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SULFIDE MAY DIRECTLY MODIFY CYTOPLASMIC HEMOGLOBIN DEOXYGENATION IN *SOLEMYA REIDI* GILLS

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Summary

The clam *Solemya reidi*, which survives in sulfide-rich sediments, houses intracellular sulfide-oxidizing bacteria as symbionts in its gills. The gill bacteriocytes also contain a high concentration of cytoplasmic hemoglobin. Although the *in situ* hemoglobin optical spectrum was not altered in the presence of hydrogen sulfide, hemoglobin deoxygenation was significantly slowed and incomplete when sulfide was present. A sulfide-mediated decrease in oxygen consumption rate, a shift in intracellular pH or the conversion of hemoglobin to an unusual derivative could all slow *in situ* hemoglobin deoxygenation. However, under low sulfide levels at which deoxygenation is incomplete, oxygen consumption rate was not inhibited, intracellular pH decreased by less than 0.1 units and the only hemoglobin derivatives present were deoxyhemoglobin and oxyhemoglobin. These results and preliminary measurements of the isolated gill hemoglobin dissociation rate constants suggest that sulfide or a rapidly formed oxidation product may directly influence the rate of *Solemya reidi* gill hemoglobin deoxygenation.

Key words: sulfide, cytoplasmic hemoglobin, gills, clams, oxygen, intracellular pH, respiration rate, symbiosis, *Solemya reidi*.

Introduction

The clam *Solemya reidi*, which survives in sediments where hydrogen sulfide concentrations can reach nearly 2 mmol l^{-1} (Lee *et al.* 1992), houses intracellular sulfide-oxidizing bacteria as symbionts in specialized gill cells called bacteriocytes (Felbeck, 1983). The symbionts utilize hydrogen sulfide to fix carbon dioxide into organic compounds for translocation to the host (Felbeck, 1983; Fisher and Childress, 1986; Anderson *et al.* 1987). This symbiosis requires both oxygen and hydrogen sulfide, molecules which are chemically reactive with each other; in addition, hydrogen sulfide is a poison of aerobic respiration (Nicholls, 1975; National Research Council, 1979). The gills of *Solemya reidi* also contain cytoplasmic hemoglobin (Kraus *et al.* 1992) in bacteriocytes as well as in symbiont-free ciliated cells. In fact, hemoglobin appears to be a common feature in bivalve/sulfide-oxidizing bacteria symbioses (Wittenberg, 1985), although a common role of the hemoglobins within the symbioses is not yet apparent (Kraus, 1995). Gill hemoglobin of the related species *Solemya velum* reversibly binds sulfide *in vivo* as ferric hemoglobin sulfide (Doeller *et al.* 1988). The three cytoplasmic gill hemoglobins of the symbiont-containing *Lucina pectinata* are only found in the bacteriocyte domain and react differentially to hydrogen sulfide *in vitro*: hemoglobin I reversibly binds sulfide as ferric hemoglobin sulfide, whereas hemoglobins II and III are refractory to sulfide (Kraus and Wittenberg, 1990; Kraus *et al.* 1990). Hemoglobin in *Solemya reidi* gills does not react *in vivo* with sulfide to form an identifiable sulfide–hemoglobin derivative; however, symbiont cytochrome c_{552} is reduced by hydrogen sulfide and the rate of reduction may be slowed in the absence of a functional hemoglobin (Kraus *et al.* 1992). These data suggest that intracellular gill hemoglobin may play a pivotal role in the delivery of oxygen and/or sulfide in bivalve/sulfide-oxidizing bacteria symbioses.

In this paper, we report the effects of sulfide on the kinetics of hemoglobin reactions with oxygen in the gills of *Solemya reidi*. To determine whether the effects of sulfide are restricted to hemoglobin or are mediated by changes in gill metabolism, we looked for ferryl hemoglobin formation in the presence of sulfide and measured the effects of sulfide on intracellular pH and gill oxygen consumption rate.

Materials and methods

Animal collection and maintenance

Solemya reidi Bernard were collected by Van Veen grab at the Hyperion sludge outfall in Santa Monica Bay, CA, USA, from a depth of 50–100 m. Animals were maintained in sulfide-

rich marine mud at 8–10 °C and were used within 1 month after collection. All experiments were performed at 10 °C.

Sample preparation

Gills of *Solemya reidi* were excised and rinsed in 0.4 μm Millipore-filtered artificial sea water (ASW, 32 ‰, approximately pH 8, Tropic Marin). For respirometric measurements, gills were freed from the adjoining hypobranchial tissue and cut into sections of approximately 100 filaments, approximately 80 mg fresh mass. For spectrophotometric measurements, individual filaments were cut from the central ligament of the gill and placed as a continuous layer of overlapping single filaments (individual filaments are about 40 μm thick; Powell and Somero, 1985; Fisher and Childress, 1986) on a piece of 100 mesh stainless-steel screen (see Kraus *et al.* 1992). On the back of the screen, black plastic adhesive tape (electrical tape) with a 1 mm×7 mm opening served as an aperture for the transmitted light beam. For experiments in which filaments were bathed in humidified gas, the layer was covered with a gas-permeable membrane (MEM 213, 25 μm thick, Mempro, Troy, NY, USA). For experiments in which filaments were bathed in liquid, the layer was covered with a second piece of 100 mesh stainless-steel screen.

Optical spectrophotometry of gills

Optical spectra from gill filaments were acquired using a recording spectrophotometer equipped with a scattered transmission accessory and a digital data acquisition and analysis system (Cary 14, Aviv Associates, Lakewood, NJ, USA). One or more layers of Parafilm (American Can Company, Greenwich, CT, USA) were used to attenuate the reference beam and to partially balance light scattering. Optical spectra were recorded from 400 to 650 nm at 1 nm intervals. Mixtures of air, nitrogen and carbon monoxide prepared using a mass-flow controller (Tylan Corporation, Torrance, CA, USA) were humidified and passed through the spectrophotometer cuvette at a flow rate of 100 ml min^{-1}. Hydrogen sulfide gas was added to the humidified gas mixture from a glass syringe driven by a syringe pump (Harvard Apparatus, Southnatick, MA, USA). Oxygen partial pressure in the sample cuvette was monitored with a sulfide-insensitive polarographic oxygen sensor (Orbisphere 2110, Geneva, Switzerland) positioned in the floor of the cuvette.

