Reference: *Biol. Bull.* **182:** 435–443. (June, 1992)

Hydrogen Sulfide Reduction of Symbiont Cytochrome c_{552} in Gills of *Solemya reidi* (Mollusca)[a]

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Abstract. The gill of the protobranch clam *Solemya reidi* houses a dense population of intracellular symbiotic chemoautotrophic sulfur-oxidizing bacteria that fix carbon dioxide into sugars and supply the carbon nutrition of the host. The gill is divided into a bacteriocyte (cells with intracellular symbionts) domain and a domain of mitochondria-rich, symbiont-free ciliated cells. Optical spectra, recorded separately from each domain, are dominated by hemoglobin. Only oxygenated and deoxygenated hemoglobin were detected in the gill. In sharp contrast to the gill of the congener *Solemya velum*, ferric hemoglobin sulfide was not detected, suggesting that this species, if formed, is short lived. The spectral contribution of hemoglobin may be cancelled or subtracted in difference spectra. Difference spectra of each gill domain in nitrogen minus the same tissue in air show a complement of reduced cytochromes, demonstrating that both symbiont and mitochondrial cytochromes are reduced by endogenous substrate. Difference spectra of the bacteriocyte domain exposed to hydrogen sulfide (air containing 1.4 torr hydrogen sulfide minus air) show only the contribution of reduced symbiont cytochrome c_{552}. The extent of reduction increases monotonically with ambient p_{H_2S}, suggesting that, by analogy with some free-living sulfur-oxidizing bacteria, cytochrome c_{552} is near the point of entry of electrons into the symbiont electron transport chain. Difference spectra of muscle or of the ciliated domain under these same conditions show reduced cytochrome c_{550}, cytochrome *b* and cytochrome oxidase, suggesting that host mitochondria may accept electrons from hydrogen sulfide.

Received 30 December 1991; accepted 2 March 1992.

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Introduction

Sulfide-oxidizing symbiotic associations between invertebrate hosts and chemoautotrophic bacteria were first recognized in members of the dense animal communities found at deep ocean hydrothermal vents (reviewed in Jones, 1985), and subsequently have been found in a number of animal species inhabiting coastal and deep sediments with disequilibrium mixtures of oxygen and sulfide (reviewed in Southward, 1987; Fisher, 1990; Childress and Fisher, 1992). The bacterial symbionts oxidize sulfide or other reduced compounds and fix carbon dioxide into nutrients that are translocated to the host (Fisher and Childress, 1986). The gigantic size of some symbiont-harboring animals attests to the effectiveness of the symbiotic association.

Symbionts in association with mollusks (clams, mussels, and a snail) are housed intracellularly in specialized cells—bacteriocytes—of the large and extensively modified gill. Within each cell they must be supplied with large influxes of hydrogen sulfide and oxygen, but must be protected from excessive hydrogen sulfide that would inhibit bacterial terminal oxidases and host mitochondrial cytochrome oxidase (Nicholls, 1975; Wilson and Erecinska, 1978; see Somero *et al.*, 1989, and Childress and Fisher, 1992, for reviews). This condition must be met in the naturally occurring steady state where hydrogen sulfide entry is matched by utilization, and the cytoplasmic concentration of hydrogen sulfide, and possibly oxygen, is probably very low (in the micromolar range; Childress, 1987; Wittenberg and Kraus, 1991). These cytoplasmic concentrations of hydrogen sulfide and oxygen may not be sufficient to support the fluxes of hydrogen sulfide and

oxygen to the symbiont, and we suggest that cytoplasmic sulfide-reactive and oxygen-reactive hemoglobins may facilitate diffusion of their ligands through the cytoplasm (Doeller *et al.*, 1988; Kraus and Wittenberg, 1990; Wittenberg and Kraus, 1991).

Hemoglobin is a nearly constant feature of symbioses between mollusks and sulfide-oxidizing chemoautotraphic bacteria (Wittenberg, 1985) and may reach concentrations as high as 1.5 m*M* in the bacteriocyte (Kraus and Wittenberg, 1990; for a possible exception see Dando *et al.*, 1986). Two abundant, high oxygen affinity, cytoplasmic hemoglobins have been isolated from the symbiont-containing gills of *Lucina pectinata* (formerly named *Phacoides pectinatus*) (Read, 1962, 1965). One of these, hemoglobin I, a monomer, reacts reversibly with oxygen but also reacts rapidly and reversibly with hydrogen sulfide in the presence of oxygen to form ferric hemoglobin sulfide (Kraus and Wittenberg, 1990; Kraus *et al.*, 1990). It may be called "sulfide-reactive." The other, the "oxygen-reactive" hemoglobin, is most probably an $alpha_2beta_2$ tetramer made up of hemoglobins II and III (Kraus and Wittenberg, 1990; Kemling *et al.*, 1991). It reacts reversibly solely with oxygen. These two hemoglobins may deliver their respective ligands to the symbiont.

