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

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Mitochondrial F₁F₀-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_0 -ATPase in the mPTP is increasingly sustained, the substitution of the natural cofactor Mg^{2+} by Ca^{2+} in the F_1F_0 -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_0 -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_0 -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_0 -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_0 -ATPase activities and a failed relationship between the Ca^{2+} -dependent F_1F_0 -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_0 -ATPase insensitivity to NaHS may allow mussels to cope with environmental sulfide.

Keywords

F_1F_0 -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_0 -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_0 -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_0 -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.

1 Accordingly, the formation of the mPTP was only described in sea urchin gametes [19], hypothesized
2 in the opisthobranch mollusk *Aplysia* [20] and undetected in crustaceans [21]. Clues of pore forming
3 properties of F₁F₀-ATPase dimers were found in model organisms, namely *Drosophila* and yeast
4 [22]. To our knowledge, there are no reports in bivalve mollusks, whose mitochondrial F₁F₀-ATPase
5 mechanism exhibits some astonishing similarities to mammals [23]. Sulfide accumulates below
6 mussel farms [24] and has a great impact on benthic populations [5].
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11 The present work aims at casting light in a quite unexplored field, by testing sulfide effects on the
12 mitochondrial F₁F₀-ATPase activated either by the natural cofactor Mg²⁺ or by Ca²⁺ in the midgut
13 gland of *Mytilus galloprovincialis*, a widely cultivated species in the Mediterranean Sea.
14 Interestingly, bivalve mollusks constitute emerging animal models to study molecular mechanisms,
15 such as those involved in cancer [25], muscle contraction [26], inflammation [27] and aging [28],
16 other than having a recognized role in ecotoxicology [29]. The results may also contribute to improve
17 the knowledge of sulfide physio-pathological roles and of the mitochondrial responsiveness to sulfide
18 in invertebrates under aerobic conditions.
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29 2. Materials and Methods

30 2.1. Chemicals

31 NaHS, oligomycin (a mixture of oligomycins A, B and C) and Fura-FF were purchased from Vinci-
32 Biochem (Vinci, Italy). Na₂ATP was obtained from Sigma–Aldrich (Milan, Italy). Quartz double
33 distilled water was used for all reagent solutions.
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40 2.2. Animals

41 Adult specimen of commercial size (mean average weight 20 g, >25 mm shell length) of mussels
42 *Mytilus galloprovincialis* Lamark were obtained from coastal culture plants in the Northern Adriatic
43 Sea and transported alive in aerated seawater tanks to the laboratory. Approximately 60 mussels were
44 used, divided into pools of 10-15 animals each. According to the Italian law, the use of commercially
45 available bivalve shellfish for research purpose does not require any approval.
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55 2.3. Preparation of mitochondrial fractions

