

Redox regulation of mitochondrial sulfide oxidation in the lugworm, *Arenicola marina*

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SUMMARY

Sulfide oxidation in the lugworm, *Arenicola marina* (L.), is most likely localized in the mitochondria, which can either produce ATP with sulfide as a substrate or detoxify it *via* an alternative oxidase. The present study identified selective activators of the energy-conserving and the detoxifying sulfide oxidation pathways respectively. In the presence of the ROS scavengers glutathione (GSH) and ascorbate, isolated lugworm mitochondria rapidly oxidized up to 100 $\mu\text{mol l}^{-1}$ sulfide with maximal oxygen consumption rates but did not produce any ATP in the process. Under these conditions, salicylhydroxamic acid (SHAM), which is an inhibitor of the alternative oxidase of plant mitochondria, completely blocked oxygen consumption whereas inhibitors of complex III and IV had hardly any effect. By contrast, dehydroascorbate (DHA) enabled the mitochondria to gain ATP from sulfide oxidation even if the sulfide concentration far exceeded the threshold for inhibition of cytochrome oxidase. In the presence of dehydroascorbate, respiratory rates were independent of sulfide concentrations, with a respiratory control ratio of 2.1 ± 0.2 , and both oxygen consumption and ATP production were completely inhibited by myxothiazol and sodium azide but only marginally by SHAM. The present data indicate that a redox mechanism may contribute to the regulation of sulfide oxidation in lugworm mitochondria *in vivo*. Thus, mitochondria are presumably much more sulfide resistant in a cellular context than previously thought.

Key words: redox regulation, sulfide oxidation, *Arenicola marina*, alternative oxidase, ATP production, glutathione, ascorbate, dehydroascorbate.

INTRODUCTION

The lugworm, *Arenicola marina* (L.) inhabits sandy to muddy intertidal flats, where it is regularly confronted with considerable amounts of sulfide as well as hypoxia during low tide (Grieshaber et al., 1994; Grieshaber and Völkel, 1998). This species is highly tolerant towards both stress factors and is therefore often used to study the underlying adaptational strategies. The mitochondria of the body wall musculature are the main site of sulfide oxidation in *A. marina*, producing thiosulfate as the dominant aerobic end product that is excreted into the seawater (Völkel and Grieshaber, 1994; Hauschild et al., 1999).

The first step in mitochondrial sulfide oxidation is presumably catalyzed by a sulfide:quinone oxidoreductase (SQR) bound to the inner mitochondrial membrane. The SQR oxidizes sulfide to persulfides and transfers the electrons to the ubiquinone pool (Theissen and Martin, 2008). Thus, H_2S can be used as a respiratory substrate to drive ATP synthesis (Völkel and Grieshaber, 1997). A putative sulfur dioxygenase and a sulfurtransferase in the mitochondrial matrix convert the persulfides, produced by the SQR, to thiosulfate (Hildebrandt and Grieshaber, 2008).

In isolated mitochondria, the energy-conserving sulfide oxidation pathway *via* cytochrome oxidase is inhibited by sulfide concentrations exceeding $10 \mu\text{mol l}^{-1}$. At higher sulfide concentrations, the electrons are transferred to oxygen *via* an alternative terminal oxidase (AOX), presumably resembling the enzyme found in plant mitochondria as it is equally cyanide insensitive and also inhibited by salicylhydroxamic acid (SHAM) (Völkel and Grieshaber, 1996). A DNA sequence similar to the plant AOX has been detected in some invertebrates such as *Crassostrea gigas*, but the function of the respective gene product remains to be proven (McDonald and Vanlerbergh, 2004).

The position of the AOX in the respiratory chain is unknown, and different studies have demonstrated varying rates of mitochondrial sulfide oxidation in *A. marina* (Völkel and Grieshaber, 1994; Völkel and Grieshaber, 1996; Völkel and Grieshaber, 1997). Sulfide detoxification capacities apparently change with the season, as does the redox environment of the lugworm tissue (Völkel and Grieshaber, 1994; Keller et al., 2004). Therefore, the redox state of the cell may take part in the regulation of mitochondrial sulfide oxidation.

In the present study, we demonstrate that reduced glutathione (GSH) and ascorbate, which are both major cellular antioxidants (Wu et al., 2004; Linster and Van Schaftingen, 2007), as well as the oxidized form of vitamin C, dehydroascorbate (DHA), are able to selectively activate the detoxifying and the energy-conserving sulfide oxidation pathways in isolated mitochondria.

MATERIALS AND METHODS

Animals

Specimens of *A. marina* from the intertidal flats near Texel, The Netherlands, were purchased from Zeeaashandel Arenicola (Texel, The Netherlands). Lugworms were held at $16 \pm 2^\circ\text{C}$ in artificial seawater (36‰) without sediment in darkened and aerated tanks for up to two weeks.

Purification procedures

A mitochondrially enriched particulate fraction was prepared from the body wall musculature of *A. marina*, as described in Völkel and Grieshaber (Völkel and Grieshaber, 1997).