Sulfide concentration

Of the two major sulfide species that exist at physiological pH, H_2S and HS^-, H_2S is primarily responsible for inhibition of aerobic respiration by reversibly binding to the a_3 heme of cytochrome oxidase (Nicholls, 1975; National Research Council, 1979). The ambient concentration of total sulfide and each sulfide species is necessarily different from the respective intracellular concentration as a result of several factors. H_2S is 2.7 times more permeable than HS^- through the body wall of the marine echiuran worm *Urechis caupo* (Julian and Arp, 1992) and is much more permeable through cell membranes (Jacques, 1936). Factors such as membrane potential, probably near −60 mV as reported for the gill cells of *Mytilus edulis* (Murakami and Takahashi, 1975), and an intracellular pH of 7.3 (this paper) would cause the intracellular concentration of HS^- to be much lower than the ambient concentration. In order to calculate concentrations of H_2S and total sulfide on the basis of P_{H_2S}, algorithms for pK of the H_2S/HS^- couple and H_2S solubility as a function of temperature, salinity and pH were used [equation 3, Millero, 1986; corrected coefficients in the salinity portion of equation 3 are −0.060581, +0.037953 and −0.00602340 (F. Millero, personal communication); equations 15 and 19, Millero and Hershey, 1989)].

The effects of sulfide on intracellular hemoglobin

Gill filaments in the spectrophotometer were oxygenated using air and deoxygenated using nitrogen. Optical spectra were recorded at each steady state, and the kinetic change from oxyhemoglobin to deoxyhemoglobin was recorded at 437 nm, the Soret peak in the difference spectrum of oxyhemoglobin minus deoxyhemoglobin. Gills were reoxygenated and exposed to hydrogen sulfide, 0.025–4 mmHg P_{H_2S} (1 mmHg=0.133 kPa) (4.6–767 μmol l^{-1} H_2S, 58.4 μmol l^{-1} to 9.3 mmol l^{-1} total sulfide), in air for 10–15 min, sufficient time for cytochrome c_{552} reduction (Kraus *et al.* 1992), after which optical spectra were recorded. Gills were then deoxygenated in the presence of hydrogen sulfide. Kinetic changes were followed and optical spectra were recorded as the trace approached an asymptote. Fractional saturation of hemoglobin with oxygen under these conditions was calculated as the ratio of the difference in optical density between two wavelengths to the maximum optical density difference observed in difference spectra of gills equilibrated with air minus gills equilibrated with nitrogen. Wavelength pairs for this calculation were 412 nm and 437 nm, 437 nm and 470 nm, and 560 nm and 580 nm, and were chosen to minimize the relatively small spectral contribution of symbiont cytochrome c_{552} (Kraus *et al.* 1992).

Single-wavelength optical density traces of gill hemoglobin oxygenation and deoxygenation were fitted with single- or double-exponential rate equations, using the Durbin–Watson statistic as a best-fit criterium (On-Line Instrument Systems, Inc. Kinfit software, Bogart, GA, USA). Because the kinetics of hemoglobin oxygenation and deoxygenation *in vivo* are governed by a combination of factors, such as molecular rate constants of hemoglobin association and dissociation with oxygen and the rates of oxygen diffusion and oxygen consumption (Colacino *et al.* 1987), the calculated rate constants which characterize each trace are termed *in vivo* rate constants to reflect the complexity of the system and to distinguish them from the molecular rate constants.

Ferryl hemoglobin formation

The possibility that gill hemoglobin formed the ferryl derivative (Fe^{4+}) in the presence of sulfide was investigated in two ways. First, because ferryl hemoglobin does not form the carbon monoxide adduct without prior reduction to the ferrous

state (Aviram *et al.* 1978), the presence of ferryl hemoglobin may be indicated by slower kinetics of hemoglobin–carbon monoxide binding in the presence of sulfide than in its absence. For these experiments, gills were equilibrated in ASW at pH 8 with nitrogen alone or in the presence of hydrogen sulfide at 1 mmHg P_{H_2S} (192 μmol l^{-1} H_2S, 2.3 mmol l^{-1} total sulfide) and then exposed to carbon monoxide at 20 mmHg P_{CO}. The kinetics of carbon monoxide hemoglobin (HbCO) formation was monitored at 420 nm, the peak of the HbCO spectrum, and an optical spectrum was recorded at equilibrium. Second, direct ferryl hemoglobin formation was attempted by exposing gills in nitrogen-equilibrated ASW to stepwise additions of hydrogen peroxide, 0.5–5 mmol l^{-1}, for 10–20 min at each step and then recording optical spectra.

Measurement of intracellular pH

To measure intracellular pH (pHi), gill cells were loaded with the pH indicator 6-carboxyfluorescein (6-CF; Molecular Probes, Eugene, OR, USA), chosen because its absorbance spectrum and that of hemoglobin show minimal overlap (Wittenberg *et al.* 1987; see Fig. 5). To load gill cells with dye, gill filaments were incubated in the presence of 700 μmol l^{-1} 6-CF as the diacetate ester in ASW buffered with 10 mmol l^{-1} Hepes and 10 mmol l^{-1} Pipes at pH 6.3 for 15–45 min at room temperature (20 °C). An incubation pH of 6.3 was necessary to increase the concentration of the uncharged permeant species in order to facilitate entry through cell membranes for initial dye loading (Thomas *et al.* 1982), and the combination of Hepes and Pipes allowed pH buffering from pH 5.8 to 8.5. After washing in air-equilibrated ASW at pH 7.7 for approximately 1 h in a shaker bath at 10 °C, dye-loaded filaments were mounted on the gill holder assembly and placed in 6.7 ml of the same solution in the spectrophotometer cuvette with a 4 ml gas space above. The solution was stirred at 460 revs min^{-1} with a stainless-steel turbine positioned just below the liquid–gas interface for equilibration of liquid with inflowing gas mixtures. Solution or ambient pH (pHa) was continually recorded with a combination pH electrode (Ross 8104, Boston, MA, USA) positioned in the floor of the cuvette. Optical spectra were recorded and ratios of optical density at 495 nm, the pH-sensitive peak of the dye *in vivo*, to optical density at the isosbestic point *in vivo* were calculated.

