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The Role of Vestimentiferan Hemoglobin in Providing an Environment Suitable for Chemoautotrophic Sulfide-Oxidizing Endosymbionts

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Abstract

Vestimentiferan blood contains an extracellular hemoglobin which binds both sulfide and oxygen. This hemoglobin binds sulfide with a high affinity (50% saturation at 11.2 μM) resulting in very low levels of free sulfide in the blood (less than 1% of total sulfide) until the blood sulfide exceeds 60% of saturation. A vestimentiferan saline solution was developed from analyses of *Riftia pachyptila* blood and used as incubation medium for vestimentiferan trophosome homogenates. These homogenates, containing intact symbiotic bacteria, fix carbon at an appreciable rate in the presence of sulfide, but are inhibited by free sulfide concentrations greater than about 100 μM. When trophosome homogenates are incubated in dilute vestimentiferan blood containing sulfide, rates of carbon fixation are several times higher than in a saline solution without blood and fixation is not inhibited by total sulfide concentrations that completely inhibit autotrophic carbon fixation in the saline incubations. However, sulfide does inhibit fixation once the blood sulfide levels exceed 70% saturation. We propose that vestimentiferan blood provides a high sulfide concentration at low activity (concentration of free sulfide), allowing high rates of autotrophy by the chemoautotrophic endosymbiotic bacteria, while protecting them from inhibition by sulfide.

Keywords: Chemoautotrophy, endosymbionts, hemoglobin, saline, symbiosis, Vestimentifera

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1. Introduction

Symbioses between marine invertebrates and endosymbiotic chemoautotrophic sulfur bacteria were first discovered at deep sea hydrothermal vents, and these associations are thought to be the main sources of organic carbon for these communities (Cavanaugh et al., 1981; Felbeck, 1981; Jannasch and Mottl, 1985). Similar associations are now well documented in three phyla of worms and in four orders of bivalves from a variety of habitats where reduced chemical species are present (Felbeck et al., 1983; Cavanaugh, 1985; Brooks et al., 1987). In most of the bivalves examined the bacteria are housed in cells within the gills in close proximity to the ambient sea water and the potential metabolites; carbon dioxide, sulfide, and oxygen (Felbeck et al., 1983; Distel and Felbeck, 1987). On the other hand, all vestimentiferans examined to date harbor abundant endosymbiotic, sulfide-oxidizing, chemoautotrophic bacteria in an internal organ, the trophosome. This organ is highly vascularized and is located in the trunk with no close connections to the ambient sea water. The trophosome comprises about 15% of the wet weight of the hydrothermal vent tube-worm, *Riftia pachyptila*, and hemoglobin-containing vascular and coelomic fluids account for at least another 30% (Childress et al., 1984). The bacterial volume is between 15 and 35% of the total volume of the trophosome (Powell and Somero, 1986). Because this organ is located in a coelomic cavity in the trunk of the animal, metabolites must be transported to the bacteria through the circulatory system, in the blood. Vestimentiferan hemoglobins bind hydrogen sulfide and oxygen independently and reversibly, preventing spontaneous oxidation of the sulfide while transporting it to the trophosome for use as an electron donor by the chemoautotrophic endosymbionts (Arp and Childress, 1983; Childress et al., 1984; Fisher and Childress, 1984; Arp et al., 1987). The high affinity of the blood for sulfide also protects the animal cytochrome *c* oxidase system from poisoning by this potentially toxic molecule (Powell and Somero, 1983; 1986).

In 1985 communities of animals were discovered on the Louisiana Slope of the Gulf of Mexico with taxonomic affinities to the invertebrates found at many hydrothermal vents (Kennicutt et al., 1985). Specifically, 2 species of vesicomyid clams (*Calyptogena ponderosa* and *Vesicomya cordata*), 2 species of vestimentiferan tube worms (*Lamellibrachia barhami* and an undescribed member of the family Escarpiidae), and a mytilid closely related to *Bathymodiolus thermophilus* have been found associated with the hydrocarbon seeps in this area. The clams and vestimentiferans contain sulfide-oxidizing chemoautotrophic symbionts, and the mussel harbors methan-

otrophic symbiotic bacteria (Childress et al., 1986; Brooks et al., 1987). Specimens of the undescribed escarpiid up to 2 m in length have been collected, with a maximum tube diameter of about 1.5 cm. This site is relatively shallow (600 to 700 m depth), when compared to other sites where vestimentiferans live, and therefore these animals can be maintained alive at ambient pressure (mussels have been maintained alive for over 1 year at ambient pressure in the laboratory and some small vestimentiferans were kept alive for almost a month under the same conditions). The experiments described here were done using tissue from specimens of the undescribed escarpiid, trawled from the Louisiana slope in 1986, and maintained alive on board ship for up to 3 days.

