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THE SULPHIDE-BINDING PROTEIN IN THE BLOOD OF THE VESTIMENTIFERAN TUBE-WORM, *RIFTIA PACHYPTILA*, IS THE EXTRACELLULAR HAEMOGLOBIN

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SUMMARY

The sulphide-binding protein that occurs in high concentrations in the vascular blood and coelomic fluid of the hydrothermal vent tube-worm *Riftia pachyptila* Jones is the haemoglobin. Sulphide binding does not occur at the oxygen-binding sites of the haem, but may occur *via* thiol–disulphide exchange at the interchain disulphide bridges on the macromolecule. We have confirmed the report that vascular blood is heterogeneous for two haemoglobins (FI and FII) that are different in M_r, but we conclude that the coelomic fluid is homogeneous for the lower M_r haemoglobin FII, in the intact, living animal. These two haemoglobins occur naturally in the living animals, and FII is not a dissociation product of the higher M_r FI. The sulphide-binding capacities of the two haemoglobin species differ by about a factor of two. Consequently, the vascular blood and the coelomic fluid also have different sulphide-binding capacities. These differences in sulphide-binding capacity may have important ramifications for the physiology of this unusual animal.

INTRODUCTION

The tube-worm *Riftia pachyptila*, a member of the phylum Vestimentifera, was first discovered living in dense assemblages around the Galapagos hydrothermal vents in 1977 (Corliss *et al.* 1979; Jones, 1981, 1985). Water entering the vent environment through fissures contains high levels of hydrogen sulphide, and sulphide concentrations around the tubes of the worms reach approximately $0{\cdot}2\,\mathrm{mmol\,l^{-1}}$ (Edmond, Von Damm, McDuff & Measures, 1982; Hessler & Smithey, 1983; Johnson, Beehler, Sakamoto-Arnold & Childress, 1986). Although sulphide poses a threat to aerobic respiration due to its capacity to inhibit the reversible binding of oxygen by haemoglobin (Hb) and the activity of cytochrome-c-oxidase (National Research Council, 1979), *Riftia* flourishes in this environment.

Riftia is unusual in that it derives metabolic energy from the oxidation of sulphide by chemolithoautotrophic bacteria that live symbiotically inside the trunk of the animal (Felbeck, 1981; Cavanaugh *et al.* 1981; Cavanaugh, 1985; Felbeck, Powell,

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Hand & Somero, 1985). The bacteria require sulphide, as well as oxygen and carbon dioxide, for the production of carbon compounds which are then made available to the animal. Our initial question was: how are these essential gases transported to the internal bacteria? Previous work has shown that the vascular blood and coelomic fluid of *Riftia* have the capacity to bind and store large quantities of oxygen and carbon dioxide (Arp & Childress, 1981; Wittenberg, Morris, Gibson & Jones, 1981). Further, we have reported that both of these Hb-containing fluids contain proteins that bind sulphide with a high affinity, and have a mean capacity for sulphide of 1·3 mmol sulphide per mmol haem (Arp & Childress, 1983; Childress, Arp & Fisher, 1984; Arp, Childress & Fisher, 1985). The ability of *Riftia* body fluids simultaneously to bind and potentially transport these three gases is well established. The specific identity and properties of the sulphide-binding protein were the subject of this study.

Riftia has two distinct body fluids that both contain high concentrations of large, extracellular Hbs which have several characteristics in common with annelid extracellular, high relative molecular mass (M_r) Hbs (Terwilliger, Terwilliger & Schabtach, 1980; Arp & Childress, 1981). These include: intact M_r values in the millions; a two-tiered, hexagonal array of submultiples; subunit M_r values of 15 000 and 30 000; a haem content of 1 mol of haem per 23 000 g protein; and similar amino acid composition of purified fractions (Terwilliger *et al.* 1980; Terwilliger & Terwilliger, 1985). The circulating vascular blood has an average haem concentration of 3·5 mmol l^{-1} and the blood constitutes more than 4 % of the mass of the animal, whereas the coelomic fluid has an average haem concentration of 1·9 mmol l^{-1} and the coelomic fluid constitutes approximately 26 % of the mass of the animal (Childress *et al.* 1984). Mixed blood samples (a combination of coelomic fluid and vascular blood) have been reported to show heterogeneity of Hb content, with a 1 700 000 M_r Hb (FI) and a 400 000 M_r Hb (FII) both present (Terwilliger *et al.* 1980). The high concentration of Hb in these body fluids made it a likely candidate for the sulphide-binding protein, even though simultaneous binding of oxygen and sulphide by a Hb molecule has never been described.

The objectives of this study were: (1) to determine if the sulphide-binding protein in the vascular blood and coelomic fluid of *Riftia* is the Hb molecule itself or a different protein; (2) to establish if sulphide binding was occurring at the haem portion of the molecule, and, if not, to explore other possible binding mechanisms; and (3) to examine the distribution of the Hbs, FI and FII, in the vascular blood and coelomic fluid of the intact animal and to compare their biochemical and functional characteristics.

MATERIALS AND METHODS

Source of materials

The experiments described throughout this report utilized body fluids obtained from live specimens of *Riftia* collected during the March, 1985 Galapagos hydrothermal vent expedition. The two types of Hb-containing fluids, vascular blood and

coelomic fluid, were collected and stored separately. Although a great deal of effort was put into keeping the coelomic fluid Hb and the vascular blood Hb separate during collection, this was not always possible. Damage to the intricate vascular network during collection of the animals by the submersible's manipulators, or during dissection, may have caused inadvertent mingling of Hbs in some of the samples. Similarly, it was difficult to get a sample of vascular blood out of the animal without contamination from the extremely abundant coelomic fluid that bathes the vascular system. The Hb composition of each body fluid sample is mentioned in the description of each experiment.

The experiments in this study were performed at sea, with fresh material, or in the laboratory using frozen material (all samples were frozen in liquid nitrogen at sea and stored at −80°C in the laboratory unless otherwise noted). The saline buffer used throughout these experiments and referred to as *Riftia* buffer is an average of the ionic compositions of the coelomic fluid and the vascular blood as determined at sea on fresh blood using an ion chromatograph (N. K. Sanders, personal communication). The formula is as follows (in $mmol\,l^{-1}$): NaCl, 400; KCl, 3; $MgCl_2$, 31; $CaCl_2$, 11; Na_2SO_4, 32; Tris-HCl buffer, 50; pH 7·5 at 22°C.

