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SULPHIDE OXIDATION AND OXIDATIVE PHOSPHORYLATION IN THE MITOCHONDRIA OF THE LUGWORM *ARENICOLA MARINA*

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Summary

Oxygen consumption, ATP production and cytochrome *c* oxidase activity of isolated mitochondria from body-wall tissue of *Arenicola marina* were measured as a function of sulphide concentration, and the effect of inhibitors of the respiratory complexes on these processes was determined. Concentrations of sulphide between 6 and 9 μmol l^{-1} induced oxygen consumption with a respiratory control ratio of 1.7. Production of ATP was stimulated by the addition of sulphide, reaching a maximal value of 67 nmol min^{-1} mg^{-1} protein at a sulphide concentration of 8 μmol l^{-1}. Under these conditions, 1 mole of ATP was formed per mole of sulphide consumed. Higher concentrations of sulphide led to a decrease in ATP production until complete inhibition occurred at approximately 50 μmol l^{-1}. The production of ATP with malate and succinate was stimulated by approximately 15 % in the presence of 4 μmol l^{-1} sulphide, but decreased at sulphide concentrations higher than 15–20 μmol l^{-1}. Cytochrome *c* oxidase was also inhibited by sulphide, showing half-maximal inhibition at 1.5 μmol l^{-1} sulphide. Sulphide-induced ATP production was inhibited by antimycin, cyanide and oligomycin but not by rotenone or salicylhydroxamic acid. The present data indicate that sulphide oxidation is coupled to oxidative phosphorylation solely by electron flow through cytochrome *c* oxidase, whereas the alternative oxidase does not serve as a coupling site. At sulphide concentrations higher than 20 μmol l^{-1}, oxidation of sulphide serves mainly as a detoxification process rather than as a source of energy.

Key words: *Arenicola marina*, lugworm, mitochondrial sulphide oxidation, ATP production, oxidative phosphorylation.

Introduction

The lugworm *Arenicola marina* inhabits marine intertidal flats. In this environment, the pore water of the sediment can contain considerable amounts of sulphide (Groenendaal, 1979; Völkel and Grieshaber, 1992). During low tide, the concentration of sulphide accumulating within the lugworm burrow can reach approximately 30 μmol l^{-1} (Völkel *et al.* 1995). Sulphide is highly toxic, mainly as a result of its inhibition of cytochrome *c* oxidase (National Research Council, 1979). *A. marina*, like many other marine invertebrates, is highly sulphide-tolerant (for reviews, see Vetter *et al.* 1991; Vismann, 1991; Völkel and Grieshaber, 1995). One of its main strategies for tolerating high levels of sulphide is its ability to oxidize sulphide to thiosulphate (Völkel and Grieshaber, 1992, 1994*a*). Sulphide oxidation is localized in the mitochondria of the body-wall musculature (Völkel and Grieshaber, 1994*a*). Recently, we have shown that mitochondrial sulphide oxidation in *A. marina* is linked to the respiratory electron transport chain (Völkel and Grieshaber, 1996). Sulphide is believed to be oxidized to thiosulphate by a sulphide oxidase. Inhibitor studies indicate that electrons from sulphide enter the respiratory chain at the level of ubiquinone or ubiquinol-cytochrome-*c* oxidoreductase (complex III). The electrons are then transferred to oxygen *via* cytochrome *c* oxidase (complex IV) or *via* an alternative terminal oxidase which branches off from the electron transport chain. The path of electron flow depends on the sulphide concentration: at concentrations lower than 10 μmol l^{-1}, the electrons from sulphide are probably channelled through cytochrome *c* oxidase. At higher sulphide concentrations, the cytochrome *c* oxidase pathway is blocked and the electrons are transferred to oxygen by the alternative, sulphide-insensitive oxidase. The existence of an alternative oxidase enables the lugworm to oxidize sulphide even at high tissue levels of sulphide (Völkel and Grieshaber, 1996).

Mitochondrial sulphide oxidation has also been demonstrated in the sulphide-tolerant clam *Solemya reidi* (Powell and Somero, 1986; O'Brien and Vetter, 1990). In this species, electrons from sulphide oxidation enter the respiratory chain at the level of cytochrome *c*. In contrast to *A. marina*, therefore, mitochondrial sulphide oxidation is inhibited by high

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sulphide concentrations. Powell and Somero (1986) have shown that mitochondrial sulphide oxidation in *S. reidi* is coupled to the synthesis of ATP. The rate of ATP production was maximal at sulphide concentrations of 10–20 μmol l^{-1}, whereas higher sulphide concentrations caused an inhibition of ATP production. In *S. reidi*, sulphide oxidation is thought to be coupled to oxidative phosphorylation solely by electron flow through complex IV. Similar mechanisms have been found in liver mitochondria of the fishes *Fundulus parvipinnis* and *Citharichthys stigmaeus* (Bagarinao and Vetter, 1990) and in mitochondria from the polychaete worm *Heteromastus filiformis* (Oeschger and Vismann, 1994).

Preliminary results have revealed that mitochondrial ATP production in *A. marina* is stimulated by 10 μmol l^{-1} sulphide (Völkel and Grieshaber, 1994*b*). However, it is not clear whether ATP can also be synthesized at high sulphide concentrations. This could be the case if electron flow through the alternative terminal oxidase were coupled to oxidative phosphorylation. An energy-conserving electron flow through alternative terminal oxidases has been demonstrated in some bacteria (Parsonage *et al.* 1986; Puustinen *et al.* 1989) although, in general, ATP production through alternative pathways is a matter of debate (Palmer, 1981; Paget *et al.* 1988; Uribe and Moreno-Sanchez, 1992). It is also not known whether sulphide-based electron transport in the mitochondria of *A. marina* is coupled to oxidative phosphorylation through more than one coupling site. In contrast to other sulphide-tolerant species such as *S. reidi* and *F. parvipinnis* (Powell and Somero, 1986; Bagarinao and Vetter, 1990), complex III participates in sulphide oxidation in the mitochondria of *A. marina*. One might expect, therefore, that in the mitochondria of *A. marina* more ATP would be synthesized per mole of sulphide oxidized than in the other species mentioned above. In the present study, therefore, we have identified the respiratory complexes associated with sulphide-stimulated oxidative phosphorylation in *A. marina*.