56 From dissected mussels, the digestive glands (hepatopancreas) were quickly removed, pooled (10-15
57 animals for each pool), repeatedly rinsed in ice-cold medium A (0.25 M sucrose, 5 mM
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1 Tris(hydroxymethyl)-aminomethane (Tris), 5 mM ethylenediamine tetraacetic acid (EDTA), pH
2 7.4) and gently dried on blotting paper. Whenever detected, the crystalline stylus was promptly
3 removed. Then tissues were weighted and stored in small vials in small amounts of medium A in
4 liquid nitrogen until use. Immediately after thawing, excess medium was decanted and the digestive
5 glands were homogenized in medium B (0.25 mM sucrose, 0.5 g/L fatty acid-free bovine serum
6 albumin (BSA), Tris 24 mM, pH 7.4), in the proportion 11 mL medium B for each g (wet mass) of
7 tissue, by Braun homogenizer Type 853202 at 450 rpm for 1 min. The mitochondrial fraction was
8 obtained by stepwise centrifugation (Sorvall RC2-B, rotor SS34). The homogenate was centrifuged
9 at 1,100xg for 8 min; the obtained supernatant was filtered through four gauze layers and further
10 centrifuged at 16,800xg for 10 min to yield the raw mitochondrial pellet. The latter was resuspended
11 in medium B and further centrifuged at the same speed for 10 min to obtain the final mitochondrial
12 pellet which was resuspended by gentle stirring using a Teflon Potter Elvehjem homogenizer in a
13 small volume of medium B, thus obtaining a protein concentration of 10-12 mg/mL. All steps were
14 carried out at 0-4 °C. Protein concentration was determined by Bio-Rad Protein Assay kit II with
15 BSA as standard according to the colorimetric method of Bradford [30]. Mitochondrial preparations
16 were then stored in liquid nitrogen until use. The stability of mitochondrial preparations in liquid
17 nitrogen was previously evaluated as a function of storage time. Results indicated that the
18 mitochondrial Mg-ATPase activity was unaffected even after a year [31].
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33 Prior to storage, the respiratory activities were polarographically evaluated [32] on freshly prepared
34 mitochondrial membranes as previously described [33], to check their functionality. These tests,
35 combined with the failed detection of the Na,K-ATPase activity, a known marker of plasma
36 membranes [34], witnessed the quality and the virtual absence of contamination of mitochondrial
37 preparations [35].
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46 *2.4. Mitochondrial F₁F₀-ATPase activity assays*

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48 Immediately after thawing, mitochondrial preparations were used to evaluate the F₁F₀-ATPase
49 activity. The ATP hydrolysis capability was assayed in a reaction medium (1 mL) containing 0.15
50 mg mitochondrial protein and 75 mM ethanolamine-HCl buffer pH 8.9, 5.0 mM Na₂ATP and 2.0
51 mM MgCl₂ for the Mg²⁺-activated F₁F₀-ATPase assay, and in the same buffer at pH 8.9 plus 5.0
52 mM Na₂ATP and 2.0 mM CaCl₂ to evaluate the Ca²⁺-activated F₁F₀-ATPase activity. After 5 min
53 preincubation at 30 °C, the reaction, carried out at the same temperature, was started by adding the
54 substrate Na₂ATP and stopped after 5 min by adding 1 mL of ice-cold 15% (w/w) trichloroacetic acid
55 aqueous solution. Once the reaction was blocked, vials were centrifuged for 15 min at 3,500 rpm
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(Eppendorf Centrifuge 5202). The concentration of inorganic phosphate (Pi) hydrolyzed by known amounts of mitochondrial protein in the supernatant, which indirectly detects the F₁F₀-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 F₁F₀-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 F₁F₀-ATPase, which selectively blocks the F₀ subunit, which is currently used in F₁F₀-ATPase assays [37], ensured maximal F₁F₀-ATPase inhibition. The F₁F₀-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 F₁F₀-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* F₁F₀-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 Mg²⁺ and Ca²⁺-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

1 [ATP], K' is a constant value. By plotting $\log v_0/(V_{max}-v_0)$ versus $\log[ATP]$ a straight line is obtained,
2 whose slope is Hill coefficient ($-n_{Hi}$). In case $|n_{Hi}| \neq 1$ multiple binding sites for the substrate can be
3 involved [39] even if the n_{Hi} value cannot stoichiometrically correspond to the binding sites.
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6 To calculate the kinetic parameters (V_{max} and K_m) in the presence of different concentrations of ATP
7 substrate, enzyme activity data were fitted to the Lineweaver-Burk equation in which the reciprocal
8 of the reaction rate ($1/v$) was plotted as a function of the reciprocal concentration of ATP, raised to a
9 power (n_{Hi}) which corresponds to Hill coefficient. This corrective procedure allows the building of a
10 linear Lineweaver-Burk plot, and consequently the calculation of V_{max} and K_m values from the
11 intercept with y and x axis, respectively, even when n_{Hi} is $\neq 1$.
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17 Correlation coefficients were never lower than 0.96 thus confirming the linearity of all plots.
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22 2.6. mPTP assay

