Sulfide consumption by mussel gill mitochondria is not strictly tied to oxygen reduction: measurements using a novel polarographic sulfide sensor

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Summary

Some organisms that survive in environments rich in hydrogen sulfide possess specific metabolic pathways for sulfide oxidation and subsequent use of reducing equivalents in oxidative phosphorylation, a process called chemolithoheterotrophy. This process is dependent on ambient oxygen partial pressure and environmental sulfide exposure. To define accurately the kinetics of sulfide metabolism and its dependence on cellular conditions, we have developed a polarographic sulfide sensor (PSS) to measure sulfide concentrations directly and continuously under physiological conditions.

The ribbed mussel Geukensia demissa, an inhabitant of sulfide-rich coastal sediments, consumes sulfide in a chemolithoheterotrophic metabolic strategy. Gill mitochondria use sulfide as respiratory substrate for ATP production, and sulfide consumption is sufficiently rapid and so kinetically complex that only continuous real-time detection captures these events. Under normoxic conditions, oxygen and sulfide consumption are matched. Under hypoxic to anoxic conditions, however, sulfide consumption continues without commensurate oxygen consumption, and these results can be duplicated at higher oxygen conditions by selective blockade of terminal oxidases. These metabolic capabilities depend on prior environmental sulfide exposure, which suggests substantial mitochondrial metabolic plasticity. The recent finding that endogenous sulfide is a critical cell signaling molecule in all organisms suggests that the metabolic pathways that tightly control cellular sulfide levels are widespread. Sensors that accurately report sulfide concentrations under physiologically relevant conditions are valuable tools with which to explore the expanding role of sulfide in biological systems.

Key words: sulfide, mitochondria, oxygen, sensor, ribbed mussel, Geukensia demissa.

Introduction

Hydrogen sulfide, H₂S, is a common constituent of several natural and man-made aquatic habitats, such as reduced intertidal sediments, hydrothermal vents, pulp mill effluents and sewage outfalls. Hydrogen sulfide binds to the hemoglobin porphyrin ring, disrupting oxygen delivery (Berzofsky et al., 1971), and it also binds with high affinity to the ferric iron in the a3 heme of cytochrome c oxidase (Nicholls, 1975), preventing oxygen reduction. Hydrogen sulfide is thus a potent toxin of aerobic cellular respiration. Using systems to prevent sulfide poisoning, animals from sulfide-rich habitats exhibit aerobic respiration with sulfide-sensitive cytochrome oxidases (Somero et al., 1989; Arp et al., 1995; Grieshaber and Völkel, 1998).

Besides being a potent respiratory toxin, hydrogen sulfide is also a source of reducing equivalents, and many organisms possess mechanisms that enable them to take advantage of sulfide’s reductive poise. Most organisms that use sulfide to their energetic advantage are chemolithoautotrophic prokaryotes, with metabolic strategies to capture energy from sulfide oxidation to support oxidative phosphorylation and carbon dioxide fixation (Atlas, 1996). A select few prokaryotes are chemolithoheterotrophs that obtain reduced carbon compounds heterotrophically and utilize the reducing potential of hydrogen sulfide for oxidative phosphorylation (Kuenen et al., 1985). Mitochondria from some metazoans also possess this ability (Powell and Somero, 1986; reviewed in Grieshaber and Völkel, 1998). We have shown that the gills of the ribbed mussel Geukensia demissa use sulfide-based ATP production to support ciliary beating, thus exhibiting metazoan chemolithoheterotrophy (Doeller et al., 1999, 2001). In this case, electrons resulting from sulfide oxidation are fed into the mitochondrial electron transport chain, most likely at cytochrome c, for the production of ATP, with ATP/oxygen atom and sulfide/oxygen molecule ratios both of one (Parrino et al., 2000; Doeller et al., 2001).

The process of sulfide-based metazoan chemolithoheterotrophy is influenced by several factors, one being oxygen. The initial steps of sulfide oxidation catalyzed...
Environmental sulfide exposure also influences the process of sulfide-based metazoan chemolithoheterotrophy. Intact gills from *Geukensia demissa* maintained in low sulfide conditions exhibited a decreased oxygen consumption rate in response to sulfide; gills from *Mytilus edulis* collected from and maintained in sulfide-free conditions exhibited an even lower oxygen consumption rate in response to sulfide (Lee et al., 1996). In this paper, we also address the dependency of mussel gill mitochondrial sulfide consumption on oxygen partial pressure with simultaneous measurements of oxygen and sulfide consumption.

Sulfide disappearance from solution in a closed container (i.e. respirometer chamber) can result from abiotic sulfide oxidation, catalyzed by a number of chemical agents (Chen and Morris, 1972). If biological samples are present in the chamber, enzymatic sulfide oxidation (sulfide consumption) and sulfide binding to molecules such as transport proteins, will also contribute to a decrease in solution sulfide concentration.

The rate of sulfide disappearance can be measured indirectly or directly. Indirect methods include the measurement of oxygen consumption rate, often combined with the rate of production of sulfide oxidation products such as thiosulfate (for intact mussel gills, see Doeller et al., 1999, 2001). Direct methods to measure solution sulfide levels include HPLC (Fahey et al., 1981) and colorimetric assays (Svenson, 1980; Cline, 1969). These measurements are not typically in real time, however, since samples are taken at specified intervals and processed for later determination of sulfide content. Because sulfide consumption in intact gills and by isolated mitochondria can be rapid, these are not methods of choice.

In this study we describe the development of a sulfide respirometric method to obtain continuous real-time measurements of sulfide consumption, accomplished using a polarographic sulfide sensor fitted into the sensor port of a respirometer. With sulfide respirometry, the kinetics of changes in sulfide concentration in physiological solutions such as seawater and mitochondrial respiration buffer are continuously recorded. Sulfide respirometry indicates that gill mitochondrial sulfide consumption shows conformity to oxygen partial pressure, exhibiting multiphase kinetics, and it also occurs in the absence of oxygen. Additionally, the process of sulfide-supported chemolithoheterotrophy in gills is dependent on environmental sulfide exposure.

**Materials and methods**

*Animal collection, maintenance and sample preparation*

*Geukensia demissa* Dillwyn 1817 were collected from an intertidal marsh on Dauphin Island, AL, USA and maintained in 200 liter Nalgene tanks containing a 20 cm bottom layer of sulfide-productive habitat sediment, also collected from Dauphin Island, and an upper 20 cm layer of 20 p.p.t. artificial seawater (ASW; Tropic Marin, Wartenberg, Germany). Mussels (<100 per tank) were inserted into the sediment as they are found in their habitat. The sediment was periodically seeded with short sections of dried linguini pasta as a source of complex carbohydrates to stimulate bacterial sulfide production. Routine sulfide levels in water samples taken just above the sediment surface were 100–500 µmol l⁻¹. The overlying water was circulated through a 6 liter external gravel filter at approximately 0.2 l min⁻¹. Mussels held in sulfide-free conditions were maintained in Nalgene tanks filled only with 20 p.p.t. ASW and an under-gravel filter at the bottom. Sulfide was undetectable in these tanks. Water parameters monitored 2–3 times weekly were 20±2°C, pH 7.8, <0.25 mg l⁻¹ ammonia, and 0–0.1 mg l⁻¹ nitrite. Mussels were fed twice weekly with 15–20 ml of finely homogenized TetraMin fish food (TetraWerke, Melle, Germany). Fish food present in the digestive diverticula was evidence of feeding by mussels. Gills were prepared for experimentation according to Doeller et al. (2001). Gill mitochondria were isolated according to Pannuto et al. (2000).

