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ATP PRODUCTION FROM THE OXIDATION OF SULFIDE IN GILL MITOCHONDRIA OF THE RIBBED MUSSEL *GEUKENSIA DEMISSA*

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Summary

The ribbed mussel *Geukensia demissa* inhabits intertidal *Spartina* grass marshes characterized by sulfide-rich sediments. Sulfide poisons aerobic respiration, and *G. demissa* may cope in this seemingly inhospitable environment by oxidizing sulfide in gill mitochondria. Well-coupled mitochondria isolated from *G. demissa* gills were used to investigate sulfide oxidation and ATP synthesis. State 3 respiration, maximally stimulated by 5 μmol l^{-1} sulfide with a P/O ratio of 0.89 and a respiratory control ratio (RCR) of 1.40, remained refractory to sulfide at higher concentrations except in the presence of salicylhydroxamic acid (SHAM), an inhibitor of alternative oxidases. Sulfide-stimulated ATP production was 3–5 times greater than that stimulated by malate and succinate, respectively, giving an ATP/sulfide ratio of 0.63. The inhibition of sulfide-stimulated respiration and ATP production by the complex III inhibitors myxothiazol and antimycin A, respectively, suggests that electrons enter the electron transport chain before complex III. Combined with *in vivo* evidence for electron entry at cytochrome *c*, these data suggest that more than one type of sulfide-oxidizing enzyme may function in *G. demissa* gills. The SHAM-sensitive pathway of electron flux may be a critical component of a physiological strategy to tolerate sulfide. We conclude that *G. demissa* exploits the energy available from its reduced environment by using sulfide as a respiratory substrate for cellular ATP production.

Key words: sulphide, ATP, *Geukensia demissa*, mussel, mitochondria, gill, alternative oxidase, salicylhydroxamic acid, bioluminescence, oxidative phosphorylation.

Introduction

The ribbed mussel *Geukensia demissa* inhabits coastal salt marshes characterized by the dense growth of *Spartina* grass and black sulfide-rich intertidal sediments. The mussels attach in clusters with byssal threads to each other and to rhizomes of *Spartina* grass, burrowing deep in the sediment below the layer of redox potential discontinuity (RPD) (Ruppert and Fox, 1988). The RPD layer defines the transition from the oxic zone of the intertidal sediment to the reduced, anoxic zone where hydrogen sulfide production predominates (Fenchel and Riedl, 1970). Hydrogen sulfide is formed by sulfate-reducing bacteria participating in a consortium of anaerobic bacteria responsible for the carbon mineralization occurring in estuarine marshes (Jørgensen, 1982; Fenchel and Finlay, 1994, 1995). Hydrogen sulfide is ubiquitous in the sediment (Jørgensen, 1977) and soluble in the water column, occurring predominantly as H_2S and HS^- (Millero, 1986).

Hydrogen sulfide poisons aerobic respiration, binding with high affinity to the ferric (FeIII) heme of cytochrome a_3 (Nicholls, 1975; National Research Council, 1979). A strategy by mussels to keep their valves closed might exclude sulfide-containing waters and prevent sulfide poisoning, but this behavior would probably require sustaining extended intervals of anaerobiosis and limited exchange of oxygen, water, nutrients and metabolites across the gill surface (Somero et al., 1989). In fact, *G. demissa* maintain their valves agape during tidal emersion while exposed to sediment pore water having sulfide concentrations typically near 1 mmol l^{-1} and as high as 8 mmol l^{-1} (Lee et al., 1996). Because the distribution and behavior of *G. demissa* suggest a preference for this seemingly inhospitable sulfide-rich environment, mechanisms to detoxify sulfide are probably present in the animal.

In some sulfide-tolerant organisms, the mitochondrion has adapted as a sulfide sink, oxidizing sulfide to innocuous species and, under certain conditions, coupling sulfide oxidation to oxidative phosphorylation (for a review, see Grieshaber and Völkel, 1998). Previous work with excised gills of *G. demissa* has shown that sulfide-stimulated oxygen consumption was limited by the addition of the mitochondrial complex IV inhibitor cyanide (Lee et al., 1996), and that sulfide increased the level of reduction of mitochondrial cytochrome *c* and

stimulated ciliary beat frequency (Doeller et al., 1999). These data suggest that, in *G. demissa* gills, sulfide oxidation contributes to mitochondrial electron flux and supports ATP consumption.

In the present study, mitochondria isolated from the gills of *G. demissa* were used to investigate whether sulfide oxidation is coupled to ATP synthesis, thereby yielding energy in the process. The respiration rate in the presence of carbon substrates as a function of sulfide concentration and the rates of respiration and ATP production with sulfide as the sole respiratory substrate were determined using well-coupled gill mitochondria. In addition, the path of electron flow from the oxidation of sulfide through the electron transport system was investigated using inhibitors of mitochondrial electron flux.

Materials and methods

Animal collection and maintenance

Geukensia demissa Dillwyn were collected from intertidal marshes on Dauphin Island, Alabama, USA, and maintained in mud-filled aquaria filled with 20 ‰ artificial sea water (Tropic Marin), as described previously (Doeller et al., 1999). Sediment sulfide production was periodically stimulated by injections of a mixture containing cellulose and calcium sulfate into the sediment. Animals were fed weekly with finely homogenized DoroMin fish food (TetraWerke, Germany).

