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CHEMOLITHOHETEROTROPHY IN A METAZOAN TISSUE: SULFIDE SUPPORTS CELLULAR WORK IN CILIATED MUSSEL GILLS

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Summary

Hydrogen sulfide, a common constituent of marine intertidal sediments, is both a potent toxin of aerobic cellular respiration and an electron-rich molecule used by some prokaryotic organisms as a source of energy. In ciliated gills from *Geukensia demissa*, a marine mussel from sulfide-rich sediments, sulfide oxidation supports cellular work. Evidence for this comes from measurements of ciliary beat frequency (f_{CB}) as a measure of ATP turnover rate, the rate of gill oxygen consumption ($\dot{M}_{O_2}$) as a measure of ATP production rate, and mitochondrial cytochrome redox state as an indicator of the path of electron flow. Results from experiments performed in the presence and absence of the mitochondrial complex III inhibitor antimycin A to limit endogenous carbon substrate oxidation showed that exposure to sulfide stimulated oxygen consumption and ciliary beating, with cytochrome *c* being the dominant reduced species. These results, along with the resultant $f_{CB}/\dot{M}_{O_2}$ ratio, are qualitatively and quantitatively consistent with the hypothesis that electrons from sulfide oxidation support mitochondrial ATP production. We propose that *Geukensia demissa* gills use sulfide as a respiratory substrate when given the choice and thus function metabolically as facultative chemolithoheterotrophs. Similar conclusions could not be drawn for the ciliated gills from *Mytilus edulis*, a marine mussel from aerated habitats, or for the ciliated lungs from the phylogenetically distinct leopard frog *Rana pipiens*.

Key words: sulphide, gill, ciliary beat frequency, oxygen consumption rate, mitochondrial cytochrome, antimycin A, mussel, *Geukensia demissa*, *Mytilus edulis*, *Rana pipiens*.

Introduction

Sulfide is ubiquitous in diverse natural environments such as deep-sea hydrothermal vents, cold seeps and intertidal estuaries. It is toxic to aerobic metabolism, acting as a high-affinity ligand for the ferric (FeIII) iron in the a_3 heme of cytochrome *c* oxidase and poisoning the mitochondrial electron transport chain (Nicholls, 1975; National Research Council, 1979). However, many organisms inhabiting sulfide-rich environments respire aerobically and possess detoxification mechanisms that allow them to coexist with environmental sulfide (for reviews, see Somero et al., 1989; Grieshaber and Völkel, 1998). Sulfide is an electron-rich compound, capable of donating up to eight electrons per mole when oxidized fully to sulfate. Sulfide-oxidizing chemolithotrophic bacteria such as the thiobacilli use electrons from sulfide oxidation to fuel the production of ATP (for reviews, see Kelly, 1982, 1985). In these bacteria, electrons enter the electron transport chain at cytochrome *c*, resulting in a molar ratio of ATP produced per oxygen atom consumed (P/O ratio) of 1. Mitochondria from animals living in high-sulfide environments may also use sulfide as a respiratory substrate. Powell and Somero (1986) were the first to demonstrate that mitochondria isolated from gills of *Solemya reidi*, a bivalve from sulfide-rich sediments which has intracellular sulfide-oxidizing bacterial symbionts in its gills (Felbeck, 1983), exhibit coupled respiration and ATP production in the presence of sulfide as the sole respiratory substrate, with a P/O ratio of 1. This mitochondrial sulfide oxidation to thiosulfate released four electrons per sulfide which then entered the electron transport chain at cytochrome *c* (Powell and Somero, 1986; O'Brien and Vetter, 1990). Symbiont-free organisms also have the potential to gain energy from sulfide, with sulfide-stimulated ATP production reported in mitochondria isolated from the liver of the sulfide-tolerant California killifish *Fundulus parvipinnis* (Bagarinao and Vetter, 1990), the head-down deposit-feeding polychaete *Heteromastus filiformus* (Oeschger and Vismann, 1994) and the lugworm polychaete *Arenicola marina* (Völkel and Grieshaber, 1996, 1997). Thus, it appears that metazoan mitochondria have the potential to use sulfide as a respiratory substrate to support ATP production. Can mitochondria function in this capacity in intact cells to support cellular work (Doeller, 1995)?

To investigate this question, we worked with two species of symbiont-free mussel, *Geukensia demissa*, the ribbed mussel, and *Mytilus edulis*, the Atlantic blue mussel. *Geukensia demissa* inhabits sulfide-rich intertidal sediment of *Spartina* grass beds (Lee et al., 1996). In contrast, *M. edulis* inhabits rocky intertidal shores with wave action and aeration, and probably does not encounter sulfide. Ciliated mussel gills function to pump water through the mantle cavity and are thus in contact with ambient sea water. Mussel gills respire aerobically, which suggests that *G. demissa* gills may possess mechanisms to avoid sulfide toxicity. Previous studies in our laboratory have demonstrated sulfide-stimulated oxygen consumption of *G. demissa* gills compared with non-stimulated or inhibited oxygen consumption of *M. edulis* gills (Lee et al., 1996). Clemmesen and Jørgensen (1987) determined that ciliated cells, which make up approximately 40 % of the mass of the *M. edulis* gill, consume up to 90 % of the total oxygen consumed by the gill. In addition, using dimensional analysis and the value of 3 ATP molecules hydrolyzed per lateral ciliary beat, Clemmesen and Jørgensen (1987) calculated that, in *M. edulis* gills with lateral cilia beating at 10 Hz, nearly 90 % of the ATP produced by a working lateral ciliated cell is consumed to support ciliary motion. This suggests that ciliary beat frequency, as an indicator of ATP hydrolysis, may also accurately reflect the production of ATP by mitochondria, or ATP turnover.