Two cytoplasmic hemoglobins occur in nearly equal concentrations in the symbiont-harboring gill of *Solemya velum*, congeneric with *Solemya reidi* of this study (Doeller *et al.*, 1983, 1988). Approximately half the total hemoglobin within the bacteriocyte domain of living gill filaments reacts reversibly to form ferric hemoglobin sulfide in the presence of sulfide and oxygen; the balance remains oxygenated (Doeller *et al.*, 1988). We infer that the hemoglobins of *Solemya velum*, like those of *Lucina*, may deliver their ligands hydrogen sulfide or oxygen to the symbiont.

Here we present optical spectra of the symbiont-harboring bacteriocyte and the mitochondria-rich ciliated domains of the gill of *Solemya reidi*, and compare these spectra with those of additional symbiont-free tissues. We find that, in contrast to the gill of the congeneric species *Solemya velum*, and in contrast to purified *Lucina pectinata* hemoglobin, the gill of *Solemya reidi* never displays the spectrum of ferric hemoglobin sulfide. Optical spectra ascribed to the bacterial symbiont, weakly apparent in other species, are seen with remarkable clarity in the gill of *Solemya reidi*.

Materials and Methods

Animals

Solemya reidi individuals were collected by Van Veen grab sampling at the Hyperion sludge outfall in Santa Monica Bay, California, from a depth of 50–100 m, and were maintained in cold (5–10°C) seawater. Animals were 3–5 cm long, and gill wet weight averaged 1.5 g. Experiments were completed within 3 weeks of animal collection.

Optical spectrophotometry

Optical spectra were acquired with a Cary model 14 recording spectrophotometer equipped with a Cary scattered transmission accessory and an Aviv digital data acquisition and analysis system (Aviv Associates, Lakewood, New Jersey). Optical spectra were recorded from 650 to 350 nm at 0.5 nm intervals. Experiments were performed at room temperature (22–24°C). This is well above the habitat temperature of *Solemya reidi* from the collection site (<10°C).

Optical spectra of gills and other tissue

Gills of *Solemya reidi* were excised and rinsed in 0.25 μm millipore-filtered seawater. Individual gill filaments were cut from the central ligament of the gill. For some experiments, filaments were divided along the chitinous rod to separate the small ciliated mitochondria-rich domain from the larger bacteriocyte domain. Whole gill filaments or filament domains, each about 40 μm thick (see micrographs in Powell and Somero, 1985; Fisher and Childress, 1986), were placed as a continuous overlapping layer, two to three filaments or about 80 to 120 μm thick on a gas-permeable membrane window (MEM 213, 25 μm thick, General Electric Corp., Schenectady, New York). The layer was covered with a second membrane, and the assembly placed in the previously described gas perfusion cuvette (see Doeller *et al.*, 1988). Each gill preparation was used for no longer than four hours, and for each new experiment, filaments were freshly cut from the gill. Other tissues, foot, adductor and pallial muscles, hypobranchial gland, and nerve trunks or ganglia were examined as thin layer samples. Pallial muscles, if cut free, contract and become thick. A useful preparation was obtained by leaving a portion of the mantle with its fringing pallial muscle attached to a fragment of the valve. A window cut in the valve allowed the light beam to pass through the muscle. A single layer of parafilm (American Can Co., Greenwich, Connecticut) was used to attenuate the Cary reference beam and partially balance light scattering.

Gas delivery

Mixtures of air, oxygen, nitrogen, and carbon monoxide were prepared using a Tylan mass flow controller (Carson, California). Gas mixtures were humidified and passed through the 5 ml spectrophotometer cuvette at a flow rate of 100 ml/min. Hydrogen sulfide was added to the humidified gas mixture from a glass syringe driven by a syringe pump (Harvard).

Protocol

For each experiment, tissue hemoglobin was first oxygenated and then deoxygenated by equilibration of the tissue with air and nitrogen, respectively. Optical spectra were recorded after each equilibration. The difference between these spectra was dominated by the contribution of the difference: oxyhemoglobin minus deoxyhemoglobin. The magnitude of this difference, together with an estimate of the tissue thickness, provided an estimate of hemoglobin concentration in each tissue sample. Subsequently, the samples of each tissue were exposed to mixtures of air and nitrogen in declining increments of 10% air, from 100% air to 0% air, and spectra were recorded at each step. The p_{O_2} at which hemoglobin in each sample was just detectably deoxygenated was noted and was used in subsequent experiments with hydrogen sulfide to minimize spontaneous sulfide oxidation. This oxygen pressure, which is influenced by hemoglobin oxygen affinity, rate of oxygen consumption and tissue thickness, was typically near 30 torr (20% air) for gill preparations and near 90–105 torr (60–70% air) for pallial muscle preparations.

Hydrogen sulfide concentration in the natural environment of *Solemya reidi* is large and variable, 0.1–3 m*M* hydrogen sulfide (Childress and Lowell, 1982), but the animal may control the concentration of sulfide in the ventilatory water current by changing the construction of the burrow and its own placement within the burrow. The partial pressure range of hydrogen sulfide used in most these experiments (0.2–2 torr) is equivalent to 30–300 μM dissolved hydrogen sulfide (Millero, 1986). These concentrations are near the range reported to support sulfide-dependent carbon fixation by *Solemya velum* gill (200 μM; Cavanaugh, 1983a) or sulfide-dependent carbon dioxide net uptake by *Solemya reidi* (50–200 μM; Anderson *et al.*, 1987).