Assay of mitochondrial respiration

Mitochondrial oxygen consumption ($\text{nmol O}_2 \text{mg protein}^{-1} \text{min}^{-1}$) was measured polarographically in an oxygraph respirometer

(Oroboros; Innsbruck, Austria) at 15°C. The reaction mixture (2 ml) contained incubation medium (450 mmol l⁻¹ glycine, 250 mmol l⁻¹ sucrose, 20 mmol l⁻¹ Tris, 1 mmol l⁻¹ EGTA, 0.2% BSA (fatty acid free), 5 mmol l⁻¹ K₂HPO₄, 3 mmol l⁻¹ MgCl₂ and 100 mmol l⁻¹ KCl, pH 7.5) and 0.1–0.7 mg ml⁻¹ of mitochondrial protein. In order to calculate the mitochondrial respiratory rates, the incubation medium was assumed to contain 269 μmol l⁻¹ O₂ at air saturation (Reynafarje et al., 1985).

Malate (8 mmol l⁻¹), succinate (4 mmol l⁻¹), or sulfide (5–100 μmol l⁻¹) was added as a respiratory substrate either in the presence of 1 mmol l⁻¹ ADP (state 3 respiration) or without ADP (state 4 respiration). The respiratory control ratios (RCRs) were determined by dividing the state 3 by the state 4 respiration rate. For inhibitor studies, 2–10 μl of the respective stock solution was added to the assay, resulting in a final concentration of 5 μmol l⁻¹ myxothiazole, 0.05 mmol l⁻¹ KCN, 1 mmol l⁻¹ NaN₃ or 0.5 mmol l⁻¹ SHAM. KCN and NaN₃ were dissolved in distilled water, SHAM was dissolved in dimethylsulfoxide, and myxothiazole was dissolved in ethanol. The solvents did not influence mitochondrial sulfide oxidation in the concentrations applied. To evaluate the effect of the cellular redox state on mitochondrial sulfide oxidation, either 2.5 mmol l⁻¹ GSH + 2.5 mmol l⁻¹ ascorbate or 1 mmol l⁻¹ dehydroascorbate (DHA) was added to the assay prior to the sulfide injections. As KCN reacts with DHA, NaN₃ was used to inhibit cytochrome oxidase in DHA containing assays.

The oxygen affinities of the terminal oxidases were determined according to Gnaiger et al. (Gnaiger et al., 1995). Mitochondria were allowed to completely consume the oxygen contained in the respiration medium, and the oxygen concentration was recorded at one-second intervals. Signal corrections were applied using DatLab 2.1 (Oroboros) to account for the time response of the polarographic oxygen sensor, as well as for the oxygen leak and blank oxygen consumption. Oxygen consumption rates were calculated from the corrected signal and plotted against the oxygen concentration. *K_m* (Michaelis–Menten constant) values were calculated by non-linear least-square analysis of the data fitted to the Michaelis–Menten equation using the enzyme kinetics module of SigmaPlot version 9.01 (Systat Software; Erkrath, Germany). Data are given as means ± standard deviation of the results from three independent experiments. Cytochrome oxidase was analysed using malate (8 mmol l⁻¹) as a substrate in the presence of 1 mmol l⁻¹ ADP and 0.5 mmol l⁻¹ SHAM. The energy-conserving and the detoxifying sulfide oxidation pathways were activated by 1 mmol l⁻¹ DHA or 2.5 mmol l⁻¹ GSH + 2.5 mmol l⁻¹ ascorbate, respectively.

Measurements of ATP production

ATP production was determined using a coupled enzyme assay (Powell and Somero, 1986), as described in detail by Bagarinao and Vetter (Bagarinao and Vetter, 1990). The reaction mixture (1 ml) contained 10 mmol l⁻¹ glucose, 3.5 U ml⁻¹ hexokinase, 1.75 U ml⁻¹ glucose-6-phosphate dehydrogenase, 0.5 mmol l⁻¹ NADP⁺, 50 μmol l⁻¹ ADP, 20 μmol l⁻¹ P₁,P₅-di(adenosine-5')pentaphosphate and 40–200 μg ml⁻¹ mitochondrial protein in the incubation medium. Assays were performed in duplicate at 15°C. The reaction was started by the addition of sulfide (5–100 μmol l⁻¹). Inhibitors and activators were applied at the same concentrations as for respiratory studies. The ATP/S ratio was determined by calculating the total concentration of ATP produced after 5 μmol l⁻¹ sulfide had been completely consumed, which was verified by high performance liquid chromatography (HPLC) (see below).

Controls were performed using heat-inactivated mitochondria (30 min at 85°C).

Substrate solutions

Sulfide stock solutions (1–10 mmol l⁻¹) were prepared in deoxygenated distilled water immediately before use in assays. The sulfide concentration was analysed photometrically using a commercial sulfide test based on Methylene Blue production (Spektroquant; Merck, Darmstadt, Germany). Samples were fixed with 50 mmol l⁻¹ zinc acetate in 150 mmol l⁻¹ NaOH prior to analysis.

Determination of sulfur compounds

The concentrations of sulfide and thiosulfate in the mitochondrial suspensions were determined using the monobromobimane HPLC method, as described in Völkel and Grieshaber (Völkel and Grieshaber, 1994).

Determination of ascorbate

Samples were stabilized by adding the same volume of 0.1 mol l⁻¹ perchloric acid. The ascorbate concentration was analysed by HPLC (Reiber et al., 1993).

Statistical analyses

Data are given as means ± standard deviation of the results from 3 to 12 different preparations, each comprising approximately 10–15 animals. Significant differences between means were evaluated by *t*-tests at the *P*<0.05 level using a statistical software package (SigmaStat version 3.1, Systat Software).