In the present study, the effects of sulfide on the ciliary beat frequency as a measure of ATP turnover, the rate of oxygen consumption as a measure of ATP production and mitochondrial cytochrome oxidation/reduction (redox) state as an indicator of the path of electron flow in excised gills of *G. demissa* and *M. edulis* were examined under different conditions of energy supply and demand. 5-Hydroxytryptamine (5-HT, also known as serotonin), a hormonal stimulant of lateral ciliary activity (Aiello, 1960), was used to increase ATP demand by the lateral cilia. In experiments to limit the use of endogenous substrate, gills were treated with antimycin A, an inhibitor of mitochondrial complex III at cytochrome *b* directly upstream from cytochrome *c*, the proposed entry site of electrons from sulfide oxidation (O'Brien and Vetter, 1990). In some experiments, ciliated lungs from the leopard frog *Rana pipiens* were also used.

Materials and methods

Collection and maintenance of animals

Geukensia demissa were collected from intertidal *Spartina* grass beds located on Dauphin Island, Alabama, USA, with sediment sulfide levels routinely near 1 mmol l^{-1} but as high as 8 mmol l^{-1} (Lee et al., 1996). Animals were maintained in 75 l aquaria with aerated artificial sea water (ASW; Tropic Marin) at 20 ‰ salinity. Aquaria contained sulfide-generating sediment to a depth of 15 cm, and animals were partially buried within this sediment. *Mytilus edulis* were obtained from sulfide-free habitats (Ocean Resources, Inc., Isle au Haut, Maine, USA) and maintained in sediment- and sulfide-free aerated ASW aquaria at 30 ‰ salinity. The aquaria room was maintained at 23±1 °C. Mussels were used within 2 months of collection.

Excised ciliated gill preparation

Whole gills were excised from living mussels and placed in Millipore-filtered (0.45 μm) ASW at maintenance salinity and room temperature (20–22 °C) for at least 1 h prior to experimentation to allow excess mucus to clear. Gills were then cut into 1 cm^2 sections. For ciliary beat frequency measurements, gill sections were separated into individual demibranchs and carefully cleaned to remove any remaining mucus.

Measurement of ciliary beat frequency

Video microscopy system

Ciliary beat frequency measurements were made using a computerized video microscopy system (Fig. 1) which provides accurate near-real-time measurement of frequencies

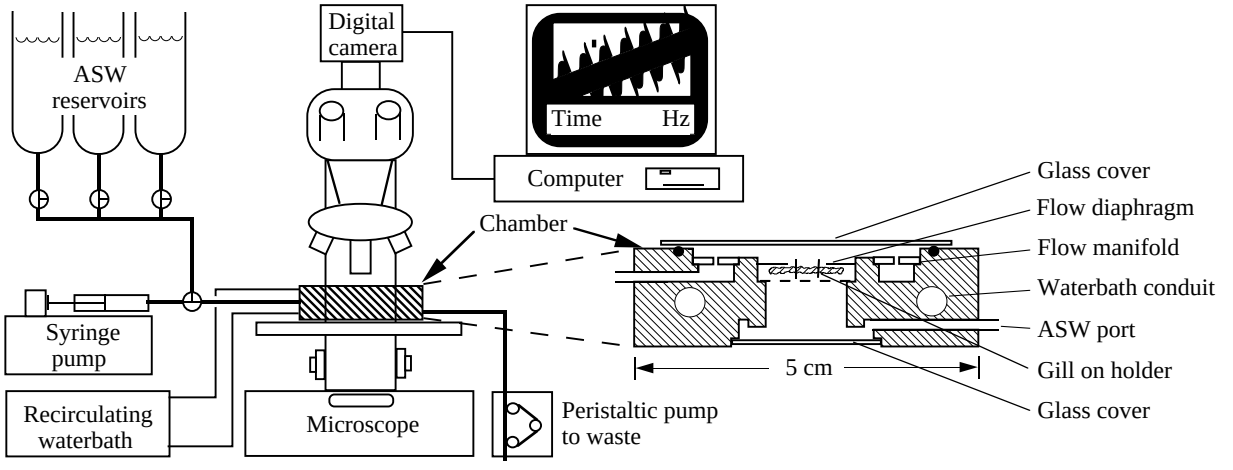

Fig. 1. Video microscopy system for the measurement of ciliary beat frequency, with the 5 cm wide flow-through chamber. The figure on the computer screen represents the metachronal waveform of the lateral cilia on a single gill filament. ASW, artificial sea water.

from 2 to 50 Hz (Gaschen, 1997). A piece of gill demibranch was mounted in a temperature-controlled flow-through chamber on the microscope stage (Fig. 1). Flow through the chamber from the ASW reservoir was gravity-fed at 600 µl min^{-1}, metered by a peristaltic pump (Pharmacia P-3) located downstream from the chamber. An injection port upstream from the chamber allowed the continuous injection of specific treatments into the air-equilibrated inflow stream using a syringe pump (Harvard Apparatus 11). Experiments were performed at 20 °C. A digital video camera (Electrim EC1000HR) was mounted on the camera port of a microscope (Zeiss Universal) trinocular head. The intensity of a single pixel located near the apex of the mussel gill lateral ciliary metachronal wave (Gray, 1930) was sampled by subarray scanning approximately every 5 ms. After 256 samples had been collected, intensity values were sent to an IBM-compatible computer and passed to a software-based fast Fourier transform. The mean magnitude of the resulting power spectrum was displayed on the computer monitor and updated every 2 s, giving ciliary beat frequency in near-real time. A comparison of system-calculated frequencies with set light-emitting diode frequencies up to 50 Hz yielded a linear regression of y=–0.009417+0.99836x, r^2=0.99946. The slope 0.99836 was not significantly different from 1 (P>0.2), indicating the accuracy of the system. System-calculated *G. demissa* lateral gill ciliary beat frequencies of 5–25 Hz differed by less than 5 % from frequencies determined by standard techniques using a stroboscope, indicating that our system is comparable with established methods.

