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Gill Hemoglobin May Deliver Sulfide to Bacterial Symbionts of *Solemya velum* (Bivalvia, Mollusca)

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Abstract. Two different hemoglobins occur in nearly equal concentrations in the gill of the bivalve mollusc, *Solemya velum* (total hemoglobin concentration is 200 μM/kg wet weight gill). A spectrophotometric study of intact gill filaments demonstrates that in the absence of sulfide, the gill hemoglobin may be oxygenated and deoxygenated, with part (5–20%) in the aquoferric form. In the presence of sulfide, about half of the gill hemoglobin is rapidly and reversibly converted to ferric hemoglobin, which then binds sulfide to form ferric hemoglobin sulfide (ferric hemoglobin with sulfide ligated to the heme iron in the distal ligand position); the balance continues to bind oxygen as oxyhemoglobin. *S. velum* inhabits reduced marine sediments where oxygen and hydrogen sulfide meet, and houses a dense population of intracellular chemoautotrophic sulfur-oxidizing symbiotic bacteria in its gill. We suggest that gill hemoglobins may mediate sulfide and oxygen delivery to the bacterial symbiont. Because sulfide is the dominant electron donor to fuel the *Solemya*/bacteria symbiosis, a cytoplasmic sulfide-binding protein that prevents the spontaneous reaction of sulfide with oxygen may be of utility in the nutrition of the animal.

Introduction

Intracellular chemoautotrophic bacteria, symbionts in the gills of certain molluscs, use the energy derived from the aerobic oxidation of sulfide (or other reductants) to fix carbon dioxide into organic compounds. Such bacterial symbionts form the primary base of the food chain of the dense populations of molluscs and giant tube worms at the deep sea hydrothermal vents (Cavanaugh *et al.*, 1981; Cavanaugh, 1985; Felbeck *et al.*, 1985). These symbiotic associations supply the majority of the carbon nutrition of the host (Cavanaugh, 1983; Felbeck, 1983; Dando *et al.*, 1985, 1986; Fisher and Childress, 1986; Anderson *et al.*, 1987; Southward, 1987).

Here we study the association between *Solemya velum*, an accessible small clam inhabiting the reduced sediment of eelgrass beds where oxygen and hydrogen sulfide meet, and chemoautotrophic bacteria housed in its gill (Cavanaugh, 1983). The gill obtains both sulfide and oxygen from the stream of seawater flowing through the mantle cavity, but the supply of sulfide and oxygen may be temporally separate as the animal moves within its burrow from the upper arms where oxygen predominates to the basal stem deeper in the sediment where sulfide predominates (Stanley, 1970; Jenner, 1977; Doeller, 1986). An intermittent supply suggests a need to store sulfide and oxygen in the gill. The diffusion path to the most remote bacterium housed in gill filament bacteriocytes is long (10–15 μm; Cavanaugh, 1983; Doeller, 1986), suggesting a need for facilitation of sulfide and oxygen diffusion to meet the demand by the symbionts.

Cytoplasmic hemoglobin is characteristic of the gills of most bivalves housing sulfur-oxidizing symbionts (Dando *et al.*, 1985; Wittenberg, 1985; *Lucinoma borealis* with symbionts may lack gill hemoglobin, Dando *et al.*, 1986; *Yoldia limatula* with few or no symbionts has gill hemoglobin at high concentration, Wittenberg, 1985; *Nucula proxima* with no symbionts has gill hemoglobin at low concentrations, Doeller, Kraus, and Smith, unpub. data). *S. velum* lacks a circulating hemoglobin (blood oxygen transport is assured by a circulating hemo-

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cyanin, Mangum *et al.*, 1987), but has hemoglobin (200 μmol/kg wet weight gill) located in the bacteriocytes as well as other cells of the gill (Doeller, 1986). We have isolated two different cytoplasmic hemoglobins in roughly equal amounts from the gill. One reacts solely with oxygen and the other reacts with sulfide as well. In this paper we will show that about half of the hemoglobin in the living gill reacts with hydrogen sulfide, and argue that the sulfide-reactive hemoglobin serves to make sulfide available to the bacterial symbionts.

Materials and Methods

Animals

Solemya velum was collected from intertidal and subtidal sandy shoals near Morehead City, North Carolina, and from tidal mud flats near Woods Hole, Massachusetts. Average animal size was 1.7 cm long, with 0.04 g gill wet weight.

Isolation of Solemya *hemoglobins*

Gills were frozen in liquid nitrogen, pulverized, and extracted by a modification of the method of Schuder *et al.* (1979). The hemoglobin was fractionated with ammonium sulfate. Two different hemoglobin fractions were obtained by successive chromatography on Sephadex G100 and Sephadex DEAE A50 (Wittenberg and Wittenberg, 1981). The fraction emerging first from the DEAE column will be called Fraction I; that emerging second will be called Fraction II. Fraction I will be shown to react with sulfide.