Free-living chemoautotrophic sulfide-oxidizing bacteria flourish in environments in which both oxygen and sulfide are present at very low concentrations and therefore low activities (Nelson and Jannasch, 1983; Nelson et al., 1986). In nature this environment is normally restricted to a very narrow interface between oxic and anoxic environments (Jørgensen and Revsbech, 1983). We propose that inside the vestimentiferans this environment is provided by the vascular and coelomic fluids, which contain extracellular hemoglobins that bind sulfide and oxygen with a high affinity (Arp and Childress, 1981, 1983; Childress et al., 1984; Arp et al., 1987). We demonstrate that this results in very low levels of free sulfide in the blood, until the blood sulfide exceeds 60% of saturation. Homogenates of vestimentiferan trophosome containing intact symbiotic bacteria fix carbon at an appreciable rate in the presence of low concentrations of sulfide (Belkin et al., 1986), but are inhibited by moderate sulfide concentrations. However, when trophosome homogenates are incubated in dilute vestimentiferan blood containing sulfide, rates of carbon fixation are several times higher and fixation is not inhibited by total sulfide concentrations that completely inhibit autotrophic carbon fixation in the saline incubations. We conclude that vestimentiferan blood provides a high sulfide concentration at low activity, allowing high rates of autotrophy by the chemoautotrophic endosymbiotic bacteria, while protecting them from inhibition by sulfide.

2. Methods

Collection and maintenance of animals

The vestimentiferans were collected using a 10 m otter trawl from the R.V. *Gyre* at 600 to 700 m depth on the Louisiana Slope in the Gulf of Mexico (27° 41′ N; 91° 32′ W), during March 1986. Immediately upon recovery, the

tube-worms were placed in buckets of chilled (7.5°C) seawater where they were maintained at this temperature, with daily changes of sea water, until used (no more than 3 days). The vestimentiferans used in this study are in an undescribed genus, either in or closely related to the family Escarpiidae (pers. comm., M.L. Jones, Smithsonian Mus. of Nat. Hist.). The escarpiids used in this study were collected during 2 separate trawls, and were recovered from both trawls in intertwined masses of approximately 50 individuals of both escarpiids and *Lamellibrachia barhami*. Only animals which were undamaged and responsive were used for the trophosome preparations.

Preparation of Riftia saline

The ionic composition of both vascular and coelomic blood of *Riftia pachyptila* was determined for Na^+, K^+, Ca^{+2}, SO_4^{-2}, Mg^{+2}, and Cl^- during the "Galápagos '85" hydrothermal vent expedition. The concentrations of these ions in the freshly collected bloods were determined using cation and anion columns (Wescan) in an HPLC system with conductimetric detection. Sodium and potassium were determined using a 0.05 M nitric acid eluent, Ultrex grade (Baker Chemical Co.), at pH 2.5 and pumped at 1.5 ml/min. Calcium and magnesium concentrations were determined on the same column using an ethylene diamine (0.033 ml/l) eluent pumped at 1 ml/min. Chloride and sulfate ions were separated on the anion column with a 0.004 M phthalic acid eluent (pH 4.7) pumped at 2 ml/min.

The concentrations of these ions in the vascular and coelomic bloods were quite similar and an average of the two was used as the basis for the "*Riftia* Saline" in this study. The formula for the saline also reflects a slight reduction in the sodium ion concentration to balance the ionic charge of the saline, and the replacement of 50 mM NaCl with 50 mM HEPES buffer. The saline was titrated to pH 7.5 with NaOH before use. One liter of *Riftia* saline contains: 20.48 g NaCl (0.4 M); 0.194 g KCl (2.6 mM); 6.22 g $MgCl_2 \cdot 6H_2O$ (30.6 mM); 1.65 g $CaCl_2 \cdot 2H_2O$ (11.2 mM); 4.53 g Na_2SO_4 (31.9 mM) and; 11.915 g HEPES (50 mM).