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25 Immediately after the preparation of mussel midgut gland mitochondrial fractions, fresh
26 mitochondrial suspensions (1mg/mL) were energized in the assay buffer (130 mM KCl, 1 mM
27 KH_2PO_4 , 20 mM HEPES, pH 7.2 with TRIS), incubated at 30 °C with 1 μ g/mL rotenone and 5 mM
28 succinate. To evaluate NaHS effect, selected NaHS doses were added to the mitochondrial
29 suspensions before mPTP evaluation. mPTP opening was induced by the addition of low
30 concentrations of Ca^{2+} (10 μ M) as $CaCl_2$ solution at fixed time intervals (1 min). The calcium
31 retention capacity (CRC), whose lowering indicates mPTP opening, was
32 spectrofluorometrically evaluated in the presence of 0.8 μ M Fura-FF. The probe has different
33 spectral properties in the absence and in the presence of Ca^{2+} , namely it displays excitation/emission
34 spectra of 365/514 nm in the absence of Ca^{2+} (Fura-FF low Ca^{2+}) and shifts to 339/507 nm in the
35 presence of high Ca^{2+} concentrations (Fura-FF high Ca^{2+}). mPTP opening, was evaluated by the
36 increase in the fluorescence intensity ratio (Fura-FF high Ca^{2+})/(Fura-FF low Ca^{2+}), which indicates
37 a decrease in CRC [40]. All measurements were processed by LabSolutions RF software.
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52 2.7. Calculations and statistics

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55 Statistical analyses were performed by SIGMASTAT software. The analysis of variance followed by
56 Students–Newman–Keuls' test when F values indicated significance ($P \leq 0.05$) was applied.
57 Percentage data were arcsin-transformed before statistical analyses to ensure normality.
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3. Results and discussion

3.1. Ca^{2+} - and Mg^{2+} -dependent F_1F_0 -ATPase kinetic parameters

As first approach, experiments aimed at pointing out the kinetic properties of the F_1F_0 -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_0 -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_0 -ATPase is inhibited by succinate- O_2 oxidoreductase activity, which generating the Δp contrast the H^+ -pumping F_1F_0 -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_0 -ATPase coupled to H^+ pumping [13]. The Ca^{2+} -dependent F_1F_0 -ATPase of swine heart [13] and pea stem mitochondria [47] are fully sensitive to oligomycin, a specific inhibitor of F_0 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_0 -ATPase shows a better value than the Ca^{2+} -dependent F_1F_0 -ATPase (0.85 ± 0.13 vs 0.37 ± 0.08). Since we do not have the protocol for F_1F_0 -ATPase isolation and purification from mussel mitochondria, the results obtained refer to the F_1F_0 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

1 cooperativity [51]. The mussel midgut gland F_1F_0 -ATPase activated by Mg^{2+} or Ca^{2+} show similar
2 Hill coefficients, namely 1.72 ± 0.20 and 1.71 ± 0.22 respectively (Fig. 2A,B), thus suggesting that, in
3 both cases, ATP can also bind to a two catalytic sites to yield high rates of catalysis. However, Mg^{2+}
4 and Ca^{2+} do not sustain ATP hydrolysis by the F_1F_0 -ATPase with the same efficiency. Accordingly,
5 even if the K_m values are similar for the two differently activated ATPases, the Mg^{2+} -activated F_1F_0 -
6 ATPase shows a 57% higher V_{max} value than the Ca^{2+} -activated enzyme (Fig. 2C,D), which indicates
7 that, when activated by Mg^{2+} , the enzyme hydrolytic activity is more efficient, as in mammals [13,14].
8 On this basis, the mussel oligomycin-sensitive Ca^{2+} -dependent F_1F_0 -ATP(hydrol)ase could be
9 enlisted in the mitochondrial bioenergetic regulation machinery, since its affinity for ATP is the same
10 as the companion Mg^{2+} -activated enzyme.
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21 *3.2. NaHS effect on the mitochondrial F_1F_0 -ATPases and permeability transition pore*