Isolation of gill mitochondria

The procedures for isolating mitochondria were modified from those of Burcham et al. (1984). Demibranch gills weighing 2.5–3.0 g from 3–5 animals ranging from 5 to 9 cm in length were excised, blotted dry and suspended in 10 ml of isolation buffer [530 mmol l^{-1} sucrose, 1 mmol l^{-1} EGTA, 0.5 % defatted bovine serum albumin (BSA) and 20 mmol l^{-1} Hepes adjusted to pH 7.5 on ice] per gram of tissue. The gills were homogenized (Brinkmann polytron PT 10/35, setting 1) for 10 s, and the homogenate was filtered through one layer of cheesecloth. The filtrate, brought back to original volume with isolation buffer, was further homogenized with one complete pass of a glass–Teflon homogenizer at 100 revs min^{-1}, then centrifuged at 1600 ***g*** in a refrigerated centrifuge (2–4 °C, Sorval RC2-B) for 10–15 min until a tight pellet formed. The low-speed supernatant was centrifuged at 7100 ***g*** for 15 min. The high-speed supernatant was gently decanted, leaving behind the brown mitochondrial pellet, and was replaced with 15 ml of fresh isolation buffer. The upper yellow layer of the mitochondrial pellet was carefully removed by gentle irrigation of the fluid over the pellet with a Pasteur pipette. The pellet was resuspended to 1 ml with respiration buffer (303 mmol l^{-1} sucrose, 90 mmol l^{-1} KCl, 1 mmol l^{-1} EGTA, 4 mmol l^{-1} KH_2PO_4, 0.5 % defatted BSA and 20 mmol l^{-1} Hepes adjusted to pH 7.5 at 20 °C) and stored on ice prior to experiments.

Mitochondrial respirometry

Mitochondrial respiration rates were determined at 20 °C in a closed dual-chambered respirometer with data-acquisition and analysis software (Oroboros Oxygraph; Paar KG, Graz, Austria). The stirred (500 revs min^{-1}) reaction mixture (2.0–2.5 ml) contained mitochondria at a protein concentration of 0.1–0.6 mg ml^{-1}. Background oxygen consumption was measured in the second chamber in the absence of mitochondria. Oxygen consumption resulting from the spontaneous oxidation of sulfide was not detectable at the sulfide concentrations used in this study.

The respiratory control ratio (RCR) and P/O ratio were determined using malate, succinate or sulfide as substrate. Respiratory states, RCR and P/O ratio are defined in Table 1 according to Chance and Williams (1955). For carbon substrates, state 1 respiration rate was determined upon the addition of mitochondria to the respirometer chamber. State 3 respiration was initiated with the addition of 4 mmol l^{-1} malate or 2.5 mmol l^{-1} succinate and an injection of 82.5–165 nmol of ADP. For studies with sulfide, experimental sulfide concentrations were below 100 µmol l^{-1}, the most likely tissue concentration since measured tissue sulfide concentrations in gills exposed to up to 1 mmol l^{-1} ambient sulfide remained below 100 µmol l^{-1} (J. E. Doeller, D. W. Kraus and M. K. Grieshaber, in preparation). Repeated injections of sodium sulfide at a concentration that maximally stimulated respiration without being inhibitory were necessary because sulfide is oxidized rapidly by these mitochondria. State 3 respiration was initiated with an injection of 82.5 nmol of ADP, followed immediately by an injection of 5 µmol l^{-1} sodium sulfide. Subsequent injections of 5 µmol l^{-1} sodium sulfide were made until ADP was exhausted and state 4 respiration was achieved. The state 3 sulfide/O_2 ratio was calculated as the amount of sulfide added during state 3 respiration divided by the amount of oxygen consumed during that period.

The effects of sulfide on carbon-supported mitochondrial state 3 respiration were determined by adding sulfide in the presence of 2.5 µmol of ADP and malate or succinate. Subsequent injections of sulfide were made after the initial sulfide addition had been consumed, as indicated by the end of the sulfide-stimulated respiratory burst.

The effects of sulfide on mitochondrial respiration were also determined in the presence of mitochondrial electron transport inhibitors in an effort to determine the path of electron flow during sulfide-stimulated respiration. The mitochondrial bc_1 complex (complex III) inhibitors myxothiazol (30 µmol l^{-1}) and antimycin A (50 µmol l^{-1}) and the cytochrome *c* oxidase inhibitor cyanide (1 mmol l^{-1}) were each used separately with state 3 mitochondria respiring either carbon substrates or sulfide. To determine whether an alternative oxidase may operate during carbon-substrate- or sulfide-stimulated respiration, salicylhydroxamic acid (SHAM; 1 mmol l^{-1}), an inhibitor of alternative terminal oxidases, was added to a state 3 mitochondrial suspension before or after sulfide addition. Respirometric controls for the inhibitors included the addition of ethanol, used as the solvent for myxothiazol and SHAM.

Measurement of ATP production

The production of ATP by mitochondria was measured as

bioluminescence produced by the firefly luciferin–luciferase reaction (Lemasters and Hackenbrock, 1976, 1979). A luminometer (Monolight 1500, Analytical Luminescence Laboratories, San Diego, CA, USA) operating in the kinetics mode followed changes in the quantity of ATP by counting photons emitted over timed intervals during the course of each experiment. The bioluminescent-generated signal, recorded as relative light units (RLUs), was calibrated using ATP standards.

Kinetic experiments of mitochondrial ATP production were performed in the following sequence. An optimized ATP assay mixture containing firefly luciferase and luciferin from Sigma or from Analytical Luminescence Laboratories was reconstituted and diluted 40-fold or fivefold, respectively, with respiration buffer. A 100 µl volume of mitochondria diluted in respiration buffer to 2.0–33.0 µg mitochondrial protein ml^{-1} was added to the luminometer cuvette. To this suspension, substrates, adenylates and/or inhibitors were added using Hamilton syringes to a total volume of no more than 115 µl. After the cuvette had been placed in the luminometer, 100 µl of the ATP assay mixture was automatically injected into the cuvette upon chamber closure, and the luminescence reaction was recorded for slightly over 5 min. The kinetics data-gathering protocol for all studies consisted of 30 count intervals at 10 s per interval with a 1 s delay between each count interval. Sample points represent the total number of photons counted per interval. Each control had precisely the same volume as the treatment by including the appropriate solvent without the chemical agent. All luminescence reactions were performed 2–4 times at 20 °C in the course of an experiment, and corresponding traces were averaged.

Mitochondrial ATP production rate was determined with 4 mmol l^{-1} malate and 2.5 mmol l^{-1} succinate and with sulfide supplied in 0.1 nmol injections. With substrates present, ATP production was initiated by an addition of ADP, typically 1.5 nmol, just prior to inserting the cuvette into the luminometer. To control for the presence of endogenous substrates, ATP production in the absence of added substrate was determined for each mitochondrial preparation and was subtracted from ATP production with the substrate included. To calibrate the RLU traces, ATP, usually a 0.1 nmol standard, was added to the reaction mixture containing diluted mitochondria and substrate. The maximum RLU value was equated with the added ATP.