Optical density ratios of the hydrolyzed diacetate ester of 6-CF were calibrated in buffered ASW over a solution pH range of 5.8–8.5. The effects of sulfide on the calibration curve were determined by equilibrating the solution of 6-CF with 1 mmHg P_{H_2S} (192 μmol l^{-1} H_2S, 205 μmol l^{-1} to 7.0 mmol l^{-1} total sulfide). Optical density ratios of 6-CF loaded in gill cells were calibrated following the method of Ellington (1993): after recording optical spectra of dye-loaded gill filaments under experimental conditions (see below), the experimental solution was exchanged with ASW containing a high $[K^+]$ (290 mmol l^{-1} NaCl, 160 mmol l^{-1} KCl, 7.5 mmol l^{-1} $CaCl_2$, 25 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} Hepes, 10 mmol l^{-1} Pipes, pH 7.8; Ellington, 1993), and the gills were exposed to the cation ionophore nigericin (10 μg ml^{-1}; Molecular Probes, Eugene, OR, USA) to allow equilibration of intracellular and solution pH. Solution pH was titrated from 6.2 to 8.2 with microliter additions of 5 mol l^{-1} NaOH or 5 mol l^{-1} HCl through an injection port in the cuvette stopper. Optical spectra were recorded at each pH following a 10–15 min equilibration period traced at 495 nm.

To assess the effect of sulfide on intracellular pH (pHi), optical spectra of dye-loaded gill filaments in ASW at pH 8.0 were recorded before and after the addition of 0.5 or 1 mmHg P_{H_2S} (96 or 192 μmol l^{-1} H_2S, 1.2 or 2.3 mmol l^{-1} total sulfide) to the inflowing gas stream. To assess pHi regulation, pHa was varied stepwise from 6.2 to 8.2 and optical spectra at equilibrium were recorded at each step. To assess the effect of pH on intracellular hemoglobin saturation with oxygen, dye-loaded gill filaments were first equilibrated at 7 mmHg P_{O_2}, then pHa was varied stepwise and optical spectra were recorded. At 7 mmHg P_{O_2}, hemoglobin is approximately half-saturated, and a change in hemoglobin oxygen-affinity and/or intracellular P_{O_2} should be reported as a change in fractional saturation. Using undyed filaments, the kinetics of hemoglobin deoxygenation was also determined at pHa values of 8.0–6.3, which correspond to pHi values of 7.3–6.7, respectively.

Respirometry of gills

The oxygen consumption rate of excised intact *Solemya reidi* gills was measured in a temperature-controlled dual closed-chamber polarographic respirometer (Oxygraph 60697, Cyclobios Paar, Graz, Austria; Haller *et al.* 1994). Gill pieces were placed on a 60 mesh stainless-steel screen disc positioned just above the magnetic stir bar in the respirometer chamber containing 5 ml of filtered ASW, pH 8. Gill wet and dry masses averaged 86.0±31.7 mg (N=21) and 19.7±7.2 mg (means ± S.D.; N=21), respectively. After control oxygen consumption rates had been established, sulfide was added with a microliter syringe through the injection port to final concentrations of 50–600 μmol l^{-1} Na_2S (4.1–49 μmol l^{-1} H_2S, 0.02–0.26 mmHg P_{H_2S}). The effects of sulfide on oxygen consumption rates were determined near full air saturation and 15 % air saturation (155 mmHg and 20 mmHg P_{O_2}, respectively).

Data presentation

Data are presented as mean ± standard deviation, with repetition number (N) in parentheses. Comparisons were made using the Student's t-test and significance was assigned at $P \leqslant 0.05$.

Results

Optical spectra of gill heme proteins

Optical spectra of *Solemya reidi* gills in ASW at pH 8 exposed to hydrogen sulfide, 0.2–7 mmHg P_{H_2S} in air (27–956 μmol l^{-1} H_2S, 513 μmol l^{-1} to 18 mmol l^{-1} total sulfide), showed a spectral change proportional to P_{H_2S} that was ascribed only to the reduction of bacterial cytochrome c_{552}, as reported in Kraus *et al.* (1992). In air-equilibrated

ASW, the optical spectrum of gill hemoglobin remained unaltered by sulfide, indicating that a direct interaction between heme iron and sulfide is not detectable (Kraus *et al.* 1992). However, hemoglobin deoxygenation, traced at a single wavelength, appeared to be incomplete in the presence of sulfide (see *Effect of sulfide on the kinetics of hemoglobin deoxygenation*). A spectrum taken as the deoxygenation trace approached an asymptote indicated that two hemoglobin derivatives were present in the presence of nitrogen and sulfide (Fig. 1A). In the difference spectrum of gills equilibrated with air and sulfide minus gills equilibrated with nitrogen and sulfide (Fig. 1B), the contribution of sulfide-reduced bacterial cytochrome c_{552} is removed and the two hemoglobin derivatives present under nitrogen and sulfide conditions in Fig. 1A can be tentatively identified. The difference in amplitude of the two difference spectra suggests that the second hemoglobin derivative in the sulfide and nitrogen spectrum was either oxygenated hemoglobin or a derivative with similar spectral features, such as ferryl hemoglobin (Fe^{4+}; Wittenberg *et al.* 1972).