Materials and methods

Animals

Specimens of *Arenicola marina* L. (Polychaeta) (0.5–2 g) were collected from intertidal flats near Zierikzee, The Netherlands, between April and December 1995. They were kept in the laboratory for up to 6 weeks in darkened tanks with aerated artificial sea water (35 ‰) at 15 °C.

Isolation of mitochondria

Mitochondria were isolated from the body-wall musculature according to Schroff and Schöttler (1977) with slight modifications: the isolation medium consisted of saccharose (0.25 mol l^{-1}), glycine (0.55 mol l^{-1}), Tris (40 mmol l^{-1}), EGTA (1 mmol l^{-1}) and bovine serum albumin (BSA) (0.2 %), pH 7.5. For the determination of the protein content, a sample of each preparation was prepared as described by Völkel and Grieshaber (1996). Protein content was measured according to Bradford (1976) using BSA (fraction V, Sigma) as a protein standard.

Assay of mitochondrial respiration

Mitochondrial oxygen consumption was measured in a glass chamber at 15 °C using a Clark oxygen electrode as described by Völkel and Grieshaber (1994*a*). The chamber (volume 3.1 ml) was filled with incubation buffer and mitochondria (mitochondrial protein content was 0.1–0.4 mg ml^{-1}). The incubation buffer consisted of saccharose (0.25 mol l^{-1}), glycine (0.45 mol l^{-1}), Tris (40 mmol l^{-1}), EGTA (1 mmol l^{-1}), BSA (0.2 %), KCl (0.1 mol l^{-1}), $MgCl_2$ (3 mmol l^{-1}) and K_2HPO_4 (5 mmol l^{-1}). The pH of the buffer was adjusted to 7.5 at 15 °C using 1 mol l^{-1} HCl. Respiratory control ratios (RCRs) and ADP/O ratios were determined according to Chance and Williams (1956) in the presence of succinate (4 mmol l^{-1}), malate (8 mmol l^{-1}) or sulphide (6–9 μmol l^{-1}) either with or without ADP (0.03–0.13 mmol l^{-1}). For inhibitor studies with salicylhydroxamic acid (SHAM), 10 μl of a stock solution (see below) was added to the assay. Controls were performed by adding the solvent without inhibitor. For sulphide experiments, oxygen consumption in the presence of sulphide but in the absence of mitochondria was determined and was subtracted from the respective values in the presence of mitochondria.

Measurement of ATP production

ATP production was measured using a coupled-enzyme method (Powell and Somero, 1986) described in detail by Bagarinao and Vetter (1990). The measurements were performed at 25 °C using a dual-wavelength spectrophotometer (ZWS II, Sigma, Berlin, Germany) at 340 and 400 nm. The pH of the assay mixture containing mitochondrial incubation buffer with glucose (10 mmol l^{-1}), NADP (0.5 mmol l^{-1}), ADP (0.05 mmol l^{-1}), P^1P^5-di(adenosine-5′) pentaphosphate (0.02 mmol l^{-1}), hexokinase (5 U; 1 U is the amount of enzyme converting one micromole of substrate per minute) and glucose-6-phosphate dehydrogenase (2 U) in a total volume of 2 ml was adjusted to 7.5 at 25 °C using 1 mol l^{-1} NaOH. Mitochondrial protein in the assay mixture amounted to 0.04–0.08 mg ml^{-1}. The reaction was started by the addition of 10 μl of malate (2.5 mmol l^{-1}), 10 μl of succinate (1.25 mmol l^{-1}) or 5–10 μl of sulphide (2.5–170 μmol l^{-1}) (final concentrations). To evaluate the effect of different sulphide concentrations, the rate of ATP production of each preparation was measured in the presence and absence of carbon substrates at successive sulphide concentrations. Each sulphide concentration was tested in a separate assay. In some cases, two assays were performed per sulphide concentration for each preparation. Variation between duplicate measurements was 4.6±4.2 % (mean ± S.D; N=45). For inhibitor studies, 10 μl of the respective inhibitor stock solution (the concentration depending on the desired concentration in the assay) or solvent (control) was added to the assay prior to the addition of the substrate. After 2 min of equilibration, malate, succinate or sulphide was added to start the reaction. The inhibition is given

as a percentage of ATP production in the presence of the respective solvent but in the absence of the inhibitor.

Cytochrome c *oxidase*

Cytochrome *c* oxidase activity was measured at 25 °C according to Hand and Somero (1983) with slight modifications: the assay mixture (1 ml) consisted of Tris (50 mmol l^{-1}, pH 7.5), NaCl (50 mmol l^{-1}) and reduced cytochrome *c* (0.1 mmol l^{-1}). The reaction was initiated by the addition of 10 µl of mitochondria (mitochondrial protein in the assay mixture amounted to 1.1–1.7 µg ml^{-1}). The inhibition by sulphide was determined by adding 5–10 µl of a sulphide stock solution, the concentration depending on the desired concentration in the assay.

Sulphide solutions

Sulphide stock solutions (0.9–20 mmol l^{-1}) were prepared by adding washed crystals of $Na_2S{\cdot}9H_2O$ to nitrogen-saturated distilled water. The sulphide solutions were neutralized using 1 mmol l^{-1} HCl. Sulphide concentrations were checked using the Methylene Blue method (Gilboa-Garber, 1971).

Inhibitors

Stock solutions of rotenone (0.3 mmol l^{-1}, Sigma) and salicylhydroxamic acid (90 mmol l^{-1}, Sigma) were dissolved in dimethylsulphoxide. Antimycin (15 mmol l^{-1}, Sigma) and oligomycin (1.3 mmol l^{-1}, Sigma) were dissolved in ethanol. KCN (50 mmol l^{-1}, Merck) was dissolved in incubation buffer.