RESULTS

Mitochondrial respiration and ATP production

The mitochondria isolated from the body wall tissue of *A. marina* were tightly coupled when respiring with malate as a substrate with state 3 respiratory rates of 7.48±2.46 nmol O₂ mg⁻¹ protein min⁻¹ and an RCR of 6.93±3.71. They produced 51.63±6.34 nmol ATP mg⁻¹ protein min⁻¹.

In the absence of any activator, maximal rates of state 3 oxygen consumption and ATP production were achieved with 5–10 μmol l⁻¹ sulfide (RCR=2.44±0.58), with both rates decreasing with increasing sulfide concentration (Fig. 1A). During complete oxidation of 5 μmol l⁻¹ sulfide, mitochondria produced 7.50±0.84 μmol l⁻¹ ATP, corresponding to a ratio of 1.50±0.17 molecules of ATP produced per molecule of H₂S consumed. Sulfide-induced oxygen consumption was partially inhibited by either myxothiazole, SHAM or KCN. By contrast, ATP production was completely blocked by myxothiazole or KCN but was insensitive to SHAM (Fig. 1D).

Effects of redox-competent molecules

GSH, ascorbate and DHA strongly diminished substrate inhibition of sulfide oxidation in the lugworm mitochondria. In the presence of 1 mmol l⁻¹ DHA or 2.5 mmol l⁻¹ GSH + 2.5 mmol l⁻¹ ascorbate, oxygen consumption rates were constant and independent of sulfide concentration within the tested range of 5–100 μmol l⁻¹ H₂S (Fig. 1B,C). They were similar to the maximal state 3 respiratory rates that were achieved at low sulfide concentrations only, without the modulators. Although the resulting rates of sulfide oxidation were similar with DHA or GSH + ascorbate, two different pathways were activated.

In the presence of DHA, oxygen consumption rates were stimulated by the addition of ADP even at high sulfide concentrations; original recordings are depicted in Fig. 2A (trace 2) for 50 μmol l⁻¹ sulfide. State 3 and state 4 respiratory rates were constant when up to 100 μmol l⁻¹ sulfide was added as a substrate (Fig. 1C), resulting in an RCR of 2.07±0.18. Accordingly, the lugworm mitochondria produced ATP during sulfide oxidation in the presence of DHA