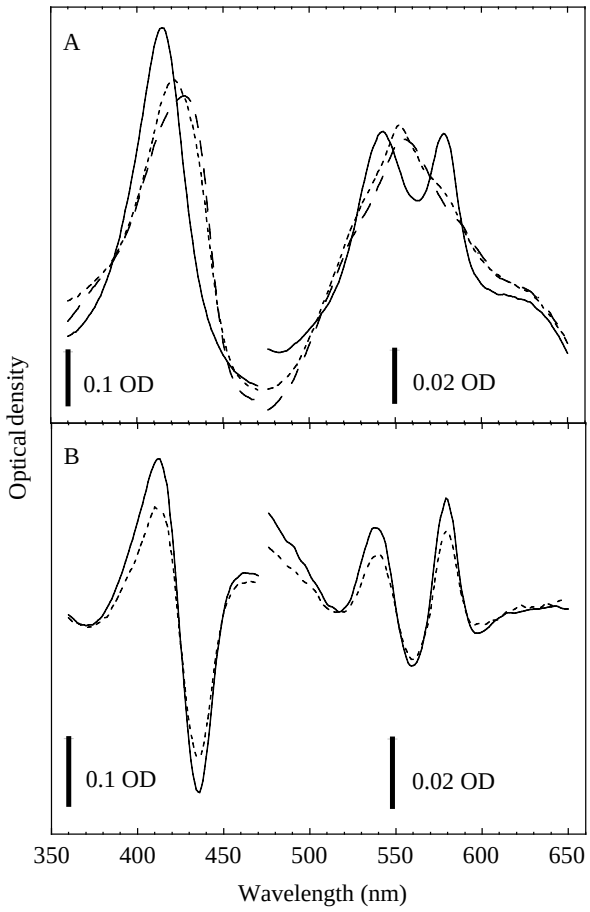

Fig. 1. Optical spectra of *Solemya reidi* gills. Traces in the visible region have been amplified fivefold. (A) Direct spectra of gills equilibrated with air (solid line), with nitrogen (dashed line), and with nitrogen and 0.4 mmHg P_{H_2S} (dotted line) (1 mmHg=0.133 kPa). To match the scattering of the gill tissue approximately, the reference cuvette contained a dilute solution of whole milk. The peak at 552 nm in the nitrogen and sulfide spectrum is attributable to reduced bacterial cytochrome c_{552}. (B) Difference spectra of gills equilibrated with air minus the same gills equilibrated with nitrogen (solid line) and of gills equilibrated with air and sulfide at 0.4 mmHg P_{H_2S} minus the same gills equilibrated with nitrogen and sulfide at 0.4 mmHg P_{H_2S} (dotted line). The contribution of sulfide-reduced bacterial cytochrome c_{552} is removed and the amount of oxyhemoglobin remaining after deoxygenation was 22.1 % in this experiment. OD, optical density units.

Oxyhemoglobin or ferryl hemoglobin formation

An unremarkable carbon monoxide hemoglobin spectrum, observed in the presence of nitrogen and 1 mmHg P_{H_2S} (192 μmol l^{-1} H_2S, 2.3 mmol l^{-1} total sulfide) within 60 s after the addition of carbon monoxide (spectrum not shown), accounted for more than 95 % of the total hemoglobin present in the gill. The kinetics of carbon monoxide hemoglobin formation remained monotonic and the *in vivo* reaction rate was not slowed in the presence of sulfide. In a direct spectrum taken 20 min after adding 5 mmol l^{-1} hydrogen peroxide to *S. reidi* gills equilibrated with nitrogen, the visible region gave no clear indication of substantial ferryl hemoglobin formation. The difference spectrum of gills in nitrogen plus 5 mmol l^{-1} hydrogen peroxide minus gills in nitrogen alone showed a feature in the Soret region which suggested that a small amount of the deoxyhemoglobin, less than 7 %, was converted to a species with a maximum at 420 nm. This wavelength corresponds to the peak of ferryl hemoglobin observed in surviving anaerobic *Aplysia californica* nerve and muscle tissue (B. A. Wittenberg *et al.* 1965; J. B. Wittenberg *et al.* 1965). However, this spectral shift was not observed in *S. reidi* gills in the presence of nitrogen plus sulfide. Because conversion of *S. reidi* gill hemoglobin to ferryl hemoglobin is not evident in the presence of nitrogen and sulfide, we argue that, in addition to deoxyhemoglobin, the other hemoglobin derivative present under these conditions is oxyhemoglobin.

In the presence of sulfide, the relative amount of oxyhemoglobin remaining when deoxygenation approached an asymptote was proportional to the partial pressure of hydrogen sulfide in the nitrogen stream (Fig. 2). At 0.025 mmHg P_{H_2S} (4.8 μmol l^{-1} H_2S, 58 μmol l^{-1} total sulfide), oxyhemoglobin was detectable at 2.2 %, and a maximum of 20–25 % oxyhemoglobin was reached at approximately 0.5 mmHg P_{H_2S} (96 μmol l^{-1} H_2S, 1.2 mmol l^{-1} total sulfide). After sulfide removal, gill hemoglobin could be completely deoxygenated, indicating reversal of the sulfide effect.

Effect of sulfide on the kinetics of hemoglobin deoxygenation

Deoxygenation of *S. reidi* gill hemoglobin *in vivo* was typically complete within 150 s (Fig. 3). The deoxygenation trace was best fittted with a single-exponential rate equation, giving an *in vivo* rate constant of 29.2±13.9 ms^{-1} (N=16). Because differences in preparation thickness or diffusion distance contributed to the variability of the average *in vivo* rate constant, *in vivo* rate constants are hereafter reported as fractions of control values.

In the presence of sulfide, the deoxygenation trace became

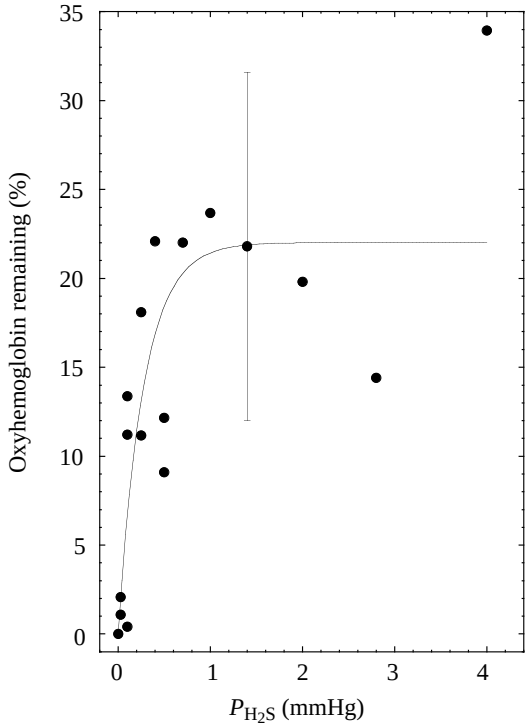

Fig. 2. Oxyhemoglobin, as a percentage of total hemoglobin in the *Solemya reidi* gill, remaining as the deoxygenation event approached an asymptote, as a function of ambient P_{H_2S}. Data point at 1.4 mmHg P_{H_2S} is an average of seven experiments with a standard deviation error bar; all other data points represent single experiments.

more complex than the single monotonic event observed when gills were equilibrated with nitrogen alone. At 0.025–0.1 mmHg P_{H_2S} (4.8–19 μmol l^{-1} H_2S, 58–234 μmol l^{-1} total sulfide), deoxygenation was initially as rapid as that of controls, then the rate slowed (Fig. 3). At P_{H_2S} values above about 0.1 mmHg, this slow rate became much more pronounced and began to dominate the deoxygenation trace. At 0.3 mmHg P_{H_2S} (58 μmol l^{-1} H_2S, 690 μmol l^{-1} total sulfide) and above, deoxygenation typically did not reach completion within 300 s and the trace was best fitted with a double-exponential rate equation, indicating at least two simultaneous kinetic events. The *in vivo* rate constant characterizing the dominant slow kinetic event approached a limit averaging 0.26±0.12 (N=16) times the control values (Fig. 4). The effect of sulfide on deoxygenation was reversible. Subsequent to sulfide removal under aerated conditions, assessed by monitoring the reoxidation of cytochrome c_{552} (requiring less than 10 min), the *in vivo* rate constant of hemoglobin deoxygenation returned to the control value.