Rates of oxygen dissociation

These were determined using a Gibson-Durrum stopped-flow spectrophotometer (Olson, 1981). The rate of oxygen dissociation from hemoglobin was determined by displacement of oxygen with carbon monoxide. A solution containing oxyhemoglobin (3 μM) and oxygen (20 μM) was mixed rapidly with a solution of carbon monoxide (1 mM) in the same buffer. Temperature was 20.0°C. The rate of the observed kinetic event was independent of carbon monoxide concentration (0.5–1 mM) and the rate of combination of carbon monoxide with deoxyhemoglobin is too fast to measure in our apparatus at these carbon monoxide concentrations.

Gas mixtures

Mixtures of oxygen, nitrogen, and carbon monoxide were prepared using a Tylan (Carson, CA) mass flow controller, humidified in a bubbler, and passed through the cuvette at a flow of 100 ml/min. Hydrogen sulfide and hydrogen cyanide were added to the humidified gas mixture from a gas-tight syringe (Hamilton) driven by a syringe pump.

Acquisition of optical spectra

In some experiments, optical spectra were acquired with a microspectrophotometer (Colacino and Kraus, 1984) and recorded from 440 to 400 nm at 2 nm intervals. Temperature was controlled at 20 ± 0.5°C. In subsequent experiments, optical spectra were acquired using a Cary model 14 recording spectrophotometer equipped with a Cary scattered transmission accessory and an Aviv digital data acquisition and analysis system (Lakewood, NJ). Optical spectra were recorded from 650 to 360 nm at 0.5 nm intervals. These experiments were performed near 24°C.

Optical spectra of purified hemoglobins

These were acquired using a thin-layer cell modified from Gill's (1981) design. Oxygenated and carbon monoxide hemoglobin were generated by exposing the hemoglobin to these gases. Deoxyhemoglobin was generated by exposing hemoglobin in a solution containing dithiothreitol to oxygen-free nitrogen. Dithiothreitol serves to reduce ferric hemoglobin to ferrous (deoxygenated) hemoglobin. Ferric hemoglobin sulfide was prepared from *Solemya* Hb I in two independent ways. In the first, ferric hemoglobin was exposed to hydrogen sulfide (0.4 torr) in nitrogen. In the second, oxyhemoglobin was exposed to a gas mixture containing oxygen (2 torr), hydrogen sulfide (0.4 torr), balance nitrogen.

Optical spectra of gill filaments

Freshly collected animals in seawater were cooled over ice. The gills were exposed after cutting the fused mantle tissue to open the valves. For experiments using the microspectrophotometer, 3–5 individual gill filaments (about 60–100 μm thick) were placed between two gas-permeable membranes (Teflon, 6 μm thick, Dilectrix Corp.) and held in a gas-tight cuvette (Colacino and Kraus, 1984). The ambient oxygen pressure or hydrogen sulfide pressure to achieve volume-average steady state half saturation of the total gill hemoglobin with oxygen or of about half the gill hemoglobin with hydrogen sulfide was calculated from absorbance changes at the wavelength pairs 415 and 430 nm, and 420 and 430 nm, respectively. The time to reach unchanging absorbance was less than 20 min. To obviate any complexities of dealing with oxygen and sulfide together during steady state measurements, the effect of sulfide on the gill hemoglobin was studied in gills held in nitrogen.

For experiments using the Cary spectrophotometer, approximately 30–50 individual gill filaments were cut

free from the central ligament of the gill and allowed to form a continuous layer of partially overlapping filaments (about 5–10 filaments or 100–200 μm thick) on the gas-permeable membrane window (MEM 213, 25 μm thick, General Electric Corp., Schenectady, NY) of the cuvette used in the Cary spectrophotometer. The sample was covered with a second membrane and the assembly was placed in a gas-tight cuvette. A single layer of parafilm was used to attenuate the Cary reference beam and to balance light scattering. The spectral contribution of hemoglobin in intact gill filaments is partially obscured by contributions from bacteria, mitochondria, other cell components, and by wavelength-dependent optical distortions. These unchanging contributions were cancelled by taking difference spectra constructed with computer assistance (Wittenberg and Wittenberg, 1986).

Results

Properties of the purified hemoglobins

Each component eluted from the Sephadex G100 column in a position near that expected for a monomeric hemoglobin of molecular weight about 17,000. The rate of oxygen dissociation, k′, for Hb I was 65 s^{-1}. This rate, although rapid, is characteristic of gill hemoglobins of many clams (Wittenberg, unpub.). The dissociation rate for Hb II was 35 s^{-1}. The combination rate constants have not yet been determined.

