The Multi-hemoglobin System of the Hydrothermal Vent Tube Worm Riftia pachyptila

I. REEXAMINATION OF THE NUMBER AND MASSES OF ITS CONSTITUENTS*

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The deep-sea tube worm Riftia pachyptila Jones possesses a well developed circulatory system and a large coelomic compartment, both containing extracellular hemoglobins. Fresh vascular blood is heterogeneous and contains two different hemoglobins (V1 and V2), whereas the coelomic fluid is homogeneous and comprises only one hemoglobin (C1). Their molecular weights have been determined by scanning transmission electron microscopy mass mapping (STEM) and by multi-angle laser light scattering (MALLS). Both methods yielded approximately the same molecular weights with masses significantly higher than the literature data for V1, V2, and C1 had $M_r$ of $3396 \pm 540 \times 10^3$, $393 \pm 71 \times 10^3$, and $410 \pm 51 \times 10^3$ by STEM, and $3503 \pm 13 \times 10^3$, $433 \pm 8 \times 10^3$, and $380 \pm 4 \times 10^3$ by MALLS, respectively. Transmission electron micrographs of V1 are typical of an hexagonal bilayer hemoglobin (HBL Hb). When subjected to dilution or osmotic shock, V1 dissociates into halves and one-twelfth subunits like annelid HBL Hbs. V1 is resistant to urea treatment, indicating that hydrophobic interactions play a small role in its quaternary structure. Conversely, V1 Hb is rather unstable in solution without denaturant, a property which seems to be characteristic of vestimentiferan HBL Hbs and could be explained by an important number of hydrogen bonds.

Deep-sea hydrothermal vents are characterized by unusual chemical and physical parameters, including high pressures, high temperature gradients, and high concentrations of toxic elements such as sulfides and heavy metals (1). In this extreme environment, flourishing animal communities live. Riftia pachyptila Jones (2–4) is the most spectacular and one of the best studied species of these communities. This animal is strictly endemic to this ecosystem and inhabits sulfide-rich environments at depths of about 2600 m. This autotrophic organism is devoid of digestive tube and derives the energy and nutrients, Riftia has to supply them with $O_2$, $H_2S$ and $CO_2$. Transportation of these inorganic nutrients is facilitated by several different extracellular hemoglobins (Hbs) present in the well developed circulatory system and the large coelomic compartment of Riftia. These Hbs have a high affinity for oxygen (8, 9), an ability to reversibly bind sulfide (10–13), and a moderate ability to combine with carbon dioxide (14). Although the functional properties of Riftia Hbs have been well studied (reviewed in Ref. 15), there are some uncertainties on their number, distribution between the body compartments, and structures (8, 11, 16). Terwilliger et al. (8) described two Hbs with different molecular weights in the blood of Riftia; a large one with $M_r$ of about $1700 \times 10^3$ (FI), and a small one of $M_r$ $400 \times 10^3$ (FII). The $M_r$ of FI was unusual when compared to HBL Hbs from annelids (17) or other vestimentifera (16, 18, 19). Arp et al. (11) concluded that the FII fractions from vascular and coelomic fluids were identical and that these compartments may therefore be confluent at the molecular size of the lower $M_r$. However, this possibility was excluded on the basis of statistical results of their distribution between the two compartments (20). To date, these conclusions have not been confirmed with structural data.

Indeed, the number and the molecular weight of the different Hbs and their distribution between the different body compartments remain unclear in Riftia and other vestimentifera. Hence, the aim of the present work was first to identify and purify Riftia Hbs, and then to evaluate their relative molecular masses using the most accurate techniques to date: scanning transmission electron microscopy (STEM) mass mapping and multi-angle laser light scattering (MALLS). With the determination of the complete polypeptide chain composition of these Hbs, reported in a companion study (27), these mass estimations will allow us to elaborate proper models of the quaternary structure of these multimeric proteins.

**EXPERIMENTAL PROCEDURES**

Animal Collection—Specimens of Riftia pachyptila were collected during three distinct oceanographic cruises “HERO’91,” “HERO’92,” and “9°50’EPR/SPRING’95” to the hydrothermal vent sites located on the East Pacific Rise at 13°N (12°46’N-103’56”W and 12°50’N-103’57”W) and 9°N (9°48’9’50”N and 104’17”W) at a depth of about 2600 m. Animals were plucked from the substrate by the manipulators of the American subsurface “Alvin” or the French subsurface “Nautile” and stored in a thermally insulated basket until they were brought to the surface.