Oxygenation of gill hemoglobin *in vivo* was typically complete within 20 s. The oxygenation trace was best fitted with a single-exponential equation, giving an *in vivo* rate constant of 220±92 ms^{-1} (N=18). In the presence of sulfide, the *in vivo* rate constant of oxygenation was 183±70 ms^{-1} (N=18), not significantly different from the control value.

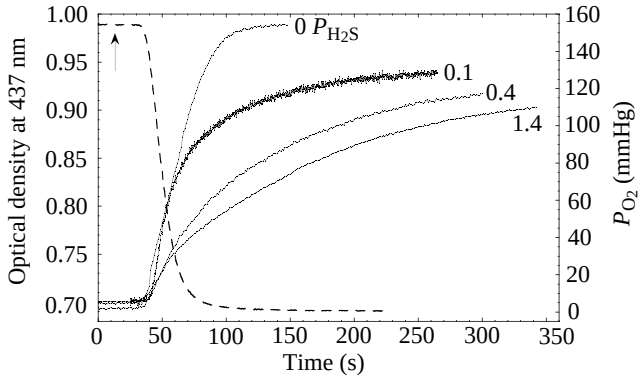

Fig. 3. Changes in hemoglobin optical density and cuvette P_{O_2} during deoxygenation events in *Solemya reidi* gill filaments as a function of time. Solid lines (left-hand axis) are traces of optical density at 437 nm, the peak wavelength of the deoxyhemoglobin minus oxyhemoglobin difference spectra. Traces represent separate experiments at different values of P_{H_2S} (shown at the end of the trace). Dashed line (right-hand axis) is P_{O_2} in the cuvette. Deoxygenation using nitrogen gas starts at the arrow.

Effect of sulfide on intracellular pH

Optical spectra of 6-carboxyfluorescein (6-CF) in gill cells obtained at different solution or ambient pH (pHa) in the presence of nigericin are shown in Fig. 5. The pH-sensitive peak of 6-CF, 493 nm, and the isosbestic wavelength, 462 nm (Molecular Probes, Eugene OR, USA), shifted to 495 nm and close to 475 nm, respectively, in gill cells (Fig. 5). The concentration of 6-CF loaded into gill cells was typically

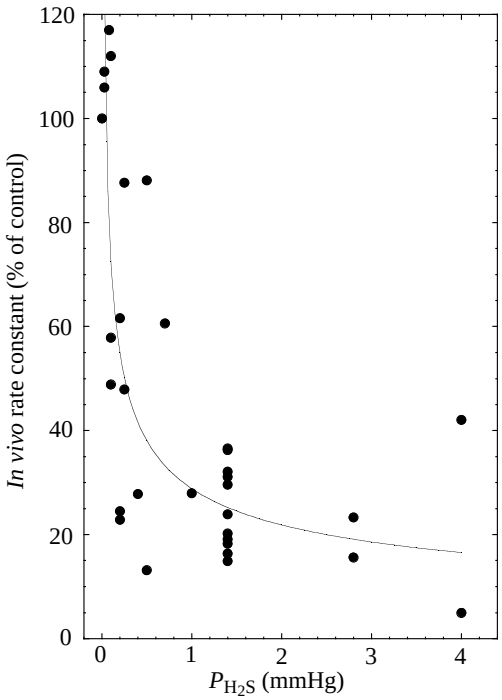

Fig. 4. Change in the *in vivo* rate constant, as a percentage of the control value, of the *Solemya reidi* gill hemoglobin deoxygenation event as a function of ambient P_{H_2S}. Data points represent individual experiments.

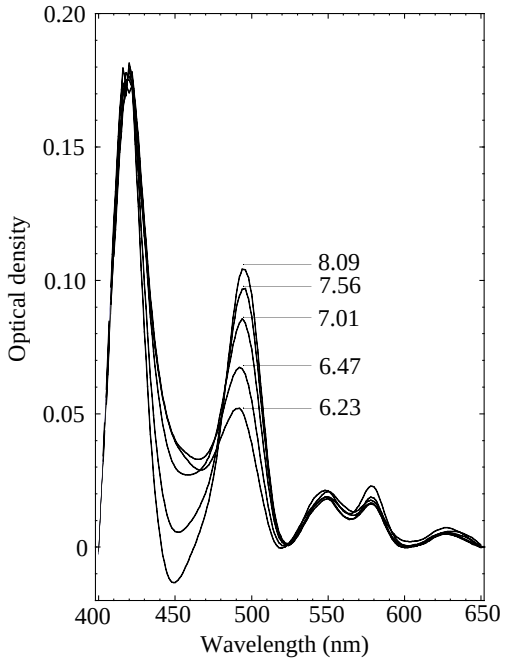

Fig. 5. Optical absorbance spectra of *Solemya reidi* gill filaments loaded with 6-carboxyfluorescein at an intracellular concentration of 330 μmol l^{-1}, equilibrated at different solution pH (shown on graph) with nigericin in aerated buffered high-K^+ artificial sea water.