Hemoglobin isolation

Hemoglobin was purified following the general methods of Schuder *et al.* (1979) and Appleby *et al.* (1983), with modifications introduced by Kraus and Wittenberg (1990). The procedure involved extraction of the powdered sample under an atmosphere of carbon monoxide and argon, followed by molecular exclusion and ion exchange chromatography. All steps were carried out under carbon monoxide-saturating conditions to maintain hemoglobin in a carbon monoxide-ligated state until isolation was complete. This minimizes oxidation of ferrous *Solemya* hemoglobin and obviates cross-linking of hemoglobin to itself and other tissue components.

Results

Hemoglobin identification and concentration

Cytoplasmic hemoglobin dominates the optical spectra of the bacteriocyte and ciliated domains of the gill, as well as of all other tissues examined, including foot, adductor and pallial muscles, hypobranchial gland, and nervous tissues. In each tissue, optical difference spectra of tissues equilibrated with nitrogen minus the same tissue equilibrated with air display well-resolved features at 412, 435, 540, and 580 nm, diagnostic for hemoglobin (the difference spectrum of the gill bacteriocyte domain is shown in Fig. 1A). These maxima are the same as those in the deoxyhemoglobin minus oxyhemoglobin difference spectrum of purified *Solemya reidi* hemoglobin. The concentration of hemoglobin in several tissues (Table I) was calculated from the difference in optical density at 435 nm and 412 nm in these difference spectra, taking tissue thickness estimated with a dissecting microscope and using the extinction coefficient ΔEmM = 135, obtained from the dominant fraction of hemoglobin isolated from *Solemya reidi* gills. Hemoglobin concentration calculated from difference spectra reflects only the hemoglobin that reversibly binds oxygen. Hemoglobin concentrations *in situ* are comparable with values obtained using hemoglobin isolated from samples of whole gills and of remaining tissues combined (Table I) and with the concentration of hemoglobin reported by Powell and Arp (1989) for gills of *Solemya reidi* (130 μM).

Optical spectra of the bacteriocyte domain of the gill

Exposure to sulfide: hemoglobin. We now examine the spectral changes induced by exposing the bacteriocyte domain of the gill to hydrogen sulfide. The bacteriocyte domain was first equilibrated with 20% air, sufficient oxygen to just saturate the hemoglobin in these samples, then hydrogen sulfide (about 0.2 torr) was added to the humidified gas. During equilibration with sulfide, absorbance was recorded continuously at either 420 nm or 430 nm, wavelengths near the maxima of ferric hemoglobin sulfide or deoxyhemoglobin, respectively. Absorbance exhibited a monotonic change with time, reaching an asymptote within 80 $\pm$ 30 s (n = 6). At steady state, an optical spectrum was recorded. Sulfide partial pressure was then increased incrementally to 2 torr, with the same procedure repeated at each step. Difference spectra of gills in 20% air containing sulfide minus gills in 20% air alone did not produce any feature that could be ascribed to sulfide ligation to hemoglobin (see Doeller *et al.*, 1988; Kraus and Wittenberg, 1990), but instead revealed an apparently single spectral entity with characteristics of a reduced minus oxidized cytochrome c_{552}, discussed below. Thus, we cannot detect the formation of ferric hemoglobin sulfide in the bacteriocyte domain of the gill of *Solemya reidi*.