*Polarographic sulfide sensor design and calibration*

The polarographic sulfide sensor (PSS; Fig. 1), based on a microsensor used to measure sulfide levels within bacterial mats (Jeroschewski et al., 1996), was designed with dimensions equal to that of the polarographic oxygen sensor (POS; model 2120 Orbisphere, Geneva, Switzerland) used in the Oroboros Oxygraph respirometer (Innsbruck, Austria), in order to place the PSS into the POS port for sulfide respirometry. The housing was machined from polyether ether ketone (PEEK, Victrex USA Inc., Rockford, MI, USA). Both anode and cathode were fashioned from platinum wire (1 and 0.5 mm diameter, respectively; Blankinship Porter, Birmingham, AL, USA) and cemented into the PEEK housing with epoxy (Scotch-Weld 2216, 3M, St Paul, MN, USA). The electrolyte, consisting of 0.05 mol l⁻¹ K₃[Fe(CN)₆] in alkaline carbonate buffer pH 10 (Fig. 1, insert), was held in the sensor tip reservoir with a two-layer membrane made of a H₂S-permeable membrane (MEM 213, 25 µm thick, MemPro, Troy, NY, USA), cemented (Silicone Adhesive RTV 167, GE, Waterford, NY, USA) to a H₂S-impermeable membrane (25 µm thick saran, mylar or FEP). The membrane was held...
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onto the PSS tip between an O-ring and an adapter ring. The term sulfide refers to the total of H$_2$S, HS$^-$ and S$^{2-}$. As H$_2$S diffuses into the electrolyte, it dissociates into H$^+$ and HS$^-$, which reduces ferricyanide to form ferrocyanide and sulfur. The anode current is created as ferrocyanide donates an electron to the anode, polarized at 0.1·V, and ferrocyanide is then restored to ferricyanide. Sulfide-dependent changes in anode current were converted to proportional voltage with a modified POS meter, and the output voltage was recorded digitally (Virtual Bench, National Instruments, Austin, TX, USA). Relative H$_2$S permeabilities of various membranes were determined as PSS signal magnitude and response time.

In general, the PSS was calibrated with stepwise increases in sulfide concentration while positioned in the respirometer chamber containing 3·ml stirred (500·r.p.m.) 20·mmol·l$^{-1}$ Tris-buffered zero-grade argon (<0.0003·kPa oxygen)-equilibrated analytical grade purified water (Solution 2000, Jasper, GA, USA), pH·7.0, 20°C. Sulfide stocks of 10·mmol·l$^{-1}$ are made by dissolving fresh crystals of Na$_2$S in 20·mmol·l$^{-1}$ Tris-buffered analytical grade water. First, the buffer solution in a 20·ml Pyrex syringe was vigorously sparged with argon for at least 10·min to achieve anoxia. Liquid from the syringe was then used to dissolve the crystals in a conical Pyrex centrifuge tube filled with a continuous stream of argon, pH was adjusted to 7 with dilute HCl, and the solution was immediately drawn back into the syringe free of bubbles and sealed with a rubber serum stopper. Samples of the anoxic Na$_2$S stock were obtained with a gas tight syringe (Hamilton, Reno, NV, USA) through the stopper and injected into the respirometer. Dilute sulfide stocks of 0.1·mmol·l$^{-1}$ were made by injecting the concentrated stock into a syringe containing anoxic buffered water through the sealing stopper. Stock solutions were calibrated with the standard 2-PDS assay (Svenson, 1980) Samples of the sulfide stock were dissolved into an excess of the reagent 2-PDS so that all sulfide reacted to form stoichiometric amounts of the product 2-thiopyridone. The optical density at 343 was divided by the extinction coefficient of 8.08·mmol·l$^{-1}$·cm$^{-1}$ to determine the concentration of the product 2-thiopyridone (Jensen et al., 2000; path length was 1·cm). The expected concentration of sulfide was regressed against the measured concentration of 2-thiopyridone, yielding a slope that was typically within ±2% of ideal.

To compare the PSS with a standard colorimetric method in terms of its ability to accurately follow the kinetics of changing sulfide concentration, the sulfide contents of 5·ml air-equilibrated solutions were assayed by both the PSS and the 2-PDS method (Svenson, 1980). To also determine if the presence of either the PSS or a POS would differentially alter chamber sulfide levels, sulfide solutions were placed in three Pyrex chambers, one with the PSS, one with a POS, and one a blank chamber without sensors. The chambers were unstoppered to allow rapid sample removal while allowing sulfide concentration to decline smoothly by volatilization and oxidation processes. After the initial addition of 100·μmol·l$^{-1}$ Na$_2$S into the chambers, 20·μl discrete samples were removed at periodic intervals and immediately injected into the 2-PDS reagents. Reaction mixtures were incubated and read at 343 nm to determine sulfide concentration as a function of time.

To determine pH effects on the PSS signal, the pH of the solution to receive the sulfide stock was adjusted with
20 mmol l\(^{-1}\) Tris, Heps or MES to cover pH within the range of 5.5 to 8.5. The anoxic sulfide stock solution was also prepared with 20 mmol l\(^{-1}\) of the same buffer at the tested pH. At each pH, the PSS signal was recorded after equilibration with stepwise additions of sulfide stock aliquot samples.

Because of the instability of sulfide in aerated solutions, even in the presence of metal chelators such as DTPA, PSS signal drift was determined in a flow-through system in which spontaneous sulfide oxidation was limited by syringe pump (model 22, Harvard Apparatus, Holliston, MA, USA) delivery of sulfide stock at a constant rate to a 3 ml constant volume chamber, with excess volume removed by aspiration. The sulfide stock solution was 7.5 mmol l\(^{-1}\) Na\(_2\)S in 500 mmol l\(^{-1}\) Tris-buffered anoxic analytical grade water, pH 7.0. Delivery rates were adjusted between 0.1 and 0.2 ml h\(^{-1}\) to create a steady state sulfide concentration between 20 and 100 \(\mu\)mol l\(^{-1}\), determined by 2-PDS. PSS signal drift was measured for up to 10 h under aerated conditions.