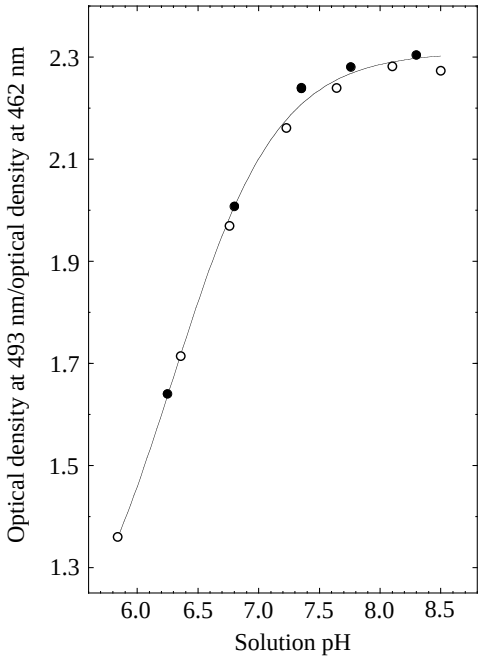

Fig. 6. Calibration curve of 6-carboxyfluorescein in air-equilibrated buffered artificial sea water with (filled circles) and without (open circles) sulfide at 1 mmHg P_{H_2S}, using ratios of optical density at 493 nm to that at 462 nm. Data are fitted with the Henderson–Hasselbalch equation, giving a pK of 6.3 (pK≈6.4, Molecular Probes).

approximately 300 μmol l^{-1}, estimated using a 0.004 cm pathlength and an extinction coefficient of 83 l mmol^{-1} cm^{-1} (Molecular Probes, Eugene, OR, USA). Sulfide appears to have little effect on the optical density changes of 6-CF in response to pH (Fig. 6). Optical density ratios of intracellular 6-CF are less than those of 6-CF in solution, partly as a result of background optical absorbance and light scattering of tissue elements (Fig. 7).

Intracellular pH of *S. reidi* gill cells was 7.3±0.1 (N=5) in ASW at pH 8.0. Intracellular pH of *Mytilus edulis* gills, measured using the same procedure, averaged 7.4, similar to that reported by others for *Mytilus edulis* (Fan *et al.* 1991). In the presence of 0.5 or 1 mmHg P_{H_2S} (96 or 192 μmol l^{-1} H_2S, 1.17 or 2.3 mmol l^{-1} total sulfide), pHi declined to 7.2±0.04 (N=3). Solution pH of buffered ASW also declined by about 0.1 units when equilibrated with 1 mmHg P_{H_2S} (192 μmol l^{-1} H_2S, 1.9 mmol l^{-1} total sulfide). Gill cytoplasm maintains pHi between 6.8 and 7.3 over a range of pHa from 6.6 to 8.0 (Fig. 8).

Under hypoxic conditions where hemoglobin is partially saturated with oxygen, oxygen percentage saturation increased by 6 and 11 % as pHi decreased from 7.4 to 6.7 (Fig. 9). The kinetics of hemoglobin deoxygenation and reoxygenation were unaltered in gills in which pHi was shifted from 7.3 to 6.8 (data not shown).

Effect of sulfide on gill respiration

At air saturation (approximately 155 mmHg P_{O_2}), the oxygen consumption rate of excised intact *S. reidi* gills was

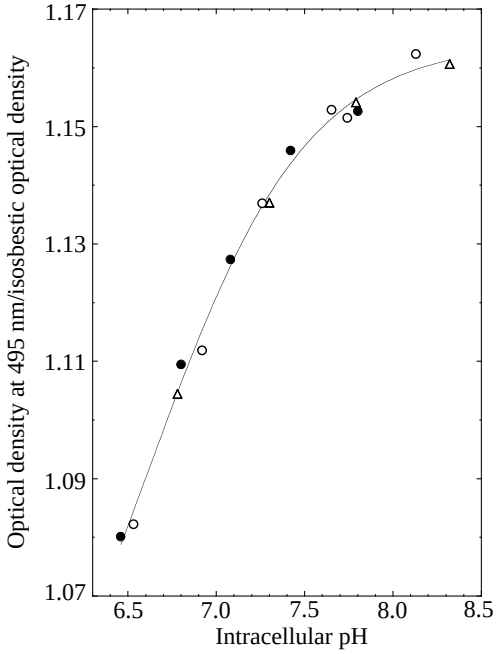

Fig. 7. Calibration curve of intracellular 6-carboxyfluorescein in *Solemya reidi* gills in air-equilibrated buffered artificial sea water, using ratios of optical density at 495 nm to that at the isosbestic wavelength (475 nm). Data are fitted with the Henderson–Hasselbalch equation, giving a pK of 6.4 (pK≈6.4, Molecular Probes). Different symbols represent separate experiments.

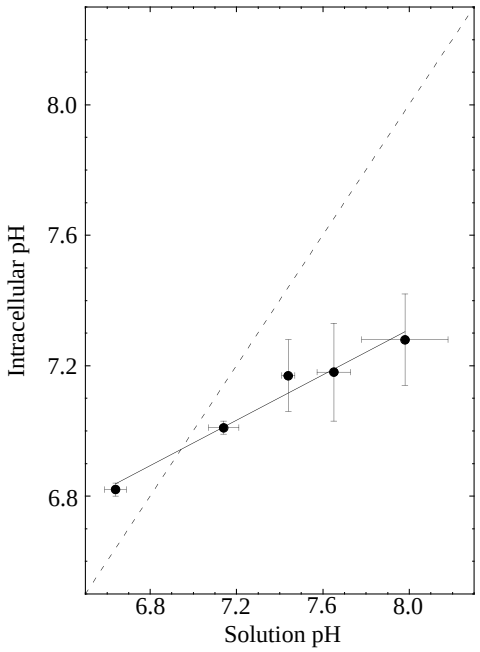

Fig. 8. Intracellular pH of *Solemya reidi* gills as a function of solution pH. Dashed line is the iso-pH line. Each point represents 3–7 replications with standard deviations.

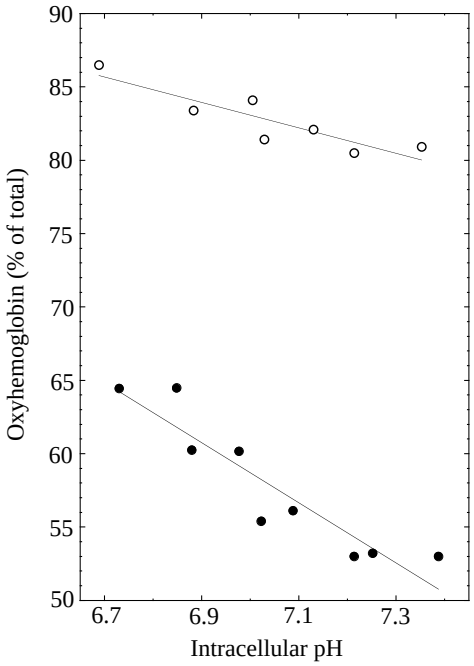

Fig. 9. The amount of oxyhemoglobin, as a percentage of total hemoglobin, in *Solemya reidi* gills as a function of intracellular pH. Gills were held in buffered artificial sea water equilibrated at 5 % air (approximately 7 mmHg P_{O_2}). Results from two separate experiments are shown. Differences in hemoglobin percentage saturation probably reflect differences in the oxygen diffusion characteristics of each preparation.