Experimental protocol

The lateral cilia of the mussel gill, located along each filament near the entrance to the interfilament canal, transport ambient water through the gill, and the beat frequency of the lateral cilia is a basic property of the gill pump (Jørgensen et al., 1986). Gill lateral ciliary beat frequency, which declined slowly after gill excision in the absence of 5-HT, was stabilized at beat frequencies from approximately 10 to 25 Hz with exogenous application of 5-HT at 10^{-8} mol l^{-1} to 10^{-5} mol l^{-1}, respectively, and remained stable for at least 4 h. The ASW reservoir contained the appropriate concentration of 5-HT (Fig. 1). Sulfide (30 mmol l^{-1} Na_2S stock) was added at concentrations of 50, 100, 150, 200, 300 and 500 µmol l^{-1} with the syringe pump (Fig. 1). For experiments with antimycin A, gills were first stimulated with 5-HT, then 10 µmol l^{-1} antimycin A (5 mmol l^{-1} stock in ethanol) was injected into the 5-HT-containing inflow stream for at least 15 min. In our investigations, antimycin A blockade of electron flow through complex III is irreversible (B. K. Gaschen, D. W. Kraus and J. E. Doeller, unpublished observation). The syringe containing antimycin A was then replaced with a syringe containing sodium sulfide for injection. During any experiment, ciliary beating was allowed to stabilize, requiring at least 15 min, prior to the beginning of the next experimental treatment. To determine ciliary beat frequency under each set of experimental conditions, at least 10 frequency determinations were recorded at five different locations on the gill. Each experiment was performed at least three times.

Measurement of the rate of oxygen consumption

Respirometry studies were performed using a dual closed-chambered respirometer (Oroboros Oxygraph, model 67097, Paar, Graz, Austria) as described by Lee et al. (1996). Briefly, a 1 cm^2 section of excised gill was mounted onto a 64 mesh stainless-steel screen (Small Parts) holder designed to allow unimpeded ciliary activity (Doeller et al., 1990). The mounted gill was inserted into a chamber containing 6 ml of stirred (500 revs min^{-1}) ASW at 20 °C. The second chamber containing an identical apparatus minus the gill served as a control for blank oxygen consumption rates. During experiments, microliter additions of stock solutions of 5-HT, antimycin A and Na_2S were made through an injection port on top of each chamber using a Hamilton syringe, and rates were measured within 5 min following additions. After each experiment, the gill piece was dried for at least 48 h at 70 °C and weighed. Rates of oxygen consumption rates are reported as pmol O_2 s^{-1} mg^{-1} dry mass. Each value is the mean of at least five measurements.

Measurement of cytochrome oxidation/reduction state

Spectrophotometric studies were performed using a computer-controlled Cary 14 recording spectrophotometer with a scattered transmission accessory (Aviv Associates, Lakewood, NJ, USA) according to the method of Kraus et al. (1996). Briefly, a section of gill approximately 1 cm×0.5 cm was held between two pieces of 100 mesh stainless-steel screen (Small Parts) and placed in the light beam of the spectrophotometer sample cuvette. The 10 ml cylindrical quartz cuvette contained 5 ml of filtered ASW stirred with a stainless-steel turbine at 500 revs min^{-1} and maintained at 20 °C with a waterbath plumbed to the cuvette jacket. A polarographic oxygen sensor (model 2110, Orbisphere) was positioned in the floor of the cuvette to report solution P_{O_2}, and the stopper provided gas inlet and outlet ports, an injection port and the stirring motor shaft. Gas mixtures were prepared and delivered as described by Kraus et al. (1996). Microliter additions of stock solutions of 5-HT, antimycin A and Na_2S were made through the injection port using a Hamilton syringe. To quantify percentage reduction of mitochondrial cytochromes *c*, *b* and *c* oxidase (*aa*$_3$) under experimental conditions, optical density differences at wavelength pairs of 550 and 540 nm, 560 and 580 nm, and 607 and 590 nm, respectively, were compared with optical density differences representing full cytochrome reduction, taken from the difference spectrum of gills under anoxic conditions minus the same gills under aerated conditions. Wavelength pairs in the Soret portion of the spectrum were not used because, although extinction coefficients in this region are larger than in the visible portion, sufficient overlap exists to prevent accurate quantification of cytochrome redox state.

Ciliated frog lung

Ciliated lungs from the leopard frog *Rana pipiens* were used for comparative purposes as an example of a ciliated tissue from a phylogenetically distinct animal with no history of sulfide exposure. *Rana pipiens* (obtained from Charles D. Sullivan Co., Inc., Nashville, TN, USA) were maintained in damp aquaria at room temperature (23 °C), fed and used within 1 week. Lungs were excised from double-pithed frogs and washed in Millipore-filtered frog Ringer's solution (Eshel et al., 1985) to remove blood and mucus. For ciliary beat frequency experiments, the external surface of a lung was removed, and the remaining internal ciliated trabeculae were suspended on a stainless-steel screen in the flow-through microscope chamber and bathed with frog Ringer's solution. For oxygen consumption experiments, a section of dissected lung was placed onto a stainless-steel screen support within the respirometer chamber containing 4 ml of stirred frog Ringer's solution. Measurements of ciliary beat frequency and rate of oxygen consumption were taken in the presence and absence of 100 μmol l^{-1} Na_2S and 10 μmol l^{-1} antimycin A. Experiments were performed at 20 °C and were repeated at least three times.

Data presentation

Values are presented as mean ± standard deviation (number of repetitions). Comparisons were made using the paired two-sample *t*-test (Statview).