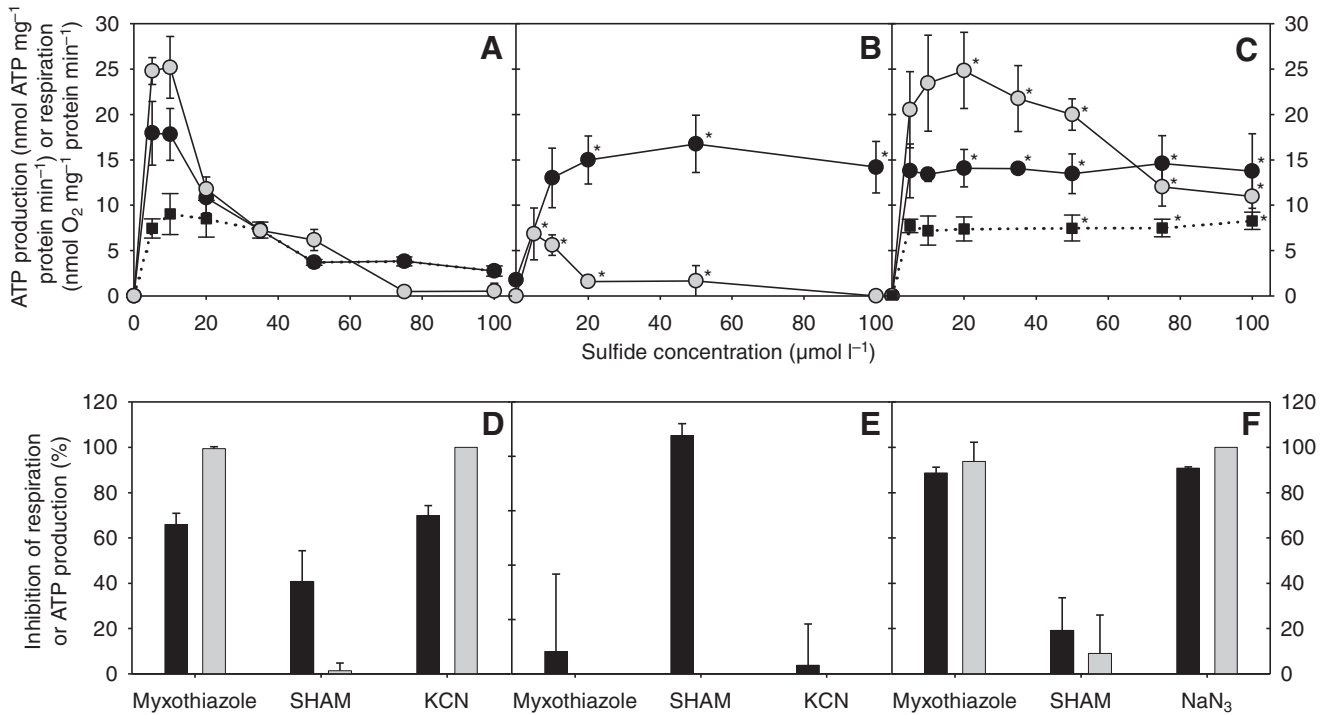


Fig. 1. Regulation of mitochondrial sulfide oxidation by glutathione (GSH), ascorbate and dehydroascorbate (DHA). (A–C) Initial rates of ATP production (gray circles) and oxygen consumption in state 3 (black circles) and state 4 (black squares) with sulfide (5–100 μmol l⁻¹) added as a substrate. (A) Sulfide oxidation in the absence of redox-competent molecules. (B) Sulfide oxidation in the presence of 2.5 mmol l⁻¹ GSH and 2.5 mmol l⁻¹ ascorbate. Oxygen consumption rates were identical in the presence and in the absence of ADP. (C) Sulfide oxidation in the presence of 1 mmol l⁻¹ DHA. *Significantly different ($P > 0.05$) from corresponding data points without redox-competent molecules. (D–F) Inhibition (%) of mitochondrial respiration (black bars) and ATP production (grey bars) with sulfide added as a substrate by 5 μmol l⁻¹ myxothiazole, 0.05 mmol l⁻¹ KCN, 1 mmol l⁻¹ NaN₃ or 0.5 mmol l⁻¹ SHAM. (D) Inhibition of oxygen consumption (state 3) and ATP production with 5–10 μmol l⁻¹ sulfide added as a substrate in the absence of redox-competent molecules. (E) Inhibition of oxygen consumption with 10–100 μmol l⁻¹ sulfide added as a substrate in the presence of 2.5 mmol l⁻¹ GSH and 2.5 mmol l⁻¹ ascorbate. ATP production rates were too low to study the effects of inhibitors. (F) Inhibition of oxygen consumption (state 3) and ATP production with 5–50 μmol l⁻¹ sulfide added as a substrate in the presence of 1 mmol l⁻¹ DHA. Data are given as means ± s.d. of the results from 3 to 12 different preparations, each comprising approximately 10–15 animals.

(Fig. 1C and Fig. 2B). Rates of state 3 respiration and ATP production were significantly increased by DHA at sulfide concentrations exceeding 10 μmol l⁻¹. Both rates were equally inhibited by myxothiazole and NaN₃ but were hardly affected by SHAM (Fig. 1F). DHA changed neither the oxygen content nor the ATP concentration in the assay when added separately. To prove whether DHA acted as an electron acceptor for sulfide oxidation, the concentrations of possible substrates and products were determined. During complete oxidation of 100 μmol l⁻¹ sulfide in the presence of 1 mmol l⁻¹ DHA, 44.52 ± 2.56 μmol l⁻¹ thiosulfate accumulated in the mitochondrial suspension and 93.53 ± 7.87 μmol l⁻¹ O₂ was consumed whereas only 1.1 ± 0.7 μmol l⁻¹ ascorbate was detectable.

GSH and ascorbate significantly increased sulfide-induced oxygen consumption rates at high sulfide concentrations (≥ 20 μmol l⁻¹; Fig. 1B). However, respiratory rates were not stimulated by the addition of ADP, and the mitochondria produced significantly less ATP during sulfide oxidation than without GSH and ascorbate. Oxygen consumption was insensitive towards myxothiazole and cyanide but was completely inhibited by SHAM (Fig. 1E). A similar effect was achieved with either GSH or ascorbate added separately; however, oxygen consumption rates were maximal and remained constant until the sulfide added had been completely consumed, only if both reductants were present. Glutathione disulfide (GSSG), the oxidized form of glutathione, had no effect on mitochondrial sulfide oxidation rates.

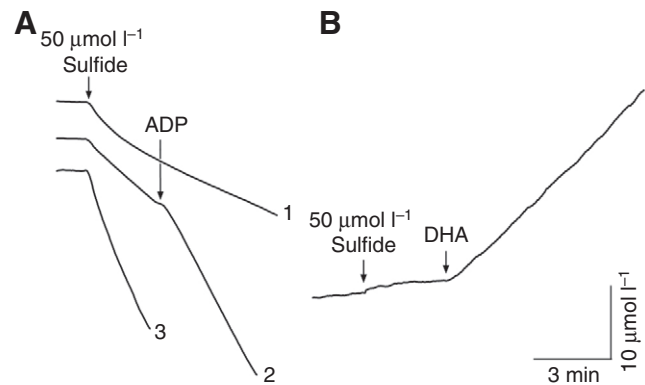


Fig. 2. Original recordings of (A) oxygen concentrations (μmol l⁻¹) and (B) ATP concentration (μmol l⁻¹) calculated from the NADPH absorbance at 340 nm in mitochondrial suspensions from body wall tissue of *Arenicola marina*. (A) Effect of redox-competent molecules on oxygen consumption with sulfide as a substrate. The assays contained 0.45 mg ml⁻¹ mitochondrial protein and either 1 mmol l⁻¹ dehydroascorbate (DHA) (trace 2) or 2.5 mmol l⁻¹ GSH plus 2.5 mmol l⁻¹ ascorbate (trace 3). The reactions were started by the addition of 50 μmol l⁻¹ sulfide. In the presence of DHA, the oxygen consumption rate could be stimulated by ADP. (B) Activation of ATP production with 50 μmol l⁻¹ sulfide as a substrate by DHA. The amount of mitochondrial protein present was 0.16 mg ml⁻¹. Additions are indicated by arrows.