Preparation and analysis of Riftia pachyptila blood

The *Riftia pachyptila* blood used in these experiments was collected from living worms during the 'Galápagos '85' Hydrothermal Vent Expedition and kept frozen at –70°C until used. This blood was a mixture of coelomic and vascular bloods from the same animal. Bound sulfide was removed from the blood by acidifying to pH 5.5 with HCl and purging the chilled blood (7°C)

with a stream of nitrogen for 24 hr. The blood was diluted with vestimentiferan saline solution before use in the trophosome homogenate incubations.

The heme content of the blood mixture was determined from the absorbance of a cyanomet hemoglobin derivative (Tentori and Salvati, 1981). The relative proportion of the small and large aggregates of hemoglobin in the blood mixture was determined by separating the 2 components on a gel column (TSK-50, 7.5 mm×300 mm) in an HPLC system (*Riftia* saline as eluent pumped at 0.3 ml/min) with detection at 415 nm.

We determined the affinity of separate aliquots of this blood for sulfide by equilibrium dialysis of the blood in 30 mM citric acid phosphate buffer with 600 μM carbon monoxide, at 6.0°C and pH 7.5. Carbon monoxide (CO) was used to deoxygenate the dialysate so that CO would bind to the heme groups. We believe it is unlikely that the state of the heme (saturated with CO) affected the sulfide binding properties of the blood since sulfide binding does not affect the affinity of the heme for oxygen, indicating an absence of interaction between the heme and the sulfide binding site (Childress et al., 1984). Samples of the blood in dialysis tubing were allowed to come to equilibrium (16 hr) with various concentrations of sulfide in the dialysate and the concentrations of sulfide in both blood and dialysate were analyzed with a gas chromatograph using thermal conductivity and photoionization detectors (Childress et al., 1984). The difference between the sample sulfide and the dialysate sulfide was taken to be the amount of sulfide bound. The heme content of these dialyzed samples was also determined and the binding capacity expressed as a molar ratio of sulfide bound per heme. This was done to compensate for small volume changes in the dialyzed sample and relates sulfide binding to hemoglobin quantity. Sulfide is not bound to the heme group (Arp et al., 1987).

Preparation of the trophosome homogenates

Trophosome was dissected free from the anterior portion of the trunk of the vestimentiferans, and tissue containing bacteria was separated from the major blood vessels and gonads also present in this organ. A portion of the tissue (0.49–0.71 g) was quickly weighed on a motion compensated shipboard balance system (Childress and Mickel, 1980) and then submerged in 20 ml of deoxygenated (nitrogen purged), *Riftia* saline solution. The tissue was gently homogenized for 5–10 sec in a chilled, loose fitting Dounce type ground glass tissue homogenizer, to rupture the bacteriocytes and disperse the symbionts. A portion of this homogenate (0.1 ml) was fixed in 3% glutaraldehyde in

0.1 M phosphate-buffered 0.35 M sucrose (pH 7.35) for later counting of the intact bacteria, using epifluorescence microscopy (Hobbie et al., 1977).

$NaH^{14}CO_3$ incubations

The incubations were conducted in 10 ml glass syringes fitted with low dead volume teflon valves, which were kept at 7.5°C in a water jacketed chamber. Prior to preparation of the homogenate, 6 syringes were loaded with incubation media (pH 7.5) consisting of either 5 ml of *Riftia pachyptila* blood diluted with *Riftia* saline (~1:1) or *Riftia* saline solution alone, variable concentrations of sulfide (4–3000 μM), and a marble for mixing the incubation medium. To confirm that stimulated carbon fixation was through autotrophic pathways, 10 mM DL-glyceraldehyde (a feedback inhibitor of RuBP carboxylase, Stokes and Walker, 1972) was added to 1 syringe in most of the experiments. To commence the incubations, $NaH^{14}CO_3$ was added to the trophosome preparation and 2.5 ml of the labeled preparation was drawn into each of the 6 syringes which already contained incubation media with the desired levels of sulfide. Sulfide and inorganic carbon concentrations in the blood and saline stock solutions were determined using a gas chromatograph (Childress et al., 1984) and activity of $NaH^{14}CO_3$ was determined by scintillation counting of replicate samples of the incubation media stabilized with hyamine hydroxide. After all syringes had been filled and the contents mixed (a process which took between 4 and 7 min) replicate samples (0.1 ml) were removed from each syringe and acidified for scintillation counting of the fixed carbon. Replicate samples (0.1 ml) were similarly removed and assayed from each syringe at 10 to 20 min intervals for the next 60 to 70 min. The incubations were treated as having commenced at the time of the first sample, and fixation rates were calculated after subtracting these values from the values measured at the later time points. Carbon fixation rates were calculated following the methods of Strickland and Parsons (1972) from the measured concentration of inorganic carbon in the incubation media, the measured specific activity of that inorganic carbon pool, and the amount of acid-stable ^{14}C found in the samples. Experiments in which the highest measured carbon-fixation rate was less than 0.2 μmol/g/h were considered to contain at best only marginally viable symbionts, and are not reported here.