One of the purified hemoglobins, Hb I, when exposed to hydrogen sulfide (0.4 torr) at an oxygen pressure (2 torr) low enough to partially desaturate the hemoglobin, formed a spectral entity unequivocally identified as ferric hemoglobin sulfide; that is, ferric hemoglobin with sulfide ligated to the heme iron atom in the distal ligand position (Keilin, 1933). The product is identified by the following properties. The optical spectrum is identical to that of the product formed by reaction of ferric *Solemya* Hb I with hydrogen sulfide (see Fig. 2C, trace 2) and is closely similar to the spectrum reported by Keilin (1933) for ferric human hemoglobin sulfide. The spectrum is also nearly identical to those of ferric *Lucina pectinata* hemoglobin sulfide and ferric sperm whale myoglobin sulfide (Kraus and Wittenberg, in prep.). There was an insufficient quantity of isolated *Solemya* hemoglobin to allow chemical determination of the stoichiometry between sulfide and heme. Identity of ferric *Lucina pectinata* hemoglobin sulfide and ferric sperm whale myoglobin sulfide was established by demonstrating that one mole of sulfide was bound per mole heme (Kraus and Wittenberg, in prep.). The identity of ferric *Lucina* hemoglobin sulfide was confirmed by electron paramagnetic resonance (EPR) spectroscopy which gave the expected signature (Berzofsky *et al.*, 1971; Kraus and Wittenberg, in prep.).

In contrast to Hb I, *Solemya* Hb II remained in the oxygenated form when exposed to mixtures of hydrogen sulfide and oxygen. Neither purified hemoglobin formed the green compound, sulfhemoglobin, under the above conditions, although under these conditions, whale myoglobin was converted to sulfmyoglobin (Berzofsky *et al.*, 1971). In this report, the hemoglobin components will be called the sulfide-reactive and sulfide-unreactive hemoglobins.

Oxygen pressure to half saturate the gill hemoglobin in situ

The ambient oxygen pressure required to achieve volume-averaged half saturation of hemoglobin in respiring gill filaments was 6 ± 1 torr at 20°C (n = 26; Fig. 1A). This value reflects a balance between oxygen entry and oxygen utilization and will depend accordingly on the length of the diffusion path both external and internal to the tissue. It is not to be regarded as a fixed parameter of the reaction *per se*.

Hydrogen sulfide pressure to half saturate the gill hemoglobin in situ

The ambient hydrogen sulfide pressure required for half maximal spectral change in the Soret region of gill filaments exposed to hydrogen sulfide in nitrogen was 1.5 ± 0.5 torr at 20°C (n = 6; Fig. 1B). Not all of the gill hemoglobin reacts with sulfide, the balance remaining as deoxyhemoglobin; in these experiments, we did not determine the fraction reacting. The rate of sulfide consumption in the closely related clam *Solemya reidi* is large and of the same order as oxygen uptake (Anderson *et al.*, 1987). Accordingly, the value of 1.5 torr P_{H_2S} also depends on a balance between sulfide entry and utilization and on the diffusion path, and cannot be regarded as a fixed parameter of the reaction *per se*.

Identification of hemoglobin species formed in the gill

Oxyhemoglobin. We use difference spectra to examine the reaction of hemoglobin *in situ*. Figure 2A, trace 1, demonstrates that most of the gill hemoglobin reacted reversibly with oxygen. Carbon monoxide was used to convert the tissue hemoglobin to a common liganded form. Taking extinction coefficients determined for isolated *Solemya* hemoglobin, we estimate the amount of hemoglobin (hemoglobin concentration times pathlength for each preparation) in the tissue both from the direct spectrum of gill filaments exposed to carbon monoxide and from the difference spectrum: the spectrum of gill filaments in carbon monoxide minus the spectrum of the same array of gill filaments in air. This estimate agrees with an independent estimate from the difference