Results

Mussel gill ciliary beat frequency as a function of sulfide concentration

In the absence of 5-HT, gills from each mussel had a ciliary beat frequency near 5 Hz approximately 1 h after excision. Low 5-HT concentrations, 10^{-8} to 10^{-7} mol l^{-1}, stabilized ciliary beat frequency near 10 Hz in approximately 10 min (see Fig. 5); similarly, 10^{-5} mol l^{-1} 5-HT stabilized ciliary beat frequency near 25 Hz over a similar time course. The beat frequency of mussel gill lateral cilia in the presence of 5-HT is shown as a function of sulfide concentration in Fig. 2. The effect of sulfide on ciliary beat frequency was more pronounced in *Mytilus edulis* gills than in *Geukensia demissa* gills, especially at low sulfide concentrations. In the presence of low 5-HT concentrations, *G. demissa* gills exhibited little decrease in ciliary beat frequency, whereas *M. edulis* ciliary beat frequency decreased significantly (P<0.0005) at 50 μmol l^{-1} Na_2S and fell to near zero at 100 μmol l^{-1} Na_2S. In the presence of 10^{-5} mol l^{-1} 5-HT, *G. demissa* ciliary beat frequency was unaffected by 50 μmol l^{-1} sulfide, whereas *M. edulis* ciliary beat frequency exhibited a significant (P<0.0005) reduction. Sulfide concentrations of 100 μmol l^{-1} and higher caused a similar decline in ciliary beat frequency in the gills from both mussel species, although ciliary beat frequency was not fully inhibited in either, even at 500 μmol l^{-1} sulfide.

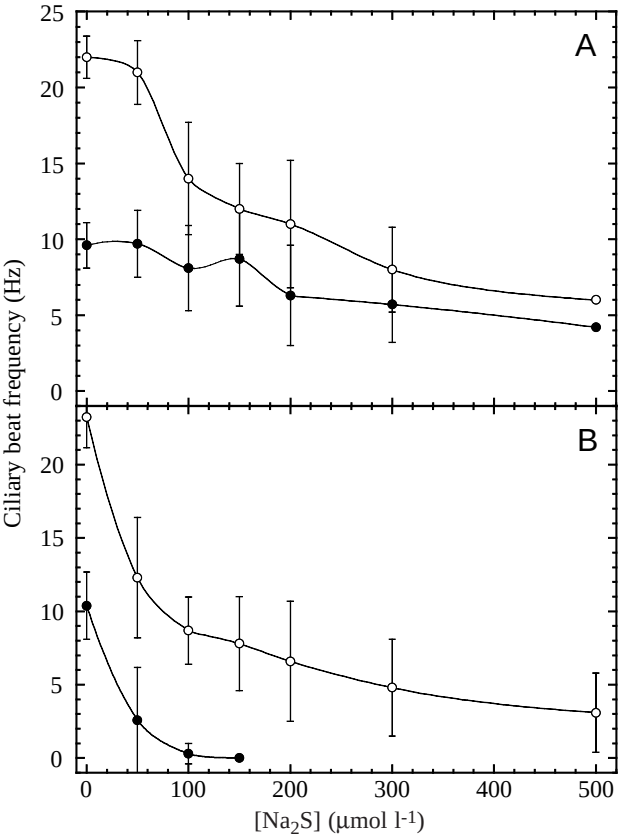

Fig. 2. Lateral ciliary beat frequency of mussel gills in the presence of low (10^{-8} to 10^{-7} mol l^{-1}; filled circles) and high (10^{-5} mol l^{-1}; open circles) concentrations of 5-HT as a function of sulfide concentration. Values are means ± S.D., N=3. (A) *Geukensia demissa*. (B) *Mytilus edulis*.

Effects of sulfide on ciliary beat frequency, rate of oxygen consumption and the $f_{CB}/\dot{M}_{O_2}$ *ratio of ciliated tissue*

Fig. 3 presents ciliary beat frequencies (Fig. 3A) and rates of oxygen consumption (Fig. 3B) of mussel gills stimulated with a low concentration of 5-HT and the ciliated lung of *R. pipiens*, all exposed to 100 μmol l^{-1} Na_2S, as fraction of the control value. Control values are given in Table 1. We have also calculated the ratio of ciliary beat frequency (f_{CB}) to rate of oxygen consumption ($\dot{M}_{O_2}$), termed the $f_{CB}/\dot{M}_{O_2}$ ratio (Fig. 3C). Changes in this ratio may reflect changes in the ratio of ATP produced per oxygen consumed, i.e. the mitochondrial P/O ratio, if the rate of ATP hydrolysis is assumed to be equal to the rate of ATP production. This assumption is supported by the observation using ^{31}P-nuclear magnetic resonance (NMR) spectroscopy that ATP levels of mussel gills remain near control values under the conditions of these experiments (J. E. Doeller, D. W. Kraus and W. R. Ellington, preliminary results). In *G. demissa* gills in the presence of 100 μmol l^{-1} Na_2S, ciliary beat frequency remained unchanged while $\dot{M}_{O_2}$ nearly tripled; the resultant $f_{CB}/\dot{M}_{O_2}$ ratio decreased to approximately 0.3 times the control value. In contrast, in the presence of 100 μmol l^{-1} Na_2S, *M. edulis* gills exhibited a large decrease in ciliary beat frequency to approximately 0.1 times the control value, while

