do not have the protocol for F_1F_O-ATPase isolation and purification from mussel mitochondria, the results obtained refer to the F_1F_O ATPase activity in isolated mitochondria.

Further experiments aimed at casting light on the enzyme kinetics, a quite unexplored field in mussel mitochondria. The binding change mechanism for the F_1-ATPase [50] is known to be sustained by positive cooperativity of three catalytic sites. The ATP substrate can simultaneously fill from one to three sites during the catalysis denoting it as uni-site, bi-site, or tri-site catalysis, respectively. The rate of ATP hydrolysis is ATP concentration dependent. Steady-state catalysis studies provide evidence that the main kinetic enhancement occurs by bi-site activation inducing strong positive cooperativity [51]. The mussel midgut gland F_1F_O-ATPase activated by Mg^{2+} or Ca^{2+} show similar Hill coefficients, namely 1.72±0.20 and 1.71±0.22 respectively (Fig. 2A,B), thus suggesting that, in both cases, ATP can also bind to a two catalytic sites to yield high rates of catalysis. However, Mg^{2+} and Ca^{2+} do not sustain ATP hydrolysis by the F_1F_O-ATPase with the same efficiency. Accordingly, even if the K_m values are similar for the two differently activated ATPases, the Mg^{2+}-activated F_1F_O-ATPase shows a 57% higher V_{max} value than the Ca^{2+}-activated enzyme (Fig. 2C,D), which indicates that, when activated by Mg^{2+}, the enzyme hydrolytic activity is more efficient, as in mammals [13,14]. On this basis, the mussel oligomycin-sensitive Ca^{2+}-dependent F_1F_O-ATP(hydrol)ase could be enlisted in the mitochondrial bioenergetic regulation machinery, since its affinity for ATP is the same as the companion Mg^{2+}-activated enzyme.

3.2. NaHS effect on the mitochondrial F_1F_O-ATPases and permeability transition pore

Since mussel midgut gland mitochondria contain an ATPase activity that can be activated by both Mg^{2+} and Ca^{2+} with different kinetic properties, it seemed interesting to evaluate the effect of the sulfide donor NaHS on these enzyme activities and to search for a putative connection with the mPTP. NaHS is widely used to generate H_2S, which, once only known as mitochondrial poison, in recent years has raised increasing interest in cell biology as endogenous gaseous neurotransmitter [52] that acts on cardiovascular system and inflammation as well as on nervous systems, pain appreciation, gastrointestinal and urogenital functions, and endocrine system [53]. The effect, deleterious or on the contrary beneficial, apparently depends on the dose and on the microenvironmental conditions, which in turn include a number of variables. Accordingly, NaHS and related compounds H_2S, HS^- and S^{2-}, can also alter the function of cellular proteins and enzymes by inducing post-translational modifications especially on "redox sensor" cysteines [54]. In mammalian mitochondria the α subunits of F_1F_O-ATPase, involved in the catalytic and non-catalytic sites of F_1 domain [55], show cysteine residues at positions 244 and 294 prone to reversible *S*-sulfhydration [56]. Modification of thiol (–SH) group of cysteines by covalent bond to H_2S forms persulfide (–SSH) group [11]. Other than acting as a mitochondrial poison when blocks complex IV, sulfide can stimulate the ATP synthase by inducing *S*-sulfhydration of α subunits in a concentration-dependent manner [56]. The increase in the enzyme catalytic mechanism when the αCys244 and αCys294 are *S*-sulfhydrized under (patho)physiological conditions prevents the disulfide bond formation under oxidative stress conditions, which forms before the individual subunits (*e.g.* α and γ subunits) assemble into the ATP synthase complex [57] and alters the F_1-ATPase chemomechanical mechanism [58]. However, quite surprisingly, when increasing NaHS concentrations are tested on mussel midgut gland mitochondria, no effect on the Ca^{2+}- and Mg^{2+}-activated F_1F_O-ATPases is shown (Fig. 3). Although in eukaryotes there is a high similarity of amino acid sequences in the F_1F_O-ATPase subunits [59], the mussel F_1F_O-ATPase cysteines may be refractory to covalently bind to sulfide. Both mPTP and oxidative stress are known to be inhibited by exogenous H_2S that acts as a cell death modulator [60]. The role of Ca^{2+} in the PTP opening has also been associated with the interaction with phospholipids of the inner mitochondrial membranes where it appears to induce changes in cardiolipin (CL) packing and to increase CL susceptibility to oxidation [61]. On these bases, the peculiar CL molecular species of mussel, dominated by an extraordinary high level of 22:6n-3 exceeding 70% of total fatty acids [62] and identified as predominantly in a form with four docosahexaenoyl chains and thus easily exposed to oxidation, can help to explain the different regulation mechanisms in different taxa. The F_1F_O-ATPase is the molecular architecture proposed to coincide with the mPTP [63–65]. Recently two conformations with low and high ion-conductance have been attributed to the monomeric or dimeric form of the F_1F_O-ATPase[15,16] in presence of Ca^{2+}. Other membrane-embedded protein of IMM as adenine nucleotide translocase, could only sustain the low ion-conductance that is Ca^{2+}-dependent and bongkrekic acid sensitive [16,66]. Even if the existence of mPTP is still elusive in mussels, the detection of the Ca^{2+}-activated F_1F_O-ATP(hydrol)ase, which was shown to most likely coincide with the mPTP [14,41], strongly suggests that mussel midgut gland mitochondria possess mPTP activity whose responsiveness to sulfide was tested. Accordingly, in mussel midgut gland mitochondria the CRC decreases at increasing Ca^{2+} concentrations (Fig. 4). The NaHS treatment desensitizes the mPTP activity, even if it is ineffective on the Ca^{2+}-activated F_1F_O-ATPase (Fig. 3). Experiments carried out by adding to mitochondria the mitochondrial calcium uniporter inhibitor Ruthenium Red result in similar CRC profiles in response to subsequent 10 μM $CaCl_2$ pulses in the presence of 50 and 100 μM NaHS and of 1 μM Ruthenium Red. Regulatory role of CL can extend to adenine nucleotide translocase which constitute regulatory component of mPTP [16]. So, the Ca^{2+} uptake by mitochondria is clearly affected by NaHS (Fig. 4) and, as a consequence the mPTP, is desensitized, namely it requires higher Ca^{2+} load to open and release the cation.