**Oxygen and sulfide respirometry**

Respiration rates of excised gills and isolated mitochondria were determined in a dual-chamber respirometer as reported previously (see Doeller et al., 2001, for gills and Parrino et al., 2000, for mitochondria; Gnaiger, 2001), except that one chamber sensor port was fitted with a POS and the other with the PSS.

**Experimental procedures**

*Intact gill sulfide and oxygen consumption as a function of \(P_{O_2}\)*

To obtain nearly identical gill pieces for use in each respiration chamber, a small section (about 1 cm anterior to posterior length) of the demibranch was excised from freshly collected or sulfide-maintained mussels and then halved into two hemibranch sections. Each hemibranch section, approximately 1 cm\(^2\) and 10 mg dry mass, was placed on a perforated 316 stainless steel support in 5 ml stirred seawater in each respiration chamber. Experimental interventions were added nearly simultaneously (<30 s delay) to both PSS and POS chambers. Typically, gills were allowed to consume nearly all the oxygen, then the partial pressure of oxygen (\(P_{O_2}\)) in the chamber was elevated by lifting the stoppers, allowing air to fill a gas space above the liquid. As the desired \(P_{O_2}\) was reached, stoppers were again lowered to eliminate the gas space, and respiration measurements were resumed. Some experiments were begun at low \(P_{O_2}\) by initially flushing argon into the gas space above the liquid while the stoppers were slightly elevated. The experimental sulfide concentration used with intact gills was 100 \(\mu\)mol l\(^{-1}\), which caused maximal sulfide-stimulated oxygen consumption (Lee et al., 1996). To determine anoxic sulfide consumption rates, sulfide was injected into the chamber after \(P_{O_2}\) had reached zero. Measurements using heat-killed gills microwaved for 60 s, or chambers alone after the gills were removed, were used to determine background or spontaneous sulfide oxidation rates.

*Gill mitochondrial sulfide and oxygen consumption as a function of \(P_{O_2}\)*

Mitochondria, isolated as previously described (Parrino et al., 2000) from gills of freshly collected, sulfide-maintained or sulfide-free mussels, were added to 1–1.5 ml respiration buffer at a concentration of 0.5–2 mg protein ml\(^{-1}\). Protein was determined using the Folin–phenol reagent and bovine serum albumin to construct the standard curve (Brookes et al., 2003). Before experimental interventions to respiration chambers, the respiratory control ratio (RCR) for each mitochondrial preparation was determined as the ratio of State 3 respiration, measured with 4 mmol l\(^{-1}\) malate as substrate and 25 \(\mu\)mol l\(^{-1}\) ADP, and State 4 respiration, measured after ADP is consumed. After RCR determination, the chamber was cleaned and another aliquot sample of mitochondria added to the respiration buffer. To achieve State 3 respiration with sulfide as sole respiratory substrate, 25 \(\mu\)mol l\(^{-1}\) ADP was injected into the respiration buffer first, then sulfide was immediately injected at one of three concentrations, 5–6, 10–13, or 18–20 \(\mu\)mol l\(^{-1}\). To determine the effect of \(P_{O_2}\) on sulfide consumption rate, mitochondria were allowed to consume all the oxygen in the chamber as sulfide was injected repeatedly. To determine anoxic sulfide consumption rates, sulfide was injected at \(P_{O_2}=0\).

*Gill mitochondrial sulfide consumption with inhibited terminal oxidase enzymes*

The roles of both classical and alternative terminal oxidase pathways in sulfide consumption were evaluated using selective inhibitors. Mitochondria were first stimulated with sulfide to achieve State 3 respiration rates in non-limiting oxygen conditions. Sulfide consumption rates were then determined at three sulfide concentrations in the presence of 1 mmol l\(^{-1}\) KCN to inhibit cytochrome c oxidase, and then after the addition of 1 mmol l\(^{-1}\) SHAM to inhibit the putative alternative oxidase.

**Data presentation and statistical analysis**

For both oxygen and sulfide measurements, the respirometer software (Oroboros DatLab, Innsbruck, Austria) was used for baseline correction, derivatization of the parent traces, rate calculations, and integration of the derivative traces to determine the quantity of oxygen and sulfide consumed. Data are presented as mean ± standard deviation (s.d.; \(N=\)number of repetitions). Two-sample comparisons were made with the paired or unpaired one-tailed \(t\)-test assuming equal variance (Microsoft Excel). Significance was assigned at the 5% level.

**Results**

*Polarographic sulfide sensor (PSS) design and calibration*

The PSS geometry enabled ease of use in the respirometer (Fig. 1). In addition, the development of the two-layer membrane was critical to ensure a rapid response to changes in solution sulfide concentration. If a single H\(_2\)S-permeable membrane was placed over the electrolyte reservoir, the PSS
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response time was long, presumably due to equilibration of the large electrolyte volume with solution sulfide concentration. By placing an H₂S-impermeable membrane with a 0.5 mm opening over the electrolyte reservoir, with the opening positioned concentrically above the 1.0 mm anode, response time was shortened, presumably due to the action of the H₂S-impermeable membrane as a virtual guard ring, restricting lateral sulfide diffusion into the reservoir. Relative sulfide permeabilities of a few polymer membranes are provided in Table 1. Interestingly, PFA and FEP membranes often used on oxygen sensors exhibit very low H₂S permeability.

The PSS signal up to approximately 150 μmol l⁻¹ sulfide can be fitted by linear regression (Fig. 2). At higher sulfide concentrations, the PSS response is best fitted by a second order polynomial equation. The curvilinear nature of the calibration curve extends to at least 1 mmol l⁻¹ sulfide and is highly repeatable. Because anoxic sulfide stocks prepared early in the day gave identical calibration curves 4 h later, it is unlikely that the curvilinear response is the result of spontaneous sulfide oxidation in the stock solution or in the anoxic chamber. Results from tests using different electrolyte concentrations suggest that the nonlinear response at higher sulfide concentrations may result in part from saturation of the sensor electrolyte. Sulfide levels used in gill experiments are below 200 μmol l⁻¹ and in mitochondrial experiments are below 25 μmol l⁻¹. A typical stepwise calibration experiment (Fig. 2, insert) illustrates a response time of approximately 30 s to reach 90% of the new signal, followed by anunchanging signal indicating slow spontaneous sulfide oxidation under anoxic conditions. Replacement of the solution with fresh sulfide-free aerated buffer returned the signal to baseline in about 30 s.

The PSS accurately followed the kinetics of changing sulfide levels, as determined by the standard 2-PDS method (Fig. 3).

Although the loss of sulfide due to volatilization was typically threefold more rapid than the oxidation rate in closed chamber measurements, the presence of a PSS or POS did not appear to accelerate sulfide loss. Furthermore, the PSS sulfide consumption rate, calculated from the microamp current using Faraday’s constant, is in the pmol s⁻¹ range, thus negligible during these experiments, and, from tests of membrane permeability, H₂S will not readily cross the POS membrane. We therefore argue that the spontaneous sulfide oxidation rate would be equivalent in all three chambers, independent of sensor type.