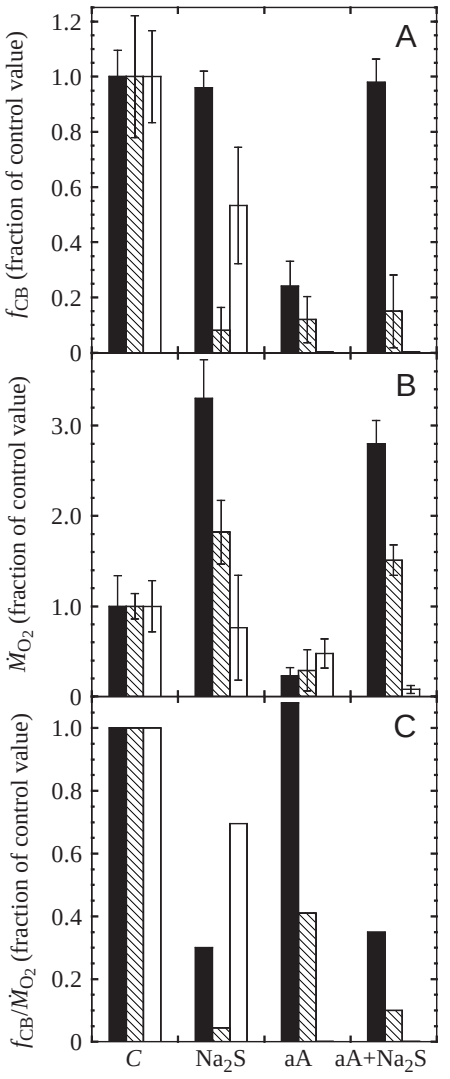

Fig. 3. Measurements of *Geukensia demissa* gills (filled columns), *Mytilus edulis* gills (hatched columns) and *Rana pipiens* lungs (open columns), presented as fraction of the control value (*C*), in the presence of 100 μmol l^{-1} Na_2S, 10 μmol l^{-1} antimycin A (aA) and a combination of the two treatments (aA+Na_2S). Control values are given in Table 1. (A) Ciliary beat frequency (f_{CB}) (*N*=3). (B) Rate of oxygen consumption ($\dot{M}_{O_2}$) (*N*=5). (C) Ratio of ciliary beat frequency to $\dot{M}_{O_2}$ ($f_{CB}/\dot{M}_{O_2}$). Values are means ± S.D.

$\dot{M}_{O_2}$ increased to approximately 1.7 times the control value; the resultant $f_{CB}/\dot{M}_{O_2}$ ratio decreased to approximately 0.1 times the control value. In *R. pipiens* lung, both ciliary beat frequency and $\dot{M}_{O_2}$ decreased significantly ($P \leqslant 0.375$ for $\dot{M}_{O_2}$; $P \leqslant 0.0005$ for f_{CB}), and the resultant $f_{CB}/\dot{M}_{O_2}$ ratio only declined to 0.7 times the control value.

Effects of sulfide on endogenous substrate-limited ciliated tissue

Inhibition of mussel gill mitochondrial complex III by antimycin A

To verify that antimycin A inhibits electron flow in mitochondria within *G. demissa* gill, optical spectra of intact gills were recorded (Fig. 4). The optical difference spectrum of anoxic gills equilibrated with nitrogen gas minus the spectrum of the same gills equilibrated with air (Fig. 4A) clearly shows the optical signature of reduced minus oxidized mitochondrial cytochromes, with absorption maxima at 445 and 607 nm for cytochrome *c* oxidase (aa_3), at 430 and 560 nm for cytochrome *b*, and at 420 and 550 nm for cytochrome *c*. The optical difference spectrum of *G. demissa* gills in the presence of 10 μmol l^{-1} antimycin A minus the spectrum of the same gills in the absence of antimycin A (Fig. 4B) shows absorption maxima at 430 and 560 nm, typical of reduced cytochrome *b*, while the maxima of cytochromes *c* and *c* oxidase downstream from the block remain unchanged. This evidence indicates that exogenous treatment of gills with 10 μmol l^{-1} antimycin A is effective on mitochondria *in situ* and leads to the singular endogenous substrate-mediated reduction of cytochrome *b* by the inhibition of electron flow at complex III. Inhibition by antimycin A, although not complete, reaches a limit at approximately 10 μmol l^{-1}, where cytochrome *b* is approximately half-reduced and ciliary beat frequency and $\dot{M}_{O_2}$ are approximately 0.2 times control values.

Table 1. *Ciliary beat frequencies and rates of oxygen consumption of* Geukensia demissa *gills,* Mytilus edulis *gills and* Rana pipiens *lungs in the absence of sulfide*

Tissue	Ciliary beat frequency Hz	Rate of oxygen consumption (pmol s^{-1} mg^{-1} dry mass)
Geukensia demissa gills[a]	9.6±1.5 (12)[b]	13.2±2.7 (8)[b,c]
Mytilus edulis gills[d]	10.4±2.3 (5)[b]	16.5±2.3 (5)[b,c]
Rana pipiens lungs	8.6±1.9 (7)[e]	8.8±2.5 (9)

Values are means ± S.D. (*N*).
[a]Value in the presence of 10^{-7} mol l^{-1} 5-HT.
[b]Similar to values reported by Clemmesen and Jørgengen (1987).
[c]Similar to values reported by Lee et al. (1996).
[d]Value in the presence of 10^{-8} mol l^{-1} 5-HT.
[e]Similar to values reported for frog palate by Puchelle et al. (1983), Spungin and Silberberg (1984) and Eshel et al. (1985).

Ciliary beat frequency

Fig. 5 shows the time course of the ciliary beat frequency of *G. demissa* gill stimulated with a low concentration of 5-HT followed by treatment with 10 μmol l^{-1} antimycin A and 100 μmol l^{-1} Na_2S. In the presence of antimycin A, ciliary beat frequency decreases from approximately 10 Hz to approximately 4 Hz (Fig. 5A). When sulfide is added to the inflow stream, ciliary beat frequency recovers to 8–10 Hz. Further decrease and recovery of ciliary beat frequency can be achieved by subsequent removal and addition of 100 μmol l^{-1} Na_2S (Fig. 5A). This phenomenon was independent of the order of addition of antimycin A and sulfide (Fig. 5B).