4. Conclusions

Some main observations can be drawn from the results of the present study. First, mussel midgut gland mitochondria contain an oligomycin-sensitive F_1F_O-ATPase activity that can be activated either by Mg^{2+} or by Ca^{2+}, as in mammals. Both F_1F_O-ATPase activity are coupled, as revealed by the extent of oligomycin sensitivity. However, some differences exist between molluscan and mammalian F_1F_O-ATPases revealed by the sulfide responsiveness. Second, mussel mitochondria are able to form the mPTP. However, the connection between the Ca^{2+}-dependent F_1F_O-ATPase and the mPTP is weakened in these invertebrates, since NaHS, at least under the experimental conditions adopted, has no effect on the enzyme activity even if it inhibits the mPTP. Moreover, if, on the one hand, the "mussel model" to deepen studies on mitochondria is strengthened, due to some kinetic similarities with mammals and the easy-to-use biological material, on the other hand the hypothesis that the mPTP regulation, and perhaps the same mPTP role, may be different in different taxa, is somehow shouldered. The mPTP responsiveness to sulfide in the mussel opens a new scenario to be investigated. Among the still unsolved questions, it remains unclear if sulfide can interact with other proteins involved in Ca^{2+} homeostasis and if the F_1F_O-ATPase refractoriness can physiologically play a protective role in sulfide-rich environments. Accordingly, some sulfide-driven post-translational modifications of proteins are irreversible and can produce permanent damage to the biostructures. In this perspective the chemical un-reactivity of the bioenergetic mechanisms can be even advantageous for mussels.

Funding

This work was financed by the University of Bologna, Italy (Almaidea senior grant to AP).

Author contributions

CA, SN and MF carried out the experiments; SN planned the experimental design and supervised the experiments, SN and AP wrote the manuscript; AP VV and FT revised the text; AP funding acquisition; all authors read and approved the final text.

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Figure captions

Figure 1. Mitochondrial ATPase activity sustained by Ca^{2+} or by Mg^{2+}. The oligomycin-sensitive ATPase activity ( ) and the oligomycin-insensitive ATPase activity ( ) are expressed as percentages of the total mitochondrial ATPase activity sustained by Ca^{2+} or Mg^{2+}, respectively. Data expressed as column chart represent the mean ± SD (vertical bars) from three experiments carried out on different mitochondrial preparations. * indicates significantly different values ($P \leq 0.05$).