Analysis of sulphide-binding capacity

Measurement of sulphide concentration in Hb solutions and body fluids was done in a modified gas chromatograph as described previously (Arp & Childress, 1983). Sulphide binding was determined by dialysing a sample against a deoxygenated citric acid–phosphate buffer (pH 7·5 at 22°C) which was brought to approximately $1{\cdot}0\,mmol\,l^{-1}$ sulphide by the addition of solid Na_2S. After approximately 18 h of dialysis at 5°C, both sample and dialysate were analysed for total sulphide content. The difference between the sample sulphide and the dialysate sulphide was taken to be the amount of sulphide bound. Additionally the samples were analysed for haem concentration according to the methods of Drabkins & Austin (1935) and sulphide-binding capacity is expressed throughout as a molar ratio of sulphide bound per haem. This was done to relate sulphide binding to Hb quantity, and does not imply sulphide binding by the haem group.

Haemoglobin sulphide binding

Previous work has shown that the sulphide-binding factor in the vascular blood and coelomic fluid of *Riftia* is a protein greater than 15 000 in M_r, which is present in concentrations that allow for the high sulphide-binding capacities of these fluids (Arp & Childress, 1983; Arp *et al.* 1985). In this study we designed a series of experiments to explore the possibility that the sulphide-binding protein in the vascular blood and coelomic fluid of *Riftia* is the Hb molecule itself.

The first step was to separate and purify the Hb as much as possible while maintaining its functional state. A mixed blood sample (a combination of coelomic fluid and vascular blood) that had been stored at −20°C was ultracentrifuged at 250 000 ***g*** for 2·5 h at 5°C to separate the high M_r proteins, such as the Hb, from other proteins with lower M_r values. The sedimented protein portion (which contained all

of the reddish-brown material) was resuspended in cold *Riftia* buffer. This resuspended pellet was then applied to a Sepharose 4B gel filtration column described below. The elution profile of the blood was determined at both 280 nm, for protein absorbance, and at 415 nm, for haem absorbance. There were only two peaks, both of which showed haem and protein absorbance. These peaks were collected separately and concentrated with an Amicon ultrafiltration system under pressurized neon gas employing membranes with a 100 000 M_r cut-off.

The second step was to test samples of the whole untreated blood, the resuspended pellet, the supernatant and the concentration peaks from the Sepharose gel filtration column for sulphide-binding capacities according to the standard procedure described above.

To determine whether sulphide binding was a phenomenon common to high M_r extracellular Hbs, fresh blood was collected from specimens of the common earthworm *Lumbricus* sp. The animals were purchased from a local bait shop and bled into a test tube. This blood was spun in a table-top centrifuge for several minutes to remove debris. One portion of the blood was further purified by ammonium sulphate precipitation by stirring in 60 % ammonium sulphate at 5 °C for 2 h, and then by centrifugation at 15 000 ***g*** for 15 min at 4 °C. The sedimented pellet was resuspended in cold *Riftia* buffer. This sample, a sample of the untreated blood, a concentrated haem-containing fraction eluted from the Sepharose 4B gel filtration column and a sample of *Riftia* mixed blood for comparison, were analysed for sulphide-binding capacity according to the standard procedure.

To test for the presence of protein other than Hb in the blood we analysed the sedimented and resuspended Hb from *Riftia*, the fractions from the Sepharose gel filtration column and *Lumbricus* sp. Hb by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions according to the methods of Davis (1964). The gel was loaded in two identical patterns and after electrophoresis was sliced into two portions, one of which was stained for protein with Coomassie Blue, while the other was stained for haem with *o*-dianisidine and peroxide (Francis & Becker, 1984).

Haem involvement in sulphide binding

To test for haem involvement in sulphide binding, we altered the haem site of *Riftia* coelomic fluid Hb by exposure to carbon monoxide (CO) gas and by formation of methaemoglobin (metHb), and then tested for differences in sulphide-binding capacity. Samples of coelomic fluid were dialysed in the sulphide-containing medium described above that initially contained no CO, and then was bubbled with CO. The samples were analysed on the gas chromatograph for sulphide concentration and CO concentration. Sulphide-binding capacity was thus determined for each sample both in the presence and absence of CO. The absorbance spectra of the samples were monitored from 500 to 650 nm during the experiment, and CO-treated samples showed the previously reported spectral shift associated with HbCO formation (Terwilliger *et al.* 1980).

Formation of metHb was accomplished by the addition of the appropriate amounts of 20 mmol l^{-1} potassium ferricyanide to bring aliquots of the same coelomic fluid samples to a slightly more than equimolar concentration of potassium ferricyanide to haem. These samples were then tested for sulphide binding according to standard procedures, and the absorbance spectra from 500 to 650 nm were determined before and after the experiment.

Body fluid composition

Haemoglobin distribution

Column chromatography was employed for identification and separation of the two different M_r Hbs, FI and FII, in *Riftia* body fluids (first described by Terwilliger *et al.* 1980). The standard column used was 2·5 cm in diameter and 45 cm in length with a total bed volume of 220 ml. The filtration medium used was Sepharose 4B (fractionation range of 60 000 to 2 000 000 M_r). *Riftia* buffer was used in all cases and columns were run at 5 °C. In one experiment, 1 % Triton X-100 was added to the buffer in an attempt to dissociate the protein. Calibration markers included dextran blue (M_r 2 000 000), thyroglobulin (M_r 670 000), apoferritan (M_r 443 000), gamma globulin (M_r 158 000) and bovine serum albumin (M_r 66 000). The eluent absorbance was measured at either 415 nm for haem absorbance, 280 nm for protein absorbance, or both wavelengths for comparison. Elution profiles were constructed from absorbance of the eluent as a function of volume.

In addition, a gel filtration column used on a Gilson high performance liquid chromatograph was employed for rapid determination of M_r values of the Hbs of the different vascular blood and coelomic fluid samples. A TSK-50 gel filtration column 7·5 mm in diameter and 300 mm in length (fractionation range 10 000–2 000 000 M_r) along with a TSK guard column 7·5 mm in a diameter and 75 mm in length were run at 5 °C in *Riftia* buffer. The buffer flow rate was 0·3 ml min^{-1}, the run time was 40 min, and a 2-μl body fluid sample was used. A Gilson holochrome detector was used to measure the absorbance at 415 or 280 nm as the eluent left the column. The resulting elution profile was recorded on a chart recorder as absorbance as a function of retention time on the column, and the percentage distribution of the fractions present in the sample was calculated. The column was calibrated with thyroglobulin, gamma globulin, ovalbumin (M_r 44 000) and myoglobin (Mb) (M_r 17 000).

Dissociation experiments

Further dissociation of the two Hbs, FI and FII, from the Sepharose gel filtration column into subunits was attempted on an ion exchange column 2·5 cm in diameter and 95 cm in length with a total bed volume of 330 ml. The column was filled with Sephadex G-75 superfine beads (fractionation range 3000–70 000 M_r). The buffer used was as described in Terwilliger *et al.* (1980) and contained various reagents according to the dissociation experiment being done. Potential dissociating reagents included: dithiothreitol (DTT), sodium cholate, guanidine hydrochloride and urea. pH was also varied by titration of the buffer. The column was fully washed and

re-equilibrated when experimental conditions were altered. Calibration markers used included: albumin, carbonic anhydrase (M_r 29 000), Mb and cytochrome c (M_r 12 400).