11.74±4.10 pmol O_2 s^{-1} mg^{-1} dry mass (N=48), which compares favorably with the oxygen consumption rate of whole *S. reidi* reported by Anderson *et al.* (1987). Normoxic oxygen consumption rates rose slightly to 1.14±0.23 (N=12) and 1.18±0.24 (N=13) times control values in the presence of 50 and 100 μmol l^{-1} Na_2S, respectively, and to 1.48±0.69 (N=4) times control values in the presence of 300 μmol l^{-1} Na_2S. At 600 μmol l^{-1} Na_2S, oxygen consumption rates were the same as control values (1.00±0.53 times control values; N=3). Under hypoxic conditions (15 % air saturation; approximately 20 mmHg P_{O_2}), oxygen consumption rates dropped to 3.36±1.44 pmol O_2 s^{-1} mg^{-1} dry mass (N=24), which is 0.25±0.09 times the normoxic control value. At this P_{O_2}, gill hemoglobin is approximately 85–90 % oxygenated. Oxygen consumption rates rose significantly to 1.48±0.20 (N=14; P=0.0007) and 1.47±0.19 (N=10; P=0.0032) times the hypoxic control value in the presence of 50 and 100 μmol l^{-1} Na_2S, respectively, and rose slightly to 1.08±0.18 (N=3) times the control value in the presence of 200 μmol l^{-1} Na_2S.

Discussion

Many infaunal marine organisms survive in habitats which contain potentially toxic levels of hydrogen sulfide in sediment pore water. The vascular and cytoplasmic hemoglobins as well as mitochondrial cytochromes found in these organisms are potential sites of chemical reactions with sulfide. In the presence of sulfide, hemoglobin can show a number of specific chemical responses. A well-known reaction of vertebrate erythrocytic oxyhemoglobin and cytoplasmic oxymyoglobin is the formation of the green sulfhemoglobin and sulfmyoglobin, respectively, in which sulfide binds to a peripheral location on the tetrapyrrole ring, thereby reducing oxygen affinity by several-fold (Berzofsky *et al.* 1971; Chatfield *et al.* 1987).

A second reaction is the formation of ferric hemoglobin sulfide in which sulfide binds to the ferric heme iron. If first chemically oxidized to the ferric state, vertebrate and presumably most other hemoglobins will readily bind sulfide at the iron (Keilin, 1933). However, some cytoplasmic hemoglobins from symbiont-harboring bivalves convert directly from oxyhemoglobin to ferric hemoglobin sulfide, possibly *via* a nucleophilic attack by sulfide on the heme iron. Hemoglobin I isolated from the symbiont-harboring gill of *Lucina pectinata* undergoes this reaction under conditions of low oxygen (2 mmHg) and hydrogen sulfide (0.1 mmHg) partial pressure, with an extremely high affinity for sulfide (K_m 3.4 nmol l^{-1}) (Kraus and Wittenberg, 1990). Approximately 50 % of the cytoplasmic hemoglobin in the surviving symbiont-harboring gill of *Solemya velum* also reversibly converts to ferric hemoglobin sulfide under conditions of low oxygen (60 mmHg) and hydrogen sulfide (0.7 mmHg) partial pressure (Doeller *et al.* 1988). In these symbiont-harboring bivalves, hemoglobin is implicated in the delivery of sulfide or reducing equivalents to intracellular symbionts.

A third reaction has been demonstrated in which hemoglobin catalyzes the oxidation of sulfide, as seen in the vascular hemoglobins of two marine worms (Patel and Spencer, 1963;

Powell and Arp, 1989), perhaps without a change in equilibrium oxygen affinity (Wells and Pankhurst, 1980). A result of hemoglobin participation in such a reaction may be the formation of hematin, a ferriheme hydroxide.

A fourth reaction involves sulfide interactions with hemoglobin at non-heme sites remote from the heme, leaving optical absorbance spectra unaltered. The high molecular mass (0.4×10^6 to 1.7×10^6 Da) hemoglobins of the vestimentiferan *Riftia pachyptila* combine simultaneously with oxygen at the heme iron and with sulfide at a site on the globin (Arp *et al.* 1987). In the hemoglobin of a related genus, *Lamellibrachia*, a cysteine residue possibly not involved in intrachain disulfide bridges has been proposed as a candidate for the sulfide binding site (Suzuki *et al.* 1990). The effects of sulfide binding on the vestimentiferan hemoglobin equilibrium oxygen-affinity and the kinetics of oxygen association and dissociation remain undescribed.

Although a direct interaction between sulfide and the heme iron or tetrapyrrole ring of the cytoplasmic hemoglobin in *S. reidi* gills is not detectable spectrophotometrically (Kraus *et al.* 1992), we report here that sulfide, directly or indirectly, alters the oxygen reaction kinetics. The most prominent effect of sulfide was the significant reduction of the rate of hemoglobin deoxygenation *in vivo* (Fig. 3). Several biochemical or cellular changes mediated by sulfide or its oxidation products may slow the rate or influence the measurement of hemoglobin deoxygenation *in vivo*. Ferryl hemoglobin formation, intracellular pH and oxygen consumption rates were considered.

Biological sulfide oxidation and the associated electron transfer reactions can yield various partially reduced sulfur compounds, oxygen- and sulfur-centered free radicals, as well as hydrogen peroxide. Hydrogen peroxide has been used to convert isolated oxyhemoglobin to ferryl hemoglobin in which the iron exists in the 4+ oxidation state (Wittenberg *et al.* 1972). The reversible conversion of cytoplasmic oxyhemoglobin to ferryl hemoglobin has also been observed in surviving tissues from molluscs and annelids during exposure to physiologically created anoxic conditions (B. A. Wittenberg *et al.* 1965; J. B. Wittenberg *et al.* 1965). The physiological role for such a reaction remains unclear. However, higher oxidation states of catalases and peroxidases which exist as intermediates during their enzymatic reactions (Schonbaum and Chance, 1976) may indicate a catalytic role for hemoglobin if oxidation to the ferryl derivative is readily reversible (J. B. Wittenberg *et al.* 1965).