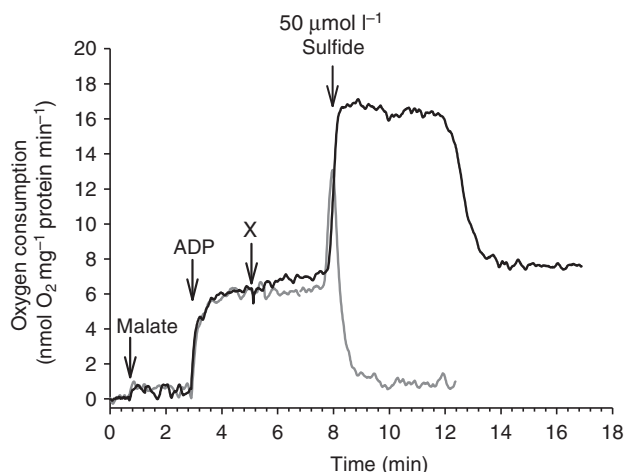


Fig. 3. Dehydroascorbate (DHA) protects mitochondrial respiration with malate as a substrate from inhibition by sulfide. Oxygen consumption rates of mitochondria from the body wall tissue of *Arenicola marina* as a function of time. Additions (indicated by arrows) were 8 mmol l^{-1} malate and 1 mmol l^{-1} ADP. Subsequently 1 mmol l^{-1} DHA was added only to the assay shown by the black line at the time marked with X. The injection of $50 \mu\text{mol l}^{-1}$ sulfide inhibited malate respiration in the absence of DHA (gray line) and, by contrast, stimulated the oxygen consumption rate in the presence of DHA (black line).

GSH, ascorbate and DHA selectively influenced sulfide oxidation without affecting the oxidation of respiratory substrates such as malate or succinate. During individual experiments, the effect of DHA is clearly visible in the course of oxygen consumption rates, as exemplified in Fig. 3. Without DHA, state 3 malate respiration was largely inhibited by $50 \mu\text{mol l}^{-1}$ sulfide (Fig. 3, gray line). The injection of 1 mmol l^{-1} DHA did not alter the rate of malate oxidation but prevented the inhibition by subsequently added sulfide (Fig. 3, black line). The oxygen consumption rate more than doubled upon sulfide addition and only decreased after the sulfide had been completely consumed.

Similarly, $50 \mu\text{mol l}^{-1}$ sulfide normally inhibited succinate respiration by $85 \pm 3\%$ (Fig. 4A). In the presence of GSH, rates of succinate respiration were not changed but could be stimulated by adding sulfide (Fig. 4B). NaN_3 completely inhibited succinate respiration whereas GSH-activated sulfide oxidation was not affected (Fig. 4C).

Oxygen affinities of mitochondrial terminal oxidases

The specific activators identified in this study make it possible to individually study either the energy-conserving or the detoxifying sulfide oxidation pathway. The oxygen affinities of the different

terminal oxidases involved in mitochondrial sulfide oxidation in *A. marina*, cytochrome oxidase and the alternative oxidase were determined. Sulfide-induced oxygen consumption rates began to decrease at a fivefold higher oxygen concentration in the presence of GSH and ascorbate than in the presence of DHA (Fig. 5). The K_m value of the alternative oxidase terminating the detoxifying sulfide oxidation pathway, which was activated by GSH and ascorbate, was $5.38 \pm 0.67 \mu\text{mol l}^{-1} \text{ O}_2$. The oxygen affinity of cytochrome oxidase was significantly higher. With malate added as a substrate, a K_m value of $1.77 \pm 0.54 \mu\text{mol l}^{-1} \text{ O}_2$ was calculated, and the energy-conserving sulfide oxidation pathway activated by DHA had a K_m value of $0.88 \pm 0.11 \mu\text{mol l}^{-1} \text{ O}_2$.

DISCUSSION

Sulfide is a potent toxin as well as a substrate of the mitochondrial respiratory chain (Grieshaber and Völkel, 1998). Thus, sulfide-adapted animals have had to evolve mechanisms to effectively exploit H_2S while simultaneously avoiding poisoning. A branched respiratory chain enables *A. marina* to use H_2S as a respiratory substrate or switch to a pathway of rapid sulfide detoxification, which is independent of the ATP demand (Völkel and Grieshaber, 1996; Völkel and Grieshaber, 1997). Selective activators of the energy-conserving and the detoxifying sulfide oxidation pathways in lugworm mitochondria have been identified.

Detoxification of H_2S

An alternative terminal oxidase similar to the extensively characterized plant AOX has been postulated for some intertidal sulfide-adapted invertebrates such as *A. marina* and the ribbed mussel *Geukensia demissa* (Völkel and Grieshaber, 1996; Kraus and Doeller, 2004). The AOX of plant mitochondria is non-protonmotive and couples the oxidation of ubiquinol to the reduction of oxygen to water (Vanlerberghe and McIntosh, 1997). It contains a non-haem di-iron center but no cytochromes and is therefore cyanide, as well as sulfide, resistant (Azcon-Bieto et al., 1989; Siedow et al., 1995).