Data treatment

Data are given as means ± standard deviation (mean ± S.D.) of the results from 3–10 different preparations, each comprising approximately 10–20 animals. Differences between means were evaluated using a statistical software package (SigmaStat, Jandel Scientific, CorteMadera, USA) and paired *t*-tests at the $P<0.05$ level.

Results

Mitochondrial respiration

Mitochondrial oxygen consumption in the presence of saturating concentrations of malate but without ADP (state 2) was approximately 5 nmol min^{-1} mg^{-1} protein (Table 1). Respiration was stimulated by the addition of ADP to approximately 19 nmol min^{-1} mg^{-1} (state 3). State 4 respiration (in the presence of malate but with all ADP consumed) was slightly higher than state 2 respiration giving a respiratory control ratio (RCR) of 3.0. The ADP/O ratio (nanomoles ADP added/nanoatoms oxygen consumed) for malate was 2.1. Respiration with succinate as a substrate (saturating concentrations) was 2.5-fold higher than malate respiration. The RCR was 2.8 and ADP/O was 1.5. State 2 and state 4 respiration in the presence of 6–9 µmol l^{-1} sulphide was significantly higher than in the presence of malate but only slightly higher than in the presence of succinate and the RCR was 1.7 and ADP/O was 1.1. Differences between assays with different levels of mitochondrial protein were negligible (data not shown).

Table 1. *Oxygen consumption rates of mitochondria from* Arenicola marina *under different conditions*

	Oxygen consumption (nmol min^{-1} mg^{-1} protein)				
Substrate	State 2	State 3	State 4	RCR	ADP/O
Malate (8 mmol l^{-1})	4.7±1.4	18.7±4.4	6.3±1.0	3.0±0.6	2.1±0.3
Succinate (4 mmol l^{-1})	12.1±3.7	35.6±12.7	12.9±4.2	2.8±0.4	1.5±0.2
Sulphide (6–9 µmol l^{-1})	18.3±5.1	30.1±8.0	16.8±5.0	1.7±0.4	1.1±0.1

State 2, in the presence of substrate; state 3, in the presence of substrate and ADP; state 4, in the presence of substrate but with all ADP consumed.

Respiratory control ratio (RCR) = state 3 respiration/state 4 respiration, ADP/O ratio = nanomoles ADP added/nanoatoms oxygen consumed during state 3.

Values are means ± S.D., data from five preparations.

Fig. 1 shows typical recordings of mitochondrial oxygen consumption. Salicylhydroxamic acid (SHAM) was used as an inhibitor of the alternative terminal oxidase. In the presence of low sulphide concentrations (4 µmol l^{-1}), neither state 2 nor state 3 respiration was inhibited by SHAM (Fig. 1A). Similarly, SHAM had no effect on state 2 and state 3 respiration with malate as a substrate (Fig. 1B). In the presence of malate, state 3 respiration could be enhanced by the addition of 4 µmol l^{-1} sulphide but, in contrast to this, respiration was inhibited by SHAM plus high sulphide concentrations (75 µmol l^{-1}) (Fig. 1B). Blind oxygen consumption (without mitochondria) was negligible in the presence of low or high sulphide concentrations (Fig. 1C).

Mitochondrial ATP production

Mitochondrial ATP production in the absence of any other substrate was stimulated by the addition of sulphide (Fig. 2). A maximal rate of ATP production (67±11 nmol min^{-1} mg^{-1} protein, $N=5$) was reached at a sulphide concentration of 7.8±0.4 µmol l^{-1}. At the same sulphide concentration, the ratio of ATP produced per mole sulphide consumed was 0.9±0.2 ($N=10$). At 11.3±1.0 µmol l^{-1} sulphide, the ATP/sulphide ratio was 0.7±0.1 ($N=4$). With increases in sulphide concentration above 15 µmol l^{-1}, the rate of ATP production gradually decreased until, above approximately 50 µmol l^{-1} sulphide, it was no longer significantly different from zero. With malate as a substrate, ATP production was 156±48 nmol min^{-1} mg^{-1} ($N=5$) in the absence of sulphide (Fig. 3). ATP production was significantly stimulated by sulphide up to a maximal rate of 174±49 nmol min^{-1} mg^{-1} ($N=5$) at a sulphide concentration of 3.8±0.5 µmol l^{-1}. At sulphide concentrations higher than 20 µmol l^{-1}, ATP production decreased significantly reaching

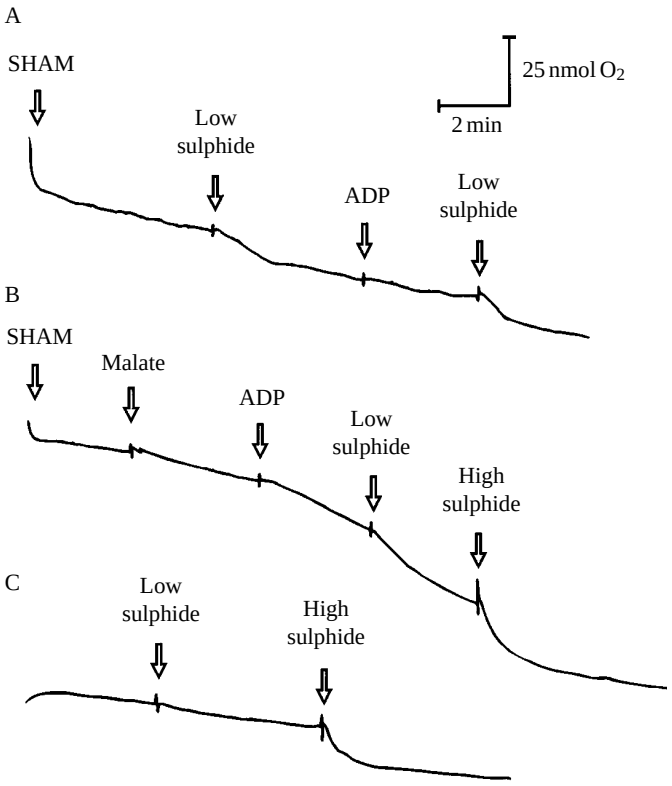

Fig. 1. Original recordings of oxygen consumption by mitochondria from the body-wall tissue of *Arenicola marina* (A,B) and oxygen consumption in the absence of mitochondria (C). The amount of mitochondrial protein present was 0.2 mg ml^{-1}. Additions (indicated by arrows) were: salicylhydroxamic acid (SHAM), 1 mmol l^{-1}; 'low sulphide', 4 μmol l^{-1} sulphide; 'high sulphide', 75 μmol l^{-1} sulphide; ADP, 0.25 mmol l^{-1}; malate, 4 mmol l^{-1}.