3. Results

The ionic compositions determined for vascular and coelomic blood from *Riftia pachyptila* are given in Table 1. The analysis was undertaken in order to develop a general saline for use in studies of the blood and symbionts, of *R. pachyptila* and other vestimentiferans. Because of the similarities of the

Table 1. Ionic composition of coelomic and vascular blood from *Riftia pachyptila*. Concentrations of the various ions are given in millimoles ± standard deviation and the number of determinations are given in parentheses.

ion	*Vascular blood*	*Coelomic blood*
Na^+	539±39 (n=8)	460±15 (n=7)
K+	2.5±1.3 (n=4)	4.8±3.7 (n=4)
Ca^{++}	12.5±1.3 (n=5)	10.1±4.4 (n=4)
Mg^{++}	28.3±2.1 (n=7)	32.4±4.4 (n=8)
$SO4^{=}_{4}$	30.3±6.2 (n=4)	33.4±2.5 (n=4)
Cl^-	464±45 (n=4)	512±15 (n=4)

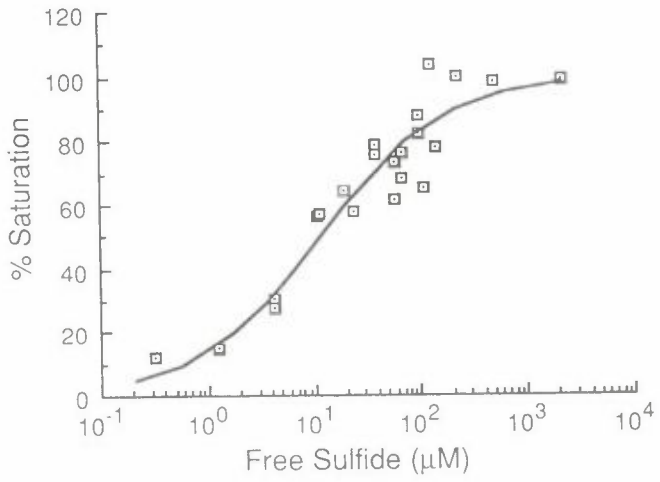

Figure 1. Sulfide binding by *Riftia* carbonyl hemoglobin as a function of free sulfide concentration. The Hill curve fitted to these data: in [% saturation÷ (100 − % saturation)] = 0.737 (in free sulfide) − 1.778, $r^2 = 0.89$, 95% confidence interval on the coefficient = ±0.129. The line shown is the relationship between the % saturation and free sulfide described by this Hill equation.

ionic composition determined for the 2 bloods, an average ion composition of these 2 bloods was used to develop the formula for the *Riftia* saline used in this study. That formula is given in the methods section.

The vestimentiferan blood incubations and sulfide binding measurements in this study were conducted using the same dilute mixture of *Riftia* coelomic and vascular bloods from a single animal. The hemoglobin in this mixture was 20% the large aggregate and 80% the smaller aggregate. The large aggregate (1.7×10^6 M_r, Terwilliger et al., 1980) is found primarily in the vascular fluid and constitutes 60–70% of the hemoglobin in this fluid. The small aggregate ($\sim 4 \times 10^5 M_r$, Terwilliger et al., 1980) is found in roughly equal concentrations in both vascular and coelomic fluids, but is the predominant (greater than 85% of the heme) form in the coelomic fluid (Arp et al., 1987). This thus mixture is intermediate in concentration of the large aggregate and presumably other properties of the vascular and coelomic fluids. In Fig. 1 the free sulfide in the blood (determined as the dialysate sulfide concentration) is plotted against the percentage of saturation of the blood with sulfide. At saturation the blood contains 1.75 HS^-/heme reflecting the relative concentrations and different binding capacities of the 2 hemoglobin aggregates. A Hill plot of these data is linear with a slope of 0.74 (significantly less than 1.0) supporting the heterogeneous nature of the observed binding (Dahlquist, 1978), and predicts that 50% saturation occurs at 11.2 μM sulfide. The result of these binding properties is that the free sulfide present at total sulfide concentrations below saturation will be quite low compared to the total sulfide pool available to the bacteria.