To determine the effects of respiratory inhibitors on ATP production, experiments were performed with malate, succinate or sulfide as respiratory substrates, each in combination with antimycin A, SHAM or cyanide. The RLU traces were calibrated using ATP standard added to the reaction mixture containing diluted mitochondria, substrate and the inhibitor or the solvent as the control. Percentage inhibition was calculated between reactions containing substrates, ADP and inhibitors and those containing substrates, ADP and the appropriate solvent as a control.

Preparation of substrates and inhibitors

Stock solutions of sulfide were prepared for each experiment by adding washed crystals of $Na_2S{\cdot}9H_2O$ to argon-saturated distilled water. Succinate stock solutions were prepared by vortexing succinate crystals in distilled water. Both malate and potassium cyanide were dissolved in respiration buffer. Stock solutions of antimycin A, myxothiazol and salicylhydroxamic acid were dissolved in 50–70 % ethanol.

Statistical treatment of data

Results are are reported as means + standard deviation (S.D.). Significant differences were calculated using unpaired one-tailed *t*-tests for Table 2 and paired one-tailed *t*-tests for Fig. 3. All statistical analyses were performed using Microsoft Excel.

Results

Mitochondrial respiration

Respiration rates indicated that mitochondria isolated from the gills of *G. demissa* were well-coupled (Fig. 1). Isolated mitochondria alone respired at a low rate that approached state 2 (see Table 1). Respiration rate increased moderately upon the addition of the substrates malate and succinate, possibly as a result of proton leaks through the inner membrane or of the

Table 1. *Respiration rates of isolated* Geukensia demissa *gill mitochondria in the presence of malate, succinate and sulfide*

	Respiration rate (nmol O_2 min^{-1} mg^{-1} protein)				
	Substrate only	State 3	State 4	RCR	ADP/O
4 mmol l^{-1} malate	1.90±0.46	10.3±2.64	3.29±1.39	3.35±0.83	2.85±0.40
2.5 mmol l^{-1} succinate	7.92±1.60	23.0±5.87	8.29±2.57	2.86±0.51	1.87±0.22
5 µmol l^{-1} sulfide	26.3±13.7	38.1±14.0	27.9±12.1	1.40±0.15	0.89±0.19

Mitochondrial respiratory state, respiratory control ratio (RCR) and ADP/O ratio used here are defined by Chance and Williams (1955). State 3 respiration is in the presence of exogenous substrate and ADP. State 4 respiration is in the presence of only substrate after ADP has been phosphorylated. RCR is equal to state 3 respiration rate divided by state 4 respiration rate. The ADP/O ratio is the number of moles of ADP added divided by the number of moles of oxygen atoms consumed during state 3 respiration. The respiration rate of mitochondria alone approaching state 2 respiration, which is respiration with endogenous substrate exhausted, was 1.23±0.80 nmol O_2 min^{-1} mg^{-1} protein (N=15).

Values are means ± S.D. N=11–19 experiments from 11 preparations for malate and succinate. N=4–8 experiments from 5 preparations for sulfide.

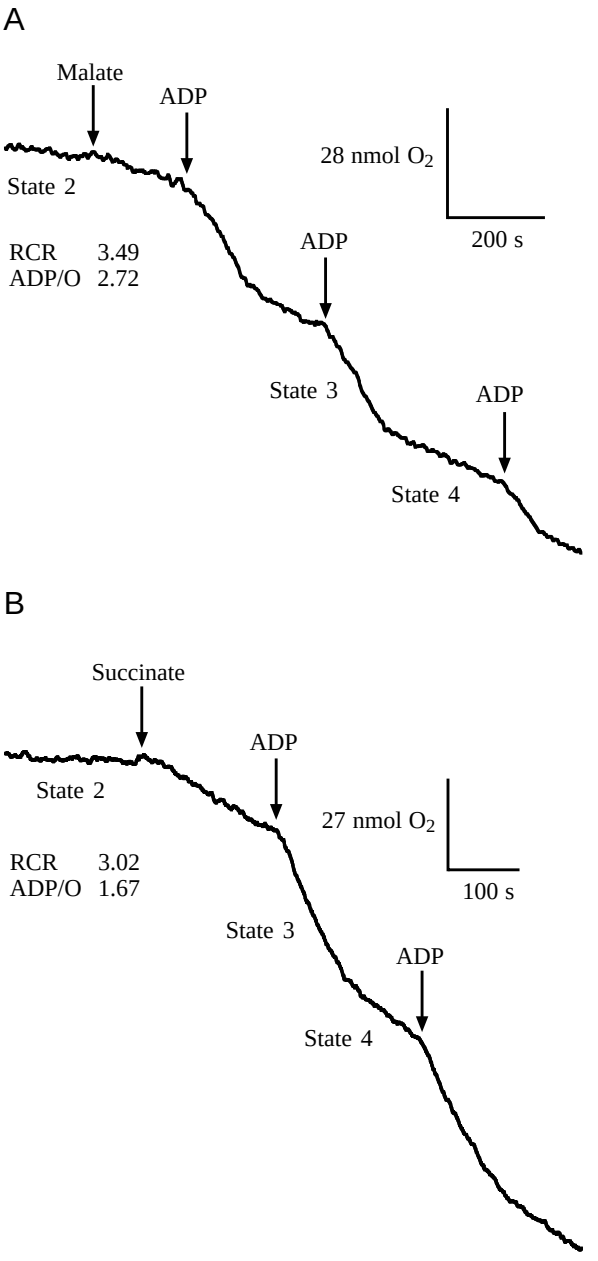

Fig. 1. Oxygraph recordings of isolated *Geukensia demissa* gill mitochondria in the presence of (A) 4 mmol l^{-1} malate or (B) 2.5 mmol l^{-1} succinate. ADP was added in pulses of 82.5 or 165 nmol. Values given for the respiratory control ratio (RCR) and for the P/O ratio are the averages for all additions of ADP per trace.

presence of a small amount of endogenous ADP recycled by potentially active ATPases. Upon the addition of ADP in the presence of substrates, respiration demonstrated a well-defined transition to state 3, then a decrease to state 4 (Fig. 1; Table 1). The respiratory control ratio (RCR) averaged near 3 for carbon substrates, and the mitochondrial P/O ratio averaged near 3 for malate and near 2 for succinate (Table 1). State 3 respiration rate in the presence of succinate was consistently approximately twofold greater than that in the presence of malate (Table 1).