The sulfide species able to diffuse across the sensor membrane is H₂S. For a given sulfide concentration, H₂S formation is favored as solution pH is lowered, leading to a rise in PSS signal (Fig. 4). The titration of sulfide to lower pH

Table 1. Comparison of O₂ and H₂S permeabilities of selected polymer films approximately 25 μm thick

<table>
<thead>
<tr>
<th>Material</th>
<th>O₂ permeability a</th>
<th>Relative H₂S permeability b</th>
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<tbody>
<tr>
<td>Polyvinylidene chloride (Saran)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Polyethylene terephthalate (Mylar)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>187</td>
<td>0</td>
</tr>
<tr>
<td>Perfluoroalkyl-tetrafluoroethylene copolymer (PFA)</td>
<td>433</td>
<td>0.01</td>
</tr>
<tr>
<td>Low density polyethylene</td>
<td>500</td>
<td>0.2</td>
</tr>
<tr>
<td>Fluorinated ethylene/propylene (FEP)</td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td>Silicone-polycarbonate copolymer (MEM-213)</td>
<td>30 000</td>
<td>1</td>
</tr>
<tr>
<td>Dimethyl silicone</td>
<td>100 000</td>
<td>1</td>
</tr>
</tbody>
</table>

aO₂ permeability is in (cm³ x mil)/(100 in² x d x atm), where mil=25 μm thick and d is day; MemPro technical bulletin, 1993. To convert given values to S.I. units [(cm³ x μm)/(cm² x s x KPa)], multiply by 4.44 x 10⁻⁹.

bPolarographic sulfide sensor (PSS) signal at 100 μmol l⁻¹ sulfide determined with tested membrane, relative to signal at 100 μmol l⁻¹ sulfide, determined with silicone membrane.

Fig. 2. Calibration of the polarographic sulfide sensor, PSS. A typical calibration curve is derived from sequential injections (inset) of Na₂S stock into anoxic 20 mmol l⁻¹ Tris at pH 7.0. A linear regression fits the PSS signals at <200 μmol l⁻¹ sulfide, whereas a second order polynomial fits the PSS signals above this concentration.

Fig. 3. The PSS accurately followed the kinetics of changing sulfide levels, as determined by the standard 2-PDS method.

Fig. 4. The sulfide species able to diffuse across the sensor membrane is H₂S. For a given sulfide concentration, H₂S formation is favored as solution pH is lowered, leading to a rise in PSS signal. The titration of sulfide to lower pH...
follows a single protonation of HS, with pK for the H₂S/HS⁻ couple near 6.9 depending on solution composition (Millero 1986; Millero and Hershey, 1989; for correct equation coefficients, see Kraus et al., 1996). Under the conditions used for the pH titration of the PSS signal, the observed pK was 6.75 (Fig. 4, insert). However, at constant solution pH, the PSS exhibits linearity up to 200 μmol l⁻¹ total sulfide.

Some general performance characteristics of the PSS are provided in Table 2. The detected sulfide species is H₂S, and ionized sulfide species do not appear to pass through the H₂S-permeable membrane. At neutral pH, with the meter amplifier at maximum gain, 0.1 μmol l⁻¹ sulfide produces a signal noticeably above baseline noise. This lower level of detection is comparable to several of the more sensitive sulfide spectrophotometry methods (see Lawrence et al., 2000; Table 1). Repeated sulfide (100 μmol l⁻¹) injections into previously cleaned anoxic chambers produced signals differing by only ±2%. The PSS functions best at pH favoring the H₂S species, but provides reliable signals up to pH 8.5. No interference was observed with other common biologically produced sulfide oxidation products (Doeller et al., 2001), nor was the PSS sensitive to oxygen, NO or H₂O₂. However, 1 mmol l⁻¹ KCN produced a small (<10 mV) positive offset of the PSS signal, presumably as HCN diffused through the membrane and altered electrolyte redox chemistry. This offset was easily subtracted as a baseline correction.

**Respiratory studies**

Upon the addition of sulfide, intact gills increased oxygen consumption rate by three- to fourfold, as previously reported (Lee et al., 1996; Doeller et al., 1999, 2001). Representative sulfide and oxygen traces over time for intact gills are shown in Fig. 5. Sulfide consumption rate of Gill sections under aerated conditions was 0.94±0.16 nmol sulfide s⁻¹ (N=7), approximately 50-fold faster than 0.020±0.005 nmol sulfide s⁻¹ (N=6), the spontaneous rate of sulfide oxidation under aerated conditions in chambers without gills or with heat-killed gills. The initial rate of gill sulfide consumption under anoxic conditions, determined as the average derivative for the first 30–50% of the trace, was 0.044±0.16 nmol sulfide s⁻¹ (N=5), approximately 5% of the normoxic gill sulfide consumption rate but approximately sevenfold faster than the spontaneous anoxic sulfide oxidation rate of 0.006±0.002 nmol sulfide s⁻¹ (N=3).

The rate of sulfide consumption was a function of both ambient oxygen tension and sulfide concentration. At higher initial oxygen levels, sulfide was consumed more rapidly. If sulfide was repeatedly injected at identical starting air saturation levels, sulfide consumption rates were consistent. Six injections of 100 μmol l⁻¹ sulfide made near 50% air saturation over 260 min showed no change in sulfide consumption rates or S:O₂ ratios (data not shown). However, normoxic sulfide consumption rates following bouts of anoxic sulfide consumption were consistently lower than pre-anoxic rates. A bolus injection of 100 μmol l⁻¹ sulfide near 50% air saturation after an anoxic sulfide consumption bout resulted in lower oxygen and sulfide consumption rates and a significantly lowered S:O₂ ratio of

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**Fig. 3.** The polarographic sulfide sensor (PSS) signal compared to the standard chemical analysis of sulfide with 2,2 dipyrindyl disulfide (2-PDS). A single injection of anoxic Na₂S stock brought the concentration to 100 μmol l⁻¹ in 3 ml air-saturated 20 mmol l⁻¹ Tris at pH 7.0. 50 μl aliquot samples of solution were removed at timed intervals and immediately added to the 2-PDS reaction mixture. Both methods reported the same change in sulfide concentration over time for intact gills.

**Fig. 4.** The polarographic sulfide sensor (PSS) signal is dependent on solution pH (5.5–8.5) with increased sensitivity of the PSS at lower pH. The titration at a specific sulfide concentrations (10–200 μmol l⁻¹) over a pH range (inset) illustrates the pK for H₂S/HS⁻ to be near 6.75 at 20°C and that the PSS is detecting only H₂S.
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0.74±0.11 (N=3) compared to the pre-anoxic ratio of 0.88±0.16 (N=8) (P=0.035).