Figure 2. Plots to obtain the kinetic parameters of the mitochondrial Mg^{2+}- and Ca^{2+}-dependent F_1F_O-ATPases. A and B) Hill plots of the Mg^{2+}-ATPase (●) and the Ca^{2+}-ATPase (○). C and D) Lineweaver-Burk plots of the Mg^{2+}-ATPase (●) and Ca^{2+}-ATPase (○). Data represent the mean ± SD from three independent experiments carried out on distinct mitochondrial preparations.

Figure 3. *In vitro* response of the mitochondrial Mg^{2+}- and Ca^{2+}-dependent F_1F_O-ATPase activities to NaHS. The F_1F_O-ATPase activities of the mitochondrial Mg^{2+}-ATPase (●) and Ca^{2+}-ATPase (○) are plotted against NaHS concentrations (logarithmic scale). Each point represents the mean ± SD from three experiments on distinct mitochondrial preparations.

Figure 4. Evaluation of mPTP opening. Representative curves of the calcium retention capacity (CRC) in mitochondrial preparations. CRC was monitored in response to subsequent 10 μM$CaCl_2$ pulses (shown by the arrows), as detailed in the Materials and Methods section, in the absence (control) and presence of 50 or 100 μM NaHS, and in the presence of the mPTP inhibitors 2 mM MgADP or 1 μM Ruthenium Red (RR). The experiments were carried out in triplicate on three distinct mitochondrial preparations.

Figure 1

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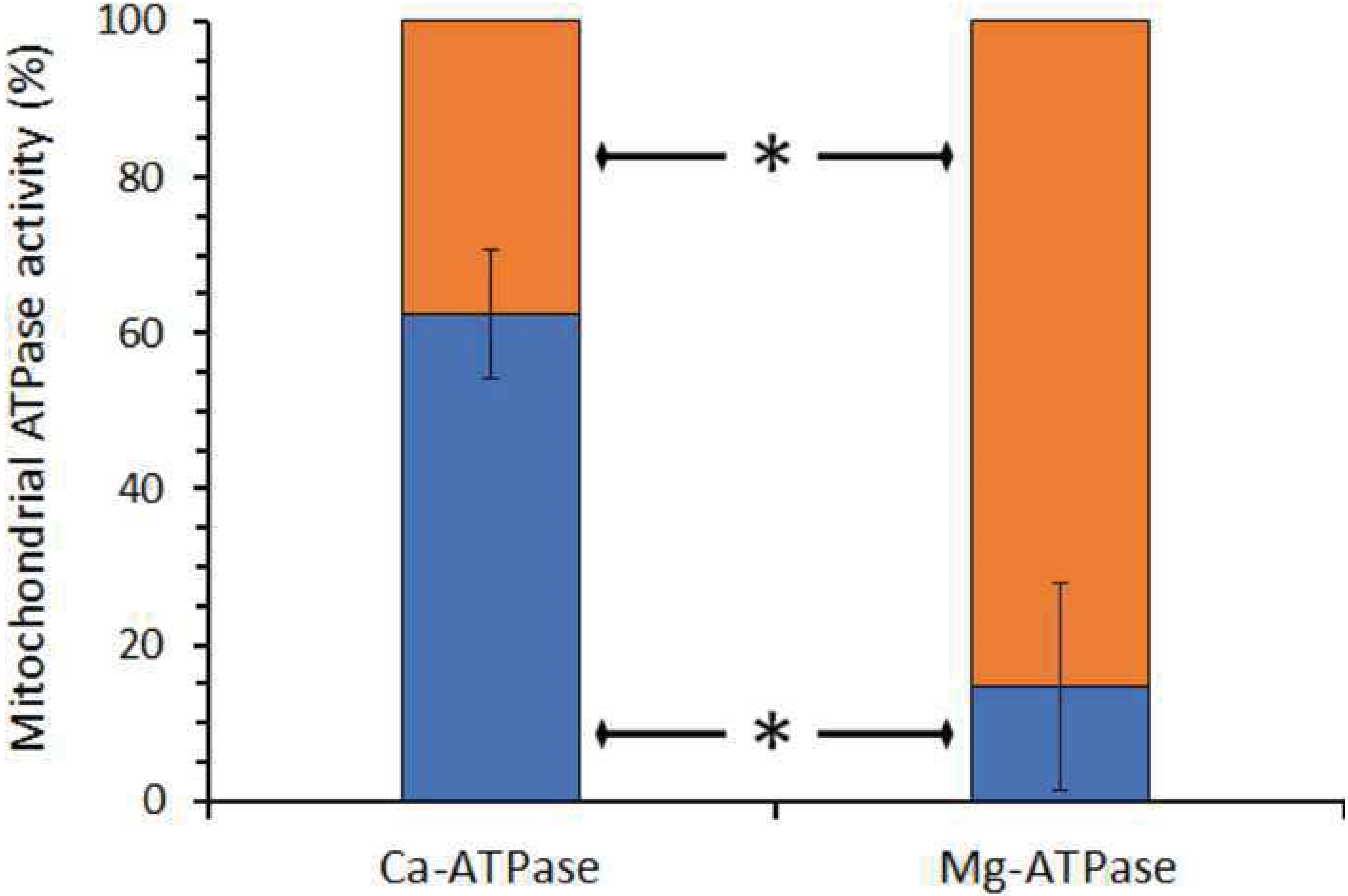