The conversion of oxyhemoglobin to ferryl hemoglobin in the presence of nitrogen and sulfide might be misinterpreted as an apparent slow deoxygenation because ferryl hemoglobin can be spectrally similar to oxyhemoglobin (Wittenberg *et al.* 1972). The rapid monotonic formation of carbon monoxide hemoglobin in the presence of nitrogen and sulfide as well as the inability of hydrogen peroxide to oxidize much of the gill hemoglobin to a recognizable ferryl species indicated that, during nitrogen equilibration in the presence of sulfide, *S. reidi* gill hemoglobin does not form the ferryl derivative but instead resists deoxygenation and remains partially oxygenated.

A change in intracellular pH in the presence of sulfide and its oxidation products could alter hemoglobin oxygen-affinity *via* a Bohr shift and thus slow the rate of deoxygenation. Intracellular pH of *S. reidi* gills was 7.3, which is comparable to the pHi of other molluscan tissues (Ellington, 1993) and to the pHi of *Mytilus edulis* gills. The intracellular sulfide concentration in *S. reidi* gills at 1 mmHg P_{H_2S} is approximately $500\,\mu mol\,l^{-1}$, partitioned as $200\,\mu mol\,l^{-1}$ H_2S and $300\,\mu mol\,l^{-1}$ HS^-, indicating that $300\,\mu mol\,l^{-1}$ H^+ is liberated by H_2S during equilibration. The measured 0.1 unit drop in pHi during sulfide exposure indicated that this proton load was effectively buffered or regulated by the gill cells. Likewise, cellular regulation limited pHi changes over a wide range of solution pH.

Hemoglobin oxygen-saturation increased slightly as pHi decreased, perhaps mediated by changes in cellular metabolism or possibly by a slight reverse Bohr effect which is consistent with slower rate of deoxygenation. However, a 0.1 unit drop in pHi resulting from exposure to high sulfide levels would only account for a 1–2 % increase in oxyhemoglobin percentage saturation, not the 20–25 % oxyhemoglobin observed at the asymptote of the deoxygenation event.

Sulfide, a potent inhibitor of cytochrome oxidase, could decrease the oxygen consumption rate of the gills. This could effectively lengthen the time for hemoglobin deoxygenation *in vivo* if deoxygenation is diffusion-limited (Colacino *et al.* 1987). However, sulfide stimulates oxygen consumption and chemoautotrophy of whole animals with a maximum effect near $100\,\mu mol\,l^{-1}$ (Anderson *et al.* 1987). At 250–500 $\mu mol\,l^{-1}$ sulfide, anaerobic pathways may be utilized (Anderson *et al.* 1990) even though oxygen consumption rates remain above control levels, perhaps reflecting the activity of uncoupled mitochondria (O'Brien and Vetter, 1990). We report that the oxygen consumption rate of *S. reidi* gills was consistently increased at 50–300 $\mu mol\,l^{-1}$ sodium sulfide, equivalent to 0.05–0.3 mmHg P_{H_2S}, partial pressures that slow hemoglobin deoxygenation. A sulfide-stimulated oxygen consumption rate should hasten hemoglobin deoxygenation *in vivo* if oxygen egress from the tissue is diffusion-limited (Colacino *et al.* 1987).

The results of these experiments suggest that the slow rate of deoxygenation of *S. reidi* gill hemoglobin *in vivo* in the presence of sulfide is not the result of changes in pHi or oxygen consumption rate.

Hydrogen sulfide has an unusual concentration-dependent capacity to either stimulate or inhibit energy metabolism of the *Solemya reidi* symbiosis. Accordingly, its role as a direct or indirect modulator of specific metabolic steps might be expected. In the hydrothermal vent crab *Bythograea thermydron*, a product of sulfide oxidation, thiosulfate, has been shown to increase the hemocyanin equilibrium oxygen-affinity (Sanders and Childress, 1992). At low ambient P_{O_2}, oxygen consumption rates of *B. thermydron* are higher in animals with elevated hemolymph thiosulfate levels, supporting the hypothesis that thiosulfate modulation of hemocyanin oxygen-affinity alters the oxygen regulatory

abilities of the crabs *in vivo* (Gorodezky and Childress, 1994). A mechanism for thiosulfate modulation of *B. thermydron* hemocyanin oxygen-affinity may be similar to that by which other inorganic anions such as Cl^- (Mangum and Burnett, 1986) modulate crustacean hemocyanin reactions with oxygen. However, a mechanism for the effect of sulfide on *S. reidi* hemoglobin remains unknown.

Preliminary results with purified hemoglobins from *Solemya reidi* gills suggest that sulfide may directly affect the hemoglobin molecule. Hemoglobin extracted from *S. reidi* gills is purified in three fractions (D. W. Kraus, in preparation). The gill hemoglobins exhibit relatively high equilibrium oxygen-affinity, with P_{50} values of about 0.3–0.5 mmHg P_{O_2} at pH 7.5 and 20 °C. All three hemoglobins have similar oxygen dissociation rate constants, about $10\,s^{-1}$ at 10 °C, comparable with that of mammalian myoglobin at physiological temperature. However, in the presence of sulfide at $600\,\mu mol\,l^{-1}$ (equivalent to 0.43 mmHg P_{H_2S}), oxygen dissociation rates diverge: the fraction I dissociation rate constant increases by 30 %, whereas the dissociation rate constants for fractions II and III decrease by five- to 15-fold. These changes in oxygen dissociation rates exhibited by the purified hemoglobin fractions in the presence of sulfide appear to support the observation that a single-rate hemoglobin deoxygenation event becomes a two-rate deoxygenation event when living *S. reidi* gills are exposed to sulfide. A mechanism for a sulfide-mediated change in reaction kinetics remains unknown when sulfide does not perturb the optical absorbance spectrum of the heme. Physiologically, such a change in the rate of oxygen unloading might prolong the intracellular oxygen store when sulfide levels increase and/or oxygen becomes limited.

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