Mitochondrial respiration

Mitochondria isolated from gills of sulfide-maintained mussels exhibited RCRs of 3.7±1.3 (N=24) for the substrate malate. Representative traces of oxygen and sulfide levels over time in the presence of gill mitochondria are shown in Fig. 6A.

The time derivatives, multiplied by −1, of these traces are the oxygen and sulfide consumption rates, shown in Fig. 6B. Bolus injections of 12.5 μmol L−1 sulfide caused brief increased oxygen consumption. At PO2 >5 kPa, the increase was threefold (Fig. 6B), and both sulfide and oxygen consumption rates exhibited single coincident peaks. As PO2 declined (4–2 kPa), the sulfide and oxygen consumption rate peaks were

<table>
<thead>
<tr>
<th>Table 2. Performance characteristics of the polarographic sulfide sensor (PSS)a prototype</th>
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<tbody>
<tr>
<td>Sulfide species detected</td>
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<tr>
<td>Detection limitsb</td>
</tr>
<tr>
<td>Accuracy</td>
</tr>
<tr>
<td>Precision</td>
</tr>
<tr>
<td>Response time to&gt;90% change in sulfide level</td>
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<tr>
<td>Signal drift</td>
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<tr>
<td>Sulfide consumption</td>
</tr>
<tr>
<td>Operational pH rangec</td>
</tr>
<tr>
<td>Interference by other compounds</td>
</tr>
<tr>
<td>AHCN gas will cause minor increase in signal (pH sensitive)</td>
</tr>
</tbody>
</table>

aPSS configured in Oxygraph respirometer with 3 ml pH-buffered sample at 20°C, electrolyte pH 10, polarizing voltage +0.1 V, 25 μm thick MEM-213.
bSamples in 50 mmol l−1 Tris, pH 6.5; samples at higher pH will have somewhat higher minimum detection limits.
cpH-dependent signal results from H2S/HS− pK of approximately 6.75 under the conditions tested.

0.74±0.11 (N=3) compared to the pre-anoxic ratio of 0.88±0.16 (N=8) (P=0.035).

Mitochondria from sulfide-maintained mussels

Mitochondria isolated from gills of sulfide-maintained mussels exhibited RCRs of 3.7±1.3 (N=24) for the substrate malate. Representative traces of oxygen and sulfide levels over time in the presence of gill mitochondria are shown in Fig. 6A. The time derivatives, multiplied by −1, of these traces are the oxygen and sulfide consumption rates, shown in Fig. 6B. Bolus injections of 12.5 μmol L−1 sulfide caused brief increased oxygen consumption. At PO2 >5 kPa, the increase was threefold (Fig. 6B), and both sulfide and oxygen consumption rates exhibited single coincident peaks. As PO2 declined (4–2 kPa), the sulfide and oxygen consumption rate peaks were
no longer coincident. Instead, sulfide consumption rates exhibited single peaks while oxygen consumption rates became kinetically complex, showing initial sulfide-stimulated high rates that decreased to sulfide-inhibited rates, then rose again as sulfide levels declined, but finally dropping as sulfide was exhausted. Interestingly, the total amount of consumed oxygen, determined from peak integral, remained constant (Fig. 6B). Multiphasic oxygen consumption rates also occurred at lower $P_{O_2}$, although with lower magnitude and for longer times. As $P_{O_2}$ decreased toward anoxia, sulfide-stimulated oxygen and sulfide consumption rates were both truncated. Once anoxia was reached, the sulfide trace followed a two-phase kinetic event (Fig. 6A) similar to that seen with whole gills (Fig. 5).

The effects of three concentrations of sulfide, each added together with ADP, on the respiration of gill mitochondria from sulfide-maintained mussels are shown as a function of time in Fig. 7. Fig. 7A,B are the parent traces, and Fig. 7C,D are the respective time derivatives (note that B and D have a compressed time scale). These mitochondria had a State 2 respiration rate of $2.1 \pm 0.5 \, \text{nmol} \, \text{O}_2 \, \text{min}^{-1} \, \text{mg}^{-1} \, \text{protein}$ ($N=24$) and exhibited relatively rapid coincident bouts of oxygen and sulfide consumption upon injections of 6.25 and 12.5 $\mu$mol l$^{-1}$ sulfide, with matched consumption rates (Fig. 7A,C). At 18.75 $\mu$mol l$^{-1}$ sulfide, sulfide consumption rate was proportionately increased (Fig. 7C) but the coincident increased oxygen consumption rate was truncated, indicating a limited sulfide-supported State 3 respiration rate that did not match the sulfide consumption rate (see Fig. 6B, below 4 kPa $P_{O_2}$). Sulfide at 18.75 $\mu$mol l$^{-1}$ inhibited the oxygen consumption rate even though the total amount of consumed oxygen remained proportional to the total amount of sulfide, with a S:O$_2$ ratio near unity. The oxygen consumption rate between sulfide injections remained elevated above the initial State 2 rate. Selected rates from respirometry experiments are given in Table 3.

In the presence of 1 mmol l$^{-1}$ KCN, the oxygen consumption rate decreased approximately fivefold from 7 to 1.5 nmol O$_2$ min$^{-1}$ mg$^{-1}$ protein (Fig. 7C), as observed previously (Parrino et al., 2000). Following KCN exposure, sulfide at all three concentrations stimulated oxygen consumption to a single limited level of about 4 nmol O$_2$ min$^{-1}$ mg$^{-1}$ protein, approximately 15% of the pre-KCN 12.5 $\mu$mol l$^{-1}$ sulfide-stimulated rate (Fig. 7B,D). Under these conditions, sulfide consumption rate also reached a limit near 15 nmol H$_2$S min$^{-1}$ mg$^{-1}$ protein, approximately 25% of the pre-KCN highest sulfide consumption rate at 18.75 $\mu$mol l$^{-1}$ (Fig. 7B,D). The limited oxygen and sulfide consumption rates resembled square waves with a duration that varied proportionally to chamber sulfide concentration (Fig. 7D). Sulfide consumption integrals, both pre- and post-KCN exposure, indicated that all sulfide was consumed at each sulfide concentration. However, the much lower sulfide-stimulated oxygen consumption rates after KCN resulted in a stoichiometric shift of the S:O$_2$ ratio to near 2.5, indicating that sulfide consumption may be uncoupled from oxygen consumption and that the reducing equivalents generated by sulfide oxidation may not be used for immediate oxygen reduction.