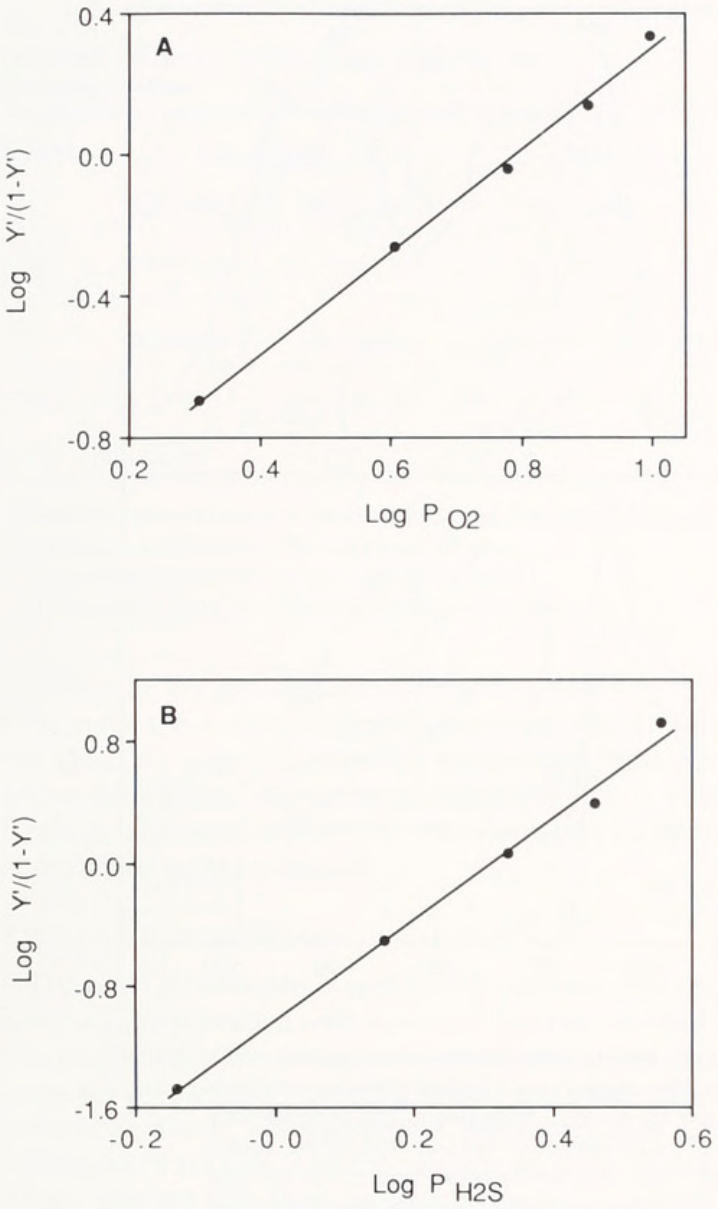

Figure 1. A. Hill plot showing the fractional saturation of the cytoplasmic hemoglobin in *Solemya* gill filaments as a function of external oxygen pressure. Data were acquired on the microspectrophotometer. Y' is defined as the fraction of the ligand-reactive hemoglobin undergoing reaction. The ambient oxygen pressure at half saturation is 6.1 torr in this representative experiment.

B. Hill plot showing the fractional ligation of sulfide to the cytoplasmic hemoglobin in *Solemya* gill filaments as a function of external hydrogen sulfide pressure. Data were acquired on the microspectrophotometer. Y' is defined as above. The ambient hydrogen sulfide pressure at half maximal spectral change is 1.8 torr in this representative experiment.

spectrum: gill filaments in air minus gill filaments in nitrogen.

Ferric hemoglobin sulfide—difference spectra. Hydrogen sulfide (0.7 torr) introduced into the flowing gas stream changed the optical spectrum of intact gill filaments either in the presence or absence of oxygen. The concentration of sulfide in water equilibrated with this gas mixture will be about 100 μM. It will be greater in buffered cytoplasm. The weak acid, H_2S, pK 7.0, decreased the pH of seawater equilibrated with this gas mixture by no more than 0.1 pH unit and is not expected to alter the pH of the strongly buffered cytoplasm appreciably.

The hydrogen sulfide-dependent reaction was followed at 435 nm, near the absorbance maximum of the difference spectrum between ferric hemoglobin sulfide and ferrous hemoglobin. Spectral change was most easily observed in gill filaments held at a gas phase oxygen pressure (about 60 torr) just low enough to bring about detectable deoxygenation of cytoplasmic hemoglobin in the thick preparation of filaments. Oxygen was removed from the gas stream after the spectral change was complete, simplifying analysis by removing the spectral contribution of oxyhemoglobin. A difference spectrum was constructed: the spectrum of gill filaments in hydrogen sulfide and nitrogen minus the spectrum of the same array of gill filaments previously exposed to nitrogen alone (Fig. 2B, trace 1). Spectral features near 422, 436, 566, and 582 nm are similar to features of the difference spectrum: purified ferric *Solemya* hemoglobin sulfide minus ferrous *Solemya* hemoglobin (Fig. 2A, trace 2). The amplitude of the difference spectrum of the gill (Fig. 2B, trace 1) corresponds to conversion of about half of the total hemoglobin in the gill to ferric hemoglobin sulfide.

Ferric hemoglobin. The feature near 405 nm in the difference spectrum (Fig. 2B, trace 1) varied in amplitude in experiments with different gills. We ascribe this feature to the removal of aquoferric hemoglobin (acid methemoglobin) initially present in the gill. This was estimated to vary from less than 5% to about 20% of the total ($n = 6$) and was estimated at approximately 10% in the particular gill figured (aquoferric hemoglobin will be converted to ferric hemoglobin sulfide). A difference spectrum: purified ferric *Solemya* hemoglobin sulfide minus the sum of the spectral contributions of ferrous *Solemya* hemoglobin (90%) and aquoferric *Solemya* hemoglobin (10%) (Fig. 2B, trace 2) is very similar to the difference spectrum of the gill (Fig. 2B, trace 1). The similarity of these two difference spectra (Fig. 2B1, 2B2) constitutes strong evidence that the form of hemoglobin generated by exposing gill filaments to sulfide is ferric hemoglobin sulfide and that a small amount of aquoferric hemoglobin is initially present in the gill.