Five separate experiments were conducted with tissue from 5 different worms. In all but the first blood incubation, one of the syringes in each experiment contained 10 mM DL-glyceraldehyde, an inhibitor of RuBP carboxylase (Stokes and Walker, 1972). The carbon-fixation rates in the syringes containing the inhibitor were always extremely low when compared to the maximum stimulated rates in that experiment (Fig. 2), which indicates that the measured, stimulated rates are due to autotrophic carbon fixation.

The results from 2 experiments conducted during the same cruise are not reported here because we have reservations about the viability of the preparations used in these experiments. These experiments show the same trends as those reported here, but the maximal stimulated carbon-fixation rates were below 0.2 μM/g/h, the levels of stimulation of carbon fixation were quite low (less than 5 times the unstimulated rates), and relatively few intact bacteria were found in the preserved homogenates. This was in contrast to the other

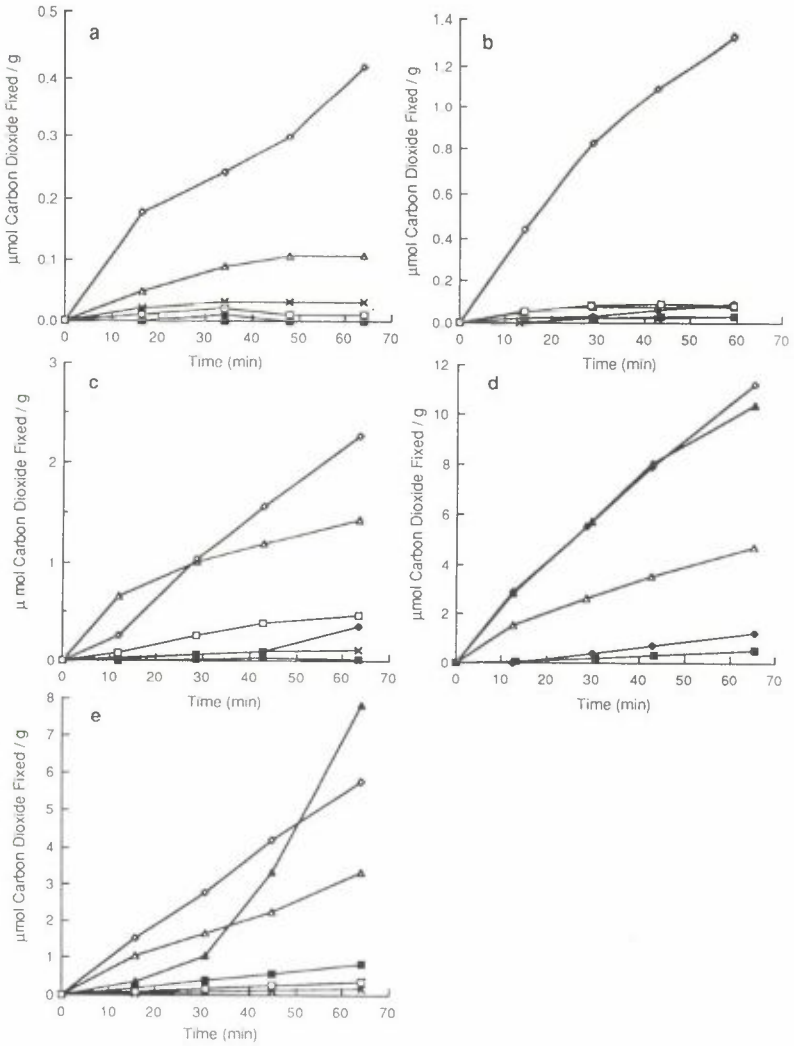

Figure 2. Rates of assimilation of $NaH^{14}CO_3$ into organic compounds by 5 trophosome preparations of seep escarpiid in saline (a,b,c) and in 33% *Riftia* blood (d,e) with different concentrations of sulfide present in the incubation media. Note the scales on the abscissae are different. (a,b,c) ranges of initial concentrations of sulfide (when final concentrations were measured those ranges are given in parentheses): x) 0; △) 25–27 µM; ◇) 95–100 µM; ◇) 460–500 µM, (270–330 µM); ■) 920–1000 µM, (570–760 µM); □) 95–100 µM with 10 mM DL-glyceraldehyde. (d) The initial and final concentrations (given in parentheses) of sulfide were: △ 70 µM, (~63 µM); ◇)375 µM, (~310µM); ▲) 725 µM, (~625 µM); ◇) 1670 µM, (~1530 µM);■) 2900 µM, (~2600 µM). (e) The initial and final (when measured) concentrations of sulfide were: x) 4 µM; △) 110 µM, (~65 µM); ◇) 280 µM; ▲) 1100 µM, (~800 µM); ■) 2195 µM, (~1950 µM); □) 110 µM with 10 mM DL-glyceraldehyde.