Figure 2

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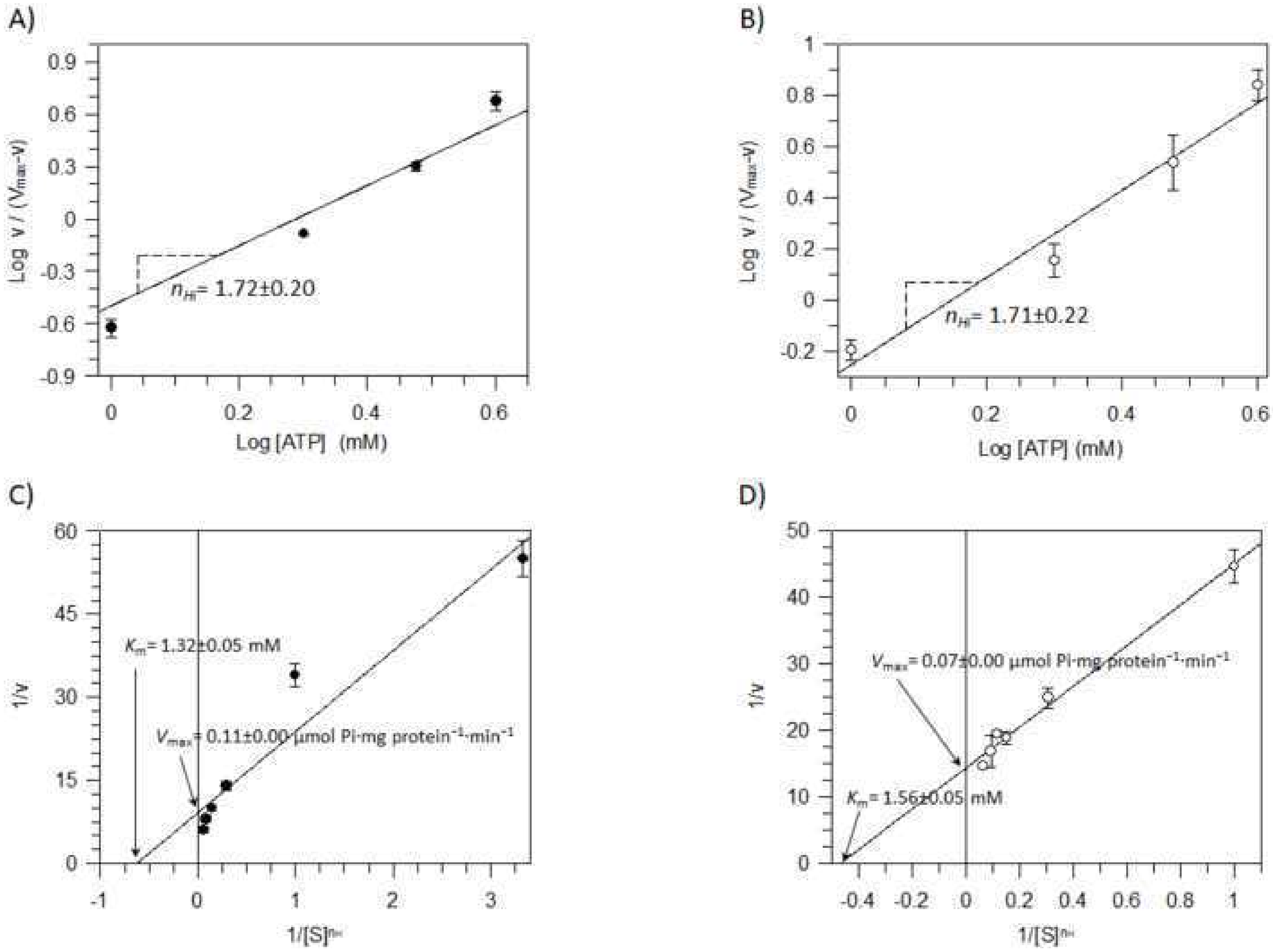