12·5 % acrylamide PAGE run under dissociating conditions [in the presence of 1 % sodium dodecyl sulphate (SDS), boiling, and 1 % β-mercaptoethanol] was performed according to the methods of Laemmli (1970). Gels of previously frozen vascular blood and coelomic fluid, as well as fractions off the Sepharose gel filtration column, were run to compare the subunit composition of the two Hbs, FI and FII.

Protein cysteine and disulphide composition

The number of free cysteine residues and exposed disulphide bridges present in the two Hbs, FI and FII, was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), either prior to or after reduction of disulphides with DTT (Ellman, 1959). Prior to our experimentation, the vascular blood or coelomic fluid had been run on the Sepharose gel filtration column described above and separated into the two Hbs, FI and FII. These separated fractions were checked for purity with the HPLC method described above. Fractions used for experimentation were at least 96 % pure for the Hb in question; the exact composition for each analysis is reported (Table 5B).

The haem content of each sample was determined according to the method of Drabkins & Austin (1935), and twice the molar haem content in DTNB was reacted with the sample for 1 h. The DTNB-reacted samples were then placed in a Centricon microconcentrator (Amicon) with a 10 000 M_r cut-off and spun at 5000 g for 2 h at 5 °C. The Hb was retained in the upper portion of the microconcentrator tube and the reacted and unreacted DTNB passed through the filter and was retained in the filtrate in the bottom portion. The Hb samples were resuspended in *Riftia* buffer at 5 °C and spun until no DTNB was detected in the filtrate. Finally the Hb sample was resuspended to the original volume and the haem content was compared to the starting amount. The filtrate was measured at 410 nm and a molar extinction coefficient of 13 600 was used to determine the molar thiol concentration (Ellman, 1959).

To determine the number of exposed disulphide bridges (those accessible without denaturation of the protein), the separated Hb fractions were reacted with ten times their haem concentration in DTT for 1 h. After reaction, the mixtures were placed in an ultrafiltration cell (Amicon) with a 10 000 M_r cut-off and the DTT was washed through with *Riftia* buffer at 5 °C. The presence of unreacted DTT in the effluent was determined by addition of DTNB which produces a bright yellow colour if thiol is present. The Hb was washed in this manner until no yellow was detected upon addition of DTNB.

After the removal of the unreacted DTT, DTNB at twice the molar haem concentration was added and reacted for 1 h. The Hb–DTNB mixture was separated and the numbers of cysteine and haem moieties were determined as above. Results are expressed as the number of disulphide bridges per mmol haem. After reduction and subsequent analysis, the Hb samples were again checked for purity on the HPLC

to determine if there had been any breakdown of the larger M_r Hb molecules into subunits.

RESULTS

Haemoglobin sulphide binding

To determine if the sulphide-binding protein in the vascular blood and coelomic fluid of *Riftia* was the Hb or another protein, we purified the Hb and tested it for sulphide binding. Initially, a sample of whole, mixed blood (a combination of coelomic fluid Hb and vascular blood Hb) was sedimented by ultracentrifugation and the resulting supernatant and resuspended pellet were tested for haem concentration and sulphide-binding ability (Table 1A). The pellet contained the majority of the haem-containing component and showed a similar sulphide-binding capacity to the mixed blood (0·94 mmol sulphide $mmol^{-1}$ haem and 0·97 mmol sulphide $mmol^{-1}$ haem, respectively). Thus, the majority of the sulphide-binding component present in the whole blood was sedimented in the haem-containing pellet.

The resuspended pellet was then run on a Sepharose 4B gel filtration column which showed the presence of two characteristic fractions: FI and FII. All filtration products eluted from this column were scanned at 280 nm for identification of protein, and 415 nm for identification of haem-containing components. All protein

Table 1. *Sulphide-binding capacity of variously treated blood samples from* Riftia *and* Lumbricus *sp.*

Sample and treatment	Haem ($mmol\,l^{-1}$)	mmol sulphide / mmol haem
(A) *Riftia* – sedimentation experiment		
control	1·44	0·97
pellet	1·08	0·94
supernatant	0·14	0·15
(B) *Riftia* – gel filtration experiment		
FI	0·16	1·83
FII	0·56	0·90
(C) *Lumbricus* sp. – sulphide-binding test		
ammonium sulphate precipitate	0·27	0
unaltered whole blood 1	0·27	0·09
unaltered whole blood 2	0·42	0·07
unaltered whole blood 3	0·55	0·17
eluted peak	0·24	0·23
Riftia control	0·63	1·09

(A) *Riftia* mixed blood centrifuged at 250 000 ***g*** for 2·5 h at 5 °C (the pellet was resuspended to the exact previous volume in 50 $mmol\,l^{-1}$ Tris standard *Riftia* buffer).

(B) *Riftia* resuspended pellet (as above) analysed on Sepharose 4B gel filtration column.

(C) *Lumbricus* sp. vascular blood precipitated in 60 % ammonium sulphate by centrifugation at 15 000 ***g*** for 15 min at 4 °C after stirring for 2 h (the pellet was resuspended as above).

All other samples were tested unaltered (1–3 are different samples from the same blood pool) or after dialysis in standard *Riftia* buffer.

peaks detectable in the eluent also showed haem absorbance (Fig. 1). This suggests that the precipitated Hb solution was pure for the two Hb fractions and did not contain any other non-haem proteins. The sulphide-binding capacities of the two fractions containing the peak haem absorbance were greater than, or similar to, the capacities of the whole blood (Table 1B).

Lumbricus sp. blood was purified by ammonium sulphate precipitation and the partially purified Hb was run on the Sepharose 4B gel filtration column. Three samples of whole blood, the ammonium-sulphate-precipitated Hb, and the Hb purified by Sepharose gel filtration were all tested for sulphide-binding ability along with a sample of *Riftia* blood as a control. Although some binding occurred in the *Lumbricus* blood sample, it was not nearly as pronounced as that of the *Riftia* blood sample (Table 1C).

We used PAGE as a further test of the hypothesis that the protein responsible for sulphide binding in *Riftia* body fluids is the Hb. We subjected fractions FI and FII (separated from *Riftia* coelomic fluid by Sepharose gel filtration) as well as the resuspended, sedimented Hb, and *Lumbricus* sp. vascular blood to non-denaturing PAGE and stained for protein and haem (Fig. 2). Virtually all of the protein in all four samples stained for both protein and haem, indicating that no major non-haem-containing protein contaminants were present.