homogenates which contained between 0.6 and 2×10^7 cells/ml. We did not standardize our data to bacterial numbers in the preparations because of difficulty in obtaining accurate counts of the very polymorphic bacteria, which tended to clump in the fixative. None the less, the numbers of bacteria estimated in the homogenates correspond to a recovery of between 6 and 20×10^8cells/g trophosome, which compares favorably to previous estimates of between 3.7 to 10×10^9 cells/g of trophosome in *Riftia pachyptila* (Cavanaugh et al., 1981; Powell and Somero, 1986).

Figure 2 shows the results of 5 carbon-fixation experiments conducted in vestimentiferan saline and dilute *Riftia pachyptila* blood. Note that the scale on the abscissa of each figure is different. In vestimentiferan saline (Figs. 2a,b,c) the maximal carbon fixation was at about 100 μM initial sulfide and sulfide levels above that inhibited carbon fixation to virtually the same degree as did DL-glyceraldehyde. The effective sulfide concentrations decrease throughout the time course of the experiments, particularly in the saline incubations since in the absence of binding proteins sulfide and oxygen are not stable together at physiological pH. The depletion of sulfide was reflected in the time course of carbon fixation in the 25 μM sulfide incubations shown in Figs. 2a and c, where the decline in the fixation rate is probably due to exhaustion of the sulfide. Final sulfide and oxygen concentrations were measured for several of the syringes in each experiment (time limitations of gas chromatographic analysis precluded measurement of final sulfide and oxygen in all syringes in each experiment). The initial and final sulfide concentrations for each of the incubations for which the final concentration was either measured or could be calculated from direct measurements, are given in the legend of Fig. 2.

As can be seen in Fig. 2, the maximal stimulated carbon fixation rate was much higher in dilute *Riftia pachyptila* blood (Figs. 2d, e) than in the saline solution. The heme contents of the blood, as diluted in the incubations, were 0.79 and 1.03 mM heme, respectively. Concentrations of sulfide that completely inhibited autotrophic carbon-fixation in the saline solution stimulated carbon-fixation maximally when hemoglobin was used to bind the sulfide, and inhibition of carbon-fixation did not occur until the sulfide concentration approached the binding capacity of the hemoglobin solution. The data from all experiments are summarized in Fig. 3 where a single rate was calculated for each of the incubations, and these rates are plotted against the concentration of sulfide in the syringes at the start of the incubations. Since the rates shown in Fig. 2 are plotted against the sulfide concentration at the

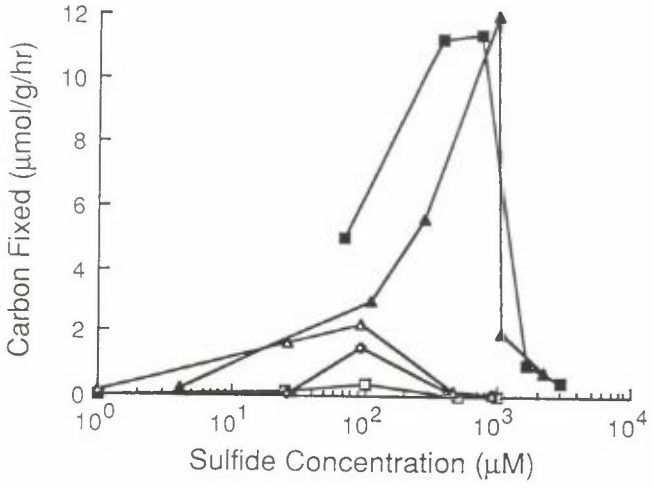

Figure 3. Rates of carbon fixation by trophosome preparations from seep escarpiid in the presence of different sulfide concentrations. Each of the rates (points) are derived from linear regressions of the data presented in Fig. 2 (see text). The open points represent experiments conducted in saline and the closed points are experiments conducted in solutions of 33% *Riftia* blood. Calculated free sulfide concentrations (in μM) in the blood incubations are the following (total [free]):■) 70 [0.31]; 375 [1.33]; 725 [5.91]; 1670 [150.7]; 2900 [1814]. ▲) 4 [0.25]; 110 [0.47]; 280 [1.21]; 975 [33.3]; 1100 [54.4]; 2195 [1117].