Ferric hemoglobin sulfide—direct spectrum. The direct spectrum of the hemoglobin species generated by exposing gill filaments to hydrogen sulfide can be reconstructed by adding to the difference spectrum: gill filaments in hydrogen sulfide and nitrogen minus gill filaments in nitrogen (Fig. 2B, trace 1) the expected sum of the spectral contributions of ferrous *Solemya* hemoglobin (90%) and aquoferric *Solemya* hemoglobin (10%) estimated to be initially present in this particular gill (Fig.

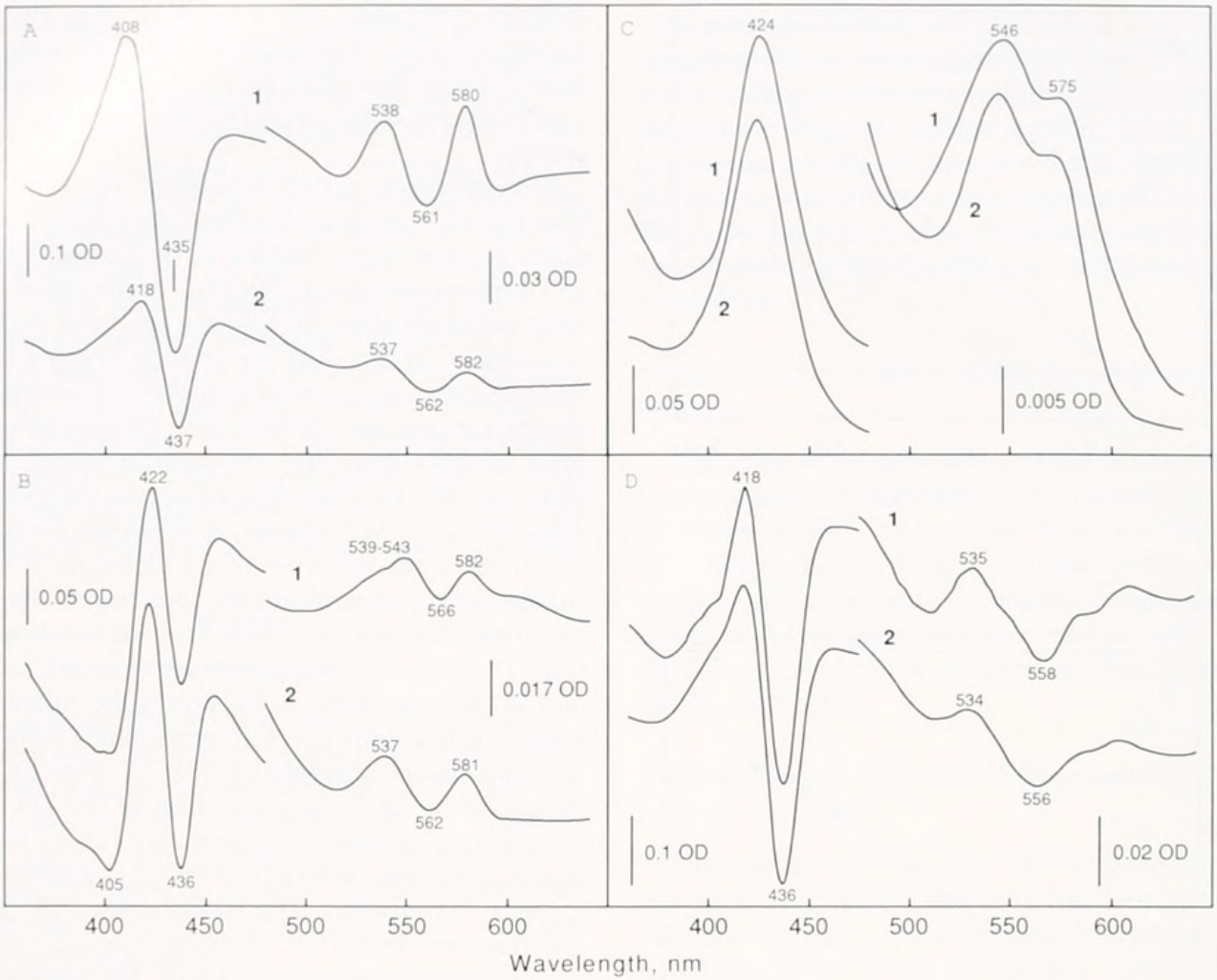

Figure 2. *Panel A. Trace 1.* Optical difference spectrum of intact gill filaments in oxygen (60 torr), balance nitrogen, minus a spectrum of the same specimen in nitrogen. *Trace 2.* Optical difference spectrum of authentic ferric *Solemya* hemoglobin sulfide minus ferrous *Solemya* hemoglobin. The ordinate scale bars in all panels are those observed in the actual spectra of the gill filaments. The amplitude of the accompanying spectra of the purified components are adjusted to an equal amount of hemoglobin in the light path. Predictably, the spectra of oxyhemoglobin and ferric hemoglobin sulfide, both low spin ferric heme derivatives with similar ligands, have similar wavelength maxima (Peisach *et al.*, 1968). It is apparent that the molar extinction of the hemoglobin derivative in trace 2 is less than that in trace 1.