a minimal rate of 21±7 nmol min^{-1} mg^{-1} at approximately 80 μmol l^{-1} sulphide. Even at sulphide concentrations as high as 150 μmol l^{-1}, however, ATP production rate remained significantly different from zero. In the presence of succinate, ATP production amounted to 119±28 nmol min^{-1} mg^{-1} (N=4) in the absence of sulphide and was significantly enhanced by sulphide up to a maximal rate of 135±23 nmol min^{-1} mg^{-1} (N=4) at a sulphide concentration of 3.9±0.6 μmol l^{-1} (Fig. 3). At a sulphide concentration of 15 μmol l^{-1}, ATP production rate decreased significantly. At 25 μmol l^{-1} sulphide, ATP production was no longer significantly different from zero. No differences between assays with different levels of mitochondrial protein were found.

To investigate the effects of different inhibitors, mitochondrial ATP production was measured in the presence of rotenone, antimycin, cyanide, salicylhydroxamic acid (SHAM) and oligomycin, and in the presence of the two solvents used, ethanol and dimethylsulphoxide (DMSO) as controls (Table 2). The production of ATP with malate, succinate or sulphide as a substrate was increased by approximately 10 % by ethanol and DMSO (data not shown). In the presence of malate, ATP production was inhibited by rotenone, antimycin, cyanide and oligomycin, but not by SHAM. With succinate as a substrate, ATP production was inhibited by antimycin, cyanide and oligomycin, but not by SHAM. In the case of rotenone, inhibition was 23 %. Sulphide-induced ATP production was inhibited by antimycin, cyanide and oligomycin, but not by rotenone or SHAM.

Inhibition of the cytochrome c *oxidase*

Cytochrome *c* oxidase activity in mitochondrial suspensions was 0.76±0.23 μmol min^{-1} mg^{-1} (N=5). The activity was inhibited by the addition of sulphide (Fig. 4). An IC_{50} value (inhibitor concentration giving half-maximal inhibition) of 1.5±0.6 μmol l^{-1} sulphide was calculated. Cytochrome *c* oxidase was inhibited by 97.5±2.7 % by cyanide (1.5 mmol l^{-1}) but only by 0.7±1.4 % by SHAM (2.9 mmol l^{-1}) (N=4).

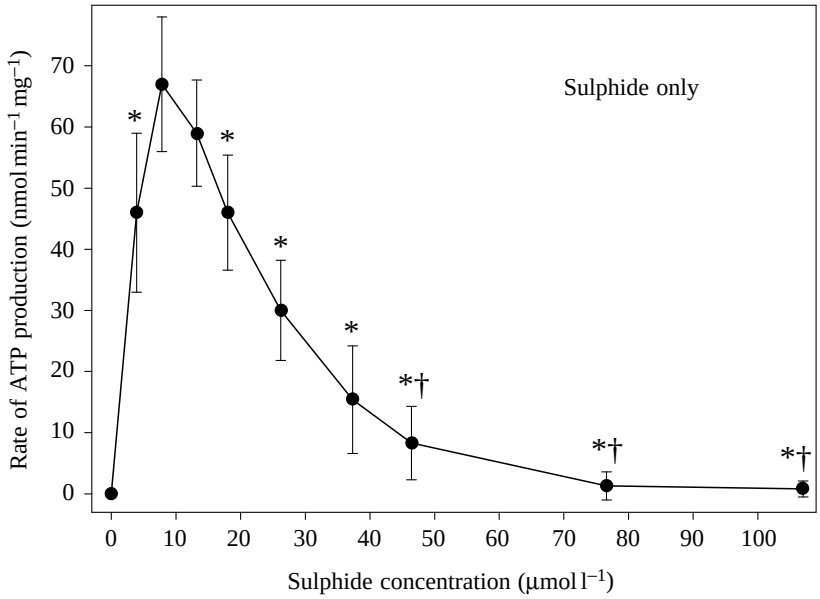

Fig. 2. Mitochondrial ATP production (nmol min^{-1} mg^{-1}) as a function of sulphide concentration in the absence of any other substrate. Data are means ± S.D. from 3–5 different preparations. *Significantly different from the highest ATP production value; †not significantly different from zero (paired *t*-test, P<0.05).

Table 2. *Inhibition of mitochondrial ATP production in* Arenicola marina

	Inhibition of ATP production (%)				
Substrate	Rotenone	Antimycin	Cyanide	SHAM	Oligomycin
Malate (2.5 mmol l^{-1})	97.8±1.3	90.3±12.0	85.2±4.5	6.4±6.8	95.1±1.8
Succinate (1.3 mmol l^{-1})	22.6±13.5	100±0.0	99.8±0.4	5.7±5.4	100±0.0
Sulphide (7–11 μmol l^{-1})	0.0±0.0	99.4±0.9	100±0.0	1.5±3.4	100±0.0

Inhibition is given as the percentage of mitochondrial ATP production (mean ± S.D., *N*=5) in the presence of the appropriate solvent without the inhibitor.

SHAM, salicylhydroxamic acid.

Inhibitor concentrations (μmol l^{-1}) were: rotenone 1.5, antimycin 75, cyanide 250, SHAM 450 and oligomycin 6.5.