These data indicate that the components of the blood and coelomic fluid of *Riftia* that bind sulphide contain predominantly one type of protein, Hb.

Haem involvement in sulphide binding

In order to determine if sulphide binding occurs on the oxygen-binding site of the Hb of *Riftia* vascular blood and coelomic fluid, we tested for sulphide-binding

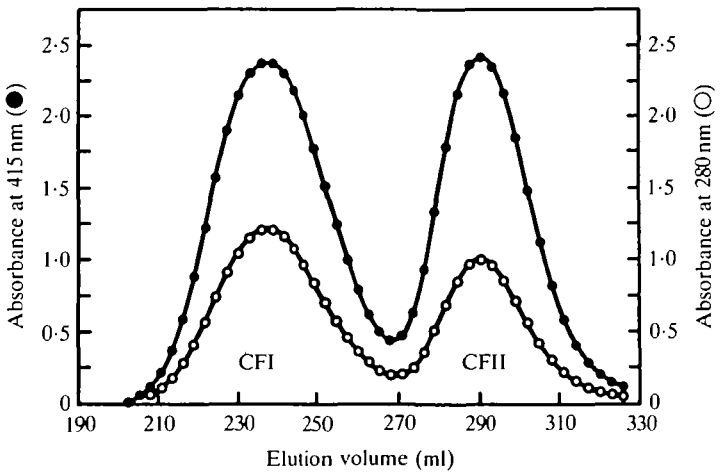

Fig. 1. Elution profiles of *Riftia* mixed blood on a Sepharose 4B gel filtration column (closed circles, absorbance at 415 nm; open circles, absorbance at 280 nm; CFI is fraction FI separated from *Riftia* mixed blood by Sepharose gel filtration and CFII is fraction FII separated from *Riftia* mixed blood by Sepharose gel filtration).

capacity under a number of conditions affecting the haem portion of the molecule. Formation of HbCO did not change sulphide-binding capacity (Table 2A, values are within the experimental error). The experiment in which metHb was induced was complicated by our discovery that metHb in *Riftia* body fluids is rapidly reduced in the presence of sulphide. MetHb formation was induced by the addition of potassium ferricyanide equal to the haem concentration. Although the blood showed the characteristic absorbance spectrum of metHb at the start of the experiment, this spectral shift was absent or reduced at the end of the experiment, and an oxyHb spectrum appeared in its place. Sulphide binding appears to be slightly augmented in the experiment reported in Table 2B. MetHb sulphide binding is not likely to be of any physiological relevance to the animal, however, as metHb is short-lived in the presence of sulphide and has never been detected in significant concentrations in the vascular blood or coelomic fluid of these tube-worms (Terwilliger *et al.* 1980; Arp & Childress, 1983).

These data, along with previous findings that the absorbance spectra of the whole blood exposed to and free from sulphide show no differences, and that oxygen-binding affinity is not affected by sulphide (Childress *et al.* 1984; Arp *et al.* 1985), indicate that sulphide binding does not occur at the haem site.

Body fluid composition

Fresh samples of coelomic and vascular blood were collected from an undamaged, living animal. The coelomic fluid of this animal, chromatographed on a Sepharose gel filtration column while at sea, contained only the low M_r fraction, FII (Fig. 3A).

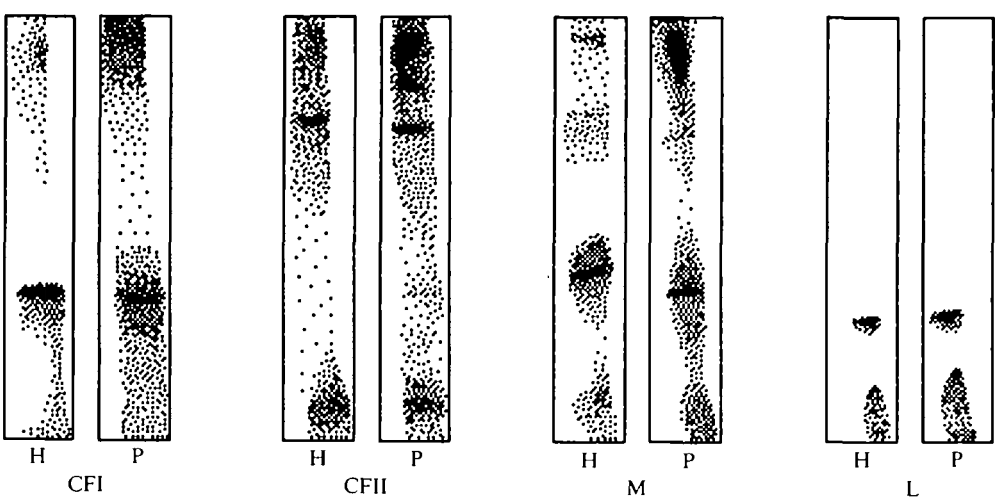

Fig. 2. Non-denaturing polyacrylamide gel electrophoresis of various Hb-containing samples comparatively stained for haem and protein (H, haem stained; P, protein stained). CFI is fraction FI separated from *Riftia* coelomic fluid by Sepharose gel filtration, CFII is fraction FII separated from *Riftia* coelomic fluid by Sepharose gel filtration, M is a mixed sample of *Riftia* blood that was sedimented by ultracentrifugation and resuspended in *Riftia* buffer as described in the text, and L is untreated *Lumbricus* sp. vascular blood.

The fresh vascular blood, however, showed the same heterogeneity (presence of the two Hb types FI and FII) as previously seen in a mixed sample of coelomic fluid and vascular blood drained from a frozen specimen (Figs 1, 3B; Terwilliger *et al.* 1980). The fact that the vascular blood is heterogeneous for FI and FII when fresh indicates that the presence of FII is not an artefact of freezing, and the vascular blood is heterogeneous in the intact, living animal. However, the coelomic fluid in the living animal appears to be homogeneous for the low M_r Hb, FII. The variability reflected in the data from freshly caught animals (Table 3) may be due in part to damage of the vascular network during collection or during sampling (as described above).