In previous studies, we provided evidence that an alternative oxidase was operational within mussel gill mitochondria (Parrino et al., 2000). Addition of SHAM, an inhibitor of alternative oxidase activity, subsequent to KCN inhibition of cytochrome oxidase further decreased the State 2 oxygen consumption inhibition while sulfide consumption increases at a low level. Note the compressed time scale in B and D.
Measurement of mitochondrial sulfide consumption

...consumption rate to approximately 5% of the pre-KCN rate (Fig. 7B,D). Exposure to sulfide at all concentrations after inhibition of both terminal oxidases did not stimulate oxygen consumption rate while the sulfide consumption rate increased to a single level of 4·nmol ·H₂S ·min⁻¹ ·mg⁻¹ ·protein, with a duration again proportional to sulfide concentration. This sulfide consumption rate was approximately 7% of the pre-KCN treated, 18.75·m mol ·l⁻¹ sulfide-stimulated rate, and about 25% of the post-KCN treated, pre-SHAM rate, although it was three- to fourfold greater than the spontaneous sulfide oxidation rate under similar PO₂ conditions. Again, sulfide consumption integrals, as with the pre-KCN integrals, indicated that all sulfide was consumed even though terminal oxidase activity was negligible.

**Mitochondria from sulfide-free mussels**

In general, gill mitochondria from sulfide-free mussels exhibited State 2 oxygen consumption rates similar to mitochondria from sulfide-maintained mussels, but exhibited much lower sulfide and oxygen consumption rates following sulfide exposure (similar to whole gills; see Lee et al., 1996). These mitochondria responded to substrates such as malate, succinate and ADP, and exhibited a malate-supported RCR of 3.8±1.8 (N=6). Typical traces of mitochondrial respiratory responses to sulfide are provided in Fig. 8. Fig. 8A,B are the parent oxygen and sulfide traces, and Fig. 8C,D are the respective time derivatives (B and D have a compressed time scale). These mitochondria had a much lower response to sulfide exposure (note that the ordinate scales of the derivative panels are about 12% of those in Fig.·7C,D for mitochondria from sulfide-maintained mussels, SMM). Both sulfide and oxygen consumption rates increased upon exposure to all three sulfide levels, but the oxygen consumption rate reached a limit at 7·nmol ·O₂ ·min⁻¹ ·mg⁻¹ ·protein. Sulfide consumption rates at 6.25 and 12.5 ·m mol ·l⁻¹ sulfide were approximately 25% of SMM mitochondrial rates, and at 18.75 ·m mol ·l⁻¹ the rate was only 8% of the SMM rate. At 18.75 ·m mol ·l⁻¹ sulfide, the oxygen consumption rate exhibited multiphasic kinetics similar to those seen in Fig.·6B for sulfide exposure of control mitochondria under low oxygen conditions. Although sulfide and oxygen consumption rates were depressed, the S:O₂ ratio remained near unity, as observed with SMM mitochondria. The addition of terminal oxidase inhibitors KCN and then SHAM resulted in matched reductions in oxygen and sulfide consumption rates at all three sulfide concentrations. The rates appeared to reach limits, with duration proportional to sulfide concentration, and in general were less than 20% of the comparable SMM mitochondrial rates (Fig. 7D). Although respiration rates were consistently lower than SMM mitochondrial rates, the S:O₂ ratio of mitochondria from sulfide-free mussels remained near unity under all conditions, and mitochondrial sulfide-stimulated respirometric measurements of oxygen and hydrogen sulfide consumption under selected conditions

<table>
<thead>
<tr>
<th>Conditions (O₂ independent region, &gt;8 kPa)</th>
<th>M₀₂ᵃ</th>
<th>Mₛₛᵇ</th>
<th>S:O₂ᵇ</th>
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<tr>
<td><strong>Sulfide-sediment mussels</strong></td>
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<tr>
<td>[Sulfide] (µmol l⁻¹)</td>
<td></td>
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<tr>
<td>5–6</td>
<td>18.2±8.7 (11:5)</td>
<td>16.6±6.2 (11:5)</td>
<td>0.85±0.16 (11:5)</td>
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<tr>
<td>10–13</td>
<td>33.6±11.4 (11:5)</td>
<td>29.2±4.7 (11:5)</td>
<td>0.81±0.11 (11:5)</td>
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<tr>
<td>18–20</td>
<td>27.1±6.2 (7:4)</td>
<td>52.5±12.0 (7:4)</td>
<td>0.96±0.15 (7:4)</td>
</tr>
<tr>
<td>+ 1 mmol l⁻¹ KCN 10–13</td>
<td>5.2±1.7 (3:2)</td>
<td>14.6±3.2 (3:2)</td>
<td>2.55±0.36 (3:2)</td>
</tr>
<tr>
<td>+ 1 mmol l⁻¹ KCN, 1 mmol l⁻¹ SHAM 10–13</td>
<td>0</td>
<td>3.6 (2:2)</td>
<td>na</td>
</tr>
<tr>
<td>Anoxia, initial rate, [Sulfide] 10–13 µmol l⁻¹</td>
<td>0</td>
<td>1.9±0.7 (5:3)</td>
<td>na</td>
</tr>
<tr>
<td><strong>Sulfide-free mussels</strong></td>
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<tr>
<td>[Sulfide] (µmol l⁻¹)</td>
<td></td>
<td></td>
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<tr>
<td>5–6</td>
<td>7.2±2.7 (3:2)</td>
<td>4.5±1.9 (3:2)</td>
<td>0.66±0.20 (3:2)</td>
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<tr>
<td>10–13</td>
<td>8.2±1.1 (3:2)</td>
<td>5.6±1.2 (3:2)</td>
<td>0.83±0.14 (3:2)</td>
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<tr>
<td>18–20</td>
<td>6.3±1.7 (3:2)</td>
<td>4.1±2.2 (3:2)</td>
<td>0.89±0.13 (3:2)</td>
</tr>
<tr>
<td>+ 1 mmol l⁻¹ KCN 10–13</td>
<td>1.3 (2:2)</td>
<td>1.5 (2:2)</td>
<td>1.1 (2:2)</td>
</tr>
<tr>
<td>+ 1 mmol l⁻¹ KCN, 1 mmol l⁻¹ SHAM 10–13</td>
<td>0.4 (2:2)</td>
<td>0.4 (2:2)</td>
<td>1.0 (2:2)</td>
</tr>
</tbody>
</table>

ᵃValue provided is the peak rate average.
bValue provided is the average ratio of paired measurements calculated from the integral of the derivative traces.
cInjected sulfide concentrations were 5–6.25, 10–12.5, or 18.75–20 µmol l⁻¹.
Values are nmol min⁻¹ ·mg⁻¹ ·protein, expressed as means ± s.d. (number of replications from: the number of mitochondrial preparations). na, not applicable.
indicating that sulfide and oxygen consumption were not uncoupled under these conditions.