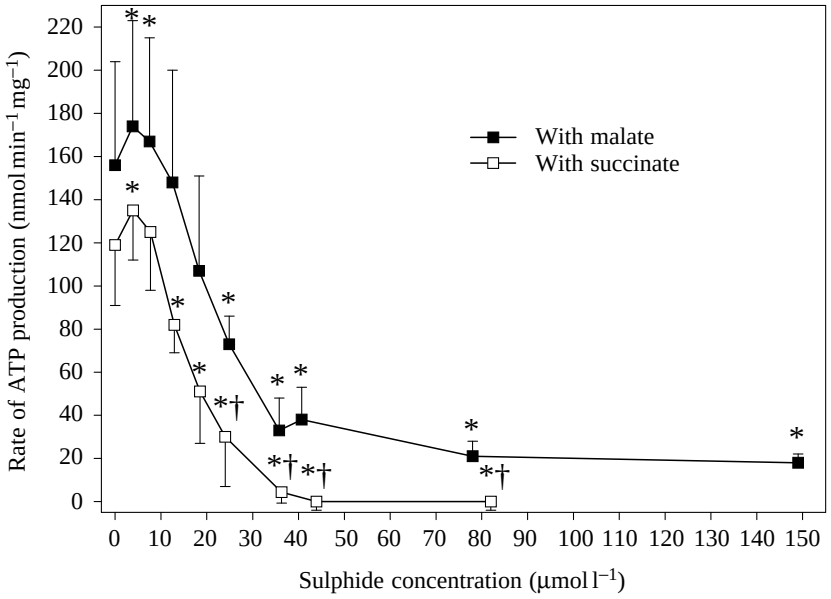

Fig. 3. Mitochondrial ATP production (nmol min^{-1} mg^{-1}) in the presence of malate (2.5 mmol l^{-1}) or succinate (1.25 mmol l^{-1}) as a function of sulphide concentration. Data are means ± S.D. from 3–5 different preparations. *Significantly different from the ATP production in the absence of sulphide; †not significantly different from zero (paired *t*-test, $P<0.05$).

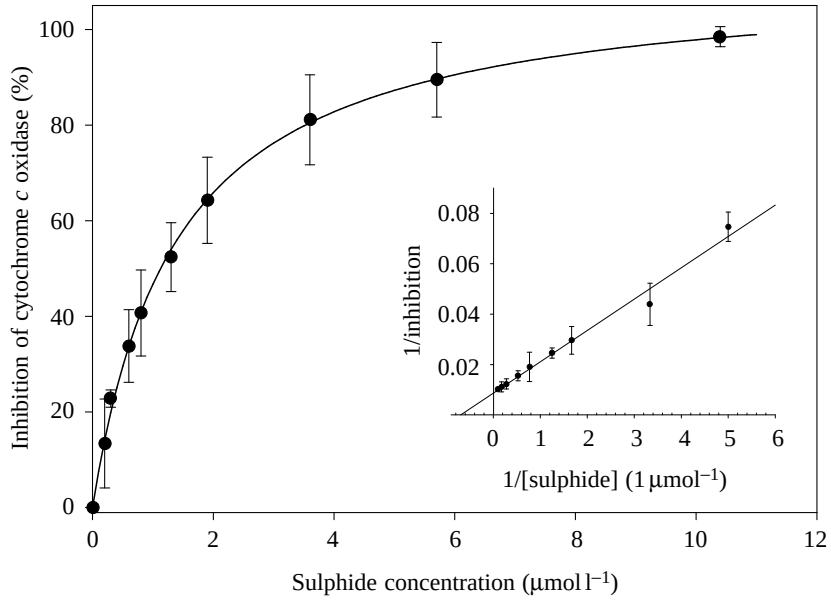

Fig. 4. Inhibition of mitochondrial cytochrome *c* oxidase activity (%) by sulphide. Data are means ± S.D. from five different preparations. Cytochrome *c* oxidase activity in the absence of sulphide was 0.76±0.23 U mg^{-1} (1 U is the amount of enzyme converting 1 micromole of substrate per minute). IC_{50} was 1.5±0.6 μmol l^{-1} sulphide. Inset shows the Lineweaver–Burk plot of sulphide-dependent inhibition.

Discussion

Mitochondrial oxygen consumption in *A. marina* in the presence of malate and ADP (state 3 respiration) was 19 nmol min^{-1} mg^{-1} and with succinate and ADP was 36 nmol min^{-1} mg^{-1} (Table 1). The RCR and ADP/O ratio were 3.0 and 2.1, respectively, in the case of malate, and 2.8 and 1.5, respectively, with succinate as a substrate. These values are in good agreement with data from isolated mitochondria from other marine invertebrates (Chen and Lehninger, 1973; Mommsen and Hochachka, 1981; Ballantyne and Storey, 1984; Burcham *et al.* 1984). The respiration rate was, however, 2.7–6 times higher than in two earlier studies on mitochondria of *A. marina* (Völkel and Grieshaber, 1994*a*, 1996). This was achieved with a slight modification of the mitochondrial incubation buffer, using EGTA instead of EDTA and adding 0.1 mol l^{-1} KCl (Nedergaard and Cannon, 1979).

Sulphide oxidation is coupled to oxidative phosphorylation

In an earlier study, we demonstrated that state 2 respiration at sulphide concentrations as high as 50 μmol l^{-1} was not stimulated by the addition of ADP (RCR=1) in *A. marina* (Völkel and Grieshaber, 1994*a*). In the present study, however, sulphide-induced oxygen consumption at sulphide concentrations of 4–9 μmol l^{-1} exhibited acceptor control (RCR=1.7; Table 1, Fig. 1A). Similar values were found in the clam *Solemya reidi* (RCR=1.8 at 20 μmol l^{-1} sulphide; Powell and Somero, 1986) and in the polychaete worm *Heteromastus filiformis* (RCR=1.4 at 5–10 μmol l^{-1} sulphide; Oeschger and Vismann, 1994). In the latter study, it was also demonstrated that, as in *A. marina*, RCR values in the mitochondria of *H. filiformis* declined at sulphide concentrations above 10 μmol l^{-1} until at concentrations above 20 μmol l^{-1} sulphide there was no acceptor control (RCR=1).