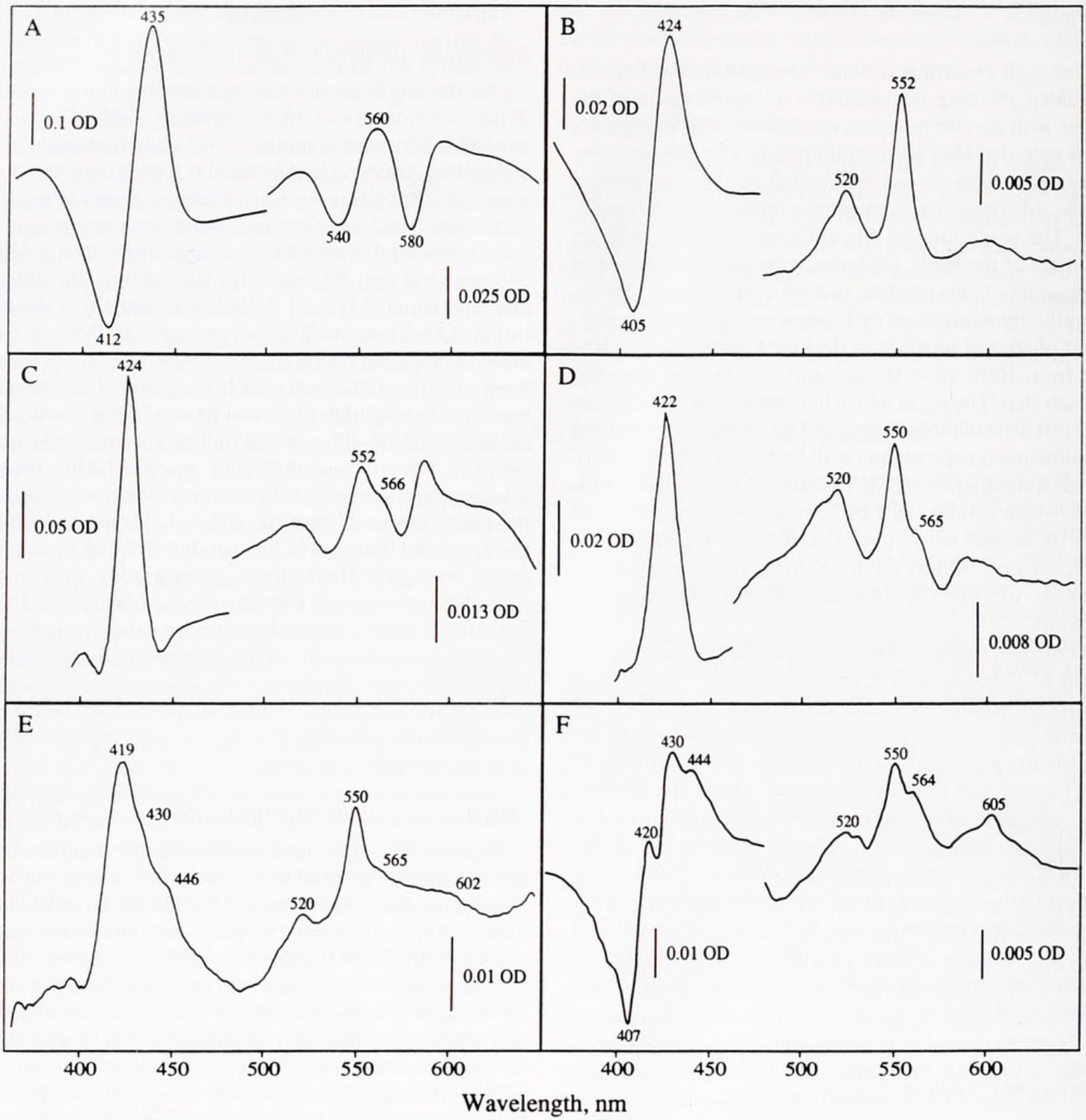

Figure 1. Optical difference spectra of living *Solemya reidi* tissues. Designated wavelength maxima are discussed in detail in text. Traces in the visible region have been amplified four-fold in Figures 1A–C, 2.5-fold in Figure 1D, and two-fold in Figure 1E.

A. Bacteriocyte domain of gill filaments equilibrated with nitrogen minus the same sample equilibrated with air. The contribution of deoxyhemoglobin minus oxyhemoglobin dominates the difference spectrum, with diagnostic maxima at 412, 435, 540, and 580 nm.

B. Bacteriocyte domain of gill filaments equilibrated with 20% air containing 1.4 torr sulfide minus the same sample equilibrated with 20% air. The dominant spectral species is identified as a reduced minus oxidized cytochrome c_{552}, ascribed to bacterial cytochrome c_{552}.

C. Bacteriocyte domain of gill filaments equilibrated with nitrogen minus the same sample equilibrated with 20% air, from which a difference spectrum of purified deoxyhemoglobin minus oxyhemoglobin, computed to be equivalent to the hemoglobin content in gills, was subtracted. The wavelength maxima indicated were confirmed in the second derivative of this difference spectrum. Features are ascribed to bacterial cytochrome c_{552} and to a cytochrome *b* (566 nm). The feature at 586 nm is not identified.

Table I

Approximate hemoglobin concentration in tissues of Solemya reidi, *determined in living tissues and from hemoglobin extracted from tissue*

Tissue	Hemoglobin concentration, μM
I. Living tissue[a]	
bacteriocyte domain of gills	450 ± 90 (21)[b]
ciliated domain of gills	120 ± 15 (4)
pallial muscle	330 (2)
hypobranchial gland	540 (2)
II. Hemoglobin extraction	
whole gill	180
combined symbiont-free tissue (foot, pallial muscle, hypobranchial gland)	170

[a] Tissue thickness was estimated with a dissecting microscope at 80 μm for bacteriocyte domain, 125 μm for ciliated domain, 300 μm for pallial muscle and 100 μm for hypobranchial gland.

[b] Numbers are given as average ± standard deviation (number of repetitions).

Exposure to sulfide: cytochrome c_{552}. The optical contribution of hemoglobin did not change in the presence of hydrogen sulfide, either aerobically or anaerobically. Consequently, the spectral contribution of oxyhemoglobin cancels in the difference spectrum: gills in 20% air containing sulfide minus gills in 20% air alone. The remaining spectrum in the bacteriocyte domain (Fig. 1B), characterized by sharp features at 405, 424, 520, and 552 nm, is identified as the difference: reduced minus oxidized cytochrome c_{552} (Pettigrew and Moore, 1987). This is unequivocally distinguishable from mitochondrial cytochrome *c* with maxima in the direct reduced spectrum at 520 and 550 nm (see Figs. 1D, E; Pettigrew and Moore, 1987), and may be ascribed to the symbiont (see Discussion). Thus, hydrogen sulfide causes reduction of symbiont cytochrome c_{552} in the gill of *Solemya reidi,* without detectable change in other heme proteins. We note that reduction of cytochrome c_{552} was not detected in gills exposed to 300 μM thiosulfate in seawater (data not shown). This may reflect the lack of uptake of thiosulfate into bacteriocyte cytoplasm.