22 Since mussel midgut gland mitochondria contain an ATPase activity that can be activated by both
23 Mg^{2+} and Ca^{2+} with different kinetic properties, it seemed interesting to evaluate the effect of the
24 sulfide donor NaHS on these enzyme activities and to search for a putative connection with the mPTP.
25 NaHS is widely used to generate H_2S , which, once only known as mitochondrial poison, in recent
26 years has raised increasing interest in cell biology as endogenous gaseous neurotransmitter [52] that
27 acts on cardiovascular system and inflammation as well as on nervous systems, pain appreciation,
28 gastrointestinal and urogenital functions, and endocrine system [53]. The effect, deleterious or on the
29 contrary beneficial, apparently depends on the dose and on the microenvironmental conditions, which
30 in turn include a number of variables. Accordingly, NaHS and related compounds H_2S , HS^- and S^{2-} ,
31 can also alter the function of cellular proteins and enzymes by inducing post-translational
32 modifications especially on “redox sensor” cysteines [54]. In mammalian mitochondria the α subunits
33 of F_1F_0 -ATPase, involved in the catalytic and non-catalytic sites of F_1 domain [55], show cysteine
34 residues at positions 244 and 294 prone to reversible *S*-sulfhydration [56]. Modification of thiol ($-$
35 SH) group of cysteines by covalent bond to H_2S forms persulfide ($-SSH$) group [11]. Other than
36 acting as a mitochondrial poison when blocks complex IV, sulfide can stimulate the ATP synthase
37 by inducing *S*-sulfhydration of α subunits in a concentration-dependent manner [56]. The increase in
38 the enzyme catalytic mechanism when the $\alpha Cys244$ and $\alpha Cys294$ are *S*-sulfhydrized under
39 (patho)physiological conditions prevents the disulfide bond formation under oxidative stress
40 conditions, which forms before the individual subunits (*e.g.* α and γ subunits) assemble into the ATP
41 synthase complex [57] and alters the F_1 -ATPase chemomechanical mechanism [58]. However, quite
42 surprisingly, when increasing NaHS concentrations are tested on mussel midgut gland mitochondria,
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1 no effect on the Ca^{2+} - and Mg^{2+} -activated F_1F_0 -ATPases is shown (Fig. 3). Although in eukaryotes
2 there is a high similarity of amino acid sequences in the F_1F_0 -ATPase subunits [59], the mussel F_1F_0 -
3 ATPase cysteines may be refractory to covalently bind to sulfide. Both mPTP and oxidative stress
4 are known to be inhibited by exogenous H_2S that acts as a cell death modulator [60]. The role of Ca^{2+}
5 in the PTP opening has also been associated with the interaction with phospholipids of the inner
6 mitochondrial membranes where it appears to induce changes in cardiolipin (CL) packing and to
7 increase CL susceptibility to oxidation [61]. On these bases, the peculiar CL molecular species of
8 mussel, dominated by an extraordinary high level of 22:6n-3 exceeding 70% of total fatty acids [62]
9 and identified as predominantly in a form with four docosahexaenoyl chains and thus easily exposed
10 to oxidation, can help to explain the different regulation mechanisms in different taxa. The F_1F_0 -
11 ATPase is the molecular architecture proposed to coincide with the mPTP [63–65]. Recently two
12 conformations with low and high ion-conductance have been attributed to the monomeric or dimeric
13 form of the F_1F_0 -ATPase [15,16] in presence of Ca^{2+} . Other membrane-embedded protein of IMM as
14 adenine nucleotide translocase, could only sustain the low ion-conductance that is Ca^{2+} -dependent
15 and bongkreikic acid sensitive [16,66]. Even if the existence of mPTP is still elusive in mussels, the
16 detection of the Ca^{2+} -activated F_1F_0 -ATP(hydrol)ase, which was shown to most likely coincide with
17 the mPTP [14,41], strongly suggests that mussel midgut gland mitochondria possess mPTP activity
18 whose responsiveness to sulfide was tested. Accordingly, in mussel midgut gland mitochondria the
19 CRC decreases at increasing Ca^{2+} concentrations (Fig. 4). The NaHS treatment desensitizes the mPTP
20 activity, even if it is ineffective on the Ca^{2+} -activated F_1F_0 -ATPase (Fig. 3). Experiments carried out
21 by adding to mitochondria the mitochondrial calcium uniporter inhibitor Ruthenium Red result in
22 similar CRC profiles in response to subsequent 10 μM CaCl_2 pulses in the presence of 50 and 100
23 μM NaHS and of 1 μM Ruthenium Red. Regulatory role of CL can extend to adenine nucleotide
24 translocase which constitute regulatory component of mPTP [16]. So, the Ca^{2+} uptake by
25 mitochondria is clearly affected by NaHS (Fig. 4) and, as a consequence the mPTP, is desensitized,
26 namely it requires higher Ca^{2+} load to open and release the cation.
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51 4. Conclusions