Ciliary beat frequency, $\dot{M}_{O_2}$ and the $f_{CB}/\dot{M}_{O_2}$ ratio

The effects of antimycin A and sulfide on ciliary beat

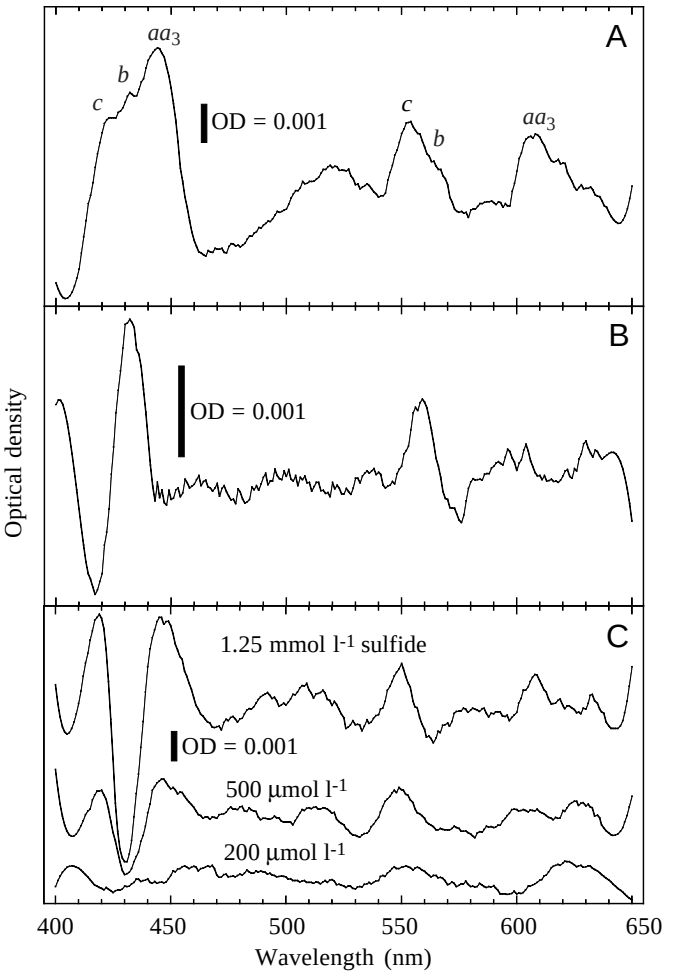

Fig. 4. Optical difference spectra of *Geukensia demissa* gills as a function of wavelength. (A) The spectrum of gills in nitrogen-equilibrated sea water minus the spectrum for the same gills in air-equilibrated sea water. See text for a description of mitochondrial cytochromes. (B) The spectrum of gills in sea water containing 10 µmol l^{-1} antimycin A minus the spectrum for the same gills in sea water. (C) The spectrum of gills in sea water containing 10 µmol l^{-1} antimycin A and sulfide at 200 µmol l^{-1} (lower trace), 500 µmol l^{-1} (middle trace) and 1.25 mmol l^{-1} (upper trace) minus the spectrum for the same gills in sea water containing 10 µmol l^{-1} antimycin A. OD, arbitrary optical density units.

frequency, $\dot{M}_{O_2}$ and $f_{CB}/\dot{M}_{O_2}$ are shown in Fig. 3. In gills from both mussel species, ciliary beat frequency and $\dot{M}_{O_2}$ decreased in the presence of 10 µmol l^{-1} antimycin A. In *G. demissa* gills, the resultant $f_{CB}/\dot{M}_{O_2}$ ratio of 1.1 times the control value possibly indicates a more tightly coupled system than that in *M. edulis* gills, with a resultant $f_{CB}/\dot{M}_{O_2}$ ratio of 0.4 times the control value. In *R. pipiens* lung, antimycin A abolished ciliary beating. In *G. demissa* gills, the addition of 100 µmol l^{-1} Na_2S restored ciliary beating and caused $\dot{M}_{O_2}$ nearly to triple, resulting in a $f_{CB}/\dot{M}_{O_2}$ ratio of 0.3 times the control value. In *M. edulis* gills, sulfide caused slight recovery of ciliary beat frequency and a significant increase in $\dot{M}_{O_2}$; the $f_{CB}/\dot{M}_{O_2}$ ratio decreased to 0.1 times the control value. In *R. pipiens* lung, ciliary beat frequency did not recover upon the addition of 100 µmol l^{-1} Na_2S.

Cytochrome redox state

Optical difference spectra of *G. demissa* gills in the presence of 10 µmol l^{-1} antimycin A and sulfide minus the spectra for same gills in the absence of sulfide are shown in Fig. 4C at three sulfide concentrations. Although the difference spectrum at 200 µmol l^{-1} Na_2S provides little evidence of reduced cytochromes, cytochrome reduction becomes clear at higher sulfide concentrations. At 500 µmol l^{-1} Na_2S, cytochrome *c* is approximately 80 % reduced and cytochrome *c* oxidase is approximately 40 % reduced, and at 1.25 mmol l^{-1} Na_2S,

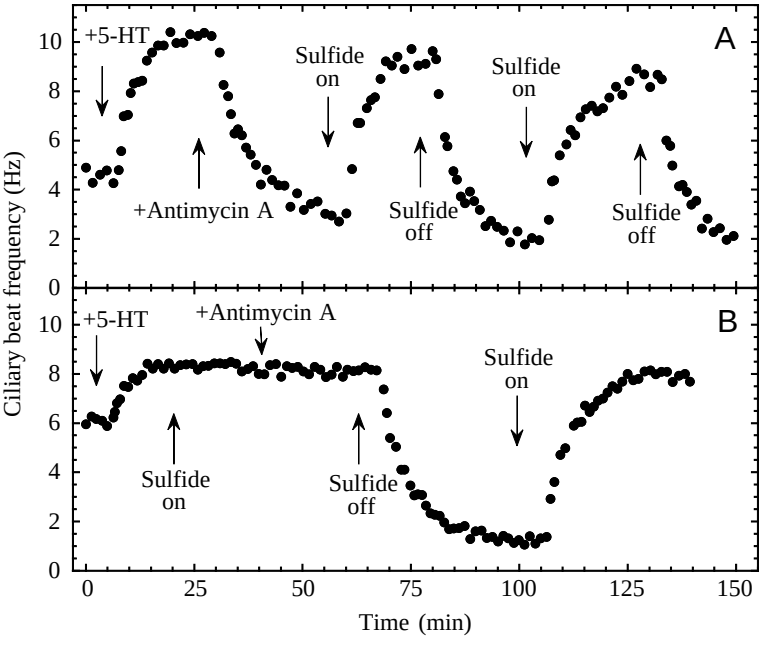

Fig. 5. *Geukensia demissa* gill ciliary beat frequency as a function of time after the addition and/or removal of 10^{-7} mol l^{-1} 5-HT, 10 µmol l^{-1} antimycin A and 100 µmol l^{-1} Na_2S. (A) Trace from an experiment in which antimycin A was added before sulfide. (B) Trace from an experiment in which sulfide was added before antimycin A.

reduction reaches 100 % and 80 %, respectively, suggesting that reducing equivalents from sulfide may be conducted from cytochrome *c* to cytochrome *c* oxidase. In the Soret portion of the difference spectra at 500 μmol l^{-1} and 1.25 mmol l^{-1} Na_2S, a clear trough at 430 nm may represent the reoxidation of cytochrome *b* under these conditions.