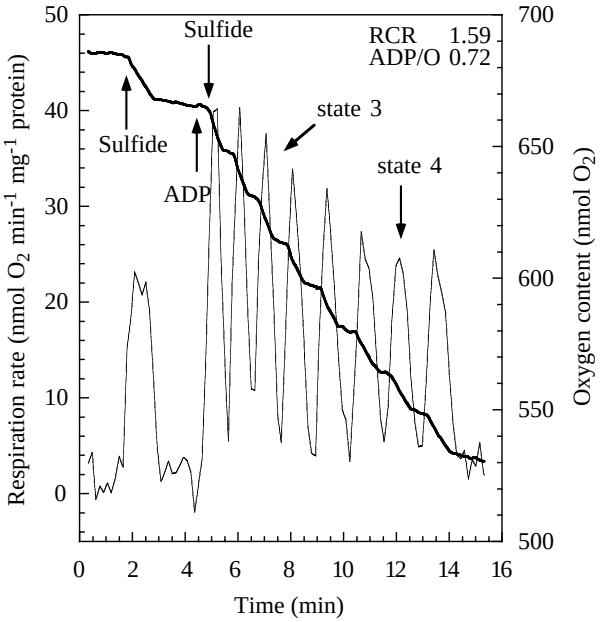

Fig. 2. Oxygraph recording (bold line) of isolated *Geukensia demissa* gill mitochondria in the presence of 82.5 nmol of ADP and repeated additions of 5 μmol l^{-1} sulfide. The bold line represents the amount of oxygen in the sample chamber containing the mitochondrial suspension and the lighter line represents the respiration rate, or the first time derivative of the bold line. RCR, respiratory control ratio.

Mitochondria supplied with 5 μmol l^{-1} sulfide as sole substrate showed a transient rise in respiration rate to 20 nmol O_2 min^{-1} mg^{-1} protein, which nearly doubled during state 3 respiration when ADP was added (Fig. 2). After repeated additions of sulfide, the transient increase in respiration rate in response to sulfide addition returned to approximately 20 nmol O_2 min^{-1} mg^{-1} protein as state 4 was achieved. The RCR for sulfide averaged 1.40, and the P/O ratio averaged approximately 1 (Table 1). The state 3 sulfide/O_2 ratio was 0.97±0.27 (*N*=16).

Respiration as a function of sulfide concentration and the effects of mitochondrial electron flux inhibitors

Carbon-substrate-supported state 3 respiration reached a maximum at approximately 5 μmol l^{-1} sulfide, rising threefold in the presence of malate and twofold in the presence of succinate compared with control values in the absence of sulfide of 11.8±4.8 nmol O_2 min^{-1} mg^{-1} protein (*N*=5) and 21.2±8.2 nmol O_2 min^{-1} mg^{-1} protein (*N*=3), respectively (Fig. 3). Sulfide-supported respiration showed a slight decline above 8 μmol l^{-1} sulfide (Fig. 3), but remained well above control values even at 90 μmol l^{-1} sulfide (data not shown).

Respiratory inhibitors were used to help characterize the path of electron flow within *G. demissa* gill mitochondria during sulfide oxidation. Myxothiazol caused an 89–97 % decrease in state 3 respiration (Table 2). State 3 respiration was not affected by SHAM alone in the presence of malate and was only slightly inhibited in the presence of succinate (Table 2). However, SHAM decreased sulfide-supported respiration by

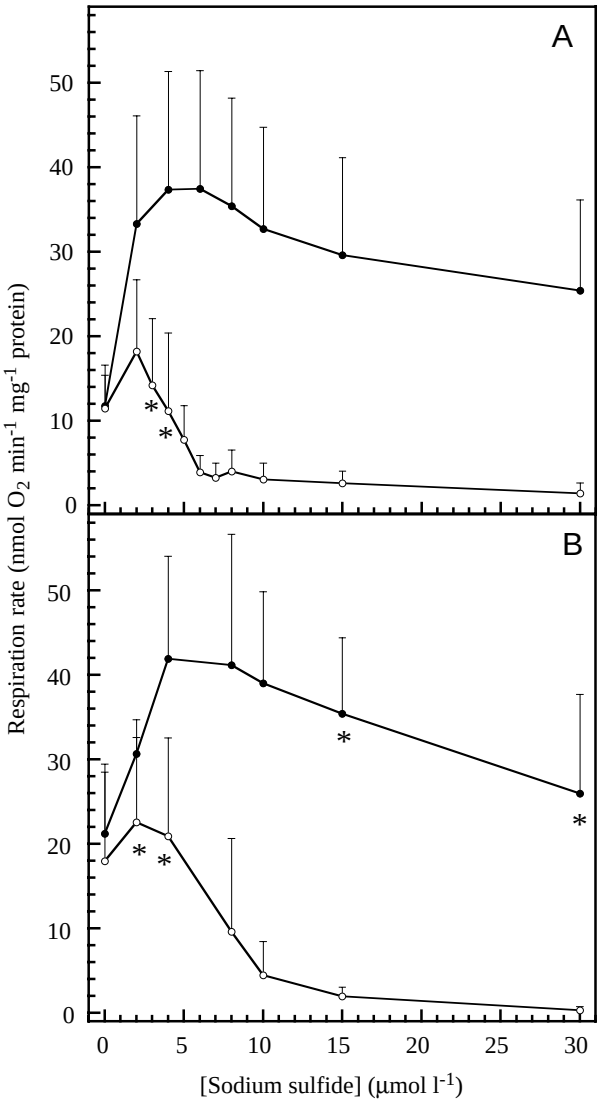

Fig. 3. Respiration rate of isolated *Geukensia demissa* gill mitochondria as a function of sulfide concentration. Mitochondria were held at state 3 respiration in the presence of 2.5 μmol of ADP and (A) 4 mmol l^{-1} malate (N=3–5) or (B) 2.5 mmol l^{-1} succinate (N=3). Sulfide was added with (open circles) and without (filled circles) 1 mmol l^{-1} salicylhydroxamic acid (SHAM). Values are means + S.D.; error bars are shown only to the high side to limit overlap. All values are significantly (P<0.05) different from control values in the absence of sulfide except for those marked with an asterisk.