The distribution of FI and FII was highly variable from animal to animal in the frozen samples, but careful examination of the distribution of these two Hbs results in the emergence of a general pattern in which the high M_r fraction, FI, is the dominant Hb in the vascular blood and the lower M_r fraction, FII, is the dominant coelomic fluid Hb (Table 3). The data from freshly collected and analysed vascular blood and coelomic fluid presented in Fig. 3A,B supported this hypothesis. The HPLC analyses showed that in the vascular blood fraction FI constitutes $62{\cdot}17 \pm 8{\cdot}90\,\%$ (mean ± S.D., $N = 18$) of the Hb present, and in the coelomic fluid fraction FII constitutes $85{\cdot}95 \pm 10{\cdot}18\,\%$ (mean ± S.D., $N = 18$) of the Hb present

Table 2. *The effect of carboxyhaemoglobin and methaemoglobin formation on sulphide binding in* Riftia *blood*

(A)	Sample number	(CO) (mmol l^{-1})	mmol sulphide / mmol haem
	30–13	0	1·53
	30–13	1·48	1·68
	25–1	0	0·92
	25–1	1·73	0·98
	19–2	0	1·03
	19–2	2·30	1·16

(B)	Sample number	Potassium ferricyanide (mmol l^{-1})	mmol sulphide / mmol haem
	30–13	0	1·53
	30–13	1·20	2·04
	25–1	0	0·92
	25–1	1·31	0·82
	19–2	0	1·03
	19–2	2·80	1·51

Sample 30–13 had 8·6 % FI, sample 25–1 had 0 % FI, sample 19–2 had 31·7 % FI.

(A) HbCO samples showed $\lambda\beta$ wavelengths characteristically shifted 2 nm, and $\lambda\alpha$ wavelengths shifted 6 nm (oxyHb, 540, 576 nm; HbCO, 538, 570 nm).

(B) Potassium ferricyanide concentrations were slightly more than equimolar to haem concentration.

(Table 3). It is particularly striking that the FII concentrations in the coelomic fluid and vascular blood are generally equivalent, while the FI concentrations are always very different (the ratio of haem concentration of coelomic fluid to haem concentration of vascular blood is $0{\cdot}15 \pm 0{\cdot}15$ for FI, and $1{\cdot}25 \pm 0{\cdot}29$ for FII; means ± S.D., $N = 18$; data from Table 3). This indicates that these compartments are not confluent at the molecular size of the high M_r Hb, FI, but that they may be at the size of the lower M_r Hb, FII.

We compared types FI and FII in the coelomic fluid with those of the vascular blood to investigate whether they are the same in both body fluids. FI and FII have similar intact M_r values in both the coelomic fluid and the vascular blood as they have similar retention times on an HPLC gel filtration column and similar elution volumes on a Sepharose gel filtration column (Table 4). When FI and FII are run on dissociating PAGE, FI in the coelomic fluid is identical to FI in the vascular blood and FII is also identical in both body fluids (Fig. 4). This gel also shows that FI and FII do not have the same subunit composition; FI contains 15 000, 30 000, and 40 000–45 000 M_r subunits, whereas, FII contains 15 000, 20 000 and 30 000 M_r subunits. These data indicate that FII is not a dissociation product of FI, but is a different Hb molecule with a different intact M_r and subunit composition.

Riftia vascular blood was run on the Sepharose gel filtration column, treated with 1 % Triton X-100, and run on the column again, to see if fraction FI dissociates into FII in the presence of this detergent. Fraction FI showed no tendency to dissociate to fraction FII (Fig. 5). Terwilliger *et al.* (1980) also reported that FI showed no

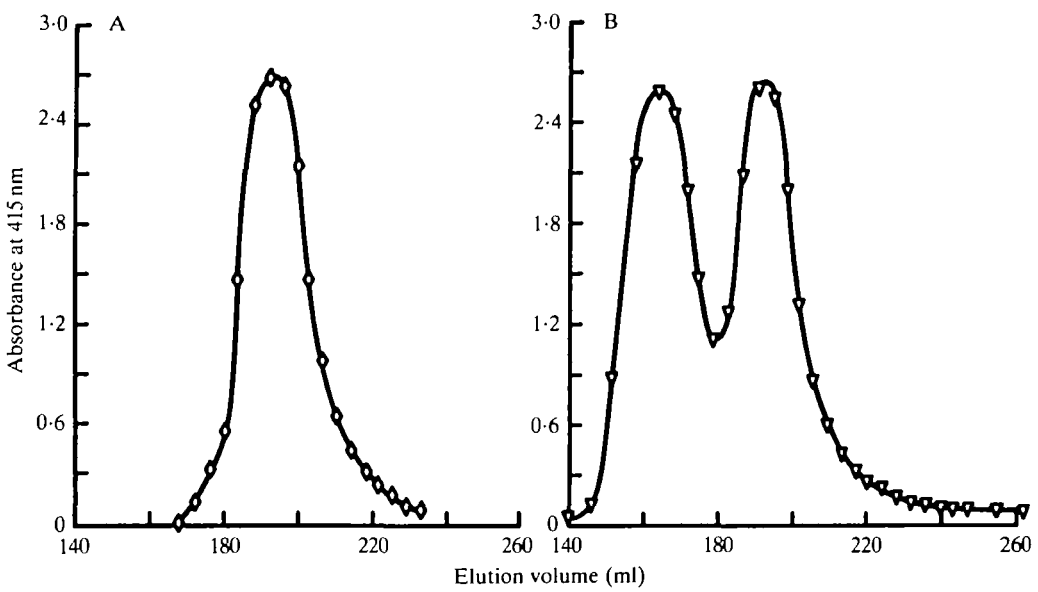

Fig. 3. Sepharose 4B gel elution profiles of *Riftia* coelomic fluid and vascular blood from a freshly killed animal. (A) *Riftia* coelomic fluid (diamonds); (B) *Riftia* vascular blood (triangles).

tendency to dissociate to FII or FII to associate to FI, that the two fractions were different on SDS PAGE and by isoelectric focusing, and that the two fractions had different amino acid compositions. Further, FII does not appear to be a degradation

Table 3. *Comparative characteristics of* Riftia *body fluids*

Animal no.	% FI	Haem FI (mmol l^{-1})	Haem FII (mmol l^{-1})	mmol sulphide / mmol haem
19–9 C	19·6	0·41	1·70	1·24
19–9 V	60·5	2·16	1·41	2·02
23–2 C	5·9	0·08	1·28	0·89
23–2 V	59·0	2·21	1·53	1·90
30–15 C	10·6	0·20	1·69	1·02
30–15 V	61·9	1·81	1·11	2·59
30–16 C	3·2	0·08	2·33	0·73
30–16 V	60·8	2·25	1·45	2·15
30–17C	11·8	0·38	2·88	1·07
30–17 V	63·6	2·92	1·67	2·01
30–18 C	28·0	0·61	1·57	1·25
30–18 V	66·9	3·26	1·62	1·48
30–22 C	15·8	0·19	1·01	0·84
30–22 V	70·6	2·68	1·11	1·76
30–23 C	6·1	0·12	1·78	0·71
30–23 V	67·5	2·62	1·26	2·49
1609–3 C	9·4	0·12	1·12	0·85
1609–3 V	60·8	1·79	1·16	1·89
1609–5 C	3·1	0·04	1·16	0·69
1609–5 V	80·7	3·02	0·72	2·34
19–8 C	0	0	1·00	—
19–8 V	63·8	1·83	1·04	—
30–21 C	14·6	0·37	2·16	—
30–21 V	34·5	1·20	2·21	—
30–10 C	7·9	0·15	1·74	—
30–10 V	62·7	1·82	1·08	—
30–11 C	31·2	0·68	1·50	—
30–11 V	65·2	2·14	1·14	—
16–1 C	15·3	0·18	1·02	—
16–1 V	67·0	2·20	1·09	—
30–30 C	10·4	0·18	1·58	—
30–30 V	58·1	1·75	1·26	—
19–3 C	36·2	0·88	1·53	—
19–3 V	59·1	1·53	1·06	—
17–1 C	23·6	0·46	1·50	—
17–1 V	56·3	1·61	1·25	—

C = coelomic fluid; V = vascular blood; dashes indicate that no data were collected for the sample.