Panel B. Trace 1. Optical difference spectrum of intact gill filaments allowed to react with hydrogen sulfide (0.7 torr) in the presence of oxygen, subsequently exposed to hydrogen sulfide in nitrogen, minus the spectrum of the same specimen previously exposed to nitrogen alone. The β-band includes a small contribution from a particle-bound (bacterial?) cytochrome *c*-like protein. This has the effect of shifting the apparent wavelength maximum toward longer wavelengths. This shift is reflected in the apparent wavelength maxima of Panel C, trace 1. *Trace 2.* Optical difference spectrum of authentic ferric *Solemya* hemoglobin sulfide minus the sum of the spectral contributions of ferrous *Solemya* hemoglobin (90%) and aquoferric *Solemya* hemoglobin (10%).

Panel C. Trace 1. Spectrum of the product formed when intact *Solemya* gill filaments are exposed to hydrogen sulfide, reconstructed by adding the computed spectrum of a mixture of ferrous *Solemya* hemoglobin (90%) and aquoferric *Solemya* hemoglobin (10%) to the difference spectrum of trace B1. *Trace 2.* Optical spectrum of authentic ferric *Solemya* hemoglobin sulfide. Traces C1 and C2 are nearly indistinguishable.

Panel D. Trace 1. Optical difference spectrum of intact *Solemya* gill filaments exposed first to a mixture containing hydrogen cyanide (0.2 torr) and oxygen (60 torr), balance nitrogen, and subsequently exposed to hydrogen cyanide (0.2 torr) in nitrogen, recorded after completion of the slow kinetic event (see text), minus the spectrum of the same specimen previously exposed to nitrogen alone. *Trace 2.* Optical spectrum of authentic ferric *Solemya* hemoglobin cyanide minus ferrous *Solemya* hemoglobin. Traces D1 and D2 are sensibly the same.

Table I

Half-times of reactions of hemoglobin in gill filaments of Solemya velum

Ligand	$t_{1/2}$, addition		$t_{1/2}$, removal	
	H_2S absent	H_2S present	H_2S absent	
O_2	1.2 ± 0.2 (21)[a] 11 ± 5 (13)[b]	1.2 ± 0.2 (15)[a]	32 ± 9 (21)[a] 21 ± 12 (14)[b]	
	O_2 present	O_2 absent	O_2 present	O_2 absent
H_2S	62 ± 23 (6)[b]	163 ± 38 (3)[a]	178 ± 72 (4)[b] 196 ± 29 (9)[a]	340 (1)[b]
CN	50 ± 16 (4)[b]	830 (2)[b]	780 (1)[b]	—

Values are given as mean ± standard deviation. Bracketed values are number of determinations. The unit is seconds.

[a] Determined using the microspectrophotometer.

[b] Determined using the Cary model 14 spectrophotometer.

2C, trace 1). This reconstructed spectrum is nearly identical to the spectrum of ferric *Solemya* hemoglobin sulfide (Fig. 2C, trace 2) generated by exposing ferric *Solemya* hemoglobin to hydrogen sulfide (0.4 torr). This is strong additional evidence for the formation of ferric hemoglobin sulfide in the gill.

Kinetics of hemoglobin reactions in the gill

Oxygen. Oxygenation of previously anaerobic gills exposed to air proceeded with $t_{1/2}$ = 1.2 s (a thin preparation examined in the microspectrophotometer) and 11 s (a thicker preparation examined in the Cary spectrophotometer; Table I). Deoxygenation proceeded with $t_{1/2}$ = 32 s and 21 s (Table I) with the respective preparations. These rates are very slow compared to the rates of the reactions of isolated hemoglobin.

Hydrogen sulfide. Spectral change in gills exposed to hydrogen sulfide (0.5–3 torr) in nitrogen occurred with $t_{1/2}$ = 163 s (these experiments were performed using the microspectrophotometer; Table I). When the gas stream was changed to air in this series of experiments, two distinct kinetic events were seen, with $t_{1/2}$ = 1.2 s and $t_{1/2}$ = 196 s, respectively (Table I). Absorption spectra at the end of the fast event indicated partial reoxygenation of the hemoglobin and those at the end of the slow event indicated fully oxygenated hemoglobin. We identify the rapid reaction as oxygenation of deoxyhemoglobin present in the tissue and identify the slow kinetic event as regeneration of oxyhemoglobin from ferric hemoglobin sulfide. We emphasize that each reaction corresponded to about half of the total hemoglobin in the gill.

In a separate series of experiments using the Cary spectrophotometer, hydrogen sulfide (0.7 torr) was introduced into the gas stream containing oxygen (60 torr), balance nitrogen. The hydrogen sulfide-dependent spectral change ($t_{1/2}$ = 62 s; Table I) was more rapid than that observed in nitrogen. When hydrogen sulfide was removed from the oxygen-containing gas stream, oxyhemoglobin was regenerated in the gill with $t_{1/2}$ = 178 s (Table I). We did not observe a fast kinetic event. We note that difference spectra showed that about half the hemoglobin was ferric hemoglobin sulfide and we assume that the balance remained oxygenated. As before, we interpret the kinetic event as regeneration of oxyhemoglobin from ferric hemoglobin sulfide.