35 % (Table 2). Fig. 3 shows that, in the presence of SHAM, the addition of sulfide had a much greater inhibitory effect on carbon-substrate-supported state 3 respiration compared with sulfide addition without SHAM. After slight respiratory stimulation at 2 μmol l^{-1} sulfide, state 3 respiration began to show significant inhibition at sulfide concentrations above 5 μmol l^{-1} and was completely eliminated at 30 μmol l^{-1} sulfide (Fig. 3). Fig. 4 further demonstrates the effects of SHAM added to mitochondria held at state 3 respiration with malate before or after the addition of 60 μmol l^{-1} sulfide, a potentially inhibitory concentration. The addition of sulfide subsequent to the addition of SHAM resulted in a 60 % decrease in state 3 respiration rate (Fig. 4B) rather than the normal stimulation (Fig. 4A). When SHAM was added after sulfide, a similar 45 % decrease in malate-supported state 3 respiration rate was observed (Fig. 4A). Cyanide caused an 81–94 % decrease in state 3 respiration rate for all three substrates (Table 2). The combination of cyanide and SHAM caused a slightly but significantly greater inhibition of respiration rate, ranging from 93 to 98 % (Table 2). An additive effect of SHAM and cyanide on carbon-substrate- and sulfide-supported state 3 respiration is apparent (Table 2), suggesting distinct sites of inhibition.

ATP production rate and the effects of mitochondrial electron flux inhibitors

The determination of a discrete amount of ATP in a biological sample is a relatively simple luminometric procedure because the enzyme reaction is rapid. The injection of 0.1 nmol of ATP into a dilute mitochondrial suspension typically resulted in maximum photon generation in approximately 30 s. Maximum photon generation, recorded as relative light units (RLUs), was a linear function of ATP quantity in the range 0.002–20 nmol of ATP (r^2=0.99961). To measure ATP production in a suspension of isolated mitochondria, the change in ATP quantity in the reaction medium was followed over a sufficient period to allow for substrate consumption and production of ATP from ADP. Without added substrate or ADP, isolated gill mitochondria generated a negligible amount of ATP; RLU values typically remained at a steady-state value below 500 (see Fig. 5). However, the addition of ADP alone resulted in a nearly linear rise in ATP concentration over the course of 5 min (Fig. 5A); this probably represents the use of endogenous substrates

Table 2. *Respiration rates of state 3 respiring* Geukensia demissa *gill mitochondria in the presence of mitochondrial inhibitors*

	Percentage inhibition of respiration rate			
	Myxothiazol, 30 μmol l^{-1}	Cyanide, 1 mmol l^{-1}	SHAM, 1 mmol l^{-1}	Cyanide+SHAM, both 1 mmol l^{-1}
4 mmol l^{-1} malate	89.0±3.11 (3)	80.8±4.53 (5)	0±0 (4)	93.1±5.22 (7)***
2.5 mmol l^{-1} succinate	96.5±2.36 (3)	90.9±7.16 (4)	8.08±2.92 (5)	97.7±1.06 (9)**
5 μmol l^{-1} sulfide	88.8±5.82 (3)	93.7±3.37 (3)	35.3±3.83 (3)	96.7±1.10 (4)*

Values are means ± S.D. with the number of experiments in parentheses.
Asterisks denote values significantly different from percentage inhibition by cyanide alone; ***P≤0.001; **P≤0.01; *P≤0.1.
SHAM, salicylhydroxamic acid.