beginning of the incubation, rates for the saline incubations with 25 μM were calculated from the first 4 times points because these rates fell off, presumably as the sulfide was depleted in the syringe (see Fig. 2a,c). The other incubation which showed a distinctly non-linear pattern of carbon fixation was the incubation in blood with 1100 μM sulfide at the start (Fig. 2e). Two rates were calculated from this line, one for the first half hour and another for the last half hour, and these are plotted in Fig. 3, against the initial sulfide concentration and the calculated sulfide concentration in the syringe after 30 min, respectively. The higher maximal stimulated fixation rates and the raised threshold for sulfide toxicity in vestimentiferan blood is evident in Fig. 3.

4. Discussion

Undiluted vestimentiferan blood can bind sulfide at concentrations up to 8.7 mM (Arp et al., 1987) and thus can contain a large pool of bound sulfide at low activity. The role of the blood in protecting the animal tissue from the

deleterious effects of sulfide while simultaneously transporting sulfide to the symbiotic bacteria in the trophosome has been previously reported (Arp and Childress, 1983; Powell and Somero, 1983; Fisher and Childress, 1984). The trophosome is a highly vascularized organ, with all symbiotic bacteria lying close (a few bacterial cells distant at most) to vascular capillaries (M.L. Jones, in press). Based on this anatomical observation and the data reported here we propose another role of vestimentiferan blood, which is to provide the symbionts with a chemical milieu (a large pool of sulfide at low activity) which both stimulates chemoautotrophy by the symbionts and protects these symbionts from sulfide toxicity.

The hemoglobin of *R. pachyptila* binds sulfide reversibly and with a high affinity. Due to the pKa of around 7.0 (Millero, 1986) and the indication that HS- is the form bound (Childress et al., 1984) the concentration of H_2S will be held to only about 31% of the free sulfide concentration in the blood at pH 7.5, which approximates physiological pH (Childress et al., 1984). For example, at half saturation, 11.2 μM total free sulfide (Fig. 1), the free H_2S concentration would be 3.5 μM. Thus, this protein is able to hold free H_2S in the vascular blood at concentrations in the low micromolar range while total sulfide concentrations in the blood are in the several millimolar range.

The apparent absence of subunit interaction in sulfide binding indicated by the low Hill coefficient is consistent with the absence of an effect of sulfide binding on oxygen binding (Childress et al., 1984). Oxygen binding in this hemoglobin shows appreciable subunit interaction (n between 2 and 3, Arp and Childress, 1981) so one would expect that if there were subunit interaction in sulfide binding it would affect oxygen binding through allosteric effects.

The binding curve presented in Fig. 1 shows some heterogeneity in binding mechanisms. For example it appears that about 10% of the bound sulfide is not readily released at pH 7.5. This may be the result of a different, more general binding mechanism than the primary one in vestimentiferan hemoglobin (Cavallini et al., 1970) or binding by methemoglobin (Smith and Gosselin, 1966). At about 80% of saturation there is scatter which we have not been able to resolve with our methods, but it may be due to different sulfide affinities of the 2 aggregates. It may also be due to variable loss of binding activity during our experiments. None the less, it is evident that at sulfide levels below 70% of saturation the free sulfide in the blood is quite low compared to the total sulfide pool available to the symbionts, which is an effective method of providing the symbionts with sufficient sulfide to

support high levels of autotrophic carbon fixation without inhibiting their metabolism.

The trophosome incubations in dilute vestimentiferan blood not only tolerate higher levels of sulfide in the incubation media but also, in general, fix carbon at a higher rate (Figs. 2,3). We have observed these phenomena in numerous trophosome preparations from other vestimentiferans as well (Fisher and Childress, unpubl.). In addition, the rates reported here for carbon fixation in dilute vestimentiferan blood are as much as 50 times higher than previously reported rates for trophosome homogenates incubated in sea water (Belkin et al., 1986) (this calculation was made based on the observation that trophosome tissue is approximately 10% protein, Fisher et al., in prep.). The possibility that this general stimulation of symbiont carbon-fixation by the blood could be due to the presence of carbonic anhydrase was tested on another cruise in paired incubations with and without acetazolamide, an inhibitor of carbonic anhydrase. There was no difference in the rates of carbon fixation in these incubations (pers. observ. [CRF, JJC]). We believe it likely that this general stimulation reflects the favorable environment around the bacteria (sulfide and oxygen tensions) which is created by the dilute blood.