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53 Some main observations can be drawn from the results of the present study. First, mussel midgut
54 gland mitochondria contain an oligomycin-sensitive F_1F_0 -ATPase activity that can be activated either
55 by Mg^{2+} or by Ca^{2+} , as in mammals. Both F_1F_0 -ATPase activity are coupled, as revealed by the extent
56 of oligomycin sensitivity. However, some differences exist between molluscan and mammalian F_1F_0 -
57 ATPases revealed by the sulfide responsiveness. Second, mussel mitochondria are able to form the
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1 mPTP. However, the connection between the Ca^{2+} -dependent F_1F_0 -ATPase and the mPTP is
2 weakened in these invertebrates, since NaHS, at least under the experimental conditions adopted, has
3 no effect on the enzyme activity even if it inhibits the mPTP. Moreover, if, on the one hand, the
4 “mussel model” to deepen studies on mitochondria is strengthened, due to some kinetic similarities
5 with mammals and the easy-to-use biological material, on the other hand the hypothesis that the
6 mPTP regulation, and perhaps the same mPTP role, may be different in different taxa, is somehow
7 shouldered. The mPTP responsiveness to sulfide in the mussel opens a new scenario to be
8 investigated. Among the still unsolved questions, it remains unclear if sulfide can interact with other
9 proteins involved in Ca^{2+} homeostasis and if the F_1F_0 -ATPase refractoriness can physiologically play
10 a protective role in sulfide-rich environments. Accordingly, some sulfide-driven post-translational
11 modifications of proteins are irreversible and can produce permanent damage to the biostructures. In
12 this perspective the chemical un-reactivity of the bioenergetic mechanisms can be even advantageous
13 for mussels.
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35 Author contributions

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

Figure 1. Mitochondrial ATPase activity sustained by Ca²⁺ or by Mg²⁺. The oligomycin-sensitive ATPase activity (■) and the oligomycin-insensitive ATPase activity (■) are expressed as percentages of the total mitochondrial ATPase activity sustained by Ca²⁺ or Mg²⁺, 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 Mg²⁺- and Ca²⁺-dependent F₁F₀-ATPases. A and B) Hill plots of the Mg²⁺-ATPase (●) and the Ca²⁺-ATPase (○). C and D) Lineweaver-Burk plots of the Mg²⁺-ATPase (●) and Ca²⁺-ATPase (○). Data represent the mean ± SD from three independent experiments carried out on distinct mitochondrial preparations.

Figure 3. *In vitro* response of the mitochondrial Mg²⁺- and Ca²⁺-dependent F₁F₀-ATPase activities to NaHS. The F₁F₀-ATPase activities of the mitochondrial Mg²⁺-ATPase (●) and Ca²⁺-ATPase (○) are plotted against NaHS concentrations (logarithmic scale). Each point represents the mean ± SD from three experiments on distinct mitochondrial preparations.

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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.