The present data indicate that, at sulphide concentrations as low as 4–9 μmol l^{-1}, sulphide oxidation in the mitochondria of *A. marina* is coupled to oxidative phosphorylation. This was confirmed by the determination of mitochondrial ATP production with sulphide as the only substrate (Fig. 2). The synthesis of ATP was stimulated by the addition of sulphide and reached a maximal rate of 67 nmol min^{-1} mg^{-1} at 8 μmol l^{-1} sulphide. This value is 5–7 times higher than the rate of ATP production in mitochondria from foot tissue of the clam *S. reidi* (Powell and Somero, 1986), from *H. filiformis* (whole animals; Oeschger and Vismann, 1994) and from liver tissue of the killifish *Fundulus parvipinnis* (Bagarinao and Vetter, 1990). This discrepancy is, however, paralleled by a much higher state 3 sulphide oxidation rate in *A. marina* mitochondria compared with mitochondria from both other species.

Cytochrome c *oxidase*

When sulphide concentrations exceeded 15 μmol l^{-1}, the ATP production rate in *A. marina* mitochondria declined significantly until it was completely inhibited at approximately 50 μmol l^{-1} (Fig. 2). Production of ATP, therefore, must be sensitive to high concentrations of sulphide, indicating that electron transport through complex IV (cytochrome *c* oxidase) is involved. Sulphide and cyanide are classical inhibitors of complex IV (Nicholls *et al.* 1972; Nicholls, 1975). Both complex IV and ATP production are inhibited by cyanide (Table 2; see Results). The sensitivity of cytochrome *c* oxidase to sulphide is demonstrated in Fig. 4. Inhibition was half-maximal at a sulphide concentration of 1.5 μmol l^{-1}, which is in the same range as in mitochondria isolated from rat lung (Khan *et al.* 1990) and from *Tubifex* sp. (Degn and Kristensen, 1981). In *A. marina* mitochondria, this enzyme was completely inhibited at approximately 10 μmol l^{-1} sulphide. It is striking that ATP production reached maximal values at a sulphide concentration of 8 μmol l^{-1}, which is sufficiently high for an almost complete inhibition of complex IV (Figs 2, 4). This may be partly due to the use of different media in the ATP and cytochrome *c* oxidase assays. Cytochrome *c* oxidase activity was measured in a hypotonic medium, which probably caused the destruction of the mitochondrial membranes. This might have interrupted the electron flow from sulphide to oxygen, so that sulphide oxidation ceased and, thus the cytochrome *c* oxidase was exposed to the same sulphide concentrations as those present in the assay medium. In contrast, the ATP assay was designed to keep the mitochondria intact. Sulphide entering the mitochondria was quickly oxidized, thus lowering the actual sulphide concentration to which the cytochrome *c* oxidase was exposed. At the same time, the lower sulphide sensitivity of ATP production compared with that of the cytochrome *c* oxidase reflects the fact that the very enzyme that is inhibited by sulphide is also involved in its detoxification. The biphasic nature of sulphide-dependent ATP production is, therefore, the result of the combined effects of saturation and inhibition kinetics.

The alternative oxidase

In contrast to ATP production, sulphide oxidation in *A. marina* mitochondria is not inhibited by sulphide but proceeds even at concentrations as high as 400 μmol l^{-1} (Völkel and Grieshaber, 1994*a*, 1996). This is due to the existence of a sulphide-insensitive, alternative terminal oxidase which branches off from the main respiratory electron transport chain (see Fig. 5). At high sulphide concentrations when the cytochrome *c* oxidase is inhibited, electrons from sulphide are transferred to oxygen *via* the alternative oxidase, thus enabling the lugworm to oxidize sulphide even at high tissue levels of sulphide (Völkel and Grieshaber, 1996). The inhibition of ATP production at high sulphide concentrations indicates that electron transport through the alternative oxidase is not coupled to oxidative phosphorylation. Sulphide-induced ATP production was not inhibited by SHAM (Table 2), which is an inhibitor of the alternative oxidase (Schonbaum *et al.* 1971). In the presence of SHAM, oxygen consumption could not be stimulated by 50 μmol l^{-1} sulphide since the alternative oxidase was inhibited by SHAM while complex IV was inhibited by sulphide (Völkel, 1995; Völkel and Grieshaber, 1996; see also Fig. 1B). There was no effect