The rate of reaction of oxyhemoglobin with hydrogen sulfide in the intact gill was much faster than the rate of reaction of purified oxy *Solemya* Hb I with hydrogen sulfide under equivalent conditions ($t_{1/2}$ ~ 400–500 s; n = 2).

Cyanide. Cyanide binds very strongly to ferric hemoglobin and is useful for experiments with intact tissues because the undissociated acid diffuses rapidly into cells. Hydrogen cyanide introduced into the flowing gas stream was used to demonstrate ferric hemoglobin formation by trapping intracellular ferric hemoglobin as ferric hemoglobin cyanide. The reaction was followed at 420 nm, near the absorbance maximum of ferric hemoglobin cyanide. A relatively rapid kinetic event ($t_{1/2}$ = 50 s; Table I) was observed in the presence of hydrogen cyanide (0.2 torr) and oxygen (60 torr), balance nitrogen. To identify the product and to simplify spectral analysis, oxygen was subsequently removed from the flowing gas stream and a difference spectrum was constructed: gill filaments in cyanide and nitrogen minus gill filaments previously exposed to nitrogen alone. This showed about half conversion of hemoglobin to ferric hemoglobin cyanide (n = 5). When gill filaments were exposed to hydrogen cyanide (0.2 torr) in nitrogen alone, only a slow kinetic event ($t_{1/2}$ ~ 830 s; Table I) was seen, and a difference spectrum constructed at the end of this event indicated complete conversion of hemoglobin to ferric hemoglobin cyanide (n = 2). The differences in each case were nearly identical to the difference spectrum: authentic ferric *Solemya* hemoglobin cyanide minus ferrous *Solemya* hemoglobin (Fig. 1D, trace 2). We suggest that the fast kinetic event reflects conversion of the sulfide-reactive hemoglobin to ferric hemoglobin cyanide. The slow kinetic event includes the conversion of both hemoglobins to the same product.

Ferric hemoglobin cyanide formation was reversed and oxyhemoglobin was fully regenerated when cyanide was removed from the oxygen-containing gas stream.

Kinetics of ferric hemoglobin formation in the presence of hydrogen sulfide

The possibility arises that sulfide interacts nonenzymatically to accelerate the rate of formation of ferric he-

moglobin. To examine this possibility, gill filaments were exposed simultaneously to cyanide and oxygen in the gas stream and the rate of conversion of oxyhemoglobin to ferric hemoglobin cyanide was estimated. Hydrogen sulfide, subsequently introduced into the gas stream, did not accelerate ferric hemoglobin formation.

Discussion

The symbiotic association between the clam *Solemya velum* and the intracellular bacterial partner depends on both oxygen and hydrogen sulfide for its nutrition. Here we ask how the hemoglobin, so abundant in the bacteria-harboring gill, interacts with these two substrates. Hemoglobin in the respiring gill is half-saturated at an ambient oxygen pressure of about 6 torr. The oxygen pressure in the oxygenated upper arms of the *Solemya* burrow might be about 50–80 torr, as it is in the U-shaped burrows of other animals inhabiting reduced sediments (Mangum *et al.*, 1975; Mangum, 1976). This oxygen pressure is more than sufficient to provide extensive oxygenation of the gill hemoglobin. The reaction of gill hemoglobin with sulfide was studied in gills held in nitrogen. Half maximal spectral change occurred at a hydrogen sulfide pressure of 1.5 torr. The hydrogen sulfide concentration in the pore water of the sediments from which these animals were collected falls within the range of 30 μM to 3 mM (Fenchel and Reidl, 1970; Howarth and Teal, 1980) corresponding very approximately to a hydrogen sulfide pressure of 0.2 to 20 torr. This suggests that a substantial fraction of the gill hemoglobin would ligate hydrogen sulfide in the basal stem of the burrow.

In this study, we use difference spectroscopy to identify hemoglobin species present in the living gill. The majority of the hemoglobin in gill filaments exposed solely to air is oxygenated. On the other hand, we observed a mixture of about equal parts of oxyhemoglobin and of an additional species in gill filaments exposed to oxygen and hydrogen sulfide at relatively low partial pressures. We seek to identify this new species. For this purpose, we generated a difference spectrum between gill filaments containing this new species and the same gill filaments previously in nitrogen and show this difference to be identical to that formed between ferric *Solemya* hemoglobin sulfide and deoxyhemoglobin. Using the difference spectrum of gill filaments, we reconstructed the direct spectrum of the hemoglobin derivative formed in the presence of sulfide. This spectrum is indistinguishable from that of authentic ferric *Solemya* hemoglobin sulfide. The only other hemoglobin derivative likely to be formed under these conditions would be sulfhemoglobin. This latter species is green, with a characteristic and unmistakable spectrum (Berzofsky *et al.*, 1971) different from any seen here. We conclude that the hemoglobin species formed in the presence of hydrogen sulfide is ferric hemoglobin sulfide; that is, ferric hemoglobin with sulfide ligated to the heme iron atom in the distal ligand position (Keilin, 1933).