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† This abbreviations used are: Hb, hemoglobin; HBL, hexagonal bilayer; bis-tris, 2-[bis(2-hydroxyethyl) amino]-2-(hydroxyethyl)-propane-1,3-diol; PIPES, Piperazine-N,N’-bis(2-ethanesulfonic acid); TEM, transmission electron microscopy; STEM, scanning electron microscopy; MALLS, multi-angle laser light scattering; FPLC, fast protein liquid chromatography.
The specimens were immediately bled after their recovery on board. Blood from the closed vascular system and coelomic fluid of *R. pachyptila* and *Arenicola marina* hemoglobin were collected and stored as described previously (14). In this study we distinguish the samples we used depending on the duration of their storage: so-called "stored" samples come from either HERO'91 or HERO'92 and have been stored at \(-40^\circ C\) until use; so-called "freshly frozen" samples come from the most recent cruise (9°50EPR/SPRING'95) and have been kept in liquid nitrogen.

**Purification Techniques**—Analytical gel filtration was performed on a 1 x 30-cm Superose 6-C column (Pharmacia Biotech Inc., fractionation range from 5 to 5000 kDa) using a low pressure FPLC system (Pharmacia). The column was equilibrated with a *Riftia* saline buffer (12) modified in our laboratory, substituting HEPES by bis-tris-propane, the pK of bis-tris-propane (6.8) being closer to the vascular and coelomic pH (7) (14). The formula is: bis-tris-propane 50 mM, 400 mM NaCl, 3 mM KCl, 32 mM MgSO4, and 11 mM CaCl2 (pH 7.0). Flow rates were typically 0.5 ml/min, and the absorbance of the eluate was monitored at 280 nm and 414 nm. The peaks were collected separately and concentrated with microconcentrator Centricon-10 (Amicon). One or two further purifications using the same protocol were performed to obtain clear fractions when necessary. The column was calibrated with the following marker proteins (Pharmacia): aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa) and purified *A. marina* hemoglobin (mean 3.730 ± 0.126 kDa) (17, 21).

**Transmission Electron Microscopy (TEM)**—TEM was performed on purified hemoglobin fractions diluted (1:600) into a 0.05 M Tris-Cl buffer (pH 7.4). Negatively stained molecules were prepared on carbon-support films following a previously described technique (22). The preparations were observed with a Philips 201 electron microscope.

**STEM**—Mass measurements were carried out at Brookhaven National Laboratory as described by Wall and Hainfeld (23). Unstained hemoglobin fractions were prepared in 0.05 M PIPES buffer, pH 7.1 mM EDTA to a final protein concentration of ca. 0.5 g/l. The STEM was operated at 40 kV, a dose level of 6–10 e/0.1 nm², and an instrumental resolution of 25 nm. The elastically scattered electron signal from the STEM large angle annular detector was used to form images. Direct measurement of particle mass was obtained by subtracting the background value of the carbon substrate from the value of the region containing particles and multiplying by a calibration factor. The latter was calculated using tobacco mosaic virus as an internal mass standard (39,000 kDa/300.0 nm) (24, 25).

**MALLS**—MALLS measurements were performed with a DAWN DSP system (Wyatt Technology Corp.) directly on-line with the FPLC system described above. The eluate was simultaneously monitored with an UV detector and a refractometer (Erma, 7512). The Debye fit method was used for molecular mass and gyration radius determinations (26). In the calculations we used two different values of \(dn/dc\), the variation rate of the refractive index as a function of concentration: \(dn/dc = 0.190\) ml/g, typical of proteins, for the larger Hb fraction, and \(dn/dc = 0.170\) ml/g, based on evidence that the smaller Hb fractions contain glycoproteins (27).

**Dissociation Experiments**—After purification of the higher \(M_r\) Hb fraction, we incubated it with 2 M, 4 M, and 8 M urea in *Riftia* saline buffer for 30 min, 2 h, 4 h, and 8 h. Isolation of the dissociation products was performed on the Superose-6 column described above, except that it was calibrated and equilibrated using a buffer containing various urea concentrations. Each peak area was determined with a Coradi planimeter.

**RESULTS**

**Purification of *R. pachyptila* Hbs**—The nomenclature previously used to name the different fractions does not differentiate between the "FII" fractions from vascular blood and coelomic fluid (8, 11). We propose a new nomenclature labeling the vascular Hbs "V" and the coelomic Hbs "C," numbered sequentially in FPLC elution order. Gel filtration of freshly frozen samples provided two hemoglobin fractions for blood (V1 and...
V2) and only one for the coelomic fluid (C1) (Fig. 1, A and B). The same experiment performed on stored Riftia fluids showed four vascular (V1–V4) and two coelomic (C1 and C2) Hb peaks (Fig. 1, C and D). The apparent molecular masses of these fractions correspond to about 3000 kDa (V1), 440 kDa (V2), 220 kDa (V3), 150 kDa (V4) for the blood, and 440 kDa (C1) and 150 kDa (C2) for the coelomic fluid. We never found hemoglobin fractions corresponding to a molecular weight of V1 or V3 in the coelomic fluid.