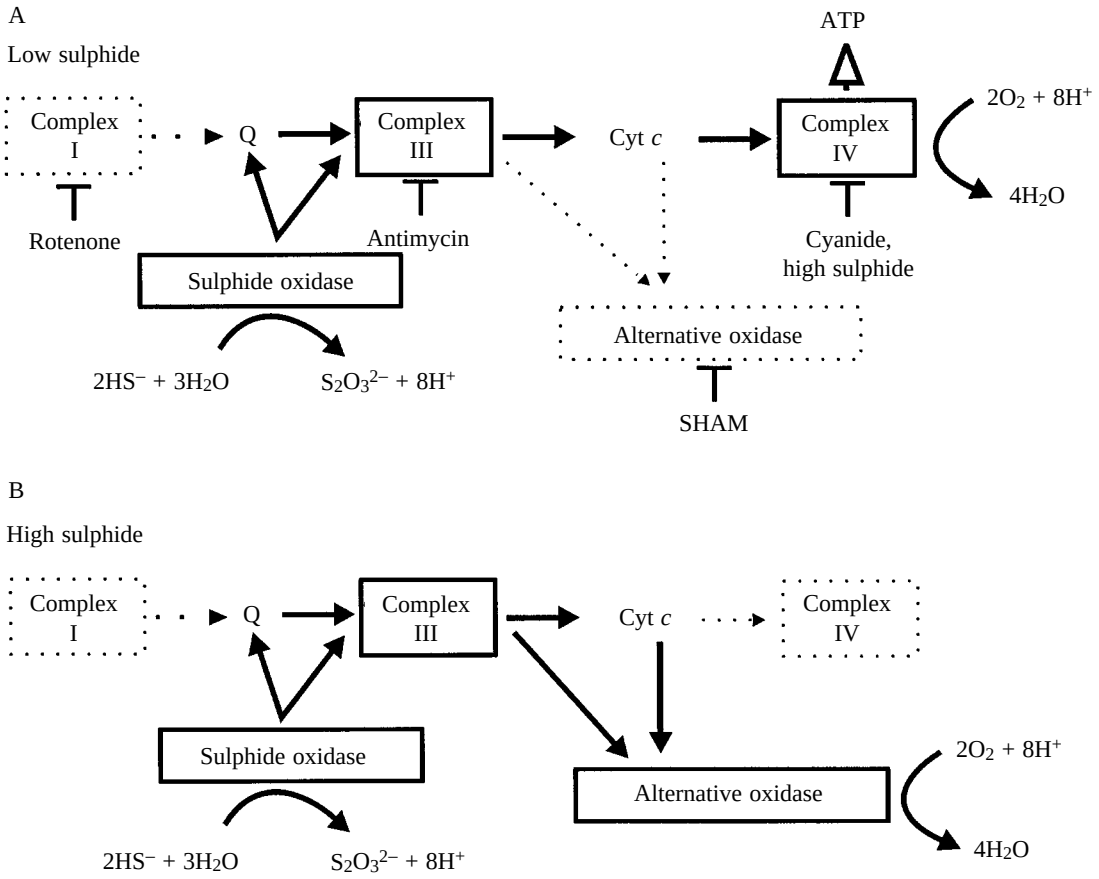

Fig. 5. Proposed model of electron transport and ATP production during sulphide oxidation in *Arenicola marina*. (A) At low sulphide concentrations, electrons from sulphide are mainly transferred to oxygen *via* cytochrome *c* (Cyt *c*) oxidase (complex IV). The electron flow through complex IV is coupled to oxidative phosphorylation. (B) At high sulphide concentrations, complex IV is inhibited and electrons from sulphide are transferred to oxygen by the alternative terminal oxidase. No ATP is synthesized during sulphide oxidation at high sulphide concentrations. A also shows the working points of the different inhibitors used in this study. Q, ubiquinone; SHAM, salicylhydroxamic acid.

of SHAM on cytochrome *c* oxidase and, therefore, mitochondrial sulphide oxidation in the presence of low sulphide concentrations (4 μmol l^{-1}) was not inhibited in its presence and acceptor control was maintained (Fig. 1A). From the present data, we conclude that in the mitochondria of *A. marina* electron transport through the alternative terminal oxidase is not coupled to ATP synthesis.

Complex III

As we have shown earlier (Völkel and Grieshaber, 1996), complex III is involved in mitochondrial sulphide oxidation in *A. marina* whereas complex I is not. Correspondingly, ATP production with sulphide as a substrate was inhibited by antimycin but not by rotenone (Table 2). Complex III and complex IV, therefore, are possible coupling sites of the respiratory electron transport chain during sulphide oxidation (see Fig. 5). If the sulphide-driven electron flow through both complexes was coupled to the synthesis of ATP, the ratio of ATP formed to sulphide consumed should have been higher than was observed in the present study (see below). In addition, at least some ATP should be synthesized during complete inhibition of complex IV since, under these circumstances, electrons from sulphide oxidation are channelled *via* complex III to the alternative terminal oxidase. It has been shown, however, that ATP production was completely inhibited by cyanide and by high sulphide concentrations (Table 2; Fig. 2). Although the concentration of cyanide (0.25 mmol l^{-1}) was sufficiently high to inhibit complex IV as well as the alternative oxidase, sulphide oxidation *via* the alternative oxidase is not sensitive to sulphide concentrations as high as 400 μmol l^{-1} (Völkel and Grieshaber, 1996).The lack of any ATP production during inhibition of complex IV indicates, therefore, that complex III does not serve as a coupling site for sulphide-driven ATP synthesis. However, the mechanisms remain to be shown by which the electrons from sulphide oxidation are channelled through complex III without the build-up of any proton gradient.

The ratio of ATP formed to sulphide consumed

In the mitochondria of *A. marina*, the maximal ratio of ATP formed to sulphide consumed was 0.9±0.2 (*N*=10). This value is within the range reported for species in which mitochondrial sulphide oxidation is coupled to ATP production only through complex IV: the clam *S. reidi* (0.5–1.2; Powell and Somero,