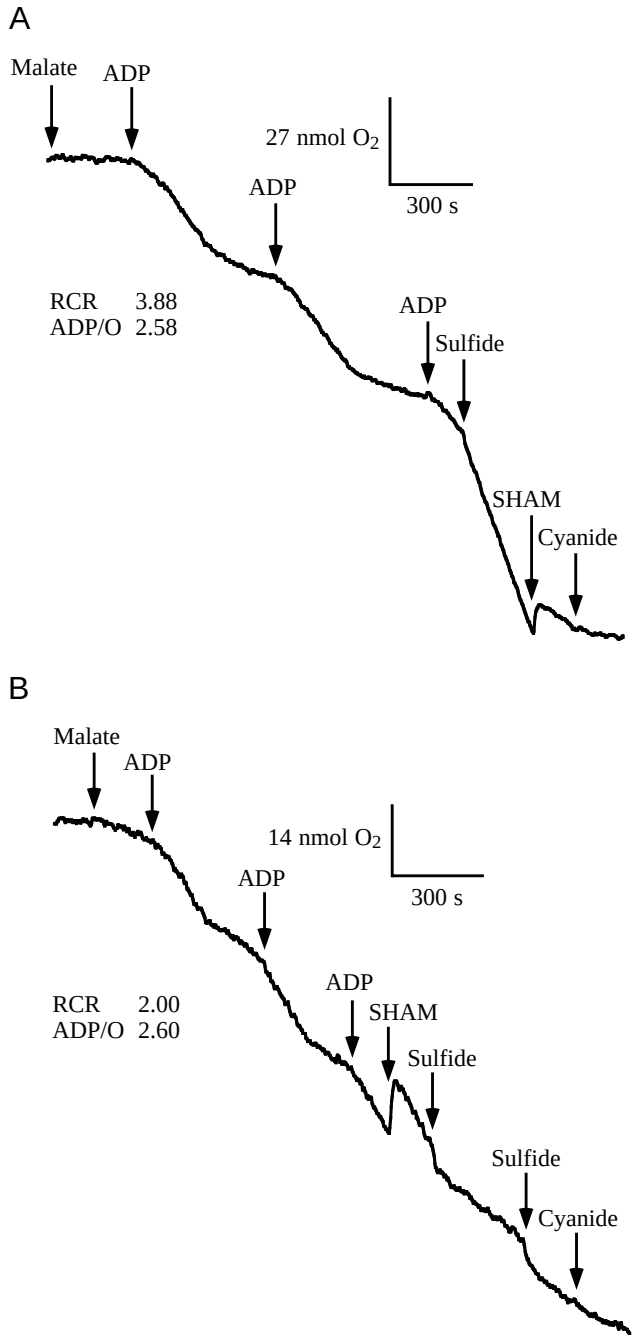

Fig. 4. Oxygraph recordings of isolated *Geukensia demissa* gill mitochondria held at state 3 respiration with 4 mmol l^{-1} malate and 2.5 µmol of ADP just prior to the addition of sulfide and salicylhydroxamic acid (SHAM). (A) Respiration rate is stimulated by the addition of 60 µmol l^{-1} sulfide and subsequently inhibited by 1 mmol l^{-1} SHAM. (B) SHAM at 1 mmol l^{-1} was added, then 60 µmol l^{-1} and 90 µmol l^{-1} sulfide. Cyanide at 1 mmol l^{-1} was added at the end of each experiment to record any additional respiratory inhibition. The first two additions of ADP were 165 nmol (A) and 82.5 nmol (B), and the subsequent averaged respiratory control ratio (RCR) and P/O ratio are given for each experiment. The steep changes in oxygen level result from the addition of a solution with an oxygen content different from that of the mitochondrial suspension in the sample chamber.

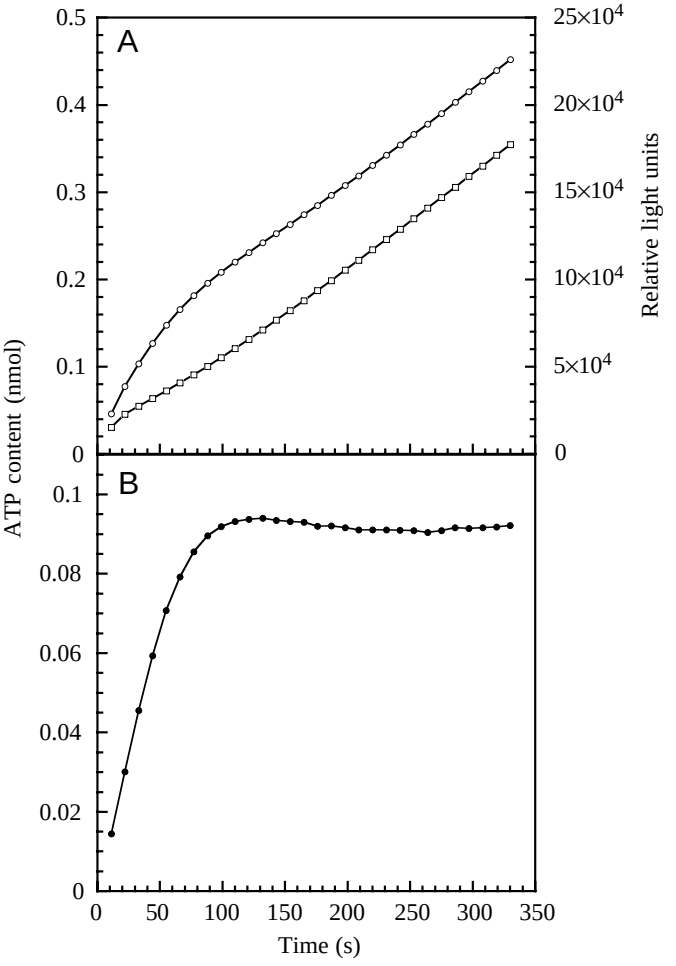

Fig. 5. Typical luminescence traces of ATP content of isolated *Geukensia demissa* gill mitochondria in the presence of sulfide. (A) ATP content as a function of time after the addition of 0.1 nmol of sulfide plus 1.5 nmol of ADP (open circles) and 1.5 nmol of ADP alone (open squares). Corresponding relative light units (RLUs) are shown on the right-hand *y*-axis. In this experiment, an injection of 0.1 nmol of ATP giving a maximum RLU of approximately 5.3×10^4 was used as a standard to calculate ATP content, assuming a linear relationship between ATP content and RLU (see text) and zero RLU at zero ATP. (B) Sulfide-stimulated ATP content as a function of time, calculated as the difference between the luminescence traces shown in A for sulfide plus ADP and ADP alone.

within the mitochondria. The addition of ADP and either malate or succinate resulted in a much steeper rise in ATP content. From this, the background rate was subtracted to show the net ATP production rate for each substrate (Table 3). The addition of ADP and a small non-inhibitory concentration of sulfide resulted in a transient rise in ATP production above background which, after sulfide had been consumed, returned to the background rate (Fig. 5A). Net sulfide-stimulated ATP production lasted approximately 2 min (Fig. 5B), comparable with the time course of sulfide-stimulated mitochondrial respiration under similar conditions (Fig. 2). Sulfide-stimulated ATP production rate, calculated from the initial linear slope of traces such as that shown in Fig. 5B, was

Table 3. *ATP production rates of state 3 respiring* Geukensia demissa *gill mitochondria in the presence of mitochondrial inhibitors*

	ATP production rate (nmol min^{-1} mg^{-1} protein)	Percentage inhibition of ATP production rate		
		Antimycin A, 50 μmol l^{-1}	Cyanide, 1 mmol l^{-1}	SHAM, 1 mmol l^{-1}
Malate	9.91±5.08 (12)	91.1±9.35 (3)	87.7±2.71 (4)	0±0 (3)
Succinate	5.75±2.61 (5)	89.4±12.2 (3)	89.7±7.0 (3)	54.8±14.1 (3)
Sulfide	29.7±10.1 (11)	90.1±0.61 (3)	95.5±0.32 (3)	66.6±0.31 (3)

Values are means ± S.D. with the number of experiments in parentheses.
SHAM, salicylhydroxamic acid.

approximately three- and fivefold greater than that stimulated by malate and succinate, respectively (Table 3). The average ratio of the amount of ATP produced to the amount of sulfide added was 0.63+0.16 (N=9).