**Dissociation of V1 Hb under Different Conditions**—To test the stability of the larger Hb we incubated purified Riftia V1 and Arenicola Hbs for 72 h at 4 °C diluted in 0.05 M Tris-Cl buffer, pH 7.5, as in Terwilliger et al. (8). Subsequent gel filtration yielded elution profiles showing three peaks (Fig. 2, A and B). The peaks corresponded to molecular masses ~3000 (native molecule), 1500, and 220 kDa.

Purified V1 Hb was also incubated with increasing concentrations of urea in saline buffer for 30 min to 8 h (Table I). After 8 h of incubation with 2 M urea, the native peak was unaltered; a small peak (400 kDa) appeared after 2-h incubation with 4 M urea and a third one (150 kDa) after 8 h. In the presence of 8 M urea after only 30-min incubation, V1 dissociated into three major components with masses ~400, 220, and 150 kDa, respectively.

**Electron Micrographs of R. pachyptila Hbs**—The appearance of V1 Hb on electron micrographs (Fig. 3A) was that of a typical HBL Hb (17). Its dimensions were: diameter 27 ± 1 nm (n = 10), height 18 ± 1 nm (n = 10). V2 and C1 Hbs appeared as ring-shaped structures (Fig. 3, C and D) with a diameter of 12 ± 1 nm (n = 10), comparable to the diameter of A. marina one-twelfth Hb (11 ± 1 nm, n = 10, Fig. 3B) or to one-third of the diameter of V1. In addition, the inset (Fig. 3A) shows an aggregation of five subunits forming an arc of a circle, indicating the dissociation of an hexagon.

**Molecular Masses of R. pachyptila Hbs by STEM**—We did not observe complete HBL structures with STEM but numerous particles corresponding to one-twelfth of the native molecule. This is observed for several other HBL Hbs, and it is probably due to instability of these molecules under STEM preparative treatment (freeze-drying). A histogram of the masses measured for 331 particles is shown in Fig. 4; the mean mass was 283 ± 45 kDa for the one-twelfth molecule. This result translates into an approximate mass of 3396 ± 540 kDa for the intact molecule. Similarly, the mass of V2 and C1 were estimated at 393 ± 71 kDa (n = 428) and 410 ± 51 kDa (n = 198), respectively. Although close, these values are statistically different (Student’s t test, 0.001 < p < 0.01), due to the high number of measurements for V2. Mean mass measurements for V3 and C2 were 80 ± 35 kDa (n = 84) and 198 ± 14 kDa (n = 14), respectively.

**Molecular Masses of R. pachyptila Hbs by MALLS**—MALLS analysis of V1 Hb yielded profiles as shown in Fig. 5A with molecular masses estimated during the elution of the peak. The mean mass of V1 was 3503 ± 13 kDa (n = 954) with a slight polydispersity as indicated by the slope of the individual estimates. The gyration radius corresponding to the mean of all dimensions of the molecule was 11.0 ± 1.1 nm for V1 Hb (Fig. 5B). Similar analysis for V2 and C1 yielded mean mass of 433 ± 8 kDa (n = 477) and 380 ± 4 kDa (n = 659), respectively. These values are statistically different (Student’s t test, 0.001 < p < 0.01). The size of V2 and C1 was too small to be determined with this technique (<10 nm, see Fig. 5B).

![Figure 2](image)

**Table I**

| Peak(s) | Mass | Incubation time |
|--------|------|-----------------|
|        |      | 30 min | 2 h | 4 h | 8 h |
|        |      | % | A\_14/A\_280 | % | A\_14/A\_280 | % | A\_14/A\_280 | % | A\_14/A\_280 |
| Urea 2 M |      | 1 | 3000 | 100.00 | 2.85 | 100.00 | 1.90 | 100.00 | 2.00 | 100.00 | 2.27 |
|         |      | 2 | 400  | 100.00 | 2.00 | 97.40 | 2.00 | 93.80 | 2.00 | 90.45 | 2.20 |
|         |      | 3 | 150  | 100.00 | 2.00 | 2.60 | ND\* | 6.20 | 2.00 | 5.72 | ND |
| Urea 8 M |      | 1 | 3000 | 77.53 | 2.04 | 68.40 | 2.00 | 71.03 | 2.10 | 73.80 | 1.25 |
|         |      | 2 | 440  | 11.95 | 2.00 | 17.10 | 2.66 | 17.46 | 2.28 | 10.71 | 2.33 |
|         |      | 3 | 220  | 3.98 | 2.33 | 5.94 | 4.00 | 3.96 | 0.60 | 5.95 | ND |
|         |      | 4 | 150  | 6.54 | 1.80 | 8.56 | 5.00 | 7.55 | 1.00 | 9.54 | 2.00 |

\* ND, not determined.
measurements of all Riftia Hb fractions as determined by FPLC, STEM mass mapping, and MALLS are summarized in Table II.