1986) and the killifish *F. parvipinnis* (0.99–1.25; Bagarinao and Vetter, 1990). According to O'Brien and Vetter (1990) the potential for ATP production would be 2.0–4.3 ATP per sulphide, assuming that sulphide oxidation is solely coupled to oxidative phosphorylation through complex IV. In their study, these authors argue that sulphide oxidation takes place outside the mitochondrial inner membrane. This derives from the fact that, in *S. reidi* mitochondria, sulphide oxidation is linked to the respiratory chain *via* cytochrome *c* (Powell and Somero, 1986). In the mitochondria of *A. marina*, however, electrons from sulphide oxidation enter the respiratory chain at the level of ubiquinone or at complex III (Völkel and Grieshaber, 1996). It currently is not known whether sulphide oxidation takes place outside or inside the mitochondrial inner membrane. If sulphide is oxidized within the mitochondrial matrix, protons that are chemically released during sulphide oxidation (see Fig. 5) must be subtracted from the biochemical proton gradient across the mitochondrial inner membrane. Depending on the form taken by the oxidized sulphide (H_2S or HS^-), the ratio of ATP produced per sulphide consumed would be 0.75 for H_2S or 1.0 for HS^-. The measured value of 0.9 is within the range of these expected values. There is insufficient evidence, however, to conclude that sulphide oxidation takes place within the mitochondrial matrix of *A. marina* since a number of other factors may contribute to the low ATP/sulphide ratio. Acceptor control with sulphide as a substrate was low in comparison with succinate or malate as a substrate (RCR with sulphide was 1.7 as opposed to 2.8 with succinate and 3.0 with malate; Table 1). This may be because sulphide itself acts as an uncoupler. The undissociated neutral H_2S molecule readily passes through the inner mitochondrial membrane, thus causing the movement of protons into the matrix as described in detail by O'Brien and Vetter (1990). Alternatively, it is possible that in the present study the sulphide added to the assay was not completely used for ATP production, since some may have been oxidized by the alternative oxidase which does not contribute to the synthesis of ATP. The regulation of electron flux between the main respiratory chain and the alternative oxidase probably depends on the sulphide concentration. Increasing sulphide concentrations cause an increasing inhibition of cytochrome *c* oxidase. As a consequence, a higher percentage of sulphide is oxidized *via* the alternative oxidase. This is indicated by a decreasing ATP/sulphide ratio at increasing sulphide concentrations (0.7 ATP per sulphide at 11 μmol l^{-1} as opposed to 0.9 ATP per sulphide at 8 μmol l^{-1} sulphide). Similarly, Bagarinao and Vetter (1990) found an ATP/sulphide ratio of 1.25 at 10 μmol l^{-1} sulphide and a ratio of 0.99 at a sulphide concentration of 20 μmol l^{-1} in the mitochondria of *Fundulus parvipinnis*, although no alternative oxidase is thought to be present in this animal. The decreasing ATP/sulphide ratio with increasing sulphide concentration could be due to an increasing uncoupling effect of sulphide. Another possible explanation would be a loss of sulphide from the assay by diffusion or chemical oxidation.

In the mitochondria of *A. marina*, the APD/O ratio in the presence of sulphide was 1.1 (Table 1). During sulphide oxidation, 1 mole of oxygen is required for the oxidation of 1 mole of sulphide (see Fig. 5; Völkel and Grieshaber, 1994*a*). The ratio of sulphide to O_2, therefore, equals 1. From this ratio, it can be estimated that 2.2 moles of ADP were consumed per mole of sulphide, which is a 2.4-fold higher value than the measured ATP/sulphide ratio. A similar discrepancy has been found during sulphide oxidation in the mitochondria of *F. parvipinnis* (Bagarinao and Vetter, 1990) and *S. reidi* (Powell and Somero, 1986). It has been ascribed to differences between the media in the respiration and the ATP assays and to methodological difficulties (Bagarinao and Vetter, 1990). The low RCR values during sulphide oxidation make it difficult to pinpoint the moment when ADP is consumed relative to the exhaustion of sulphide in the assay.

ATP production in the presence of carbon substrates

ATP production with 10 μmol l^{-1} sulphide as the only substrate was approximately 40 % of that with malate and 60 % of that with succinate. With malate or succinate as a substrate, however, ATP production was only enhanced by 14.7±7.6 % (N=9) by 4–10 μmol l^{-1} sulphide (Fig. 3). This slight but significant increase is probably caused by the feeding of additional electrons from sulphide into the respiratory chain. Malate and succinate were present at saturating concentrations and no increase in ATP production rate was achieved by further addition of the same substrate. Sulphide may permeate the mitochondrial membrane more quickly than malate and succinate, thus being able to increase the ATP production rate. It is very difficult, however, to determine which part of the ATP production is attributable to carbon substrates and which to sulphide, since both substrates compete for the respiratory chain. Sulphide-dependent ATP production, therefore, may represent from 15 % to approximately 50 % of the total maximal ATP production under these conditions.

Concentrations of sulphide between 15 and 20 μmol l^{-1} led to a significant decrease in ATP production. With succinate as a substrate, full inhibition was reached at 25–40 μmol l^{-1} sulphide (Fig. 3), indicating complete inhibition of the cytochrome *c* oxidase. In the case of malate, 80–90 % inhibition could be achieved by the same levels of sulphide. Interestingly, 10–15 % of the malate-driven ATP production remained insensitive to sulphide (Fig. 3) and inhibition by cyanide was also only 85 %, although complete inhibition was obtained by rotenone (Table 2). This may be due to some anaerobic reduction of fumarate (Schroff and Schöttler, 1977). Malate entering the mitochondria can be partly converted to fumarate by the fumarase. Under anaerobic conditions, fumarate is reduced to succinate by the fumarate reductase. This step is coupled to the synthesis of ATP (see Schöttler and Bennet, 1991). Since cyanide and high concentrations of sulphide both inhibit the last step of the respiratory chain, their effect may be similar to that of a lack of oxygen.

In conclusion, the present study indicates that *A. marina* can use sulphide directly as an inorganic energy source. Powell and Somero (1986) were the first to report a similar

mechanism in the mitochondria of the clam *S. reidi*. In the last few years, other species have been shown to use sulphide as a substrate for oxidative phosphorylation (Bagarinao and Vetter, 1990; Oeschger and Vetter, 1992; Oeschger and Vismann, 1994) suggesting that a capacity for mitochondrial sulphide oxidation coupled to the synthesis of ATP may not be restricted to a few highly specialized species but may be widespread among sulphide-tolerant species. It is still unclear whether ATP production from sulphide oxidation can contribute significantly to the energy supply of the animal. As shown in this study, energy provision in *A. marina* is only slightly enhanced by sulphide when the level of carbon substrates is high. Under conditions in which food is limited, however, sulphide-driven ATP production may gain in significance, although it is a comparatively inefficient pathway. In both cases, ATP production can only be stimulated by low sulphide concentrations. With increasing sulphide concentrations, ATP production gradually decreases and mitochondrial sulphide oxidation should be increasingly regarded as a detoxification process rather than a source of energy (see Fig. 5). Sulphide oxidation in *A. marina* is the only mitochondrial system so far known that is not inhibited by high sulphide concentrations. This enables the lugworm to detoxify sulphide even when levels in the tissues are very high, although sulphide cannot be used as an energy source under these conditions.

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