All data presented on percentage distribution of FI and FII were obtained by HPLC gel filtration.

product of FI, as body fluids taken from live animals and analysed fresh at sea showed the same characteristic presence of the two Hbs (Fig. 3).

Fractions FI and FII showed different sulphide-binding capacities (Table 5A). FI binds approximately twice as much sulphide per haem as FII. When sulphide-binding capacity is plotted as a function of the percentage of FI present, it becomes apparent that the more FI present, the greater the sulphide-binding capacity of the body fluid, and that vascular blood binds more sulphide than coelomic fluid (Fig. 6).

The fact that neither of the two Hb species contained easily accessible free cysteine residues was shown by their lack of reaction with DTNB prior to reduction.

Table 4. *Comparative characteristics of* Riftia *haemoglobin fractions FI and FII separated by Sepharose gel filtration column chromatography, and also run on HPLC gel filtration*

Body fluid	% FI	% FII	RT (HPLC)	Ve (Sepharose)
Vascular blood				
FI	100	0	28·25	161/163
FII	0	100	32·29	191/193
Coelomic fluid				
FI	100	0	28·32	166/161
FII	0	100	32·20	191/190

RT, retention time (in min); Ve, elution volume in (ml).

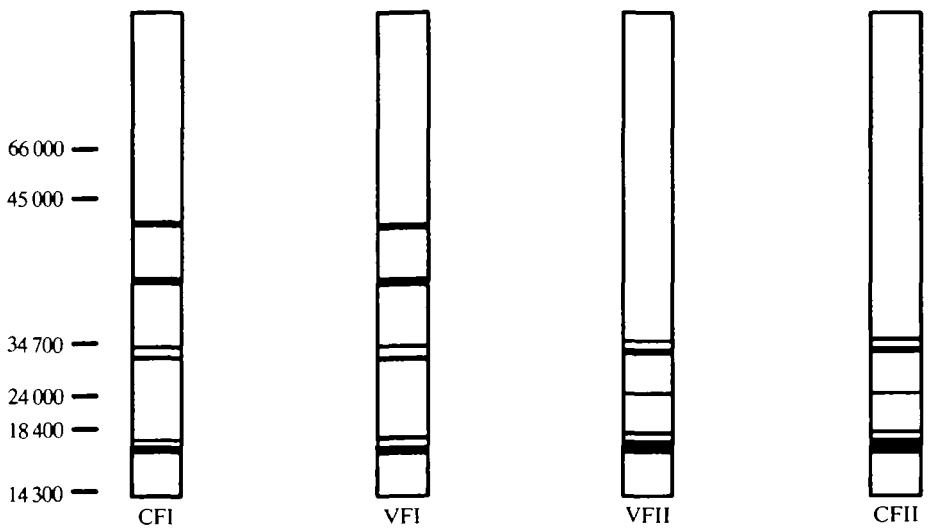

Fig. 4. 12·5 % polyacrylamide gel electrophoresis run in the presence of 1 % SDS and 1 % β-mercaptoethanol. CFI is fraction FI from *Riftia* coelomic fluid, CFII is fraction FII from *Riftia* coelomic fluid, VFI is fraction FI from *Riftia* vascular blood, and VFII is fraction FII from *Riftia* vascular blood. All fractions were separated from *Riftia* body fluids by Sepharose gel filtration, and application of known proteins to the gel are included for M_r comparisons (bovine serum albumin, M_r 66 000; egg albumin, M_r, 45 000; pepsin, M_r 34 700; trypsinogen, M_r 24 000; β-lactoglobulin, M_r 18 400; lysozyme, M_r 14 300).

However, both contained disulphide bridges which were freely reducible without denaturation of the protein. The number of freely reducible disulphide bridges was 1·13 disulphides per haem for FI and 0·49 disulphides per haem for FII (mean values; Table 5B), and sulphide-binding capacities were 1·93 and 0·87 mmol sulphide $mmol^{-1}$ haem, respectively (mean values; Table 5A). Thus, the higher M_r fraction, FI, had approximately twice as many easily reducible disulphide bridges as the lower M_r fraction, FII, the approximate ratio of sulphide binding between the two fractions.

The vascular blood and coelomic fluid samples were chromatographed by HPLC prior to and after determination of thiols and disulphides. All samples showed the same chromatographic profiles before and after reduction, indicating that the freely reducible disulphide bridges are not responsible for maintaining quaternary structure of either Hb. Similarly, whole blood run on ion exchange columns in the presence of the reducing agent DTT (1 mmol l^{-1}) at pH 10 or with the detergent sodium cholate (50 mmol l^{-1}) did not show complete break-up into subunits. Further, aggregated subunits of *Riftia* Hb did not dissociate when treated with 8 mol l^{-1} urea and 4 mol l^{-1} guanidine hydrochloride at alkaline pH during chromatography on ion exchange columns. These experiments suggest that the native Hb structure in *Riftia* body fluids is not easily disrupted and that easily reducible disulphide bridges may not be required for maintaining the higher structural state of the Hb.