In isolated lugworm mitochondria, the electron flow during sulfide oxidation splits between the classical respiratory chain and the AOX pathway, since oxygen consumption was decreased but not completely stopped by the addition of each of the inhibitors of complex III, complex IV and the AOX. The ROS scavengers, GSH and ascorbate, selectively activated the detoxifying sulfide oxidation pathway (Fig. 6A). Hardly any ATP was produced during sulfide oxidation in the presence of GSH and ascorbate. Thus, the proton translocating complexes III and IV of the respiratory chain were not involved, and sulfide oxidation *via* the AOX was insensitive towards myxothiazole, NaN_3 and cyanide, which are potent inhibitors of these enzymes. When using KCN in assays containing reactive sulfur intermediates, possible side reactions have to be taken

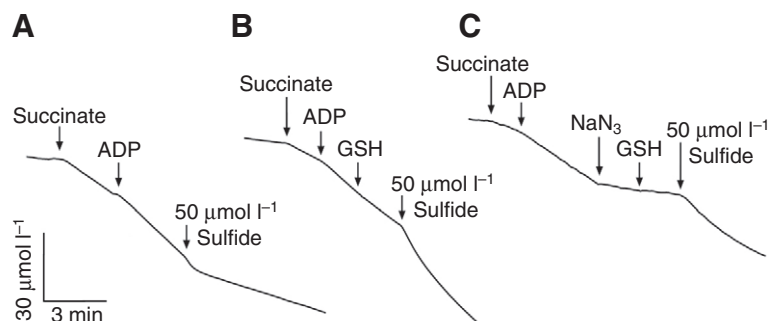


Fig. 4. Original recordings of oxygen concentrations ($\mu\text{mol l}^{-1}$) in mitochondrial suspensions from body wall tissue of *Arenicola marina*. Additions of 4 mmol l^{-1} succinate, 1 mmol l^{-1} ADP, 5 mmol l^{-1} glutathione (GSH), 1 mmol l^{-1} NaN_3 and $50 \mu\text{mol l}^{-1}$ sulfide are marked with arrows. Mitochondrial protein: (A) 0.53 mg ml^{-1} , (B) 0.48 mg ml^{-1} , (C) 0.35 mg ml^{-1} .

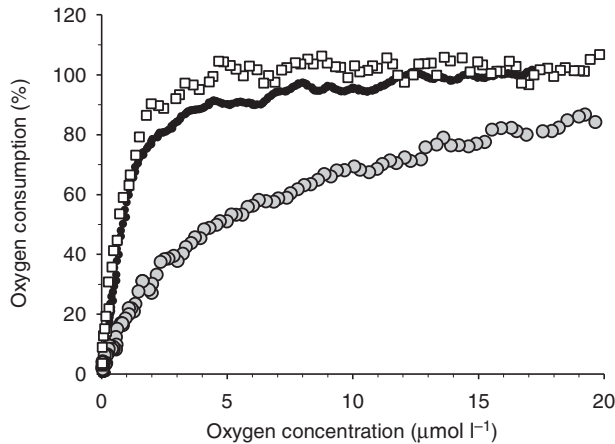


Fig. 5. Mitochondrial oxygen consumption (% of oxygen consumption rate at air saturation) as a function of oxygen concentration ($\mu\text{mol l}^{-1}$). State 3 respiration was recorded with 8 mmol l^{-1} malate added as a substrate in the presence of 0.5 mmol l^{-1} salicylhydroxamic acid (SHAM) (black dots) and with $50\text{ }\mu\text{mol l}^{-1}$ sulfide added as a substrate in the presence of 1 mmol l^{-1} DHA (open squares) or in the presence of 2.5 mmol l^{-1} GSH + 2.5 mmol l^{-1} ascorbate (gray circles). Mitochondria were allowed to completely consume the oxygen contained in the respiration medium, thus providing respiration rates at variable oxygen concentrations.

into account. In lugworm mitochondria, cyanide may act as a persulfide acceptor for the SQR and pull the reaction towards sulfide oxidation (Theissen and Martin, 2008). Nevertheless, as KCN completely inhibited sulfide-induced ATP production, the main respiratory chain was definitely blocked. The equivalent oxygen consumption rates during sulfide oxidation with either myxothiazole or cyanide added as an inhibitor further demonstrate that the AOX pathway can be stimulated irrespective of the further fate of the immediate sulfide oxidation product. The present results therefore clearly demonstrate that lugworm AOX either accepts the electrons directly from the SQR or is fed by the ubiquinone pool.

ATP production from H_2S

Not only sulfide-tolerant animals but also chicken liver mitochondria use sulfide as a substrate for oxidative phosphorylation; indirectly,

it has also been demonstrated for human cells (Powell and Somero, 1986; Bagarinao and Vetter, 1990; Völkel and Grieshaber, 1997; Hahlbeck et al., 2000; Yong and Searcy, 2001; Gubern et al., 2007). Isolated mitochondria only synthesize ATP in the presence of low sulfide concentrations; however, the inhibitory concentration of H_2S clearly depends on the cellular environment. The mussel *G. demissa*, for example, inhabits sediments that regularly contain millimolar sulfide concentrations. Pieces of gill tissue oxidize up to $200\text{ }\mu\text{mol l}^{-1}$ with maximal rates, but isolated gill mitochondria are already inhibited by $8\text{ }\mu\text{mol l}^{-1}$ H_2S (Lee et al., 1996; Parrino et al., 2000). The present study demonstrates that lugworm mitochondria use high sulfide concentrations as a substrate for chemolithotrophic ATP production in the presence of 1 mmol l^{-1} DHA (Fig. 6B).