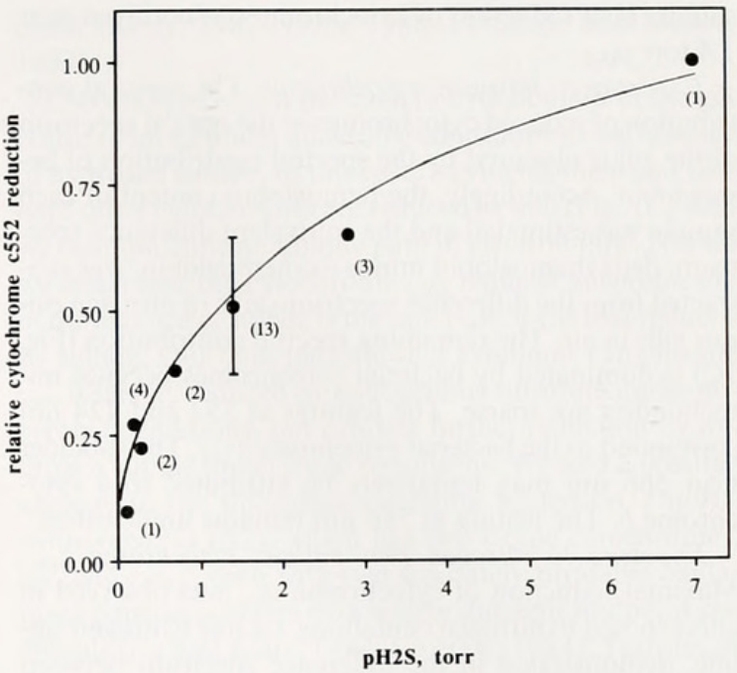

Figure 2. Relative reduction of cytochrome c_{552} in the bacteriocyte domain of living *Solemya reidi* gill filaments as a function of ambient partial pressure of hydrogen sulfide in air. Relative reduction is calculated from difference spectra as described in text. Half-reduction of cytochrome c_{552} occurs near 1.4 torr p_{H_2S}. Numbers in parentheses represent number of experiments.

The relative extent of reduction of cytochrome c_{552} in the bacteriocyte domain of aerobic gills increases monotonically with p_{H_2S} from zero in the absence of sulfide to a limit near 7 torr p_{H_2S} (Fig. 2). Relative reduction was calculated from the magnitude of the optical density differences 424 nm minus 405 nm and, separately, 552 nm minus 540 nm in difference spectra similar to Figure 1B (gills in 20% air containing sulfide minus gills in 20% air), normalized to a constant amount of hemoglobin in the optical path so as to allow comparison of samples of different thickness. Hemoglobin content was estimated from difference spectra similar to Figure 1A (gills in nitrogen minus gills in air) obtained from the same tissue sample. Reduction of cytochrome c_{552} was taken as maximal at p_{H_2S} = 7 torr and minimal in the absence of hydrogen

D. Ciliated domain of gill filaments equilibrated with nitrogen minus the same sample equilibrated with 40% air, from which a difference spectrum of purified deoxyhemoglobin minus oxyhemoglobin, computed to be equivalent to the hemoglobin content in gills, was subtracted. The wavelength maxima indicated were confirmed in the second derivative of this difference spectrum. Features are ascribed to mitochondrial cytochrome c_{550} and cytochrome *b*.

E. Ciliated domain of gill filaments equilibrated with 40% air containing 1.4 torr hydrogen sulfide minus the same sample equilibrated with 40% air. The wavelength maxima indicated were confirmed in the second derivative of this difference spectrum. Features are ascribed to mitochondrial cytochrome c_{550}, cytochrome *b*, and cytochrome oxidase.

F. Pallial muscle equilibrated with 70% air containing 1.4 torr hydrogen sulfide minus the same sample equilibrated with 70% air. Features are ascribed to mitochondrial cytochromes.

sulfide. Half reduction of cytochrome c_{552} occurred near 1.4 torr p_{H_2S}.

Exposure to nitrogen: cytochromes. The spectral contribution of reduced cytochromes in the optical spectrum of the gill is obscured by the spectral contribution of hemoglobin. Accordingly, the hemoglobin content of each sample was estimated and the equivalent difference spectrum: deoxyhemoglobin minus oxyhemoglobin, was subtracted from the difference spectrum: gills in nitrogen minus gills in air. The remaining spectral contribution (Fig. 1C) is dominated by bacterial cytochromes because mitochondria are sparse. The features at 552 and 424 nm correspond to the bacterial cytochrome c_{552}. The shoulder near 566 nm may tentatively be attributed to a cytochrome *b*. The feature at 586 nm remains unidentified.

Exposure to nitrogen plus sulfide: cytochrome c_{552}. Maximal reduction of cytochrome c_{552} was observed in gills exposed to nitrogen containing 1.4 torr hydrogen sulfide, demonstrated in the difference spectrum between this condition and gills in air (data not shown). Reduction of cytochrome c_{552} in gills exposed to nitrogen alone (Fig. 1C) represented only 81 ± 6% (n = 10) of maximum. It follows that symbiont cytochrome c_{552} is largely reduced in nitrogen alone, but is reduced still further in the presence of hydrogen sulfide. We note that the optical density differences (424 nm minus 405 nm and 552 nm minus 540 nm) are the same in gills exposed to 1.4 torr hydrogen sulfide in the presence of nitrogen and in gills exposed to 7 torr hydrogen sulfide in air, indicating that maximal reduction was achieved in each instance.