The presence of mitochondrial inhibitors had significant effects on substrate-stimulated ATP production. Antimycin A and cyanide reduced ATP production with all three substrates by approximately 90 % (Table 3). Although SHAM had no effect on malate-stimulated ATP production, it did significantly limit succinate- and sulfide-stimulated ATP production by more than 50 % (Table 3). A large proportion of succinate- and sulfide-stimulated ATP production appears to be sensitive to both SHAM and cyanide.

Discussion

Geukensia demissa *gill mitochondrial integrity*

Respirometric traces of mitochondria isolated from the gills of *G. demissa* in the presence of malate and succinate demonstrated well-defined transitions between state 3 and state 4 respiration rates. The computed functional parameters of respiratory control and P/O ratios and the respiration rates of these mitochondria were comparable with those of other bivalve mollusc gill mitochondria (Burcham et al., 1983, 1984; Powell and Somero, 1986a), indicating that the isolation protocol optimized in this study yielded intact, well-coupled and functional mitochondria.

Mitochondrial sulfide oxidation

Sulfide oxidation coupled to ATP synthesis has been observed in mitochondria isolated from several inhabitants of sulfide-rich environments, including the symbiont-harboring gills of the clam *Solemya reidi*, the liver of the killifish *Fundulus parvipinnis*, the whole polychaete worm *Heteromastus filiformis* and the body wall of the polychaete worm *Arenicola marina* (for a review, see Grieshaber and Völkel, 1998). Sulfide-stimulated ATP production rates reported for mitochondria from these organisms range from 4.46 to 67.0 nmol min^{-1} mg^{-1} protein, and ATP/sulfide ratios range from 0.5 to 1.25, values consistent with those reported here for *G. demissa* gill mitochondria.

Doeller et al. (1999) showed that sulfide-mediated ATP production supports cellular energy requirements in intact *G. demissa* gills. Their data suggest a regulatory control over sulfide oxidation by the demand for ATP, and support cytochrome *c* as the site for electron transfer from sulfide oxidation in *G. demissa* gills. In the present study, the sulfide-mediated transitions between state 3 and state 4 respiration rates and the production of ATP are evidence that isolated *G. demissa* gill mitochondria phosphorylate ADP in the presence of sulfide as the sole substrate. Sulfide-stimulated ATP production is probably the result of a contribution of electrons from sulfide oxidation to mitochondrial electron flux, as shown by the inhibition of ATP production in the presence of cyanide, a complex IV electron flux inhibitor. Thus, *G. demissa* may have adapted to its sulfide-rich environment by oxidizing sulfide in gill mitochondria and gaining cellular energy from this reduced inorganic substrate in the process.

A sulfide/O_2 ratio of 0.97 for *G. demissa* gill mitochondria suggests that the four electrons from the oxidation of one hydrogen sulfide molecule, according to the equation:

$$2H_2S + 3H_2O \rightarrow SSO_3^{2-} + 8e^- + 10H^+$$

(O'Brien and Vetter, 1990) are donated to the mitochondrial electron transport system and consumed in the reduction of O_2 to two molecules of water. This ratio is also found in whole gills (J. E. Doeller, D. W. Kraus and M. K. Grieshaber, in preparation). Assuming that ATP synthesis from sulfide oxidation is coupled only to complex IV, which translocates two protons per two electrons (Malatesta et al., 1995), and that the proton/ATP ratio is 4 [three protons translocated by ATP synthase (Nicolls and Ferguson, 1992) and one proton translocated in the exchange of ATP for ADP+P_i], the ATP/sulfide ratio and the P/O ratio would be 1 and 0.5, respectively, if the five scalar protons per sulfide oxidized do not contribute to ATP synthesis, and 2.25 and 1.125, respectively, if the scalar protons are conserved in the ATP synthesis reaction. These theoretical calculations assume that the oxidation of sulfide occurs at the cytosolic side of the mitochondrial inner membrane (O'Brien and Vetter, 1990; Völkel and Grieshaber, 1997) and that oxygen is consumed in the matrix. The ATP/sulfide ratio in *G. demissa* gill mitochondria was 0.63, and the P/O ratio for sulfide was 0.89, close to the theoretical values for ATP synthesis from sulfide oxidation coupled to complex IV without the contribution of the scalar protons.

The inhibition of sulfide-stimulated respiration and ATP production by myxothiazol and antimycin A, respectively, suggests that electrons from sulfide oxidation enter the electron transport system at a site before cytochrome *c*, which differs from the evidence from excised gill tissue (Doeller et al., 1999). The differences between the entry sites of electrons from sulfide oxidation may be explained by the possible presence of more than one sulfide-oxidizing enzyme. Sulfide oxidation is thought to occur by two enzyme systems: the sulfide quinone oxidoreductase, which plays a role in sulfide-dependent anoxygenic photosynthesis in cyanobacteria (Arieli et al., 1994) and in energy transfer in non-sulfur purple bacteria (Schütz et al., 1997, 1999), and sulfide cytochrome *c* oxidoreductase, also called flavocytochrome *c* or sulfide dehydrogenase, which plays a role in energy transfer in chemolithogrophic sulfur-oxidizing bacteria (Schneider and Friedrich, 1994; Visser et al., 1997; Sorokin et al., 1998). Some bacteria may have both enzyme types (Visser et al., 1997). Recently, the gene from a yeast mitochondrial sulfide-oxidizing enzyme that catalyzes the reduction of quinone by sulfide has been shown to have homology with genes from other prokaryotes and eukaryotes, extending the family of flavoenzymes involved in sulfur chemistry to the eukaryotes (Vande Weghe and Ow, 1999). The sulfide-oxidizing enzyme(s) in *G. demissa* gills may also belong to this family of proteins, as was suggested for *Arenicola marina* by Völkel and Grieshaber (1996).