Examination of the pattern of carbon fixation in the saline incubations in the syringes which contained ~25 μM sulfide at the start of the incubations illuminates another role of the blood, which is to stabilize the sulfide in the presence of oxygen. It is evident in Fig. 2b that no sulfide was present in the saline by the beginning of the incubation in the syringe which was primed with 25 μM sulfide 15 min earlier. In the other 2 saline experiments the rates of carbon fixation with 25 μM sulfide declined over the time course of the experiments, again presumably due to sulfide depletion. Estimates of the molar ratio for net carbon dioxide-fixation per sulfide oxidized range from 0.15 to 0.35 for live *Solemya reidi*, a chemoautotrophic symbiont-containing bivalve (Anderson et al., 1987) and 0.35 for free-living *Beggiatoa* sp. (Nelson et al., 1986). Using these molar ratios one can calculate that at most about one half of the sulfide present in the 25 μM sulfide incubation shown in Fig. 2a was oxidized by the bacteria, and the rest of the depletion was auto-oxidation.

The discrepancy between our observation of almost complete inhibition of carbon fixation by 500 μM sulfide and the report by Belkin et al. (1986) of maximal stimulated carbon-fixation by that concentration of sulfide with *Riftia pachyptila* trophosome preparations is difficult to reconcile. These differences could be due to differences in the symbionts in the 2 species of vestimentiferans. However, it is important to note when examining thee data that

the maximum stimulated rate reported in that experiment of 1.7 nmol/mg protein/h is quite low, even when compared to other experiments in that study (this rate is equivalent to 0.17 μmol/g/h, assuming 10% protein in trophosome tissue), and that the maximum stimulated rate is only 5 times the unstimulated rate (Belkin et al., 1986) (compared to 13 to 76 times, as found in this study).

Using the data from Figs. 1 and 3 we calculate (using the Hill equation fitted to the data, see Fig. 1 legend) that inhibition of carbon fixation by sulfide occurs when the blood is about 70% saturated by sulfide. Using the same data and equation we can calculate the free-sulfide concentration in the blood when inhibition occurs. In the first blood incubation (Fig. 2d) inhibition occurred between 6 and 151 μM free sulfide in the blood (these values correspond to total sulfide levels of 725 and 1670 μM respectively in that experiment). A better estimate of the free sulfide level in the blood that inhibits autotrophic carbon-fixation can be derived from the second blood incubation (Fig. 2e). In this experiment, carbon-fixation was clearly inhibited in the syringe containing 1100 μM total sulfide at the start of the incubation. Approximately half way through the incubation the preparation began fixing carbon at a much higher rate. This is most likely due to the drop in the sulfide concentration in the syringe during the incubation. The sulfide concentration in the syringe after 30 min was calculated to be 975 μM (based on a linear depletion of sulfide over the 90 min between the start of the incubation and the measurement of the final sulfide concentration). These calculations indicate that inhibition occurred at a free-sulfide level of about 50 μM in that incubation. Free-sulfide levels in the blood as low as 1.3 μM (375 μM total sulfide, Fig. 2d) are able to support near maximal rates of carbon fixation, which supports the previously reported observation that the symbionts can utilize the large pool of bound sulfide present in vestimentiferan blood (Fisher and Childress, 1984).

We believe that the hemoglobin, which also has a high affinity for oxygen, probably carries out a similar role with regard to this ligand as well. Thus the blood appears to provide an environment in the trophosome that is only found in very small microhabitats in nature, allowing for the proliferation of a type of chemoautotrophy that is often quite restricted in nature. This situation is comparable to the role of leghemoglobin, with respect to oxygen, in the root nodule symbiosis between leguminous plants and oxygen-sensitive nitrogen-fixing bacteria (Wittenberg et al., 1974; Appleby, 1984; Wittenberg, 1985). The gill hemoglobins of some molluscs with chemoau-

totrophic symbionts may be playing a similar role with respect to oxygen and sulfide (Doeller, 1984; Dando et al., 1985; Wittenberg, 1985). We see clearly here that the large size of the host tubeworm, as well as its tissue and organ levels of organization, enable it to maintain an environment suitable for its symbionts, something which they are quite unable to do for themselves and, yet, which is quite beneficial to them.

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