Discussion

Hydrogen sulfide is highly toxic to aerobic respiration, and animals from high-sulfide environments have mechanisms to avoid sulfide toxicity. Yet sulfide can potentially supply electrons to the mitochondrial electron transport chain for energy gain, and many animals may be able to benefit energetically from sulfide oxidation. Some studies have demonstrated that mitochondria isolated from the inhabitants of sulfide-containing sites exhibit coupled respiration and ATP production in the presence of sulfide as the sole respiratory substrate (Powell and Somero, 1986; Bagarinao and Vetter, 1990; Oeschger and Vismann, 1994; Völkel and Grieshaber, 1996, 1997). Here, we present the following evidence that sulfide-mediated ATP production can support cellular work in an intact tissue: in the presence of the mitochondrial complex III inhibitor antimycin A, which limits the oxidation of endogenous carbon substrates, ciliary beat frequency in the gills of *G. demissa* can be increased and decreased by the addition and removal of sulfide, respectively. The reduction in the $f_{CB}/\dot{M}_{O_2}$ ratio in the presence of sulfide and in the presence of antimycin A plus sulfide is quantitatively consistent with electrons from sulfide oxidation entering the electron transport chain at cytochrome *c*; and cytochrome *c* becomes reduced in the presence of sulfide, suggesting that cytochrome *c* may be the entry point for electrons from sulfide oxidation.

The demonstration that ciliary beat frequency is increased and decreased by the addition and removal of sulfide, respectively, in the presence of the mitochondrial complex III inhibitor antimycin A is dramatic support for sulfide use as a respiratory substrate in intact ciliated gills of *Geukensia demissa*. When electron flow from endogenous substrate is blocked at complex III, confirmed by optical spectra showing reduced cytochrome *b* in the presence of antimycin A (Fig. 4B), ciliary beating or ATP turnover rate can be maintained only in the presence of sulfide, implying that sulfide oxidation contributes to ATP production. This demonstration was only possible under the condition of limited endogenous substrate oxidation. Although inhibition of electron flow at complex III by antimycin A represents a non-physiological situation, we argue that, without such a blockade, gill mitochondria readily use sulfide as a respiratory substrate to maintain a constant ATP turnover rate and ciliary beat frequency, as is observed in the presence of sulfide alone.

In *G. demissa* gills in the presence of sulfide or sulfide plus antimycin A, ciliary beat frequency remained nearly constant, yet $\dot{M}_{O_2}$ nearly tripled and the resultant $f_{CB}/\dot{M}_{O_2}$ $\dot{M}_{O_2}$ ratio decreased to 0.3 times the control value. Any reduction in the $f_{CB}/\dot{M}_{O_2}$ ratio could suggest that sulfide may be oxidized primarily for the purpose of detoxification or that sulfide may partially uncouple mitochondrial oxidative phosphorylation. Sulfide oxidation for the purpose of detoxification is exhibited by many animals inhabiting sulfide-rich environments. Sulfide oxidation can be mediated by enzymes, heme proteins and/or mitochondria (for reviews, see Somero et al., 1989; Arp et al., 1995; Völkel, 1995; Grieshaber and Völkel, 1998). Extramitochondrial sulfide oxidation appears to be effective in protecting cytochrome *c* oxidase from sulfide poisoning (Arp et al., 1995; Völkel, 1995). Mitochondrial sulfide oxidation for detoxification may result from the uncoupling of oxidation from phosphorylation. O'Brien and Vetter (1990) argue that sulfide may uncouple *Solemya reidi* mitochondria since, given the number of protons available from the oxidation of sulfide to thiosulfate, Powell and Somero (1986) found a lower than expected ratio of ATP produced to sulfide oxidized in these mitochondria. The mechanism of mitochondrial uncoupling may involve the diffusion of H_2S across the inner mitochondrial membrane into the more alkaline matrix where proton dissociation occurs (O'Brien and Vetter, 1990). However, mitochondrial uncoupling to increase sulfide oxidation for detoxification would seem to require strict cellular regulation to avoid the production of reduced ATP resulting from either excessive uncoupling or sulfide poisoning. We argue that the $f_{CB}/\dot{M}_{O_2}$ ratio is a good indicator of the mitochondrial P/O ratio and that, in *G. demissa* gills, the reduction in the $f_{CB}/\dot{M}_{O_2}$ ratio to 0.3 times the control value is quantitatively consistent with electrons from sulfide oxidation entering the electron transport chain at cytochrome *c*, decreasing the mitochondrial P/O ratio from approximately 3 to approximately 1. This implies that, in the presence of sulfide, *G. demissa* gill mitochondria may limit carbon substrate oxidation. Sulfide inhibition of succinate dehydrogenase (Bergstermann and Lummer, 1947, as cited in National Research Council, 1979; Khan et al., 1990) may represent part of a mechanism to limit the supply of reduced coenzymes to the mitochondrial electron transport chain when oxidative phosphorylation is being powered by sulfide oxidation.