If the site of electron transfer from sulfide oxidation to the electron transport system is before cytochrome *c*, perhaps sulfide-stimulated electron flux through the coenzyme Q pool can bypass complex III or the proton pumping of complex III (Völkel and Grieshaber, 1997). In *G. demissa* mitochondria, sulfide oxidation may be coupled to ATP synthesis only at complex IV, even though electron entry is before cytochrome *c*. The lower than expected values for the ATP/sulfide ratio may be due to partial uncoupling of sulfide oxidation from ADP phosphorylation by two mechanisms. First, hydrogen sulfide may act as an uncoupler (O'Brien and Vetter, 1990). The RCR with sulfide as substrate is lower than that for succinate or malate as substrate, indicating that sulfide oxidation is only partially coupled to ADP phosphorylation. Second, electron flow to an alternative oxidase may function as an uncoupling site (Meeuse, 1975; McIntosh, 1994; Day and Wiskich, 1995).

An alternative pathway of electron flux for sulfide oxidation

Cytochrome *c* oxidase is inhibited by both sulfide and cyanide at the ferric a_3 heme, so the presence of a cyanide-insensitive terminal oxidase may also confer sulfide insensitivity within the mitochondria. In several higher plants, fungi, yeast and protista, a cyanide-insensitive alternative oxidase exists with a branch point typically from the substrate side of complex III at the level of the coenzyme Q pool; electron flux through the alternative pathway has a limited role in ATP synthesis (Moore and Siedow, 1991). Although the function of the alternative oxidase is not clear in many organisms, it is known that thermogenesis as a result of high alternative oxidase activity in floral tissues of aroid plants leads to the evaporation of insect attractants to promote pollination (Meeuse, 1975).

SHAM blockade of cyanide or sulfide-insensitive oxygen flux is often used to identify the presence of an alternative oxidase. Mitochondrial respiration in the soil amoeba *Acanthamoeba castellanii* is stimulated by low concentrations of sulfide and is only partially suppressed at higher concentrations; however, in the presence of SHAM, sulfide inhibition is enhanced at both low and high concentrations (Lloyd et al., 1981). In the lugworm *A. marina*, cytochrome *c* oxidase is sensitive to sulfide (Völkel and Grieshaber, 1997). At low or subinhibitory concentrations, the oxidation of sulfide to thiosulfate supplies reducing equivalents to the electron transport system at the level of ubiquinone/ubiquinol, before complex III, for ATP production. This path utilizes cytochrome *c* oxidase, and the resultant ATP production is unaffected by SHAM. However, if cytochrome *c* oxidase is inhibited by high sulfide concentrations, sulfide oxidation continues as electrons flow through a SHAM-sensitive sulfide-insensitive alternative terminal oxidase. In this case, sulfide oxidation is primarily a detoxification process and does not yield ATP, even though the alternative oxidase branch point is hypothesized to be after complex III (Völkel and Grieshaber, 1997).

A sulfide oxidase pathway involving an alternative oxidase may also be present in *G. demissa* mitochondria. State 3 respiration with a carbon substrate is maximally stimulated by low sulfide concentrations and remains refractory to higher concentrations of sulfide except in the presence of SHAM. This indicates that inhibition of cytochrome *c* oxidase by sulfide is evident only when the alternative oxidase pathway is blocked. In *G. demissa*, cyanide inhibition of carbon-substrate- and sulfide-supported respiration and ATP production suggests that electrons from carbon substrates and sulfide share a common path. However, cyanide inhibition under these conditions is not expected if electron flux from sulfide oxidation can be diverted to an alternative pathway when complex IV is inhibited. Our data show that SHAM inhibits 67 % of the ATP production from the oxidation of sulfide and 55 % from the oxidation of succinate. Perhaps sulfide contributes to a decrease in cytochrome *c* oxidase activity when the alternative pathway is blocked or perhaps the alternative pathway is coupled to ATP production in *G. demissa* mitochondria. SHAM suppression of succinate-stimulated ATP production suggests that a branch point to the alternative oxidase occurs after complex III. Why malate-stimulated ATP production is not inhibited by SHAM remains unclear.

In *G. demissa* mitochondria, the alternative pathway may function to detoxify sulfide and maintain concentrations subinhibitory to cytochrome *c* oxidase, allowing continuous electron flux through the main respiratory chain and energy gain from sulfide oxidation. If electrons from oxidized sulfide are donated at cytochrome *c*, the alternative oxidase may branch from the main respiratory chain at cytochrome *c*. However, if electrons are donated before complex III, the

alternative oxidase may branch at ubiquinone/ubiquinol, perhaps similar to the alternative pathways of plant mitochondria (Moore and Siedow, 1991). The alternative pathway may enhance the capacity of gill mitochondria to act as a sulfide sink and prevent the distribution of sulfide to other aerobically respiring sulfide-sensitive tissues. Compared with gill tissue, excised *G. demissa* cardiac tissue has a much lower capacity to oxidize sulfide, which may indicate a greater sulfide sensitivity of internal tissues in this animal (J. E. Doeller, D. W. Kraus and M. K. Grieshaber, in preparation). A biochemical strategy to oxidize sulfide enzymatically in the outer body tissues to protect the inner tissues from sulfide poisoning is common in inhabitants of sulfide-containing environments (Powell and Somero, 1986b; Fisher, 1990; Arp et al., 1995).

In conclusion, sulfide oxidation is coupled to ATP production in gill mitochondria of *G. demissa*. Electrons from sulfide oxidation flow through complex III of the electron transport system. The low ATP/sulfide stoichiometry and the low RCR for sulfide may indicate that the mitochondria are partially uncoupled in the presence of sulfide. *G. demissa* is exposed to chronic high ambient sulfide concentrations that would poison aerobically poised tissues. The alternative oxidase pathway seems to be essential in detoxifying sulfide and allows the gill mitochondria to act as a sulfide sink and a protective barrier for internal organs.

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