Only about half of the hemoglobin in intact gill filaments reacts to form ferric hemoglobin sulfide. The magnitude of the difference spectrum constructed at the end of the reaction with sulfide in the presence of oxygen corresponds to reaction of about half the hemoglobin in the gill. The rapid reoxygenation of deoxyhemoglobin in the gill at the end of the reaction with sulfide carried out in the absence of oxygen corresponds to the remaining half of the hemoglobin in the gill. These results suggest but do not prove that there are two differently reactive hemoglobin components in the gill. This suggestion is supported by experiments with cyanide. About half the hemoglobin reacts rapidly with cyanide in the presence of oxygen. The balance of hemoglobin, either in the presence or absence of oxygen, reacts ten times more slowly but under these forced conditions eventually is converted to ferric hemoglobin cyanide.

We suggest that the initial event in the formation of ferric hemoglobin sulfide is the conversion of ferrous to ferric hemoglobin. Difference spectroscopy of gill filaments demonstrates a standing crop of aquoferric hemoglobin, often about 10% of the total hemoglobin in the gill. When ligands for ferric hemoglobin, hydrogen sulfide or cyanide are added in the presence of oxygen, about half of the total hemoglobin is rapidly converted to the corresponding ligated ferric hemoglobin. The close similarity of the rates of reaction with the two very different ligands (Table I) suggests a common rate-limiting step which, we argue, is the formation of ferric hemoglobin. Subsequent ligation of hydrogen sulfide or cyanide will be rapid and independent of enzyme action.

The rate of formation of ferric hemoglobin sulfide ($t_{1/2}$ = 62 s) in the intact gill is much faster than the rate of the purely chemical event observed when purified Hb I is exposed to mixtures of oxygen and hydrogen sulfide ($t_{1/2} \approx$ 400–500 s), and is only 5 to 6-fold slower than the diffusion-limited ingress of oxygen in the same experimental series (Table I). Accordingly, we suggest that the conversion of ferrous to ferric hemoglobin is accelerated in the *Solemya* gill. The reverse reaction, reduction of ferric cytoplasmic hemoglobin, is known from studies of other tissues to require the action of enzyme systems (metmyoglobin or methemoglobin reductase).

Rapid conversion of ferrous to ferric cytoplasmic hemoglobin is unusual and is not encountered in tissues of any of the vertebrate and invertebrate species we have examined. In the presence of cyanide (1–10 mM, sufficient to inhibit most respiratory oxygen consumption), all hemoglobin in the tissue of the following species remained in the oxygenated form, with no detectable ferric

hemoglobin cyanide: the vertebrate tissues rat cardiac myocytes (Wittenberg and Wittenberg, 1986) and pigeon breast muscle (Wittenberg *et al.*, 1975); the molluscs *Busycon canaliculatum* radular muscle, *Aplysia californica* buccal muscle, *Spisula solidissima* nerve, *Tellina alternata* nerve; the annelid *Arenicola cristata* body wall; the echiuran *Urechis caupo* body wall; and the insect *Gastrophilus intestinalis* tracheal cells (unpub. data).

Sulfide-binding components have been demonstrated in other invertebrates participating in sulfur-oxidizing symbioses. A component of the colorless blood plasma of the giant clam *Calyptogena magnifica* of the hydrothermal vents binds sulfide (Arp *et al.*, 1984). The blood hemoglobin of the giant tube worm *Riftia pachyptila* of the hydrothermal vents binds sulfide at a site believed to be remote form the heme (Arp *et al.*, 1987). Sulfide oxidation, presumptively to thiosulfate, has been noted in the animal tissues of *Solemya reidi* and the suggestion made that thiosulfate so formed is used by the symbionts (Powell and Somero, 1985, 1986).

We consider that the two hemoglobins found in the gill of *Solemya velum* may represent a division of labor in which the sulfide-reactive hemoglobin delivers sulfide and the sulfide-unreactive hemoglobin delivers oxygen to their respective consuming centers. We note the strong analogy with the nitrogen-fixing root nodules of legumes in which legume hemoglobin mediates oxygen delivery to the nitrogen-fixing bacterial symbionts (Appleby, 1984; Wittenberg, 1985). In the absence of sulfide, we envision a dynamic balance between ferric hemoglobin formation and removal in the gill, with about 5–20% of the total gill hemoglobin in the aquoferric form. In the presence of the natural substrate sulfide or the experimentally added cyanide, this balance is shifted and about half of the total hemoglobin (perhaps the sulfide-reactive component, Hb I) becomes trapped in the ligated form. Hemoglobins that ligate oxygen and sulfide, thereby preventing these molecules from interacting spontaneously, may be key features of the *Solemya*/bacteria symbiosis.

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