In *M. edulis* gills, the large reduction in $f_{CB}/\dot{M}_{O_2}$ ratio suggests that sulfide probably uncouples mitochondrial oxidative phosphorylation. Sulfide stimulation of gill $\dot{M}_{O_2}$ is associated with some thiosulfate production (although only approximately 0.1 times that of *G. demissa* gills; J. E. Doeller, D. W. Kraus and M. K. Grieshaber, in preparation); thus, the operation of sulfide detoxification pathways may also contribute to the low $f_{CB}/\dot{M}_{O_2}$ ratio. In *R. pipiens* lung, the moderate decline in the $f_{CB}/\dot{M}_{O_2}$ ratio is probably the result of sulfide inhibition of cytochrome *c* oxidase which, in turn, limits oxygen consumption and ATP production and thus ciliary beating. Because ciliary beating was inhibited to a slightly greater extent than oxygen consumption, oxidative phosphorylation may have become partially uncoupled as well. Frog lung ciliary beating was completely inhibited by antimycin A and could not be revived by sulfide, indicating that the response of antimycin-A-treated gill tissue to sulfide is not a response of ciliated tissue in general. It is unlikely that

sulfide directly inhibits cilia because, in the presence of antimycin A, sulfide mediates full recovery of ciliary beating in *G. demissa* gills and slight recovery in *M. edulis* gills.

Finally, optical difference spectra of intact *G. demissa* gills show cytochrome *c* as the dominant reduced species in the presence of low to intermediate concentrations of sulfide (Fig. 4C). If sulfide were acting solely as a respiratory inhibitor, its effects should perhaps be observed spectrally as an initial reduction of cytochrome *c* oxidase and, in fact, the combination of sulfide with the ferric a_3 heme of cytochrome *c* oxidase produces a spectral species nearly indistinguishable in the visible region (607 nm) from reduced cytochrome *c* oxidase (Nicholls, 1975). Instead, the more extensive reduction of cytochrome *c* is strong evidence that cytochrome *c* is near the entry point for electrons from sulfide oxidation into the mitochondrial electron transport chain. Wavelength minima at 430 nm in these optical difference spectra indicate some reoxidation of cytochrome *b* under these conditions which, as a working hypothesis, may reflect sulfide inhibition of endogenous substrate oxidation as follows. Antimycin A inhibits oxygen consumption and ciliary beating, but not completely, and the continued, albeit slow, flow of electrons through complex III maintains cytochrome *b* in the reduced state. However, in the presence of sulfide, endogenous substrate catabolism is inhibited, perhaps at succinate dehydrogenase or at other sites upstream from complex III, and electrons that exit cytochrome *b* are not replaced, leading to its reoxidation.

In conclusion, we have presented evidence consistent with the hypothesis that electrons from sulfide oxidation enter the mitochondrial electron transport chain at cytochrome *c*, leading to ATP production which supports cellular work in *G. demissa* gills. Additional evidence consistent with this hypothesis includes rates of sulfide oxidation that are proportional to f_{CB} and thus closely match ATP turnover rates in intact gills (J. E. Doeller, D. W. Kraus and M. K. Grieshaber, in preparation) and coupled respiration of mitochondria isolated from *G. demissa* gills using sulfide as the sole substrate, with a P/O ratio of 1 (V. Parrino, D. W. Kraus and J. E. Doeller, in preparation). To our knowledge, this is the first report of sulfide-supported cellular work in an intact metazoan tissue. The response to sulfide of *G. demissa* heart, another hard-working but more internal tissue than the gills, includes a relatively weak sulfide oxidation capacity and a partially inhibited ATP turnover rate (J. E. Doeller, D. W. Kraus and M. K. Grieshaber, in preparation), suggesting that sulfide use as an energy source may be tissue-specific in *G. demissa* perhaps only in those tissues that experience chronic exposure to sulfide.

It is well known that many bacteria exploit sulfide-rich habitats with different metabolic strategies for generating cellular energy (Atlas, 1997). Most of these bacteria are chemolithotrophs, oxidizing reduced sulfur compounds for energy production. Many of these bacteria are also autotrophs, obtaining organic carbon from the energetically expensive process of carbon fixation. Chemolithoautotrophs can be either obligate or facultative, able to obtain carbon heterotrophically. When given a choice of substrates, bacteria often choose a preferred substrate. One mechanism enabling choice is catabolite repression, in which products of catabolic reactions regulate other cellular reactions such as metabolic pathways, membrane transport, transcription or translation, etc. When facultative chemolithoautotrophs are exposed to both inorganic and organic nutrients, they must choose between carbon catabolism and lithotrophy as a source of electrons for energy production. For example, when the facultative chemolithoautotroph *Thiobacillus intermedius* was supplied with both glucose and thiosulfate, carbon catabolism was limited by inhibition or repression, possibly by thiosulfate itself, of the glucose transport system and other carbon catabolic pathways (for a review, see Matin, 1978). These types of study demonstrate that, in bacteria with versatile metabolic pathways, carbon substrates can be spared from combustion if enough metabolic energy is available from the combustion of inorganic substrates. Under these conditions, assimilated organic carbon is used for growth and reproduction. A metabolic strategy that results in carbon sparing may provide a selective advantage.

One small and not very well-known group of bacteria are the true chemolithoheterotrophs, represented by some strains of *Thiobacillus perometabolis* (for a review, see Kuenen et al., 1985). These bacteria use reduced sulfur compounds as inorganic energy sources but are obligate heterotrophs because they cannot fix carbon dioxide. Sulfur-oxidizing chemolithoheterotrophs live in habitats with plenty of reduced sulfur compounds but also with enough organic material that carbon dioxide fixation is probably not energetically favorable. The mussel *G. demissa* may occupy an analogous niche to these bacteria. The intertidal habitat of *G. demissa* is highly reduced, with high levels of sulfide (Lee et al., 1996) and abundant organic carbon, mostly from decaying vegetation. Given this niche and the evidence presented above, we propose that *Geukensia demissa* gills use sulfide as a respiratory substrate when given the choice and thus function metabolically as chemolithoheterotrophs.

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