ATP production in *A. marina* was SHAM insensitive but was completely inhibited by myxothiazole, cyanide or NaN_3 . Therefore, the electrons from sulfide oxidation are transferred to oxygen via the respiratory chain complexes III and IV. Six protons are translocated for each electron pair so that 1.5 molecules of ATP can be produced (Hinkle, 2005). The SQR oxidizes H_2S to persulfides, corresponding to a two-electron oxidation (Theissen and Martin, 2008), and an ATP/S ratio of 1.5 was experimentally confirmed for $5\text{ }\mu\text{mol l}^{-1}$ sulfide. The ATP yield decreases with increasing sulfide concentrations to 0.9 ATP/S at $8\text{ }\mu\text{mol l}^{-1}$ H_2S and 0.7 ATP/S at $11\text{ }\mu\text{mol l}^{-1}$ H_2S , probably because a higher percentage of the electrons is transferred to oxygen via the alternative oxidase (Völkel and Grieshaber, 1997).

Redox regulation of sulfide oxidation

Reactive oxygen species (ROS) are produced mainly by mitochondria when electrons leak from a highly reduced respiratory chain (Andreyev et al., 2005). Apart from causing oxidative damage to several cellular constituents, ROS also function as signal molecules (Moran et al., 2001; Damdimopoulos et al., 2002).

Protein sulfhydryl groups are crucial to redox regulation as they can be reversibly oxidized, forming inter- or intra-molecular disulfide bridges (Ghezzi, 2005). The plant AOX is a dimer covalently linked by a disulfide bridge that has to be reduced and form a thiohemiacetal with pyruvate in order to produce the active conformation (Rhoads et al., 1998; Vanlerberghe et al., 1998). Thus, the redox state regulates the partitioning of electron flux between AOX and cytochrome oxidase in plant mitochondria (Berthold et

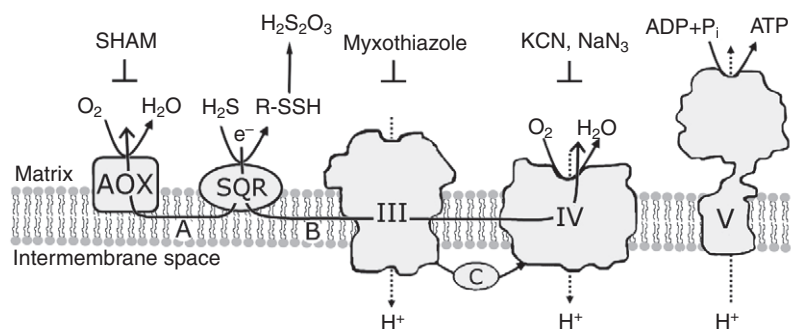


Fig. 6. Proposed model of the two different pathways for sulfide oxidation in the mitochondria of *Arenicola marina*. A membrane-bound sulfide:quinone oxidoreductase (SQR) oxidizes sulfide (H_2S) to persulfides (R-SSH; R=cysteine residue of the SQR) and transfers the electrons to the ubiquinone pool (Theissen and Martin, 2008). In the presence of GSH and ascorbate, representing reducing cellular conditions, a pathway of sulfide detoxification is activated (A). Electrons are transferred to oxygen via an alternative oxidase (AOX) without proton translocation. The energy-conserving sulfide oxidation pathway can be activated by dehydroascorbate (DHA) *in vitro* (B). Electrons are channelled to oxygen through the classical respiratory chain complexes III and IV, which transport protons across the membrane, thus allowing ATP production by complex V (ATP synthase). Inhibitors used in this study are specified above their target enzymes.

al., 2000). The lugworm AOX may be regulated in a similar way, as the detoxifying sulfide oxidation pathway was active exclusively under reducing conditions. However, sulfide but not pyruvate was essential for AOX activity in *A. marina*.

GSH and ascorbate are important ROS scavengers and are present in millimolar concentrations in mammalian cells (May et al., 1997). In the body wall tissue of *A. marina* approximately 0.6 mmol l^{-1} GSH was detected in the winter (Buchner et al., 1996) but there are no data available on the ascorbate concentration or on seasonal changes of the antioxidant status in the lugworm.

GSH and ascorbate could activate sulfide detoxification by reducing a regulatory cysteine residue. Furthermore, AOX activity could be reversibly modulated by glutathionylation. Several mitochondrial proteins form mixed disulfides with GSSG under resting conditions and are activated by reduction at a high GSH:GSSG ratio (Brigelius et al., 1983; O'Donovan and Fernandes, 2000; Ghezzi, 2005).

The finding that the AOX activity in lugworm mitochondria strongly depends on the redox state serves to resolve a discrepancy detected during former studies that obscured the exact position of the AOX in the respiratory chain. Sulfide oxidation *via* the alternative pathway was inhibited by antimycin A; therefore, complex III was thought to be involved in this pathway without any proton translocation taking place (Völkel and Grieshaber, 1996; Völkel and Grieshaber, 1997). In contrast to myxothiazole, antimycin A stimulates ROS production at complex III, which in turn evidently inhibits the AOX (Andreyev et al., 2005). Therefore, the addition of antimycin A can indirectly inhibit sulfide oxidation *via* the AOX, although complex III is not involved in the reaction.

DHA selectively activates the energy-conserving sulfide oxidation pathway in lugworm mitochondria, probably by preventing the inhibition of cytochrome oxidase. However, the mechanism of this reaction does not exclusively depend on the cellular redox state, as GSSG had no activating effect. A non-enzymatic oxidation of sulfide by DHA can be excluded since hardly any ascorbate was detectable in the assay. Furthermore, the ratio of oxygen consumed and thiosulfate produced per molecule of sulfide oxidized was comparable in the presence and in the absence of DHA (Hildebrandt and Grieshaber, 2008), whereas less oxygen would be consumed if DHA was used as an electron acceptor.