Optical spectra of the ciliated domain of the gill

Exposure to nitrogen: cytochromes. Cytoplasmic hemoglobin, at a concentration roughly one third that of the bacteriocyte domain (Table I), dominates the optical spectrum of the symbiont-free ciliated domain of the gill. A reduced minus oxidized difference spectrum (Fig. 1D) was constructed by subtracting the expected spectral contribution of cytoplasmic hemoglobin from the difference spectrum: gills in nitrogen minus the same tissue in air or 40% air. Clearly resolved spectral features at 422, 520, and 550 nm may be ascribed to mitochondrial cytochrome *c*, identified by comparison with the difference of reduced minus oxidized horse heart cytochrome *c*, which has features at 419, 520, and 550 nm (Pettigrew and Moore, 1987). The 550 nm feature in the ciliated domain difference spectrum is consistently distinct from the 552 nm feature seen in the bacteriocyte domain difference spectrum (Fig. 1B). The shoulder at 565 nm may be reasonably ascribed to mitochondrial cytochrome *b* (ubiquinone-cytochrome *c* oxidoreductase).

Exposure to sulfide: cytochromes. We next examine the spectral change produced by exposing the air-equilibrated ciliated domain to 1.4 torr hydrogen sulfide. The difference spectrum (Fig. 1E) is very similar to that of partially reduced minus oxidized mitochondria. Features at 419, 520, and 550 nm may again be ascribed to cytochrome *c*. Features at 446 and 602 nm may be ascribed to cytochrome oxidase. Small features near 430 and 565 nm may be ascribed to cytochrome *b*. All of these features become more prominent with increased hydrogen sulfide to 2.8 torr (data not shown).

Exposure to nitrogen plus sulfide: cytochromes. Further reduction of mitochondrial cytochromes by hydrogen sulfide in nitrogen compared to nitrogen alone was not observed. The optical difference: gills in nitrogen containing 1.4 torr hydrogen sulfide minus gills in nitrogen alone was relatively featureless and exhibited no peaks ascribed to mitochondrial cytochromes (data not shown).

Optical spectra of pallial muscle

Exposure to sulfide: cytochromes. The pink-colored pallial muscle is another example of symbiont-free tissue with cytoplasmic hemoglobin. In the difference spectrum of a thin piece of pallial muscle equilibrated with 70% air containing 1.4 torr hydrogen sulfide minus the same sample in 70% air alone, the spectrum of hemoglobin cancels and the remaining difference spectrum suggests reduction of a full complement of mitochondrial cytochromes: cytochrome c_{550}, cytochrome *b* and cytochrome oxidase (Fig. 1F).

Discussion

The protobranch mollusk *Solemya reidi* lives in burrows in strongly reducing sediments, with hydrogen sulfide concentrations reaching 3 m*M*. It is quite mobile and may seek appropriate sulfide concentrations (Reid, 1980; Childress and Lowell, 1982). There is no sulfide-binding protein in the circulating blood and the sulfide concentration in gills of freshly captured specimens is close to environmental (Childress, 1987), suggesting that symbionts take up hydrogen sulfide and oxygen directly across the gill. Without question, symbionts use hydrogen sulfide. Sulfide stimulates oxygen and carbon dioxide consumption of *Solemya reidi* (Anderson *et al.*, 1987) and carbon fixation in isolated gills of *Solemya velum* (Cavanaugh, 1983a). We note that on a per sulfur basis, sulfide is much more effective in stimulating carbon dioxide fixation than thiosulfate: approximately 14-fold more effective in whole *Solemya reidi* (recalculated from Anderson *et al.*, 1987), and 6-fold more effective in isolated *Solemya velum* gills (recalculated from Cavanaugh, 1983a).

The gill of *Solemya reidi* is comprised of a few hundred individual filaments held together at a central ligament and arranged in parallel somewhat like pages of a book. Each filament is divided into two major domains by a chitinous skeletal rod located near the outer edge: a rel-

atively small outer domain of mitochondria-rich ciliated cells and a much larger inner domain comprised largely of bacteriocytes (Yonge, 1939; Reid, 1980; Powell and Somero, 1985). Ciliated cells near the outer edge drive the flow of water between filaments and across the face of the inner domain. Electron micrographs of *Solemya reidi* (Powell and Somero, 1985) and of the related species *Solemya velum* (Cavanaugh, 1983b; Doeller, 1986) show that cells in the ciliated domain are densely packed with mitochondria and are free of bacteria. Conversely, bacteriocytes are densely packed with bacteria; mitochondria are sparse. Separation of the two cell types occurs also in gills of several lamellibranch mollusks of the family Lucinidae (Giere, 1985), including *Lucina pectinata* (J. B. Wittenberg, unpub.). In this study we take advantage of the separation of bacteriocyte and mitochondria-rich domains to study each separately.