**Oxygen and sulfide consumption rate apparent \( P_{50} \)**

It is possible to estimate apparent \( P_{50} \) values of oxygen and sulfide consumption (partial pressure of oxygen at half maximal rate) for mitochondria from sulfide-maintained mussels from a plot of initial sulfide-stimulated oxygen and sulfide consumption rates (Fig. 6) as a function of ambient \( P_{O_2} \) (Fig. 9). The resultant apparent \( P_{50} \) values were 2 kPa and 1 kPa for oxygen and sulfide consumption rates, respectively, indicating that the oxygen consumption rate was more sensitive to declining \( P_{O_2} \) than the sulfide consumption rate. However, the S:O\(_2\) ratio remained near unity until <0.5 kPa as anoxia was approached, where sulfide consumption continued at a low rate and oxygen consumption reached zero. At \( P_{O_2} \) above the oxygen-limiting region, KCN inhibited oxygen and sulfide consumption rates of sulfide-maintained mitochondria to about 10% and 20% of pre-KCN values, respectively, and the S:O\(_2\) ratio increased to about 2.5. The addition of SHAM further depressed oxygen consumption to near zero while sulfide consumption rate decreased to about 10% of control, a response similar to sulfide consumption under anoxic conditions where oxygen and sulfide consumption rates were unmatched. Terminal oxidase-inhibited rates from sulfide-free mitochondria were lower but coincident, indicating that sulfide and oxygen consumption remained coupled.

**Discussion**

**Polarographic sulfide sensor**

The PSS was developed to allow continuous measurements of sulfide dissolved in aqueous solutions. The PSS design is similar to a POS with several modifications such as anode and cathode metals, electrolyte solution, gas permeable membranes separating the electrolyte solution from the liquid being measured, and polarizing voltage. As such, its ease of use and reliability should be comparable to POS values.

Sulfide levels in solution are usually measured in discrete samples by various multi-step wet chemical, colorimetric or gas extraction/gas chromatography methods (Lawrence et al., 2000), or with the sulfide electrode. This bare Ag\(_2\)S electrode in combination with a reference electrode was developed to
detect S\textsuperscript{2−} in solution at strongly alkaline pH (Vesely et al., 1972). Recent work in which it was used to determine the maximum sulfide consumption rate of a protozoan over a range of steady state sulfide concentrations suggests that it may also be sensitive to submicromolar concentrations of HS\textsuperscript{−} (Searcy and Peterson, 2004). The bare Ag\textsubscript{2}S coating requires daily reconditioning to remove interfering deposits that form from constituents present in biologically relevant solutions. In contrast, the PSS continuously reports hydrogen sulfide levels in solutions near neutral pH under physiological conditions in real time. The sulfide-reactive interior components are protected from other sample components by a polymer membrane. To use the PSS accurately, solution pH must be known. As hydrogen sulfide is ionized to the hydrosulfide anion above pH 7, the proportion of hydrogen sulfide in solution decreases. Although the PSS loses sensitivity at alkaline pH, it was successfully calibrated at pH 8.5.

The concentration of hydrogen sulfide in biological tissues (Abe and Kimura, 1996; Wang, 2002) or in sulfide-rich environments where organisms survive (Lee et al., 1996; Grieshaber and Voelkel, 1998) is usually in the micromolar range, comparable to oxygen concentration. Spontaneous reaction between hydrogen sulfide and oxygen, catalyzed by numerous organic and inorganic agents (Chen and Morris, 1972), can rapidly change the solution sulfide concentration. In addition, cellular enzymatic oxidation of sulfide can also be rapid. To obtain accurate sulfide measurements under physiological time frames and condition, it is necessary to use a highly sensitive, continuously recording sulfide sensor such as the PSS. By using this novel device in a closed chamber respirometer, we have observed sulfide oxidation kinetics of representative tissues and organelles under physiological conditions.

**Respiratory studies**

**General response**

The influence of oxygen partial pressure \( P_{O_2} \) on tissue oxygen consumption rate is well known. Oxygen diffusion rate into tissue is dictated by \( P_{O_2} \), and mitochondrial cytochrome \( c \) oxidase is kinetically dependent on \( P_{O_2} \). Mussel gill oxygen consumption is independent of \( P_{O_2} \) down to about 5 kPa, where it begins to show conformity (Doeller et al., 1993). Gill sulfide consumption is also dependent on oxygen, showing conformity at about 4 kPa following a 50–100 \( \mu \)mol l\textsuperscript{−1} sulfide exposure. The lugworm Arenicola marina, another inhabitant of sulfide sediments, exhibits oxygen-dependent sulfide consumption and readily switches to anaerobic metabolic pathways (Vökel and Grieshaber, 1992, 1994). The complex kinetic responses of oxygen and sulfide consumption rates indicate interdependence of each process on the other substrate (Fig. 6). At low \( P_{O_2} \), sulfide stimulates oxygen consumption by providing reducing equivalents to cytochrome \( c \) (Doeller et al., 1999) or to ubiquinone (Parrino et al., 2000), but it simultaneously competes with oxygen for cytochrome \( c \) oxidase and in turn partially inhibits oxygen consumption. As the sulfide level declines, inhibition is partly removed and oxygen consumption rises until sulfide is exhausted, when it then falls. The throughput of electrons from sulfide oxidation to oxygen via mitochondrial electron transport also controls the rate of sulfide consumption. As \( P_{O_2} \) decreases further, the sulfide consumption rate also decreases. More evidence of the interdependence of oxygen and sulfide consumption is seen in the \( S:O_2 \) ratio being maintained near unity until anoxia is approached. Evidence from the \( S:O_2 \) ratio, rates of ATP and thiosulfate production, and cytochrome redox states supports the hypothesis that electrons from sulfide oxidation enter the mitochondrial electron transport chain, coupling sulfide consumption with ATP production (Doeller et al., 2001). However, at low \( P_{O_2} \), as sulfide consumption continues (albeit at a low level) and oxygen consumption drops to zero, it is unclear where reducing equivalents from sulfide are delivered or sequestered or if sulfide-stimulated ATP production continues.