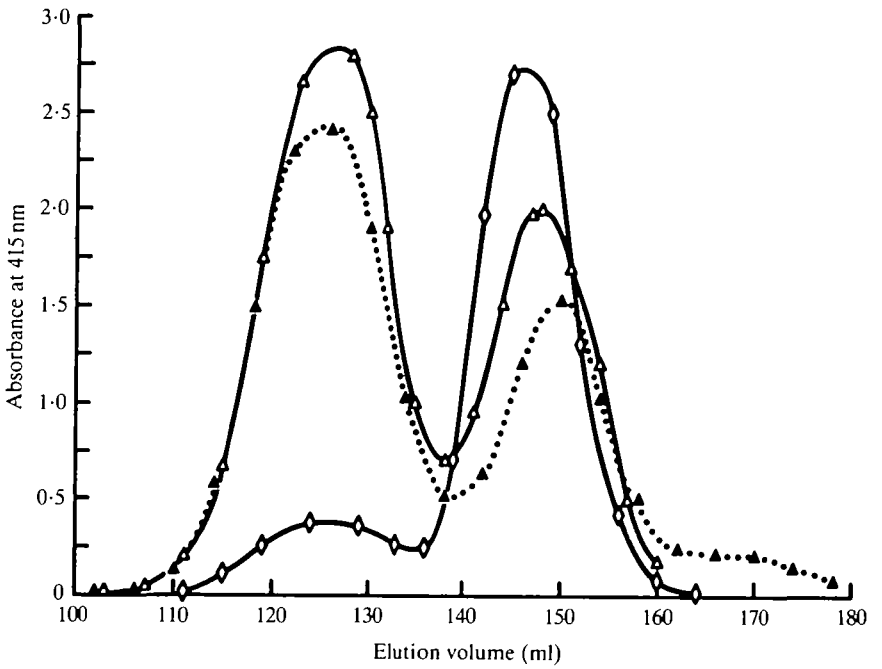

Fig. 5. Elution profiles of *Riftia* coelomic fluid and vascular blood on a Sepharose 4B gel filtration column. *Riftia* coelomic fluid (open diamonds), *Riftia* vascular blood (open triangles) and *Riftia* vascular blood treated with 1 % Triton X-100 (closed triangles).

DISCUSSION

The initial discovery of sulphide binding by the body fluids of the tube-worm *Riftia pachyptila* identified the binding factor as a protein present in both the vascular blood and coelomic fluid (Arp & Childress, 1983). The research presented here establishes that these sulphide-binding proteins are the large, extracellular Hb molecules that occur in high concentrations in both of these fluids. Hbs purified by ultracentrifugation and by column chromatography have greater or equal sulphide-binding capacities to that of whole blood. Elution profiles from chromatographic separations show that the major protein present is Hb, and haem and protein staining

Table 5. *Characteristics of the purified haemoglobins FI and FII from Sepharose gel filtration separations of* Riftia *body fluids*

(A)	Blood type and fraction	mmol sulphide / mmol haem	% FI or FII
	Mixed no. 1		
	FI	1·58 ± 0·14 (3)	—
	FII	0·78 ± 0·12 (3)	—
	Mixed no. 2		
	FI	1·83	—
	FII	0·90	—
	18–2 C		
	FII	0·88	100
	23–2 C		
	FII	0·89	93
	23–2 V		
	FI	2·39	100
	FII	0·90	100

(B)	Blood type and fraction	disulphide bridge / mmol haem	% FI or FII
	18–2 C		
	FII	0·51	100
	27–10 V		
	FI	1·02	98
	27–6 V		
	FI	1·23	100
	FII	0·46	100
	27–2 V		
	FI	1·14	100
	FII	0·50	96

(A) Sulphide-binding capacities.

(B) Distribution of disulphide bridges expressed on a haem basis (C, coelomic fluid; V, vascular blood; mixed indicates a combination of coelomic fluid and vascular blood; the percentage of FI or FII in the fluid was determined by HPLC after preparative separation by Sepharose gel filtration; dashes indicate no data were collected; numbers in parentheses are number of analyses run when greater than one; numbers are means ± S.D. when more than one analysis was done).

of PAGE gels shows that detectable proteins in these samples and in the unaltered body fluids are haem-containing. These data indicate that the protein present in the body fluids of *Riftia* capable of sulphide binding is the Hb itself. Studies of the vascular blood of *Lumbricus* sp. showed that blood and purified Hb from this annelid do not have the same capacity for sulphide binding as the body fluids of *Riftia*, although the Hb does show reduced sulphide binding (about one-quarter that of *Riftia*).

The simultaneous binding of sulphide and oxygen on a Hb molecule is an unprecedented finding. Hb exposed to sulphide typically forms a sulphhaemoglobin (sulphHb) complex which has a greatly reduced ability to bind oxygen. The only reports of sulphide binding by Hb have been in studies with vertebrates where induction of metHb formation has been shown to prevent sulphide inhibition of respiration (Smith & Gosselin, 1979; National Research Council, 1979; Torrans & Clemens, 1982). MetHb binds sulphide at the haem site and in this way removes it from possible interaction with the cytochromes. However, because the haem site is occupied, metHb is incapable of oxygen binding. To date, prevention of sulphide poisoning in invertebrates *via* metHb formation has been poorly studied. The only data available show an inability of annelid worm Hbs to form metHb or sulphHb in the presence of sulphide (Patel & Spencer, 1963; Wells & Warren, 1975; Wells & Pankhurst, 1980).

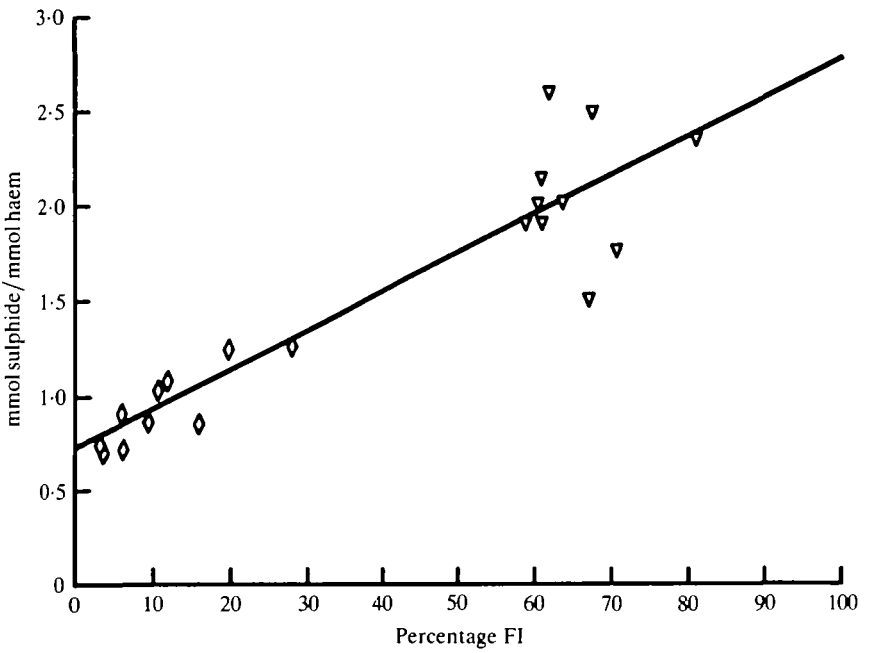

Fig. 6. Sulphide-binding capacity plotted as a function of the percentage of fraction FI in the sample being tested. The data are from Table 4; samples were either untreated coelomic fluid (diamonds) or vascular blood (triangles) tested according to the standard procedures described in the text (the equation for the regression line is $y = 0{\cdot}021x + 0{\cdot}702$, $N = 20$, $r = 0{\cdot}920$).