Cytochromes of the bacterial partner are singularly well resolved in optical spectra of the bacteriocyte domain of the *Solemya reidi* gill. Difference spectra of the bacteriocyte domain exposed to a low concentration of hydrogen sulfide in the presence of air are dominated by the spectral contribution of a hemeprotein characterized by a prominent alpha-band centered at 552 nm and identified as a *c*-type cytochrome by the positions of its wavelength maxima, 424, 520, and 552 nm (Fig. 1B). It may be called cytochrome c_{552}. Cytochromes *c* characterized by maxima between 551 and 553 nm are conspicuous components of many sulfur-oxidizing bacteria (Pettigrew and Moore, 1987). They are not known in eukaryote tissues and are easily distinguished from mitochondrial cytochrome c_{550}, observed, for instance, in the ciliated domain of the gill and the pallial muscle (Fig. 1D, E, and F). Narrow spectral bandwidths in the observed spectrum (Fig. 1B) and the absence of features not related to cytochrome c_{552} (other than a small perturbation near 600 nm) suggest strongly that hydrogen sulfide, in the presence of oxygen, has reduced a single spectrally demonstrable species, bacterial cytochrome c_{552}. The extent of this reduction is a monotonic function of ambient p_{H_2S} (Fig. 2), suggesting, but by no means proving, that hydrogen sulfide is the immediate or near immediate reductant for symbiont cytochrome c_{552}. Reduction of cytochromes by sulfide might proceed by direct electron transfer (Wilson and Erecinska, 1978) as it does in the free-living bacterium *Thiobacillus denitrificans* (Sawhney and Nicholas, 1978), or it could be mediated by flavocytochromes *c* (reviewed in Pettigrew and Moore, 1987). Reduction of *Solemya reidi* symbiont cytochrome c_{552} finds a strong parallel in the free-living sulfur-oxidizing bacteria, where soluble *c*-type cytochromes occurring in the periplasmic space are considered the main point of transfer of electrons from external reduced sulfur compounds into the bacterial cytochrome chain (Kelly, 1982, 1985, 1988; Pettigrew and Moore, 1987).

Cytochrome c_{552} in the bacteriocyte domain of the gill is also reduced under anaerobic conditions in the absence of hydrogen sulfide. In this case, a cytochrome *b* and perhaps other cytochromes are reduced as well (Fig. 1C), and we cannot define an unique path of electron flow. Nor do we assert that the cytochrome c_{552} reduced anaerobically in the absence of sulfide is the same species as that reduced by sulfide. Our data indicate that symbiont cytochrome c_{552} is largely reduced by endogenous substrate under anaerobic conditions, but exhibits further reduction by hydrogen sulfide under these conditions. We find a possible analogy in the thiosulfate-oxidizing bacterium *Thiobacillus versutus* (A2), which has two *c*-type cytochromes, c_{551} and $c_{552.5}$, each with two separately titratable oxidation/reduction centers with widely different midpoint potentials (Lu and Kelly, 1984; Lu *et al.*, 1984). Transfer of electrons at two different potentials from thiosulfate to cytochrome *c* of the bacterial electron transport chain is mediated by this multi-heme complex (Lu and Kelly, 1984; Lu *et al.*, 1984). The additional reduction of symbiont cytochrome c_{552} in *Solemya reidi* gills by hydrogen sulfide, over and above that in nitrogen alone, indicates that cytochrome c_{552} may have two oxidation/reduction centers as well.

The symbiont-free ciliated domain of the gill, made anaerobic, displays the expected spectrum of the reduced mitochondrial electron transport chain, with features ascribable to cytochrome *c*, cytochrome *b*, and cytochrome oxidase (Fig. 1D). The ultimate reductant in this instance must be endogenous substrate.

The mitochondria-rich tissues, pallial muscle with singularly well-resolved spectra and the ciliated domain of the gill, exposed to hydrogen sulfide in the presence of air, exhibit spectra of reduced mitochondria, once again with features ascribed to cytochrome *c*, cytochrome *b*, and cytochrome oxidase (Fig. 1E, F). A simple explanation is that hydrogen sulfide is serving as the reductant for the respiratory chain. Indeed, isolated gill mitochondria from *Solemya reidi* are known to oxidize sulfide with production of ATP (Powell and Somero, 1986; O'Brien and Vetter, 1990). The inference from these studies is that electrons from hydrogen sulfide enter the mitochondrial electron transport chain at the level of cytochrome *c*, with oxidative phosphorylation only at the cytochrome oxidase site (Complex IV). An alternative explanation of our observations is that hydrogen sulfide may bind to and inhibit cytochrome oxidase, with a spectral signature not easily distinguished from normal oxidation/reduction (Wilson and Erecinska, 1978). This also would lead to net observed cytochrome reduction, in this instance by endogenous substrate. Possibly both processes occur simultaneously.

Hemoglobin, presumably located in the host cell cytoplasm, is abundant in the symbiont-containing bacteriocytes of the *Solemya reidi* gill and occurs as well in the symbiont-free ciliated domain of the gill, foot, pallial and adductor muscles, hypobranchial gland, and nervous tissue (Table I). Hemoglobin concentration in these tissues is comparable to the myoglobin content of many hard-working muscles (Schuder *et al.*, 1979). The concentration of hemoglobin in the bacteriocyte domain of the gill is in the upper part of the range reported for other symbiont-housing molluscan gills (Wittenberg, 1985).