**Sink for reducing equivalents**

The effect of low \( P_{O_2} \) on oxygen and sulfide consumption is also seen at non-limiting \( P_{O_2} \) in the presence of terminal oxidase inhibitors. Previous investigations have demonstrated that KCN and SHAM limit sulfide-stimulated oxygen consumption and ATP production (Parrino et al., 2000). KCN inhibition of cytochrome \( c \) oxidase results in an 80% decreased mitochondrial oxygen consumption rate and a 50% decreased sulfide consumption rate, compared to the pre-KCN 12.5 \( \mu \)mol l\textsuperscript{−1} sulfide-stimulated rates. This apparent uncoupling of oxygen and sulfide consumption, also observed under anoxic conditions, suggests that the path of reducing equivalents from sulfide to oxygen includes a component that can either accumulate electrons or pass them onto an alternative electron acceptor. Sulfide-maintained mussels have much higher total glutathione and sulfite levels in sulfide-exposed gill tissue compared to sulfide-free mussels (Doeller et al., 2001). Gluthathione disulfide (GSSG), which can be reduced to glutathione (GSH) by glutathione reductase, may represent a competent electron acceptor if sulfide was the reductant. The total glutathione concentration, approximately 1 mmol kg\textsuperscript{−1} gill tissue, and the equilibrium GSH/GSSG redox potential, −240 mV (Sévier and Kaiser, 2002), compared to −270 mV for HS\textsuperscript{−}/S (Kelly, 1982), lends support for such consideration. Ascorbate is another agent involved in cellular redox cycling that represents a potential sink for reducing equivalents. Compared to GSH, gill tissue ascorbate concentration, approximately 50 \( \mu \)mol kg\textsuperscript{−1}, is low. However, the levels of both ascorbic acid (AA) and dehydroascorbate (DHAA) are two- to fourfold higher in gills from sulfide-maintained mussels compared to those from sulfide-free mussels (D.W.K. and J.E.D., preliminary data). The operation of a cellular sink for electrons produced from sulfide consumption under oxygen-limiting conditions may include the subsequent delivery of those electrons to mitochondria upon return to oxygenated conditions. This is evident in intact gills by the decrease in S:O\textsubscript{2} ratio after a bout of anoxic sulfide consumption.
consumption. The S:O$_2$ ratio decline might also suggest that the anoxic event mediates a persistent partial mitochondria uncoupling. The GSH/GSSG and the AA/DHAA ratios before and after sulfide exposure must be determined to evaluate the roles of GSH and AA in sulfide consumption under oxygen-limiting conditions. Both GSH and AA participate in the plant mitochondrial Halliwell–Asada pathway, which operates to deliver reducing equivalents and limit oxidative stress (Noctor and Foyer, 1998). Perhaps a similar cyclical pathway could operate in mussel gills to sequester abundant reducing equivalents. Using matrix volume determination (Walajtys-Rhode et al., 1992), total glutathione concentration in rat liver and heart mitochondria is 3–4 mmol l$^{-1}$ (Shiva et al., 2004; Shu et al., 2003), and ascorbate concentration in heart mitochondria is approximately 1.3 mmol l$^{-1}$ (Shu et al., 2003). Another possible sink for molecular sulfide under oxygen-limiting conditions may be the formation of disulfides, which occurs on free cysteine residues of sulfide binding proteins in Riftia pachyptila blood (Zal et al., 1998; Bailly et al., 2002) and trisulfide glutathione (Pruet, 1993; Moutiez et al., 1994). Further sulfide and oxygen respirometric investigations are needed to evaluate the existence and nature of a reducing equivalent/sulfide store present in sulfide-maintained mussel gills and lost after the mussels are acclimated to sulfide-free conditions.

**Alternative oxidase pathway**

Inhibition by KCN of gill mitochondria suggests that under non-limiting $P_{O_2}$, cytochrome c oxidase accounts for about 80% of the sulfide-stimulated oxygen consumption rate. A cyanide-insensitive alternative oxidase accounts for the remaining 20%, as seen by SHAM inhibition. Alternative oxidases, previously found in plants and microorganisms (Vanlerberghe and McIntosh, 1997), have been implicated in sulfide oxidation pathways in the lugworm (Völkel and Griesshaber, 1997) and in mussel gill mitochondria (Parrino et al., 2000). Mussel gill mitochondria exhibiting a 200–300% increase in oxygen consumption rate during exposure to 5–10 μmol l$^{-1}$ sulfide also exhibit a <50% inhibition at the same sulfide concentration if the alternative oxidase is first blocked by SHAM (Parrino et al., 2000). The alternative oxidase may serve as a shunt for sulfide oxidation electrons, thus allowing cytosolic sulfide concentration to be regulated and limiting inhibitory effects of sulfide on cytochrome c oxidase. Because alternative oxidases are not involved in proton pumping, sulfide consumption could continue even during low metabolic demand for ATP generation. The partitioning of electrons through each terminal oxidase may be adjustable and depend on metabolic demand and the concentration of available substrates, a potentially complex matrix. Inhibition of both terminal oxidases under non-limiting oxygen conditions creates anoxia-like conditions for sulfide and oxygen consumption, demonstrating that sulfide consumption is not totally dependent on $P_{O_2}$. With further respirometric studies, the metabolic role of each terminal oxidase could be defined.

**Mitochondria from sulfide-free mussels**

Although gill mitochondria isolated from sulfide-free mussels are stimulated by typical mitochondrial substrates, they exhibit a much weaker response to sulfide (Lee et al., 1996), and reach limits for both oxygen and sulfide consumption rates that are 25% or less of the rates exhibited by mitochondria from sulfide-maintained mussels under comparable conditions. However, the S:O$_2$ ratios are maintained near unity at all sulfide concentrations and under conditions of cytochrome c oxidase and alternative oxidase inhibition. These results suggest that specific components of sulfide metabolic pathways may be downregulated during acclimation to sulfide-free conditions, although the ability of these mitochondria to metabolize organic substrates and ADP indicates that cytochrome c oxidase activity is maintained. The limited sulfide consumption rates suggest that the activities of both sulfide oxidase and alternative oxidase have been substantially lowered. In addition, the maintenance of coupled oxygen and sulfide consumption suggests that the reducing equivalent/sulfide store concentration is also decreased, which is in agreement with lower GSH and AA levels in gills of sulfide-free mussels. Preliminary data from a 2-D gel electrophoresis proteomics study of *G. demissa* gills demonstrated that approximately 36 distinct proteins are differentially expressed in gills from sulfide-maintained mussels compared to those from sulfide-free mussels. These results indicate that specific sulfide metabolic pathway components may be inducible by environmental sulfide in animals adapted to high sulfide exposure. Identification of inducible components of sulfide metabolic pathways may help us understand how organisms adapt to the extreme conditions of high sulfide and low oxygen and also understand the role that sulfide may play in cell signaling in all biological organisms, including humans (Wang, 2002; Moore et al., 2003).

**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>ascorbic acid</td>
</tr>
<tr>
<td>DHAA</td>
<td>dehydroascorbic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
</tr>
<tr>
<td>MES</td>
<td>2-morpholinoethanesulfonic acid</td>
</tr>
<tr>
<td>PEEK</td>
<td>polyether ether ketone</td>
</tr>
<tr>
<td>2-PDS</td>
<td>2,2’-dipyridyl disulfide</td>
</tr>
<tr>
<td>POS</td>
<td>polarographic oxygen sensor</td>
</tr>
<tr>
<td>PSS</td>
<td>polarographic sulfide sensor</td>
</tr>
<tr>
<td>RCR</td>
<td>respiratory control ratio</td>
</tr>
<tr>
<td>SHAM</td>
<td>salicylhydroxamic acid</td>
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<tr>
<td>SMM</td>
<td>sulfide-maintained mussels</td>
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Preliminary results were presented during the symposium *Compensatory Responses of Mitochondria in Stressful Environments* of the sixth ICCPB, Mount Buller, Australia, February 2003. We thank the students of Cellular Physiology,
BY 416/616, fall semester 2002 for their participation in this research.

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