Riftia Hb prevents sulphide inhibition of respiration by binding free sulphide on the Hb molecule and removing it from contact with sensitive tissues (Powell & Somero, 1983). This occurs without the formation of sulphHb or metHb and does not occur at the haem portion of the Hb molecule. Further, metHb has never been observed in high concentrations in either the blood or coelomic fluid of *Riftia* and sulphHb has never been observed. Rapid reduction of metHb by sulphide in *Riftia* body fluids shown in this study may keep metHb levels low *in vivo*, and aid in maintaining Hb function.

The absence of metHb and sulphHb and the ability of sulphide-saturated Hb to bind oxygen, along with the fact that formation of HbCO does not alter sulphide-binding capacity, indicate that the binding site for sulphide is not on the oxygen-binding site of the haem portion of the Hb molecule. Data on disulphide bond distribution suggest that sulphide binding may occur at disulphide bridges. One likely mechanism for sulphide binding is thiol–disulphide exchange in which an oxidized disulphide in a protein undergoes nucleophilic attack by a reduced thiol. This results in a reduced cysteine residue and a protein–thiol mixed disulphide (Torchinsky, 1981). A change in external thiol concentration or a conformational change in the protein may allow the protein disulphide to reform, releasing the thiol. This type of interaction is well known for cellular glutathione and coenzyme A but has not been described for sulphide (Gilbert, 1982; Zeigler, 1985). For this to be a possible sulphide-binding mechanism in *Riftia* Hb three criteria must be satisfied: (1) the Hb must contain disulphide bridges; (2) these disulphide bridges must be accessible to reduction; and (3) the participating disulphides must not be required for the integrity of the macromolecular structure.

Disulphide bridges are rare in Hbs, presumably because they confer rigidity to a molecule that depends on flexibility for cooperative binding (Nascimento, Daniel & Heneine, 1982). *Riftia* Hb contains easily reducible disulphides, as do other extracellular high M_r Hbs of some annelid worms such as *Lumbricus* sp. (Waxman 1971, 1975; Vinogradov, Shlom, Kapp & Frossard, 1980). Disulphide bridges of proteins are generally classified as either exposed or buried. Exposed linkages are those that are reducible with a reagent such as DTT in the native conformation. These disulphides are most often, but not exclusively, interchain disulphide bridges. Buried disulphides are those that are susceptible to reduction only under denaturing conditions such as the presence of urea or guanidinium chloride. These are most often interchain disulphide bridges (Konigsberg, 1972; Torchinsky, 1981). The disulphide bridges described in this study were all accessible in the native form and are probably interchain disulphide bridges. The reduction of these disulphide bridges did not result in denaturation or dissociation of the Hb as all of the initial Hb sample was recovered in the pellet of the centrifugal separations after reduction, and the final HPLC chromatograms were identical to the initial HPLC chromatograms, showing the presence of the intact Hbs, FI and FII.

Although these experiments in no way prove that sulphide is bound *via* thiol–disulphide exchange *in vivo*, the initial criteria for the mechanism have been satisfied. Sulphide binding at disulphide bridges is supported by the fact that the Hb

molecules contain easily reducible disulphides, and that they may not be the sole forces holding this molecule together. The facts that simple reduction of disulphide bridges, as well as capping of the resulting free cysteine residues with *N*-ethylmaleimide, diminish sulphide binding also suggest disulphide involvement with sulphide binding (A. J. Arp & R. D. Vetter, unpublished results). Interchain disulphide bridges have been reported to be responsible for holding together the trimers, which then form the submultiples that make up the Hb molecules of annelid extracellular Hbs (Waxman, 1971, 1975; Vinogradov *et al.* 1980). In contrast, *Riftia* Hb's higher molecular structure does not appear to be solely dependent on interchain disulphide bridges, or it would dissociate into its constituent subunits when reduced.

Riftia blood has been reported to be heterogeneous in Hb composition (Terwilliger *et al.* 1980). Our recent work on carefully separated fresh and frozen blood confirms that the vascular blood is heterogeneous for the two Hbs, but indicates that the coelomic fluid is homogeneous for the lower M_r species and that there are important functional as well as structural differences between these two compartments. The purified Hb fractions FI and FII differ in their structural properties (intact M_r subunit composition) and column chromatography shows there is no tendency of FI to dissociate into FII. The sulphide-binding capacity of purified FI is slightly more than twice that of FII, and the disulphide bridge content of FI is also slightly more than twice the disulphide bridge content of FII.

Although there is some variability in the distribution of FI and FII in the paired body fluid samples from the vascular blood and the coelomic fluid collected from living specimens, it is clear that vascular blood contains predominantly FI (62%) and some FII (38%), and that the coelomic fluid contains mostly FII (86%) (Table 3). The haem concentrations and sulphide-binding capacities of the paired samples are different because of the presence of these characteristic amounts of the two Hbs with their different properties.

The structural and functional differences between the coelomic fluid and the vascular fluid could have important physiological ramifications. Further studies are being carried out to determine the relative affinities of the vascular blood and coelomic fluid Hbs for sulphide, as well as the molecular mechanism of sulphide binding and delivery. If the vascular blood proves to have a higher sulphide-binding affinity as well as capacity, it could be responsible for sulphide uptake at the plume and sulphide transport to the internally located bacteria – with the coelomic fluid functioning as a storage organ. If the sulphide affinity of the coelomic fluid is higher than that of the vascular blood, sulphide would flow from the vascular blood into the coelomic pool until it was saturated, making the coelomic fluid the likely source of sulphide for the bacteria. The discovery of the differences in the structural and functional characteristics of the two Hbs, FI and FII, and the different Hb compositions of the vascular blood and coelomic fluid, provides a new perspective on the physiology of the hydrothermal vent tube-worm *Riftia pachyptila*.

In conclusion, this report reveals that the large, extracellular Hb molecules that occur in high concentrations in the vascular blood and coelomic fluid of the hydrothermal vent tube-worm *Riftia pachyptila* bind sulphide. Sulphide binding

does not occur at the oxygen-binding site of the haem, but may occur *via* thiol–disulphide exchange at the interchain disulphide bridges on the macromolecule. Vascular blood is heterogeneous for the two haemoglobins, FI and FII, but the coelomic fluid is homogeneous for the lower M_r haemoglobin FII in the intact, living animal. The sulphide-binding capacities of FI and FII differ by about a factor of two. Consequently the vascular blood and the coelomic fluid also have different sulphide-binding capacities.

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