Only oxygenated and deoxygenated hemoglobin were detected in the bacteriocyte domain of the *Solemya reidi* gill and in other tissues (*e.g.*, Fig. 1A). Ferric hemoglobin sulfide, that is ferric hemoglobin with sulfide ligated to the heme iron atom in the distal position, was not detected under any conditions. This stands in sharp contrast to the behavior of hemoglobin in the living gill of the congeneric species *Solemya velum*, where about half of the gill hemoglobin is rapidly and reversibly converted to ferric hemoglobin sulfide when the gill is exposed to low concentrations of hydrogen sulfide in aerated seawater (Doeller *et al.*, 1988). The behavior of hemoglobin in the *Solemya reidi* bacteriocyte also stands in contrast to the reaction of the "sulfide-reactive" hemoglobin, Hb I, isolated from the symbiont-containing gill of the lucinid clam *Lucina pectinata* (Kraus and Wittenberg, 1990). Oxygenated *Lucina* Hb I reacts rapidly with hydrogen sulfide at micromolar concentration to form ferric hemoglobin sulfide. We propose that in these three symbioses, sulfide-reactive gill hemoglobin functions to deliver either hydrogen sulfide or reducing equivalents to the symbiont. As one working hypothesis, we offer that the observed large difference in the steady-state concentration of ferric hemoglobin sulfide in the two *Solemya* gills reflects very different rates of chemical reaction. Extraordinary slow dissociation of sulfide from ferric *Lucina* hemoglobin sulfide ($k_{off} = 2 \times 10^{-4}\ s^{-1}$; implying a turnover time of 5000 s) suggests that delivery of sulfide cannot be achieved by simple dissociation of the ligand. Instead, we suggest reduction of ferric hemoglobin sulfide near the peribacterial membrane surface may precede ligand delivery (Kraus and Wittenberg, 1990; Wittenberg and Kraus, 1991). We consider that this latter step may be rate-limiting in the gill of *Solemya velum* but not of *Solemya reidi*. Reduction of the major hemoglobin of the *Solemya reidi* gill, when ferric, is very much more rapid than the corresponding reduction of ferric *Lucina* hemoglobin sulfide in the presence of excess sulfide (Kraus and Doeller, unpub.). Perhaps rapid reductive removal of ferric hemoglobin in the *Solemya reidi* gill prevents accumulation of ferric hemoglobin sulfide in the tissue. As an alternative hypothesis, we offer that *Solemya reidi* gill hemoglobin may function in the transfer of reducing equivalents from sulfide to symbiont. This function is suggested by the rapid reduction of ferric *Solemya reidi* hemoglobin by hydrogen sulfide and by the lack of formation of ferric *Solemya reidi* hemoglobin sulfide *in vitro* (under the same conditions as those that lead to the formation of ferric *Lucina* hemoglobin sulfide; Kraus and Doeller, unpub.).

In summary, bacterial symbionts of the *Solemya* gill use hydrogen sulfide as the sole environmental source of reducing equivalents. We show here that symbiont bacterial cytochrome c_{552} is extensively reduced when the gill is exposed to hydrogen sulfide, and that hydrogen sulfide is the immediate or near immediate reductant for this cytochrome. This finds a strong parallel in free-living, sulfide-oxidizing bacteria that are considered to accept electrons from reduced sulfur compounds at the level of cytochrome *c*, supporting oxidative phosphorylation at the level of the terminal oxidase and reverse electron flow to NAD (reviewed in Kelly, 1982, 1985, 1988; Pettigrew and Moore, 1987). Only oxyhemoglobin and deoxyhemoglobin are detected in the gill of *Solemya reidi*, in sharp contrast to the congener *Solemya velum* where ferric hemoglobin sulfide constitutes about half of the hemoglobin in gills exposed to hydrogen sulfide and oxygen. *Solemya reidi* gill hemoglobin may participate in the symbiosis by rapid formation and reduction of ferric hemoglobin sulfide, or by transfer of electrons from sulfide to symbiont.

Acknowledgments

This work was supported in part by research grants DMB 87-03328 and DCB 90-17722 from the National Science Foundation (to JBW), in part by USPHS research grant HL19299 (to Dr. B. A. Wittenberg), and in part by the University of Alabama at Birmingham Faculty Research Grant (to DWK). Animals used in this study were collected on a cruise of the Point Sur, supported by research grant OCE 86-10514 from the National Science Foundation (to Dr. J. J. Childress). J. B. Wittenberg is a Research Career Program Awardee 1-K6-733 of the United States Public Health Service, National Heart, Lung and Blood Institute. We extend special thanks to Drs. C. R. Fisher and J. J. Childress for the invitation to DWK and JED to participate in the *Solemya reidi* collecting cruise, to the captain and crew of the Point Sur for their help in the collection of *Solemya reidi*, to Dr. R. G. B. Reid for supplying specimens of *Solemya reidi* for preliminary experiments, and to Dr. B. A. Wittenberg for continuing discussions.

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