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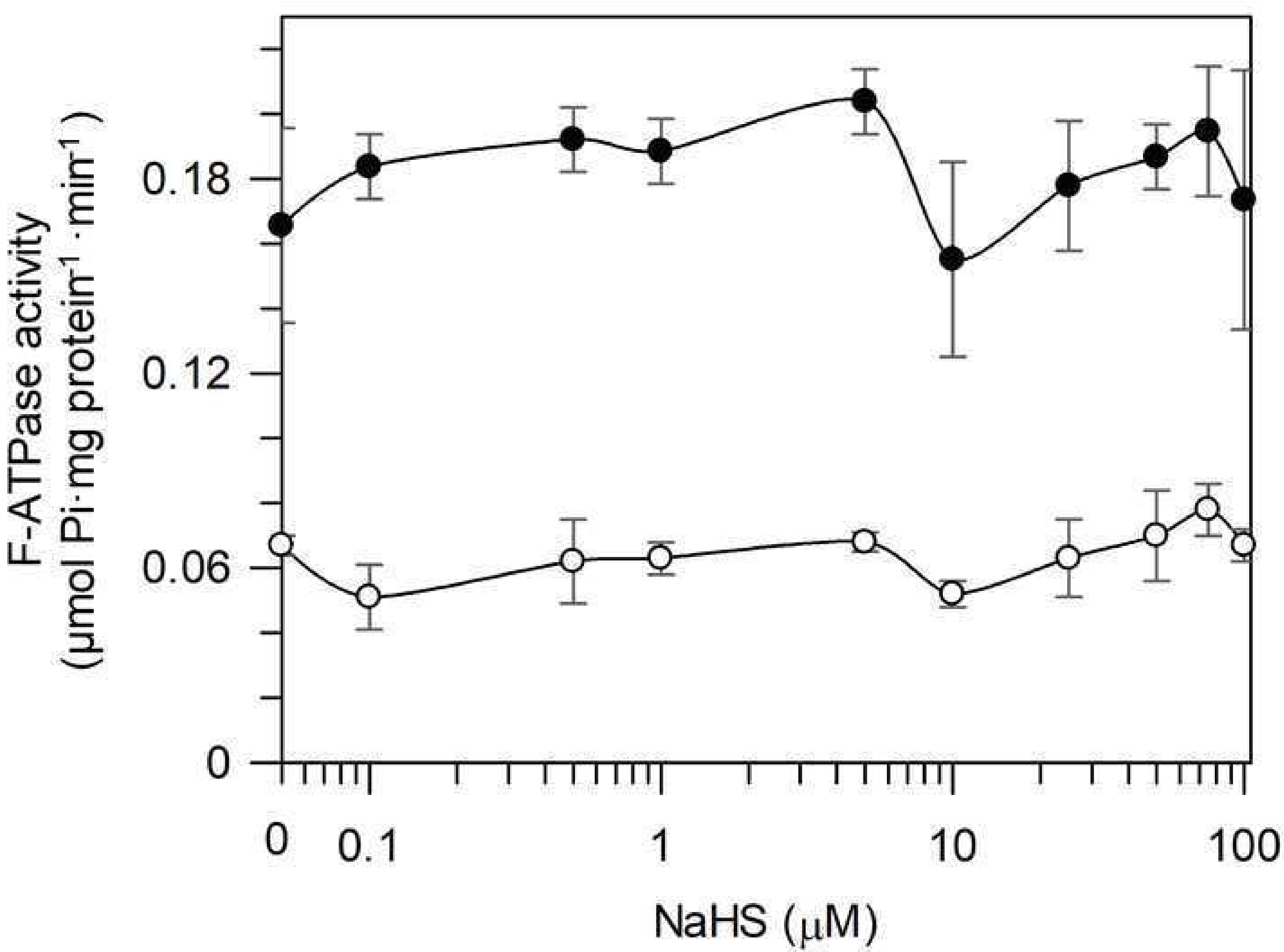

Figure 4

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