**DISCUSSION**

Gel filtration performed on freshly frozen body fluids from R. pachyptila revealed two Hb fractions for vascular blood (V1 and V2) and one for coelomic fluid (C1). Therefore we assume that V3, V4, and C2 found only in stored samples are dissociation products of V1, V2, and C1. The elution profile of stored Riftia vascular blood was similar to that obtained for Lamellibrachia sp., another vestimentiferan worm (18). However, in this last case, the body fluids were not collected separately before gel filtration, and these results were obtained on mixed fluid. Anyway, Riftia Hbs Mr values determined by FPLC are in agreement with those found in Lamellibrachia sp. (18) for V1, V2, or C1, but they are different from those reported previously for R. pachyptila larger Hb (8). Indeed, Terwilliger and co-workers (8), also working on mixed fluid, obtained two major fractions with masses around 1700 kDa (F1) and 400 kDa (FII). They interpreted the unusual mass of F1 by the pigment instability as well as the broadness of the elution peak that may contain some intact molecules whose molecular masses would correspond more closely to 3000 kDa (8), the usual mass of HBL Hbs.

Our experiments on dilution and/or osmotic shock on V1 Hb and A. marina Hb could also explain this unusual mass. Gel filtration performed after incubation of both hemoglobins in 0.05 M Tris-Cl buffer (pH 7.5) revealed in both cases three peaks which could correspond to the dissociation of the whole molecule into halves, and one-twelfth subunits. This scheme of dissociation was also found for Lumbricus terrestris HBL Hb (28, 29). V1 Hb is rather unstable and dissociates rapidly in agreement with first observations (8, 30), suggesting that the instability of R. pachyptila vascular Hb might be due to changes in pressure as the protein is brought to the surface or during work at atmospheric pressure. This relative instability might well be an inherent property of vestimentiferan Hbs, since the same phenomenon was observed for Lamellibrachia sp. (19) and for Tevnia jerichonana. Furthermore, the quaternary structure of HBL Hbs from alvinellids, which are vent annelids, is not similarly affected by pressure changes (30).

Surprisingly, V1 Hb was resistant to dissociation by urea since a small amount (about 30%, Table I) of dissociation prod-

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3 F. Zal, personal observations.
ucts were observed even at high urea concentrations. The same was true for *Lamellibrachia* sp. (19). However, V1 Hb produced the same dissociation products as *L. terrestris* HBL Hb at alkaline pH with the appearance of three peaks corresponding to subunits approximately 350, 130, and 60–90 kDa (31). As in *L. terrestris*, the 350-kDa component is not exactly one-twelfth of the native molecule since it contains some linker chains (31).

Since urea is known to destabilize hydrophobic interactions (32), these should play a small role in the quaternary structure of V1 Hb. This is consistent with the fact that V1 is rather unstable in solution without denaturant, which could be explained by an important number of hydrogen bonds.

Molecular masses of *R. pachyptila* Hbs measured by MALLS and STEM mass mapping are statistically different (Student’s *t* test, *p* < 0.001). This discrepancy may be due to the rather harsh preparative treatments necessary for STEM mass mapping as opposed to MALLS, which analyses molecules directly after the purification step. This is also reflected by the lower standard deviations obtained with MALLS. However, both methods yield a usual mass for V1, corresponding to a classical HBL Hb as found in annelids and vestimentifera, and in agreement with the TEM micrographs.

V2 and C1 Hbs had masses corresponding to the smaller ring-shaped structures revealed by electron micrographs. Similar structures are also found in pogonophoran (33). Both MALLS and STEM mass mapping provide statistically different masses for V2 and C1 suggesting that these two Hbs are distinct molecules. However, as determined by MALLS V2 is larger than C1, while the opposite was found with STEM mass mapping. Even if MALLS estimations are considered more accurate, it is therefore hazardous to conclude that V2 and C1 are indeed distinct molecules. More accurate data on the complete polypeptide chain composition of these Hbs are necessary to resolve this crucial point.

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