H₂S has been identified as a signaling molecule in mammals (Wang, 2002), and the modification of the susceptibility of cytochrome oxidase towards sulfide by DHA may be part of a regulatory cascade. Cytochrome oxidase activity is regulated in a complex manner by NO as well as CO, which favors ROS production and activates cytoprotective signalling pathways (Zuckerbraun et al., 2007). H₂S could function in a similar way. Mammalian mitochondria probably do not contain an alternative oxidase so a mechanism to protect cytochrome oxidase from sulfide inhibition would be even more advantageous than in sulfide-adapted invertebrates. As the physiological sulfide concentrations detected in different tissues are much higher than the inhibitory concentration for isolated mitochondria (Wang, 2002), a cytosolic activating factor is likely to exist in mammals as well.

The present results demonstrate that the mitochondrial sulfide oxidation pathway in *A. marina* is suitable for an effective detoxification of H₂S and can be regulated in order to meet different cellular demands. Nevertheless, additional unknown cytosolic or extracellular sulfide oxidation enzymes might exist. Furthermore, it is still unknown whether all of the enzymes involved in mitochondrial sulfide oxidation are localized in the matrix. The SQR is depicted on the matrix face of the inner

mitochondrial membrane in Fig. 6 because the optimum at pH 9 indicates a localisation in the alkaline matrix (Theissen and Martin, 2008). Thus, H₂S has to enter the mitochondria before being oxidized and also has access to its binding site at cytochrome oxidase. This arrangement facilitates a regulatory role for H₂S. Without further modulators, half-maximal inhibition of cytochrome oxidase in *A. marina* is achieved at $1.5 \mu\text{mol l}^{-1}$ H₂S (Völkel and Grieshaber, 1997), which is considerably lower than the K_m value of $9.9 \mu\text{mol l}^{-1}$ H₂S determined for SQR (Hildebrandt and Grieshaber, 2008). Therefore, respiration can be completely blocked by sulfide, and the degree of inhibition could be modified by DHA *via* a decrease in H₂S affinity of cytochrome oxidase. If the SQR is oriented towards the intermembrane space, intermediates of sulfide oxidation have to be transported across the inner mitochondrial membrane so this process could be regulated as well.

Oxygen affinities of mitochondrial oxidases

Cytochrome oxidase from lugworm mitochondria has a significantly lower K_m value for oxygen than the alternative oxidase. Plant mitochondria show the same tendency. In different tissues of the soy bean, *Glycine max*, K_m values between $1.6 \mu\text{mol l}^{-1}$ and $9.9 \mu\text{mol l}^{-1}$ O₂ were determined for the alternative oxidase, as opposed to $0.05\text{--}0.15 \mu\text{mol l}^{-1}$ for cytochrome oxidase (Millar et al., 1994; Millar et al., 1997). Thus, sulfide detoxification in *A. marina* is less effective than the energy-providing pathway *via* the classical respiratory chain at low oxygen concentrations. The oxygen affinity of mammalian complex IV is comparable to the value calculated during energy-conserving sulfide oxidation in the lugworm mitochondria ($K_m=0.08\text{--}0.8 \mu\text{mol l}^{-1}$ O₂) (Gnaiger et al., 1998). The small but significant difference between the K_m values determined with malate as a substrate and with sulfide in the presence of DHA may result from conformational changes of cytochrome oxidase in the course of redox regulation.

Possible role of redox regulation in *A. marina in vivo*

In marine sediments, high sulfide concentrations often occur in combination with hypoxia. Due to the lack of an appropriate electron acceptor, the lugworm mitochondria oxidize sulfide only at low rates during hypoxia and thus it accumulates in the tissue (Völkel and Grieshaber, 1994). When the oxygen concentration increases after an anoxic period, mitochondria produce radicals, which cause much of the tissue damage known as ischemia-reperfusion injury (Li and Jackson, 2002). The elevated sulfide concentration is beneficial during reoxygenation, as H₂S protects the cellular components from oxidative stress (Geng et al., 2004; Whiteman et al., 2005). Furthermore, the more oxidized redox state of the cells activates the energy-conserving sulfide oxidation pathway and inhibits the AOX. This pathway is particularly favorable when the oxygen supply is limited, as the available oxygen can be used for ATP production and for sulfide detoxification simultaneously.

By contrast, sulfide is rapidly detoxified *via* the AOX pathway during normoxic periods due to the reducing cellular redox state, and carbon substrates can be used for more effective ATP production.

The present study clearly demonstrates two different pathways of mitochondrial sulfide oxidation in *A. marina* and conveys a simple method to assay them separately. In contrast to previous assumptions, the partitioning of electron flux between the main respiratory chain and the AOX does not exclusively depend on the sulfide concentration but it is influenced by other physiological factors such as GSH and vitamin C. Since, in the present study, all modulators were applied in physiological concentrations, they are

likely to take part in the regulation of sulfide oxidation *in vivo*, presumably in combination with several other cellular factors, which remain to be identified.

LIST OF ABBREVIATIONS

AOX	alternative oxidase
DHA	dehydroascorbate
GSH	glutathione
GSSG	glutathione disulfide
RCR	respiratory control ratio
ROS	reactive oxygen species
SHAM	salicylhydroxamic acid
SQR	sulfide:quinone oxidoreductase

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